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BIOMIMETIC AND BIOINSPIRED ANTI-ADHESIVE AND ANTIMICROBIAL SURFACES

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ABSTRACT

Macro- and microfouling cause serious problems in many kinds of technical equipment. Consequently, there is great interest in the development of surfaces with anti-adhesive and antimicrobial properties. A range of surfaces with such properties exist in nature and artificial systems that mimic or are inspired by these natural systems could potentially be valuable technical surfaces.
SmartMembranes GmbH have demonstrated several applications of porous anodized aluminium oxides (AAO) membranes as templates for the synthesis of structurally well-defined surface nanopatterns among other things. We investigated the anti-adhesive and therewith antimicrobial properties of AAO membranes to assess their potential to prevent biofouling, using *E. coli* as a model microbe. Precision etching of aluminium membranes produced structures with pore diameters of 200-300 nm and whose porosity, topography, and hydrophobicity suggested they would exhibit strong anti-adhesive activity. Comparative experiments showed that these membranes were much more resistant to bacterial attachment and biofilm growth than stainless steel, which is widely used in the construction of industrial equipment. However, further work will be required to produce artificial surfaces that reproduce biological surfaces' ability to simultaneously suppress micro- and macro-fouling.

INTRODUCTION

Macro- and microfouling of heat exchangers used in processing industries are known to cause chronic operational problems in applications relating to energy recovery and environmental welfare (Müller-Steinhagen, et al., 2009).
During our recent attempts to optimize the operating parameters of a water-cooled frequency converter at a chemical plant in Saxony in order to minimize fouling and ensure the facility's uninterrupted availability, it was found that neither macro- nor microfouling could be prevented completely. Fouling occurred despite the use of standard anti-fouling processing techniques and devices including ion exchangers, pH adjustment, and the treatment of the cooling water with additives such as biocides, inhibitors of crystallisation, anti-conditioning agents, and inhibitors of corrosion. To determine why the system was so prone to biofouling, water from the heat exchanger's cooling circuit was sampled and subjected to microbial analysis. Despite all of the anti-fouling measures that were implemented, the cooling water was contaminated with bacteria (mainly *Bacillus* spp. and *Pseudomonas* spp.) at a level of around 10^4 detectable colony forming units (cfu) per mL. This may not sound like a high level of contamination, but it is in fact a serious problem because every individual bacterial cell can potentially adhere to a surface and initiate biofilm growth. Furthermore, *Bacillus* spp. are spore-forming bacteria and can withstand challenging conditions including high temperatures and extremes of pH. Consequently, the system was well set up to accommodate biofilm development and thus biofouling.
Biofouling is a common problem of industrial water treatment systems and in other contexts. However, the mechanistic details of the early stages of biofilm formation remain unclear (Flemming, 2011). The available data suggest that the process by which microorganisms transition from being suspended in a liquid medium to being adherent to a solid substrate can be divided into three stages (Jeyachandran, et al., 2006). These steps are:
1. ***Reversible adhesion***, whereby microorganisms are transported to the substrate by Brownian and gravitational forces, diffusion, convection, and their own active movement. The degree of adhesion between the microorganisms and the substrate depends on both electrostatic and hydrophobic/hydrophilic interactions including van der Waals forces as well as abiotic factors (Simoes, et al., 2007).
2. ***Conditioning***, in which the substrate is modified in various ways (e.g. by the uptake of water) and inorganic molecules are incorporated into a polymeric extracellular matrix that is constructed by the adhering microorganisms (Beech, et al., 2005). It is not currently clear whether the irreversible attachment of molecules from solution to the surface in a so-called conditioning film is important in this process (Hwang, et al., 2013).
3. ***Irreversible adhesion***, in which the attached microorganisms start to grow in 3D to form a biofilm (Dunne, 2002).

It has to be outlined that even the sequence of the first two steps remains controversially discussed. On one hand there are proofs that the presence of a conditional film is needed as a prerequisite for bacterial adhesion and/or such adsorbed layers improve biofilm formation (Whitehead, et al., 2010, Whitehead, et al., 2008). On the other hand we could show, that *E. coli* SM2029 is able to adhere to bare, ultraclean stainless steel surfaces as well (Wagner, et al., 2013). This brings us to the conclusion that further investigations are required to understand first stages of biofilm formation in their complexity - as biofilms in general.

