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ARTICLE TYPE

Bioinspired Passive Anti-biofouling Surfaces Preventing Biofilm Formation

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Biofilm formation enables bacteria to grow under unfavorable conditions, provides them with protection, and increases their resistance to antimicrobial agents. Once a biofilm has formed, it is difficult and in some systems impossible to treat. Strategies based on the release of biocidal agents have shown only transient efficiency. Here, we present a novel bioinspired passive approach to the prevention of surface biofilm attachment by exploiting superhydrophobic surfaces formed via the self-assembly of paraffin or fluorinated wax crystals. Our surfaces show exceptional ability to inhibit biofilm formation of both Gram-positive *Bacillus cereus* and Gram-negative *Pseudomonas aeruginosa* over a 7-day period (up to 99.9% inhibition).

Introduction

The vast majority of bacteria often grow as elaborate multicellular communities, referred to as biofilms. [1, 2] Biofilm formation represents one of the most successful strategies for survival of microorganisms in natural environments: it protects bacteria and facilitates their growth under unfavourable conditions such as turbulent flow or limited access to nutrients.[2, 3] Biofilm formation is a multistage process in which cells adhere to a surface by producing an extracellular matrix typically composed of exopolymeric substances such as polysaccharides, proteins and nucleic acids[4]. Which often surround and protect the bacteria.[5] Thus, biofilm bacteria are more resistant than planktonic cells to various antimicrobials.[6-8] Although antimicrobial agents have long provided the world with a safety net against bacterial infections, today it is clear that intensive usage of antimicrobial agents has a negative impact both on health and on the environment. Their excessive use has increased the resistance of many microbial species, and a likely mechanism underlying bacterial resistance was recently shown to be related to biofilm formation.[5, 9, 10]

Biofilms are problematic in a broad range of areas, particularly in the food, environmental and biomedical fields.[8] A considerable problem in the food industry is the formation of biofilms in dairy processing plants, particularly those formed by members of the *Bacillus* genus.[8, 11] *B. cereus* is a spore-forming pathogenic bacterium that causes two distinct types of food poisoning, the diarrheal and emetic syndromes, as well as a variety of local and systemic infections. [12] It has been shown to be capable of forming biofilms on stainless steel, plastic and glass wool.[13-16] *Pseudomonas* spp., considered to be one of the most important groups of bacteria in clinical as well as in industrial settings.[8, 17] Biofilms of *Bacillus* and *Pseudomonas* species are thus jointly regarded as the most significant microbiological problem in the food industry, because the damage they inflict on the quality and safety of food products may impact public health as well as the economy.[8] Their ubiquitous nature, combined with their ability to grow even at refrigerator temperatures, make them difficult to control.

Prevention of biofilm formation would clearly be a much more desirable option than treating it, and a wide range of bacteria-resistant surfaces has been proposed for this purpose. Examples are surfaces modified with nanoparticles (ZnO, TiO2 and carbon nanotubes) that mechanically damage the bacterial cells.[18] However, most fabrication methods rely on one of two main strategies. The first is based on the release of biocidal compounds such as silver or copper ions, various antibiotics, chlorohexidine,

or quaternary ammonium salts.[18-20] The second strategy depends on inhibition of adhesion, and various methods have been suggested for this purpose: using hydrophilic surfaces to create fully hydrated surfaces, the best-known example being polyethylene glycol (PEG) polymers.[18, 21, 22] Other methods in this category make use of zwitterionic materials,[23] novel liquid-infused structured surfaces,[24] amphiphilic block copolymer surfaces,[25] and smart stimuli-responsive materials designed as fouling-release surfaces.[18] Both strategies, however, have drawbacks. Biocidal-releasing compounds usually have only short-term efficiency because of the limited amount of the biocidal compound or the resistance to the compound developed by the bacteria.[3, 26] PEG coatings undergo oxidation damage that leads to loss of efficiency.[27] Metallic nanoparticles might have harmful effects on human tissue[28]. Therefore, in most industrial and medical applications the main strategy for preventing biofilm formation currently still relies on regular and aggressive cleaning and disinfection of bacterial contact surfaces.[8]

Previous reports have described the antifouling properties of natural surfaces such as taro leaves[29] and cicada wings,[30] and of bioinspired surfaces such as slippery surfaces inspired by the Nepenthes pitcher plant.[24] In addition, several superhydrophobic surfaces have demonstrated antibacterial properties.[31-33] Based on those reports, and on reports of the impact of micropatterning[34] and nanotopography[35-37] on bacterial adhesion, we present our findings on biomimetic superhydrophobic surfaces that are formed via the self-assembly of paraffin or fluorinated wax crystals and prevent bacterial attachment and biofilm formation on different substrates.

