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Hydrophobic Liquid-Infused Porous Polymer Surfaces for Antibacterial Applications

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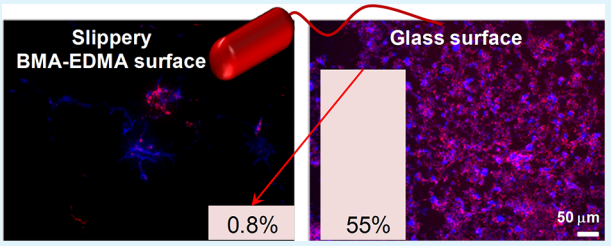
Supporting Information

ABSTRACT: Biofilms represent a fundamental problem in environmental biology, water technology, food hygiene as well as in medical and technical systems. Recently introduced slippery liquid-infused porous surface (SLIPS) showed great promise for preventing biofilm formation owing to the low surface energy of such surface in combination with its self-cleaning properties. In this study we demonstrated a novel hydrophobic liquid-infused porous poly(butyl methacrylate-co-ethylene dimethacrylate) surface (slippery BMA-EDMA) with bacteria-resistance in BM2 mineral medium and long-term stability in aqueous environments. We showed that the slippery BMA-EDMA surface prevents biofilm formation of different strains of opportunistic pathogen *Pseudomonas aeruginosa* for at least up to 7 days in low nutrient medium. Only ~1.8% of the slippery surface was covered by the environmental *P. aeruginosa* PA49 strain under investigation. In uncoated glass controls the coverage of surfaces reached ~55% under the same conditions. However, in high nutrient medium, more relevant to physiological conditions, the biofilm formation on the slippery surface turned out to be highly dependent on the bacterial strain. Although the slippery surface could prevent biofilm formation of most of the *P. aeruginosa* strains tested (~1% surface coverage), the multiresistant *P. aeruginosa* strain isolated from wastewater was able to cover up to 12% of the surface during 7 days of incubation. RAPD-PCR analysis of the used *P. aeruginosa* strains demonstrated their high genome variability, which might be responsible for their difference in biofilm formation on the slippery BMA-EDMA surface. The results show that although the slippery BMA-EDMA surface has a great potential against biofilm formation, the generality of its bacteria resistant properties is still to be improved.



KEYWORDS: *biofilm formation, Pseudomonas aeruginosa, strain specificity, SLIPS and porous polymer surface*

1. INTRODUCTION

A biofilm represents a sessile community of bacteria in which the microorganisms benefit from metabolic exchange, genetic flexibility, and protection.[1] The formation of biofilms of single bacterial species or mixed bacterial populations has been a natural evolutionary development with several selective advantages for the involved organisms.[2,3] One of such advantages is that bacteria are able to form biofilms in a wide variety of natural and anthropogenic environments and on all kinds of technical, medical or biological surfaces being in contact with water.[4,5] Thus, infections associated with biofilm growth on prostheses, catheters, and heart valves are an immense problem in medicine. In addition, biofilm resistance against antibiotics, disinfectants, and biocides is much stronger than that of planktonic bacteria, making biofilm removal more difficult. In technically used surfaces, the same resistance of mature biofilms against antimicrobial substances makes removal of biofilms, efficient disinfection, or long-term elimination of mature biofilms either impossible or very difficult.[3,5] This makes surface coatings capable of preventing formation of biofilms before their maturation one of the most promising solutions to the biofilm associated problems.