Nevertheless all three phases are highly complex processes and are influenced by the properties of the microorganisms including what species they belong to as well as the presence of conjugative plasmids and pili (Turroni, et al., 2013). Other important factors include the microorganisms' morphological features and physiological state as well as the physicochemical properties of their cell surfaces such as their net charge (van Merode, et al., 2006), Zeta potential (Weigl, 2004), hydrophobicity/hydrophilicity (Strevett and Chen, 2003), ability to recognize solid phases via surface-exposed adhesive moieties (Garnett and Matthews, 2012), and the composition of the lipopolysaccharide-containing lipid membrane (Ghequire, et al., 2014). In addition, an ability to form a polymeric extracellular matrix appears to be important (Jaglic, et al., 2014). Also relevant are abiotic factors such as the composition and physicochemical properties of the fluid (i.e. its temperature, viscosity, content of detergents, ionic strength, and pH) as well as hydrodynamic factors and shear stress. Last but not least, the substrate surface itself plays an important role: its roughness and topography both have significant effects on biofilm formation, as do its temperature, chemistry and charge (Pasmore, et al., 2002). In addition, the extended Derjaguin, Landau, Verwey, Overbeek (DVLO) theory indicates that the surface's hydrophobicity/hydrophilicity influences biofilm formation (Boks, et al., 2008). To describe the interactions between microbes and surfaces, Strevett and Chen (2003) developed the "microbial surface thermodynamic theory", which considers the substrate's surface tension and wettability (characterized in terms of contact angles) as well as the microorganisms' surface tension, which is governed by the chemistry of the cell envelope. However, this theory is somewhat controversial (Yang, et al., 2010) .

Fouling causes a number of practical problems, necessitating the development of innovative strategies to reduce macrofouling and biofilm formation. Therefore, surfaces with anti-adhesive and antimicrobial features are attracting considerable interest, and a range of different approaches have been explored for their preparation. Progress in this area was recently reviewed by (Flemming, 2011). However, it is difficult to achieve everything required to prevent both micro- and macro-fouling using just one strategy.

One possible way of addressing this challenge is to look at how similar problems are solved in nature. During the last decade there have been many attempts to characterize natural structures with properties relevant to anti-fouling efforts and develop novel materials that mimic these properties. One of the first of these studies focused on the extreme hydrophobicity of lotus leaves, which is known as the Lotus effect (Bhushan and Jung, 2011). Later attempts were inspired by the surfaces of other plants' leaves (Koch and Barthlott, 2009) and sharkskin (Su, et al., 2010). More recently, various groups have drawn inspiration from the topography of insect wings including those of butterflies (Fang, et al., 2007), cicadas (Pogodin, et al., 2013)), mosquitos (Gao, et al., 2007) and termites (Watson, et al., 2010). The cuticles of springtails have also been investigated in this context (Nickerl, et al., 2013).

The projects discussed above all sought to reproduce the properties of natural surfaces in order to develop artificial anti-adhesive surfaces that are resistant to (bio)-fouling. At this point, it is worth noting that there is a difference between biomimetic approaches that seek to simply copy natural models, and "bioinspired" approaches that attempt to improve on them (Ralston and Swain, 2009). There are many ongoing research programs aiming to create artificial surfaces whose topography resembles that of a natural surface and which may incorporate chemicals with antimicrobial properties to provide additional resistance to microbial adherence. It is clear that a very wide range of parameters and their interactions will influence the properties of such surfaces.

We have recently initiated a research program investigating the antimicrobial properties of nanostructured surfaces inspired by cicada wings and springtail cuticles.