We examined several waxes (both paraffin and fluorinated), and found that all of them formed 3D crystalline structures on a variety of substrates and thus present superhydrophobic properties (contact angle >150˚ and contact angle hysteresis <10˚) regardless of the underlying substrate type. Earlier studies by our group showed that hierarchical structures ($C_{36}H_{74}$+$C_{50}H_{102}$ and $C_{24}F_{50}$) have extremely low contact angle hysteresis and are able to support small drops,[38, 39] suggesting that they might be beneficial in a submerged environment and under flow conditions.

Here, we demonstrate that our modified surfaces passively (with no toxic influence on bacterial cells) almost completely prevent the formation of biofilms (reduction of 95.6-99.9% over a 7 d period) by two different pathogenic bacteria, *B. cereus* (Gram positive) and *P. aeruginosa* (Gram negative), both considered to be extremely problematic bacteria in clinical as well as in industrial settings. These results exceed resent developments reported on other state-of-the-art antibacterial surfaces (in comparison: 93% reduction of *P. aeruginosa* coverage over 3d period,[25] 90% reduction of *P. aeruginosa* coverage[30], and 98% reduction of *P. aeruginosa* coverage over 7 d period under flow conditions[26]). Moreover, such wax surfaces can be formed on a great variety of materials and intricately shaped surfaces, making the technology potentially feasible for various medical and industrial applications.

Experimental Section

Strains and growth media: *Bacillus cereus* strain ATCC 10987 was obtained from the laboratory collection of Michel Gohar (INRA, France). *Pseudomonas aeruginosa* PA14 was obtained from the laboratory collection of Shlomo Sela (Agricultural Research Organization, Israel). For routine growth all strains were propagated in Lysogeny broth (LB; 10 g tryptone, 5 g yeast extract, 5 g NaCl per liter) or on solid LB medium supplemented with 1.5% agar. For biofilm generation using optimal growth conditions for tested strains, bacteria were grown to the stationary phase in LB liquid medium at 37 °C in shaking culture. The generated cultures were seeded (1:100 dilution) into sterile polystyrene multidishes containing different substrates and were inoculated statically (without agitation) into fresh media (95% air / 5% CO_2 (v/v)). Biofilms of *B. cereus* ATCC 10987 were generated at 30 °C in LB liquid medium for either 24 h or 7 days. Biofilms of *P. aeruginosa* were grown in the same medium for either 24 h at 37 °C or 7 days at 30 °C. The medium was renewed every 2 days during the long-term biofilm experiment.**Development of technology for surface modification:** Surfaces were prepared by thermal deposition of n-paraffin and fluorinated waxes on glass, polystyrene and steel substrates using a Moorfield MiniLab evaporator. The waxes hexatriacontane ($C_{36}H_{74}$, 98%), pentacontane ($C_{50}H_{102}$, ≥97%) and perfluorotetracosane ($C_{24}F_{50}$) were purchased from Sigma-Aldrich (France). The deposition procedure was conducted in a vacuum chamber at 5×10^{-6} mbar. Samples were positioned on a holder 12 cm above a crucible loaded with 40−50 mg of an n-paraffin wax or 110 mg of perfluorotetracosane. The system was slowly heated from 70 °C to 120 °C. Evaporation occurred at 120 ± 5 °C within 10-15 min. Evaporated specimens were transferred to room temperature (25 °C). Hierarchical structures were prepared by thermal evaporation of a $C_{50}H_{102}$ wax layer on top of a previously evaporated $C_{36}H_{74}$ wax. Surface wetting properties were assessed by measuring contact angles with an Attension Theta tensiometer. Measurements were performed with 7 µL of high purity water (milli Q). Contact angle hysteresis was measured with alternating drops of 7 ± 13 µL. Surface imaging was performed by high-resolution scanning electron microscopy (Zeiss Ultra Plus HR-SEM). Roughness was assessed by confocal microscopy (Leica DCM 3D).

Quantification of live bacteria on the coated substrates: The above mentioned bacterial strains were grown in shaking cultures for 6 h at 37 °C in LB liquid medium. Dense cultures (10 µL) were then seeded onto 12-well plates with stainless steel, glass or polystyrene surfaces (1cm × 1 cm) and incubated at room temperature (RT) for 30 min to allow initial adhesion. Thereafter, LB media (2 mL) was added to each well and the plates were incubated at 30 °C for 24 h. The supernatant was then removed and a sterile swab was used to pick up the biofilms attached to the surfaces. The swabs were inserted into 15-mL tubes containing 2 mL of PBS and shaken for 20 min at RT. Each sample was decimally diluted and plated out on LB agar by the pour plate method. Plate counts (CFU/ml) were carried out after 24 h of incubation at 37 °C.