During the past decade, a lot of research on the development of coatings for preventing biofilm formation has been done.[6] Coatings "actively" releasing antibacterial compounds such as antibiotics,[7−11] silver nanoparticles or silver ions,[12−15] antibacterial antibodies,[16] or nitric oxide[17,18] have been reported. The primary advantage of active antibacterial surfaces is that delivery of antibacterial agents takes place directly from the surface. The disadvantage is that the release rate of agents is usually uncontrolled and decreases with time.[19] Another strategy for preventing biofilm formation is based on the bacterial resistant properties of either hydrophilic polymers such as poly(ethylene glycol),[20] poly(ethylene oxide) brushes,[21] and hydrophilic polyurethanes[22] or zwitterionic molecules.[23,24] Passive antibacterial surfaces based on toxic materials,[25−32]

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chitosan,[33] polyethylenimine derivatives[34] or antimicrobial peptides[35,36] and some other antimicrobial compounds[37] have been also reported. Surface physical cues, such as surface topology or elasticity (or combination of the physical properties with surface chemistry), have also been used to resist the biofilm formation.[38,39] Epstein et al.[38] showed that biofilm growth could be inhibited by controlling elasticity and topography of a surface. The major problem of both passive and active coatings is that adhered "conditioning" layer of proteins and dead bacteria[37] eventually leads to the loss of bacterial resistant properties making such coatings active for only limited period of time. Thus, to achieve long-term biofilm resistance, an easy detachment of the adhered conditioning layer is essential. These serious problems underline the strong need for more efficient antibacterial coatings that can both prevent initial bacterial adhesion and remove already adhered bacteria.

Recently, a slippery liquid-infused porous surface (SLIPS) was introduced by Aizenberg's group.[40] The SLIPS was prepared by infiltrating a porous fluorinated membrane with a fluorinated fluid and showed exceptional ability to prevent biofilm formation of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli* laboratory reference strains.[41] The antifouling property of SLIPS is thought to be caused by the very weak adhesion of bacteria to the fluid interface of the SLIPS and, hence, adhered bacteria can be easily removed from the surface even under weak shear forces. The authors concluded that the developed SLIPS exhibited a universal antibacterial behavior against diverse types of biofilm forming bacteria. In another report, we proved that the SLIPS showed long-term repellency against the eukaryotic cells.[42]

Here, we present a novel slippery surface based on macroporous poly(butyl methacrylate-*co*-ethylene dimethacrylate) (BMA-EDMA) films[43,44] infused with the perfluoropolyether (PFPE) fluid—slippery BMA-EDMA. The porous BMA-EDMA substrates are cheap and can be prepared in-situ by free-radical polymerization on different substrates. In addition, the morphology, thickness as well as surface chemistry of the porous BMA-EDMA surfaces can be easily tuned,[44] which makes them a suitable candidate for investigating properties of SLIPS made using such porous films. The three-dimensional morphology and stability of the surface under aqueous milieu were characterized by the water contact angle and X-ray phase contrast tomography techniques. Biofilm formation on the slippery BMA-EDMA surface was investigated using different environmental and laboratory reference *P. aeruginosa* strains. Consistent with the remarkable adaptability that allows *P. aeruginosa* to be both a ubiquitous environmental organism and an opportunistic pathogen, the *P. aeruginosa* genome is large and complex. Specifically, the *P. aeruginosa* genome contains a disproportionally large number of genes to encode factors involved in adhesion, motility, antibiotic efflux, virulence factors, and environmental two-component systems.[45] Consistent with this variability, here we demonstrate that the biofilm formation on the novel slippery BMA-EDMA surface is highly strain specific. Furthermore, toxicity assay was performed to show the bacterial response to the coating reagents. A molecular biology fingerprint method was used to discuss possible physiological differences of the strains under investigation.

2. EXPERIMENTAL SECTION

Materials and Methods. Preparation of the Slippery BMA-EDMA Surfaces. Porous BMA-EDMA polymer surfaces were prepared on glass substrate (Nexterion glass B, Schott, Mainz, Germany) by UV initiated polymerization of the polymerization mixture which contains 20% (w/v) of butyl methacrylate (BMA) (Sigma-Aldrich, Munich, Germany), 30% (w/v) of ethylene dimethacrylate (EDMA) (Sigma-Aldrich, Munich, Germany), 50% (w/v) of 1-decanol (Sigma-Aldrich, Munich, Germany), and 1% (w/v) (with respect to monomers) of 2,2-dimethoxy-2-phenylacetophenone (DMPAP) (Sigma-Aldrich, Munich, Germany), as described previously.[43,44] After washing the surface with methanol and drying with nitrogen gun, excess amount of PFPE liquid (Dupont Krytox® GPL 103, H Costenoble GmbH & Co. KG, Eschborn, Germany) was applied on the surfaces. The surfaces were tilted at an angle of ~20° for approximately 2 h to let excess of the PFPE fluid flow off the surfaces.