Ordered macro- and nanoporous aluminium surfaces have highly ordered hexagonal honeycomb or cubic structures with little variation in their pore diameters and interpore distances. Porous AAO membranes formed under self-ordering conditions exhibit a poly-domain structure in which each domain contains hexagonally ordered nanopores that have identical orientations and are separated by well-defined boundaries. Recently developed anodization techniques (e.g. mild, hard, pulse, cyclic, and guided anodization) have made it possible to strictly control the diameter, density, and aspect ratio of surface pores and even the internal pore structure of AAO membranes by selecting appropriate anodization conditions. These capabilities offer considerable scope for the templated syntheses of low-dimensional functional nanostructures, and also in the development of advanced AAO-based devices, enabling simple and cost-effective non-lithographic fabrication of extended arrays of identical structurally well-defined nanostructures (Zhang, et al., 1998). We therefore investigated the potential of these nanostructured surfaces as anti-adhesive coatings and the relationship between their surface properties and anti-adhesive behaviour.

MATERIALS AND METHODS

Biofilm experiments

Strains, media and growth conditions. We used a GFP-tagged *E. coli* SM2029 strain kindly provided by Søren Molin of the Technical University of Denmark (Reisner, et al., 2003). Due to the GFP-tag, the strain could be visualized via fluorescence microscopy on non-transparent surfaces without additional staining. Its pili enable it to attach to other cells and surfaces (Reisner, et al., 2003).

The flow cell experiments were conducted using a modified variant of the M9 minimal medium (Sternberg, et al., 1999) in which glucose (180 mg/L) was used as the carbon source rather than Na-citrate. Due to the auxotrophy of the *E. coli* mutant, sterile-filtered proline (10 mg/L) and thiamine (1 mg/L) were also added to the medium. Precultures were grown at 30 °C in shake flasks containing 20 g/L LB-medium (Lennox, Carl Roth GmbH) for 5-6 h at 300 rpm in a Compact Shaker KS 15 A (Edmund Bühler GmbH, D – Hechingen). To sustain the plasmids, 50 mg/L kanamycin was added to each preculture. The precultured bacteria were filtered through a 5 µm sterile filter (Minisart®, Sartorius Stedim Biotech S.A.) to avoid clusters in the inoculum. The filtrate was then diluted in phosphate buffered saline to 2×10^6 cfu/mL for inoculation. In all flow experiments, the fluid velocity was set to 3.0×10^{-3} m/s.

Flow cell and biofilm system. The biofilm experiments were conducted using a MicCell system (GeSim, Germany) as shown in Fig. 1.

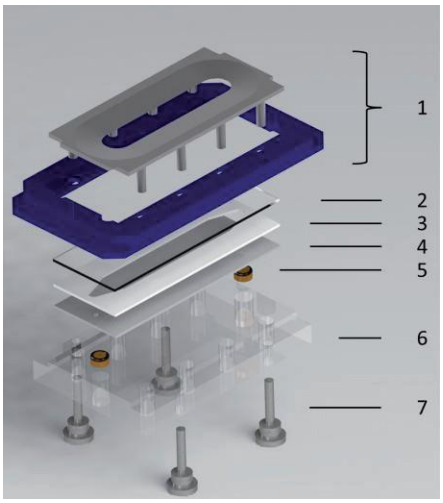

Fig. 1 Schematic diagram of the flow chamber components: (1) base plate, (2) glass slide, (3) fluidic layer, (4) substrate, (5) gasket, (6) pressure plate and (7) screws.

The flow chamber consists of a microfluidic chip (50 mm x 25 mm), a base, and a pressure plate (6). The pressure plate has two holes that enable fluid entry and exit. The holes have seals and can accommodate HPLC tubing connectors (Watson-Marlow-Bredel Pumps, Cornwall, England), and the entire assembly is held together with four screws (7). To study modified surfaces on supports with different heights in the range of 1 to 2 mm, we employed a spring-loaded pressure plate (6) with an online monitoring "window" (10 mm × 54 mm). The plate makes it possible to maintain a constant connecting pressure.