Confocal laser scanning microscopy: To visualize the constructed biofilms the substrates were removed from the wells, washed with PBS buffer, and stained by using a FilmTracerTM LIVE/DEAD Biofilm Viability Kit (Molecular Probes) according to the manufacturer's protocol. Stained samples were visualized

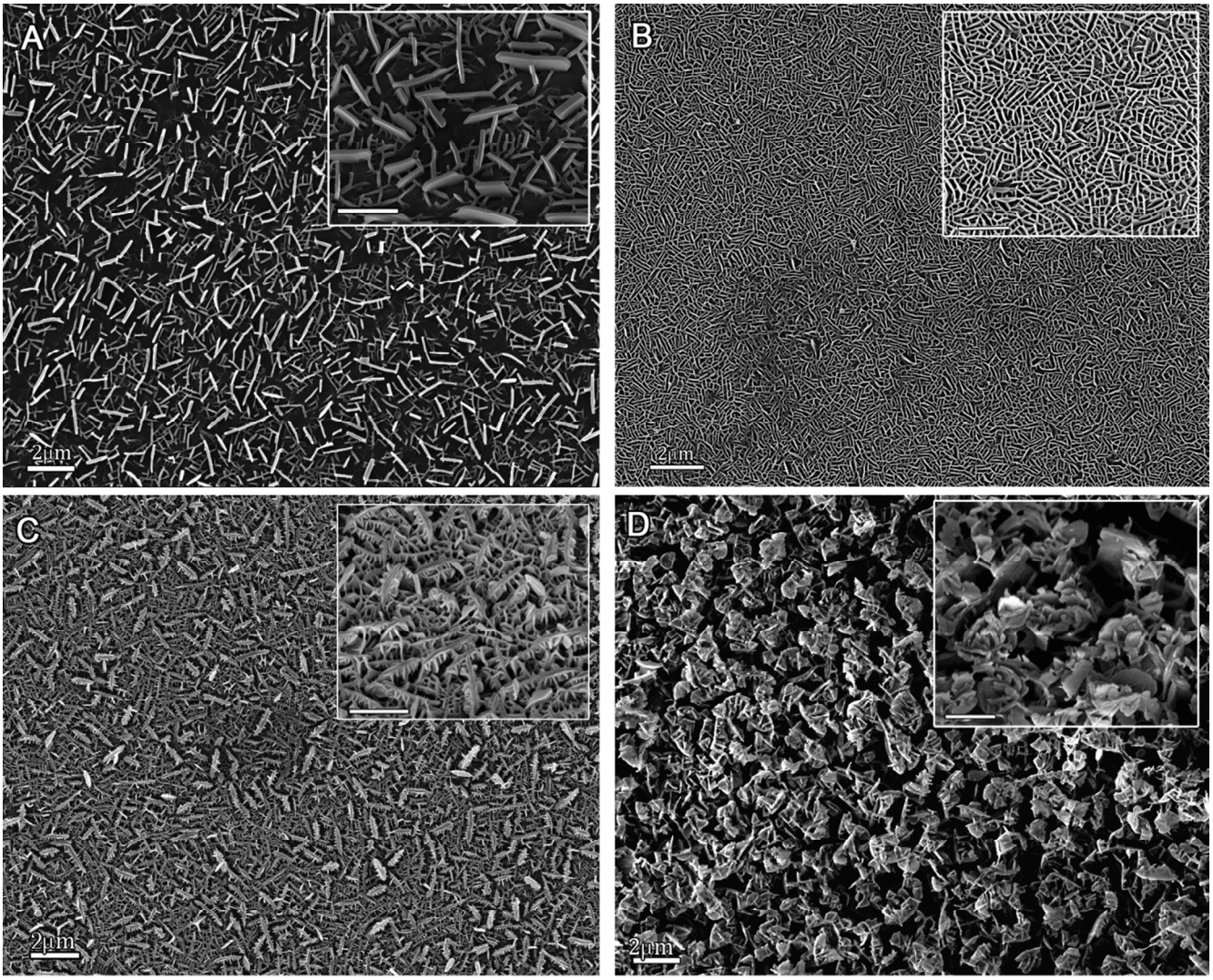

Fig. 1. HR-SEM micrographs of 3D crystalline structures thermally evaporated on glass substrates (A) $C_{36}H_{74}$ (B) $C_{36}H_{74}$+$C_{50}H_{102}$, (C) $C_{50}H_{102}$ and (D) $C_{24}F_{50/}$ Insets were acquired at a 30° tilt angle. Scale bar is 1μm

under an Olympus IX81 confocal laser scanning microscope (CLSM, Japan). Live cells were stained green and dead cells were stained red. Fluorescence emission of the stained samples was measured using an Olympus IX81 CLSM equipped with 488-nm argon-ion and 543-nm helium-neon lasers.