Scanning Electron Microscopy (SEM) Measurements of the BMA-EDMA Surface. The SEM measurements were performed using LEO 1530 Gemini scanning electron microscope (Zeiss, Oberkochen, Germany). The accelerating voltage was 2 KV for the measurement. The samples were sputtered with a 30 nm thick gold layer using a Cressington 108 auto sputter coater before the SEM analysis.

Imaging of the Slippery BMA-EDMA Surfaces Using X-ray Tomography. X-ray phase contrast tomography was performed at the nano-imaging station ID22 of European Synchrotron Radiation Facility in Grenoble (ESRF). The X-ray beam was monochromatized and focused using X-ray reflective optics to a spot size of 50 × 50 nm^2. The pink beam with an energy of 29.6 keV was used for the imaging. The sample was placed behind the focal spot and imaged onto a scintillator screen and a CCD camera a fixed distance downstream of the focal spot. Phase contrast images were obtained and input to a tomographic reconstruction algorithm based on filtered back projection. Slippery BMA-EDMA surface was prepared on 1 mm thick PMMA substrates. To prevent the evaporation of the PFPE liquid layer caused by the heat generated during the measurement, the slippery BMA-EDMA surface was fixed vertically in water in a closed PMMA vial (inner diameter 5 mm, Eppendorf, Germany) during the test. The thickness of the lubricant layer on the slippery BMA-EDMA surface was measured. We first measured the thickness of the whole slippery BMA-EDMA surface using the reconstructed X-ray tomography images. After this, the thickness of the porous polymer part of the slippery BMA-EDMA surface was measured. The thickness of the lubricant layer was obtained by subtracting the thickness of the porous polymer part from the thickness of the whole slippery BMA-EDMA surface. The resulting value is an average of the results from 5 different measurements.

Stability Characterization of Slippery BMA-EDMA Surfaces via Water Contact Angle Measurements. Three microliters of deionized water were placed on the surfaces to measure the static water contact angles of the surfaces. Two microliters of deionized water were used to measure the dynamic water contact angle of the surfaces. A UK 1115 digital camera (EHD Imaging GmbH, Damme, Germany) was used to take images of water droplets for water contact angle measurements. ImageJ software with a Dropsnake plugin was used to measure the water contact angle.

Slippery BMA-EDMA and BMA-EDMA (non-infused) surfaces were incubated in Petri dishes with BM2 mineral medium or with high nutrient medium for 7 days. The static, advancing and receding water contact angles of these surfaces were measured before and after incubation. The water contact angle hysteresis reported here is the difference between the advancing water contact angle and receding water contact angle.

Bacteria and Growth Conditions. Clinical wastewater samples were taken from the sewers close to the surgery department and from the clinics' wastewater collection pipes in a German city. Conventional API 20NE (BioMérieux, Nürtingen, Germany) was used for taxonomic identification. The gentamicin (GM; 10 μg/disc), ciprofloxacin (CIP; 5 μg/disc), imipenem (IPM; 10 μg/disc), ceftazidime (CAZ; 30 μg/disc), amikacin (AN 30 μg/disc), azlocillin (AZ 75 μg/disc), and