The fluidic chip consists of a glass slide, a fluidic layer and the substrate to be analysed. The fluidic layer (3) is pressed against a 3D polymer layer (EAH Jena, Germany) that is printed on the glass slide (2) and whose thickness and channel structure can be tuned. The fluidic layer is in turn placed on the substrate (4), which contains holes to permit liquid access, and the whole assembly is pressed together. A more detailed description of the apparatus is available elsewhere (Mulansky, et al., 2014, Wagner, et al., 2013)). Afterwards the flow cell must be screwed together so that the fluidic access points align with the holes in the substrate and leakage is eliminated. The assembled flow cell is then attached to the rest of the experimental setup, which consists of a peristaltic pump (Watson-Marlow 205S; Watson-Marlow-Bredel Pumps) that transports fluid from the medium supply through a bubble trap (Department of Systems Biology, Technical University of Denmark) and then into the flow cell. For additional information, see the work of (Sternberg and Tolker-Nielsen, 2006). All of these components are connected via silicone and PTFE tubes.

Inoculation and experimental procedure. To minimize unwanted growth of microorganisms in the inlet tubes, the test organisms are introduced directly via the inlet of the flow cell. This is done by stopping the pump and clamping the inlet tube shut in front of the bubble trap. One chamber volume (200 µL) of a diluted pre-culture containing 2 x 10^6 cfu/mL in total is introduced into the inlet using a syringe (MYJECTOR® 1 mL U-100 Insulin, Terumo Europe N.V., Belgium). Thus, the medium in the flow cell is replaced by the culture. After all the inoculum has been injected, any leakage from the needle is cleaned with ethanol and the fissure is closed with silicone glue (732Multi-Purpose Sealant, Dow Corning®, USA). The clamp on the outlet tube is then fastened and the flow cell is inverted so that the microorganisms can sediment, settling on the substrate. After a prescribed adhesion time, the flow cell is re-inverted so that the substrate is on the top of the flow chip to prevent further attachment due to sedimentation. Finally, the clamps on the inlet and outlet tubes are loosened, the pump is started, and the whole system is incubated at 30 °C for a prescribed period of time either in an incubator or directly under a tempered microscope.

Microscopic analysis of biofilm formation. The system is designed for observation using a microscope equipped with long-range objectives. For microscopic investigations and imaging of the biofilm, we used an Axioplan Imaging 2 instrument equipped with an ApoTome slider module (Carl Zeiss Micro Imaging GmbH, Germany). The GFP-labelled microorganisms were detected at a wavelength of 525/50 nm (470/40 nm excitation). Images were taken from inlet to outlet in three parallel rows, with each row featuring at least thirty pictures. We then used Fiji (ImageJ, Particle Analysis) to calculate the biofilm covered surface [%].

Surfaces. During the preparation of anodized aluminium membranes and oxide films, the most important parameters affecting the anodic oxidation process and the product's properties are the applied anodization voltage (V_{ap}) and current, pH, the identity and concentration of the electrolyte, and the temperature (T). Choi and Kim (2006) have shown that depending on the conditions employed (V_{ap}, T) and the properties of the electrolyte solution, either a continuous "barrier-type" or a "porous-type" oxide film can be obtained, as shown in Fig. 2(a). For the former, neutral or basic electrolytes that produce negligible oxide dissolution should be used