Bacterial growth analysis: Shaking cultures of *B. cereus* and *P. aeruginosa* were grown in LB in the presence of 10 mg/ml of $C_{24}F_{50}$, $C_{36}H_{74}$, $C_{50}H_{102}$ or $C_{36}H_{74}$+$C_{50}H_{102}$ paraffin powders. Control cultures without the added powders were also prepared. Samples were incubated in an orbital shaker at 37 °C / 150 rpm. Optical density at 550 nm was measured hourly for 20 h.

Results

The generated surfaces affect biofilm formation

To examine the effects of surface modification with hydrophobic wax on biofilm formation we analyzed two pathogenic bacteria, *B. cereus* (Gram positive) and *P. aeruginosa* (Gram negative), for their ability to form biofilms on stainless steel, glass and polystyrene. Each substrate was separately coated with each of the following waxes: $C_{24}F_{50}$, $C_{36}H_{74}$, $C_{50}H_{102}$, and $C_{36}H_{74}$+$C_{50}H_{102}$. Each wax formed a 3D crystalline structure with crystals of different sizes (as a function of the molecular weight), where both $C_{24}F_{50}$ and $C_{36}H_{74}$+$C_{50}H_{102}$ formed two-tiered hierarchical crystalline structures (**Table S1** and **Figure 1**).

All examined surfaces demonstrated superhydrophobic behaviour (contact angel >150° contact angel hysteresis lower than 10°), the lowest contact angel was observed for $C_{50}H_{102}$ surfaces which exhibited the lowest surfaces roughness. The hierarchical surfaces demonstrated, in addition to extremely high water contact angles (C.A >170°) and low contact-angle hysteresis (**Table S2**), an ability to support water droplets of ~100 pl (**Figure S1**) indicating elevated stability of the superhydrophobic wetting state (Cassie wetting state[40]).[41]