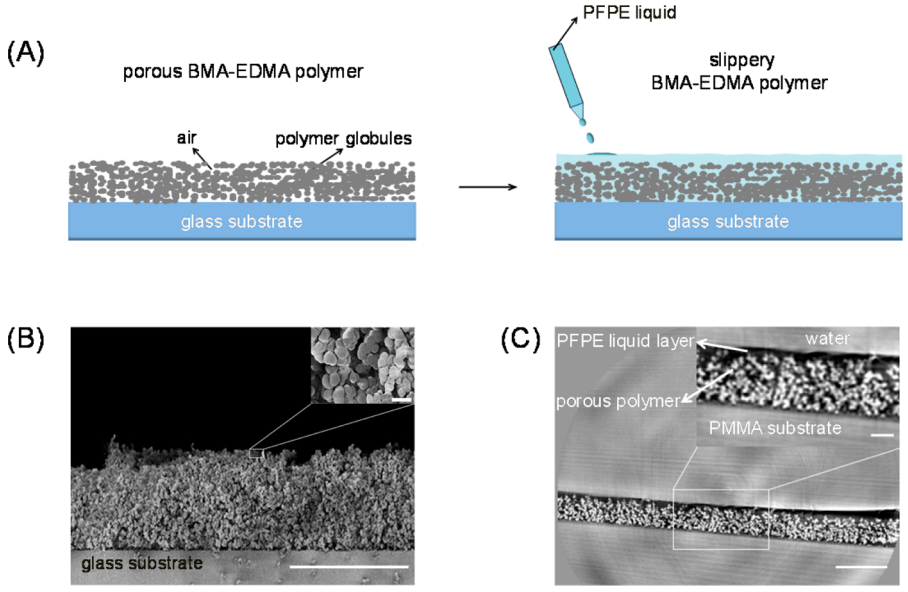

Figure 1. (A) Schematic representation of the fabrication of the slippery BMA-EDMA surface by infusion of the porous polymer with a perfluoropolyether fluid. (B) Cross section (scale bar 100 μm) and surface (inset, scale bar 2 μm) SEM micrographs showing the morphology of the porous structure of the BMA-EDMA surface. (C) Reconstructed X-ray propagation phase contrast tomography image showing the cross section of the slippery BMA-EDMA surface under water. The liquid PFPE layer is visible on the surface of the porous BMA-EDMA. Scale bar: 100 μm in the image and 20 μm in the inset.

piperacillin/tazobactam (PT 100/10 μg) resistances of the *P. aeruginosa* isolates were evaluated via antibiogram testing according to CLSI (Clinical and Laboratory Standards Institute, 2006; formerly NCCLS), wherein the zone of inhibition on MH agar (Merck, Darmstadt, Germany) was measured after 18 h incubation at 37 °C.

P. aeruginosa were grown in BM2 mineral medium consisting of 62 mM potassium phosphate buffer (pH 7.0), 7 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 10 μM $FeSO_4$, and 0.4% (w/v) glucose or 1:4 diluted high nutrient Brain Heart Infusion (BHI) broth (Merck, Darmstadt, Germany). Overnight cultures were used to start the biofilm reactor experiments to analyze the bacterial adhesion on slippery BMA-EDMA surfaces and the impact of PFPE liquid on the bacterial growth (toxicity test).

Bacterial Adhesion Assay. The slippery BMA-EDMA surfaces were incubated with bacterial suspension in an in-house constructed Plug Flow Reactor (PFR) (chamber dimensions length 29.0 cm, inner diameter 4.6 cm). Uncoated glass slides (Menzel-Gläser, Braunschweig, Germany) were used as reference for all experiments. The slides were thoroughly washed with ethanol and deionized water, and no further modification or treatments were done to the glass slides prior to the bacterial experiments. The biofilm reactor was inoculated with diluted bacterial suspensions (~10^8 CFU mL^{-1}). After a static 1 h inoculation period, a continuous flow rate of 0.94 mL min^{-1} was adjusted and kept for 7 or 14 days at room temperature (22 ± 2 °C).