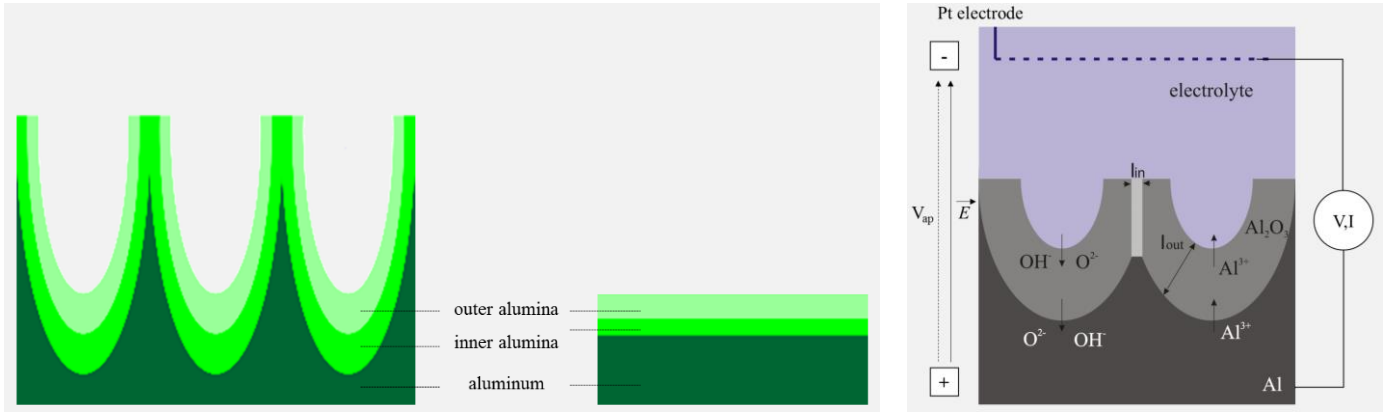

Fig. 2 (a) The structures of barrier-type (left) and porous-type (right) aluminium oxide films. (b) Electrochemical processes occurring at the anode: at the oxide/electrolyte interface, Al^{3+} ions enter into solution, while the oxide layer grows at the metal/oxide interface.

whereas acidic electrolytes are typically required for the latter (Wehrspohn, 2005). Overall, the growth of AAO films relies on a balance between electrical-field-driven oxide formation at the metal/oxide interface and oxide dissolution at the electrolyte/oxide interface, as shown in Fig. 2(b).

The FlexiPor AAO membranes used in our experiments were prepared by a two-step anodization process as described previously (Liu, et al., 2006). Briefly, high purity aluminium sheets (99.999 %) were first electropolished in a mixture of $HClO_4$ and C_2H_5OH (1:4 v/v) for 5-10 min. The polished Al sheets were then anodized in 1 wt% H_3PO_4 at 145 V (FlexiPor) or 195 V, or in 0.3 M $H_2C_2O_4$ at 50 V (SmartPor) at 3 °C. The first anodizations were usually performed over 20 h, but those conducted at 145 V lasted for only 4 hours. The anodized Al sheets were then immersed in an acid mixture (6 wt% H_3PO_4 and 1.8 wt% CrO_3) to completely remove the porous layer before being subjected to a second anodization that lasted for 30-50 h in the case of sheets initially anodized at 145 V, or for 16 h under the conditions used during the first anodizations in the case of sheets initially anodized at 195 V or 50 V. Free-standing aluminium membranes were obtained by etching away the underlying aluminium substrates with a mixture of $CuCl_2$ and HCl. The barrier layers were then removed by treatment with 5 wt% H_3PO_4 at 30 °C for 220 min for H_3PO_4-anodized AAO, or with 5 wt% H_3PO_4 at 30 °C for 47 min for $H_2C_2O_4$-anodized AAO. For aluminium membranes etched at 145 V, the underlying aluminium layer was separated using a special thinning process involving stepwise voltage reduction rather than by chemical treatment.

Contact angle measurements were performed using the sessile drop method (static θ_{stat}) with the Dataphysics Contact Angle System OCA 15 Plus. The analyte was degassed MiliQ-Water with a droplet size of 1.5 µL.

RESULTS

An effective porous aluminium etching process was established, making it possible to create aluminium membranes with a range of defined surface topographies including "over etched" surfaces with needle-like protrusions. A controlled wet chemical widening process based on treatment with 5 wt% H_3PO_4 at room temperature was used to increase the pore size of the aluminium membranes (and thus the porosity of the resulting structure). The degree of widening was tuned by varying the treatment time between 1 and 5 hours; treatments longer than 5 hours caused complete dissolution of the pore walls and structural collapse.

A three-hour etching period produced a structure with pores having a mean diameter of 230 nm; increasing the etching period to 4 h increased the mean pore diameter to 300 nm (Fig. 3).