Confocal scanning laser microscopy (CSLM) images showed that

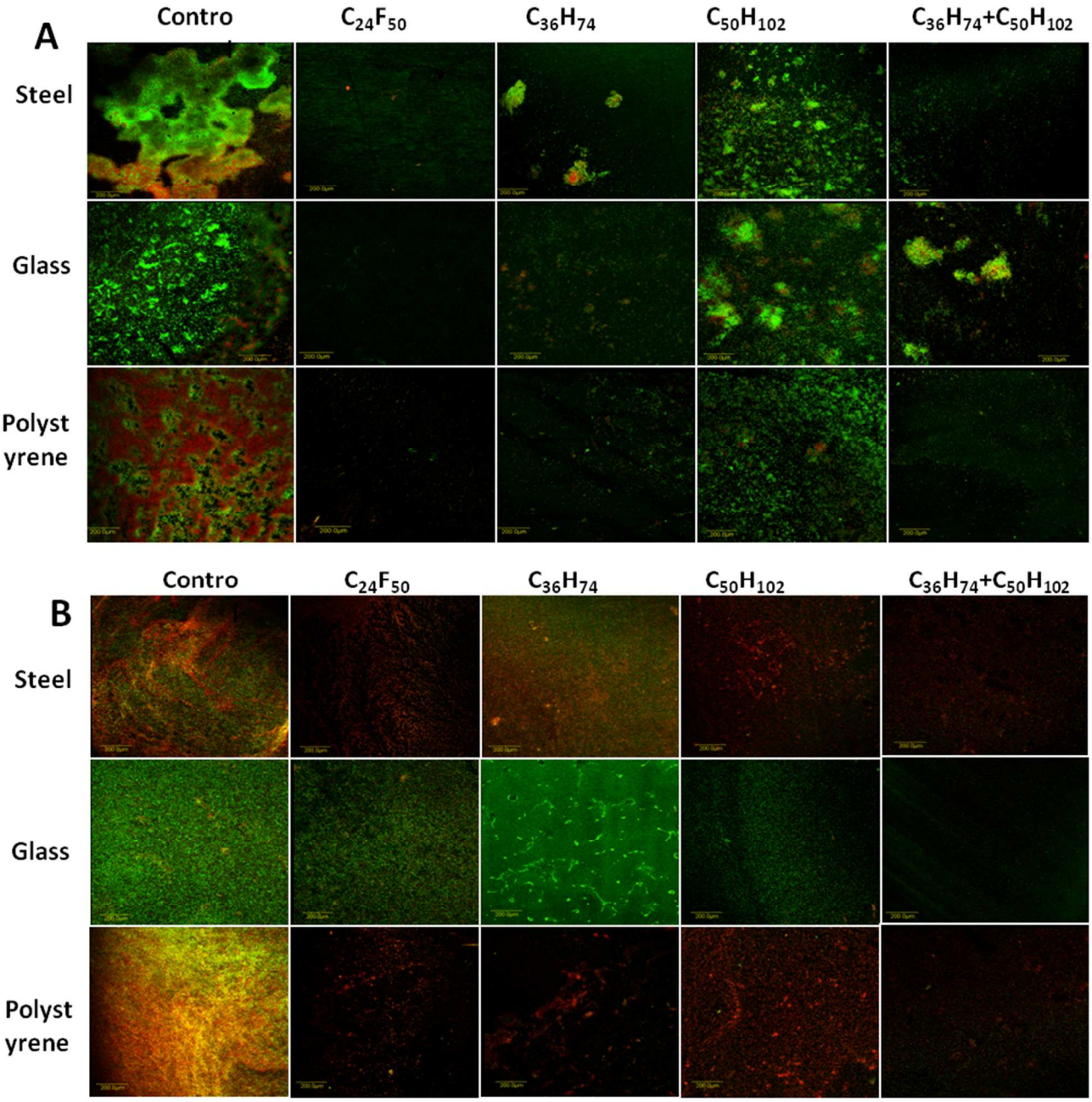

Fig.2. CSLM images of (A) *B. cereus* ATCC 10987 biofilms and (B) *P. aeruginosa* PA14 biofilms generated on $C_{24}F_{50}$, $C_{36}H_{74}$, $C_{50}H_{102}$ and $C_{36}H_{74}$+$C_{50}H_{102}$ wax surfaces thermally evaporated on steel, glass and polystyrene substrates

the cells of *B. cereus*, in comparison to their adhesion to control (non-coated) surfaces, could not adhere successfully to form confluent biofilms after 24 hours of incubation on steel, glass or polystyrene substrates coated with either $C_{24}F_{50}$ or $C_{36}H_{74}$+$C_{50}H_{102}$, while only slight inhibition was observed for $C_{36}H_{74}$ surfaces (**Figure 2A** and **Table S4A**). Over the same time span, biofilm formation by *P. aeruginosa* on substrates coated with $C_{36}H_{74}$+$C_{50}H_{102}$ was strongly prevented. However, other tested coatings, such as $C_{24}F_{50}$ and $C_{50}H_{102}$, did not significantly inhibit the formation of *P. aeruginosa* biofilms (**Figure 2B** and **Table S4B**).

To further support our results we used the plating method to quantify colony-forming units (CFU) of viable bacteria adhering

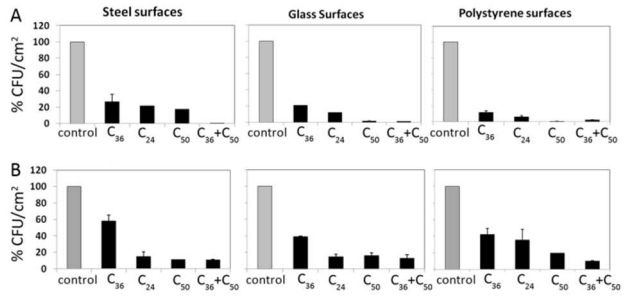

Fig. 3. Live bacterial counts on uncoated steel, glass or polystyrene surfaces (control) and on surfaces coated with $C_{24}F_{50}$, $C_{36}H_{74}$, $C_{50}H_{102}$ or $C_{36}H_{74}$+$C_{50}H_{102}$**.** Biofilms were grown in LB liquid media for 24 h (A) *B. cereus* biofilms, (B) *P. aeruginosa* biofilms.