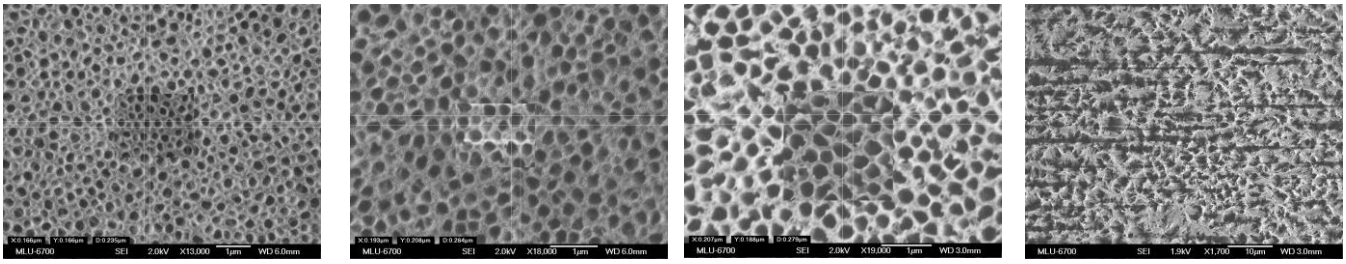

Fig. 3 Different stages of the pore widening process. Images show a FlexiPor AAO membranes after immersion for different lengths of time in 5 wt% H_3PO_4 at room temperature.

DISCUSSION

Aluminium oxide has a high energy surface and is therefore hydrophilic (Enke, et al., 2010). This was confirmed by our experiments with FlexiPor AAOs, which exhibited θ_{stat} values in the range of 25°; the corresponding value for electropolished 1.4301 stainless steel was around 80° (data not shown). Our results thus suggest that the greater the wettability of the surface, the better it is at preventing the attachment and retention of *E. coli*. This consistent with the findings of Gomes, et al. (2015), and should be borne in mind when designing technical equipment for use in areas where water films are present.

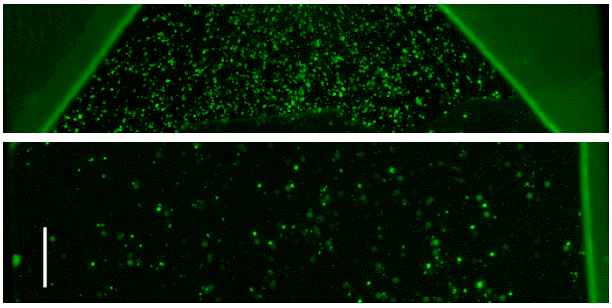

Fig. 4 Fluorescence images showing *E. coli* SM2029 biofilms after 16.5 h of dynamic cultivation in the flow chamber system. The upper image shows microcolonies on electropolished 1.4301 stainless steel; the lower shows colonies on the FlexiPor AAO. (Bar length: 1 µm).

Our results also show that the anti-adhesive properties of the FlexiPor AAO structures were superior to those of electropolished 1.4301 stainless steel (Fig. 4 and Fig. 5).

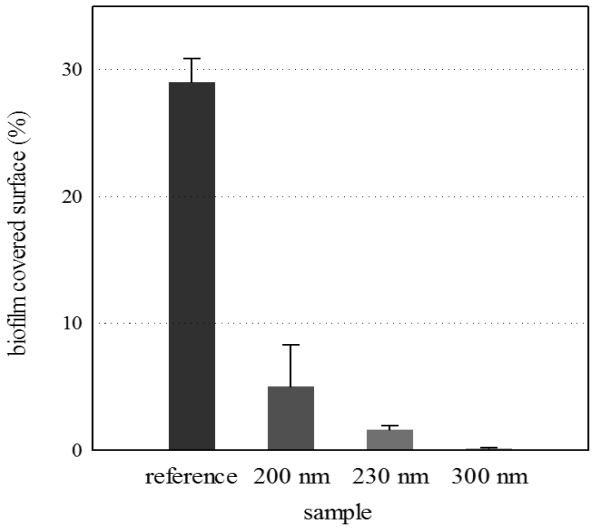

Fig. 5 Plot of the biofilm-covered surface area (%) after 16.5 h of dynamic *E. coli* cultivation on nanostructured FlexiPor AAOs as a function of pore size (nm). Electropolished 1.4301 stainless steel was chosen as a reference material because it is widely used in the construction of technical devices.