to various wax surfaces formed on different substrates. Compared to uncoated control surfaces, reductions of 80% to 99.4% were observed in the numbers of *B. cereus* cells adhering to wax (either paraffin or fluorinated) surfaces formed on glass, stainless steel or polystyrene substrates (**Figure 3A**). Of all the surfaces examined, $C_{24}F_{50}$ and $C_{36}H_{74}$+$C_{50}H_{102}$ consistently demonstrated the most significant reductions in bacterial adhesion. The adhesion of *B. cereus* to glass, polystyrene and steel substrates coated with $C_{24}F_{50}$ was reduced by 98%, 97%, and 82%, respectively. Notably, all substrates coated with $C_{36}H_{74}$+$C_{50}H_{102}$ showed significant reductions (>97%) in *B. cereus* adhesion and biofilm formation. On examining the adhesion of *P. aeruginosa* to the wax surfaces, we observed reductions of 42% to 90% in the numbers of adherent cells compared to control surfaces (**Figure 3B**). Of all the examined surfaces, the $C_{36}H_{74}$+$C_{50}H_{102}$ hierarchical structure exhibited the largest reductions in adhesion of *P. aeruginosa* to glass, steel and polystyrene substrates (by 87%, 89% and 90%, respectively). The $C_{24}F_{50}$ hierarchical structure also effectively reduced *P. aeruginosa* adhesion and biofilm formation (**Figure 3B**). These results strongly indicate that the two-tiered paraffin $C_{36}H_{74}$+$C_{50}H_{102}$ surface provided the most effective reduction of both Gram-positive and Gram-negative bacteria adhesion and hence of biofilm formation on the different types of substrates.

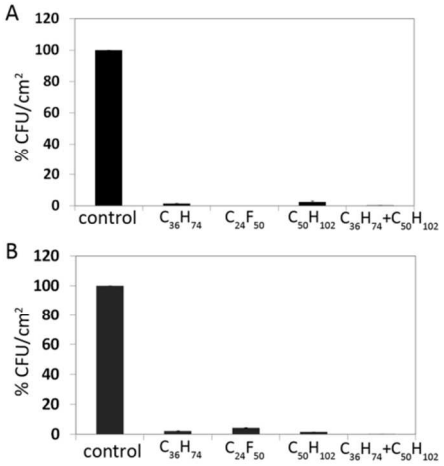

Fig 4. Live bacterial count on a steel substrate. A stainless steel substrate was left uncoated (control) or was coated with $C_{24}F_{50}$, $C_{36}H_{74}$, $C_{50}H_{102}$, or $C_{36}H_{74}$+$C_{50}H_{102}$. Biofilms were grown in LB liquid media for 7 days at 30 °C for (A) *B. cereus* or (B) *P. aeruginosa*.

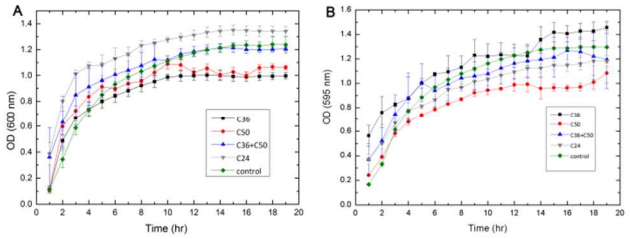

Fig 5. Effect of waxes on bacterial growth; Growth curves were analyzed in shaking cultures of (A) *B. cereus* and (B) *P. aeruginosa* in LB liquid medium at 37 °C in the presence of 10 mg wax powders used to formulate the described coatings ($C_{24}F_{50}$, $C_{36}H_{74}$, $C_{50}H_{102}$ and $C_{36}H_{74}$+$C_{50}H_{102}$).

The generated surfaces inhibit biofilm maturation

We examined the inhibitory capacity of the developed surfaces during long-term biofilm formation by *B. cereus* and by *P. aeruginosa*. All wax surfaces formed on steel substrate demonstrated strong inhibition of biofilm formation throughout the 7 days of incubation with each species (97.6-99.9% inhibition of *B. cereus* and 97.8-99.9% inhibition of *P. aeruginosa*, **Figure 4**). At the end of this incubation period there was impressive inhibition (almost 3-log) in *P. aeruginosa* biofilm formation by the $C_{36}H_{74}$+$C_{50}H_{102}$ structured surface (99.9%) and significant inhibition by both $C_{36}H_{74}$+$C_{50}H_{102}$ and $C_{24}F_{50}$ hierarchical structures of *B. cereus* biofilm formation (99.8% and 99.9% respectively). A fascinating finding was that the inhibition of mature (7-day) biofilm formation was more marked than that of young (24-hour) biofilm. This phenomenon might be explained by possible damage caused by waxed surfaces to the biofilm maturation process (a critical step during biofilm formation), with consequent inability of the bacteria to aggregate to form a confluent and mature biofilm, even if some initial adhesion (as seen after 24 h of incubation) has occurred**.**

The generated antifouling surfaces are non-toxic to bacteria

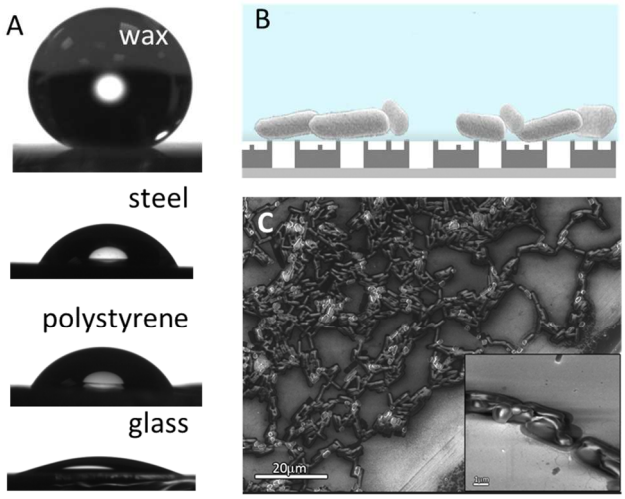

Fig .6. (A) optical images of a 7µL water droplet on various substrates, (B) a schematic describing the bacteria-surface interface on a superhydrophobic surface and (C) HRSEM micrograph of *B. cereus* on flat steel substrate.

To rule out killing effect as the cause of the dramatic inhibition of bacterial attachment that we observed on some wax surfaces, we tested the wax powders $C_{24}F_{50}$, $C_{36}H_{74}$, $C_{50}H_{102}$ and

$C_{36}H_{74}$+$C_{50}H_{102}$ for their effects on bacterial growth. Analysis of the growth curves shown in **Figure 5** yields no evidence of toxicity of the tested wax powders: bacterial growth in the presence of $C_{24}F_{50}$, $C_{36}H_{74}$, or $C_{50}H_{102}$ and $C_{36}H_{74}$+$C_{50}H_{102}$ powders was similar to that in control cultures with no added powders. It thus seems that the action of wax surfaces operates specifically through inhibition of bacterial attachment and biofilm formation, probably as an outcome of the superhydrophobic properties of the tested wax surfaces (**Figure 6**).

Discussion

It is becoming increasingly clear that most bacteria in their natural state exist as matrix-enclosed, surface-associated biofilms. In this mode of growth, the bacteria are largely protected from environmental insults as well as from various antimicrobial treatments. Since bacterial biofilms, once formed, are extremely resistant to antimicrobial treatments, the results of this study are of vast importance in the field of microbiology, as the antimicrobial coatings tested here can be used to modify any industrial or clinical surface to prevent bacterial colonization and biofilm formation.

Our approach incorporates an easily applicable coating technology based on thermal evaporation of waxes (paraffin or fluorinated), allowing the formation of superhydrophobic surfaces that prevent biofilm formation on stainless steel, polystyrene or glass substrates. These 3D crystalline wax surfaces form a Cassie wetting state (heterogeneous surface that combines wax and air pockets), reducing the contact area between a bacterium and the surface and thereby interrupting bacterial adhesion, thus preventing the initial step of biofilm formation. Our aim was to devise an approach that could be applied in substrates used in food and other industries for the development of novel surfaces that would prevent the adhesion of bacteria and consequently reduce biofilm formation. To test the efficacy of the generated surfaces, we examined the interactions of bacteria with wax-coated stainless steel, glass, and polystyrene substrates.

As shown in **Figures 2A** and **3A**, biofilm formation by *B. cereus* on $C_{24}F_{50}$-coated and $C_{36}H_{74}$+$C_{50}H_{102}$-coated surfaces of all examined substrates was significantly reduced. In the case of *P. aeruginosa*, notable reductions in biofilm formation were seen on $C_{24}F_{50}$, $C_{50}H_{102}$ and $C_{36}H_{74}$+$C_{50}H_{102}$ surfaces (**Figures 2B** and **3B**). A possible explanation might derive from differences in crystal size, density, and surface roughness. $C_{24}F_{50}$ and $C_{36}H_{74}$+$C_{50}H_{102}$ are hierarchical structures with high surface roughness (**Table S1**). Lower surface roughness results in reduced hydrophobicity (that is, lower contact angles and higher contact-angle hysteresis) and consequently reduced water repulsion. When calculating f_{SL} (the fraction of solid liquid interface,[40, 42] **Table S3**) the lowest values were received for the hierarchical structure $C_{36}H_{74}$+$C_{50}H_{102}$ (0.22) where the highest values received for $C_{36}H_{74}$ (0.6). Low f_{SL} value indicates a small contact bacteria-surface area compared with flat surface (**Figure 6A** and **B**) resulting with reduced bacterial adhesion. Furthermore, hierarchical structures are known for their higher stability of the Cassie wetting state, [41, 43] which might be beneficial for long-term antifouling capabilities.