These preliminary findings suggest that surfaces that do not provide adjacent favourable positions for cells to settle, deter the adhesion of *E. coli*. This is consistent with the conclusions of Scardino, et al. (2006), although those authors investigated diatoms rather than bacteria. Similarly, Kargar, et al. (2014) investigated adhesion and colony formation by *Pseudomonas aeruginosa* on a solid coated with close-packed 630–1550 nm monodisperse spheres of polystyrene and concluded that the reason for the coated surface's inability to accommodate multiple bacterial cells in close proximity was the lack of adjacent favourable positions.

Given the limited available data, it is difficult to draw robust conclusions about the roles played by the needles that are observed on the surfaces of FlexiPor AAO structures having a mean pore diameter of 300 nm. In our experiments these nano-needles clearly hindered the attachment of bacteria but did not kill them as was reported by Ivanova, et al. (2012), who studied the anti-biofouling properties of cicada wings with nano-pillar structures separated by around 200 nm and found that these pillars penetrated and killed the bacterial cells.

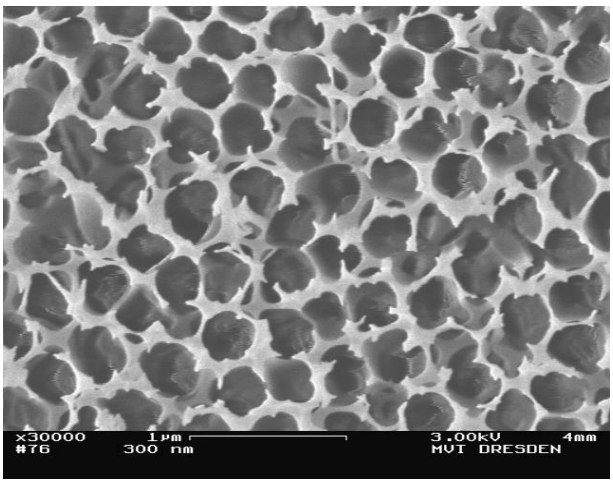

Fig. 6 Nano-needles on microstructured FlexiPor AAO (mean pore size 300 nm).

CONCLUSIONS

Anti-adhesive and therewith antimicrobial surfaces could potentially be very useful in preventing the biofouling of technical systems. We are interested in the initial stages of biofilm development, which create a foundation for the subsequent attachment of other substances and (micro)-organisms, leading to diverse problems in pieces of equipment such as heat exchangers.

Our findings provide further support for the hypothesis that nanostructures from nature can be used to guide and inspire the design of microstructures on technical surfaces that can prevent bacterial adhesion and thus the first stage of biofilm development. However, further studies will be required to fully understand the complex mechanisms that allow natural systems to prevent both macro- and micro-fouling, and to develop biomimetic or bioinspired strategies for recreating these properties on a technical scale.

OUTLOOK

We have conducted preliminary studies on the anti-adhesive properties of SmartPor surfaces, which feature pores with a diameter of around 200 nm and a pore distance of 350 nm (Fig. 7). This pore size and the surface's honeycomb-like topography makes the SmartPor surface similar to that of a springtail's cuticle.

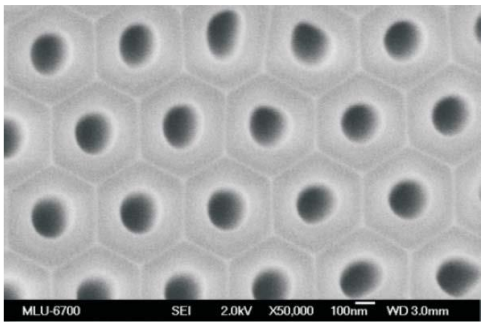

Fig. 7 SmartPor surface produced by electrochemical precision etching.

AKNOWLEGDEMENT

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NOMENCLATURE

T	temperature, °C
V	voltage, V
θ	angle, °

Subscript

ap	applied
stat	static

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