The difference observed in the adhesion of *B. cereus* and *P. aeruginosa* $C_{36}H_{74}$ surfaces can be attributed to the experimental conditions: Biofilms of *B. cereus* were generated at 30 °C while *P. aeruginosa* biofilms were grown at 37 °C. We previously reported on temperature dependant growth of $C_{36}H_{74}$ crystals[44] resulting in length, width and height parameters and only minor change in the distance between adjacent crystals (**Figure S3**) leading to increased f_{SL} values (**Table S3**) with an overall outcome of easier bacterial attachment. Furthermore, the increase in crystal size leads to similarity to the bacterial size (**Figure 6C**) and thus to increased probability of bacterial retention and adhesion[34]. The overall reduction in bacterial adhesion was similar for both species on $C_{50}H_{102}$ surfaces (an average reduction of 13-20%).

Yet, another possible factor accounting for the observed the differences in biofilm formation between the tested surfaces has to do with differences in the adherence mechanisms of Gram-negative (*Pseudomonas*) and Gram-positive (*Bacillus*) bacteria. Studies have shown that adhesion and biofilm formation by *Bacillus* species depend on the creation of a conditioning film that adsorbs to the surface.[45, 46] In *Pseudomonas* species, on the other hand, adherence and biofilm formation occur through direct bacteria-surface interactions in the absence of conditioning film.[47] As seen in **Figures 2** and **3**, on most coated surfaces the cells of *B. cereus* formed poor biofilms compared to the biofilms of *P. aeruginosa*. Spores of *B. cereus* have been shown to adhere strongly to different surfaces owing to several characteristics, including high hydrophobicity and low surface charge. In addition, the spore morphology[48, 49] is typified by long appendages that cover the spore surface and in some cases promote adhesion. In some *B. cereus* strains, however, they reduce adhesion, owing to the formation of large spore clusters that can be easily removed from the surface.[50] *P. aeruginosa*, in contrast, is not a spore-forming bacterium, and this might serve as an advantage through allowing the adhesion and colonization of the coated surfaces to occur through pili and flagella.[47]

The exceptional ability of the $C_{36}H_{74}$+$C_{50}H_{102}$ and $C_{24}F_{50}$ hierarchical structures to resist attachment and biofilm maturation of the tested bacteria independently of any specific chemical or physical feature of the cells points to the potential feasibility of using this coating as a general antifouling material for resisting biofilms formed by a broad spectrum of bacteria. Given our experimental observations, it is conceivable that the bacteria have difficulty in developing a confluent biofilm efficiently since they are not able to be in contact with substrates coated with $C_{36}H_{74}$+$C_{50}H_{102}$ and $C_{24}F_{50}$. Importantly, the $C_{36}H_{74}$+$C_{50}H_{102}$ and $C_{24}F_{50}$ wax coatings were nontoxic to the tested bacteria; thus, the action of the $C_{36}H_{74}$+$C_{50}H_{102}$/ $C_{24}F_{50}$-coated surfaces is most probably attributable to biofilm formation. This is an important requirement for suppression of bacterial resistance to the antimicrobial treatment.

Conclusions

We have demonstrated a novel approach to the prevention of surface biofilm attachment using biomimetic superhydrophobic surfaces formed via the self-assembly of paraffin and fluorinated wax crystals. We found that hierarchical structures ($C_{36}H_{74}$+$C_{50}H_{102}$ and $C_{24}F_{50}$) possesses exceptional ability to inhibit biofilm formation of the Gram-positive *B. cereus* as well

as the Gram-negative *P. aeruginosa*, both of which are recognized as among the most problematic bacteria in industrial and clinical contexts. The surfaces have been proved effective over a 7 day period, competing with state-of-the-art antibacterial surfaces

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Notes and references

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† Electronic Supplementary Information (ESI) available: Supporting tables 1,2 and 3 and supporting figures 1-3 are available. This material is available free of charge via the Internet at http://pubs.acs.org
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Our bioinspired, superhydrophobic surfaces show exceptional ability to passively inhibit biofilm formation of Gram-positive and Gram-negative bacteria over a 7-day period.