Fluorescence Staining and Quantification of Bacterial Adhesion. Staining of viable bacteria was based on a standard assay based on intracellular enzymatic reduction of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC; Polysciences Europe GmbH, Eppelheim, Germany) to red fluorescent formazan crystals. For the staining of the total cell count, the DNA specific 4′,6-diamidino-2-phenylindol dihydrochloride (DAPI; AppliChem GmbH, Darmstadt, Germany) staining was applied.[46] For maximum detection of respiring bacteria, a CTC solution was freshly prepared by adding CTC to a final concentration of 3.8 mM. Samples were removed from the reactor and gently washed with sterile cell wash buffer (5 mM Magnesium acetate, 10 mM Tris, pH 8.0), followed by incubation in the CTC staining solution upon gentle shaking in darkness at room temperature (22 ± 2 °C) for 3 h. Subsequently, DAPI stain was added to the samples to a final concentration of 11.4 μM and incubated for 10 minutes. The test surfaces were washed again with sterile cell wash buffer. The biofilm formation was analyzed by epifluorescence microscopy with 200-fold magnification using Axioplan 2 imaging system (Carl Zeiss, Oberkochen, Germany) with the filters sets for CTC (BP 546/12, FT 580, LP 590) and DAPI (G 365, FT 395, BP 445/50). Digital images of each sample were obtained with a Zeiss AxioCAm MRm camera and the AxioVision 4.6 software. The surface coverage of the respiring bacteria (CTC stained, red) of five independent images of each sample was determined with the BioFlux 200 software (Version 2.3.0.2; Fluxion Biosciences/IUL Instruments GmbH, Königswinter, Germany).

Genotypic Characterization: Random Amplification of Polymorphic DNA (RAPD) - PCR Analysis. DNA was isolated from overnight cultures of the *P. aeruginosa* strains with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) was performed with the use of a Thermocycler GeneAmp PCR System 9700 (Applied Biosystems, Darmstadt, Germany) in 50 μL reaction volumes containing 40 ng of template DNA, 10x PCR-Puffer (PeqLab, Erlangen, Germany), 20 pmol of single 10-base primer, 1.25 units of Taq-DNA polymerase (peqGold Hot Taq DNA-Polymerase; PeqLab, Erlangen, Germany), 200 μM dNTPs and H_2O. The four 10-nt primers P15 (5′AATGGCGCAG3′), 1290 (5′GTGGATGCGA3′), 1254 (5′CCGCAGCCAA3′), and 1247 (5′AAGAGCCCGT3′) were used.[47,48] The cycling parameters were as follows: initial activation of the polymerase at 95 °C for 30 seconds; 45 cycles of 94 °C for 60 seconds, 33 °C to 72 °C for 3 minutes and 72 °C for 2 minutes; and 72 °C for 10 minutes final extension. The RAPD-PCR products were separated by electrophoresis through 4% polyacrylamide (PAGE) gels using the D-Code-System (Bio-Rad Laboratories GmbH, Munich, Germany). The gels were run at 120 V in 1x TBE buffer and stained with SYBR Gold (Invitrogen, Karlsruhe, Germany). The stained gels were analyzed at 520 nm wavelength using the LumiImager Working Station (Roche Diagnostics, Mannheim, Germany). Each isolate was scored for the presence or absence (1 or 0) of each band on the polyacrylamide gel. The index of similarity (F) was calculated according to the formula of Nei and Li[49] $F_{xy} = 2n_{xy}/(n_x + n_y)$, where n_{xy} is the number of RAPD bands shared by the two samples and n_x and n_y are the numbers of RAPD bands scored in each sample.

Toxicity Test of the PFPE Liquid. The impact of PFPE liquid on bacterial suspensions was analyzed in a microtiter plate. A 2-fold serial dilution of PFPE liquid with sterile Milli-Q water (final volume 50 μL) was prepared in triplicate and 50 μL bacterial suspension of *P. aeruginosa* strains PA14, PA30, PA910, or PA49 (~ 10^6 CFU mL^{-1})

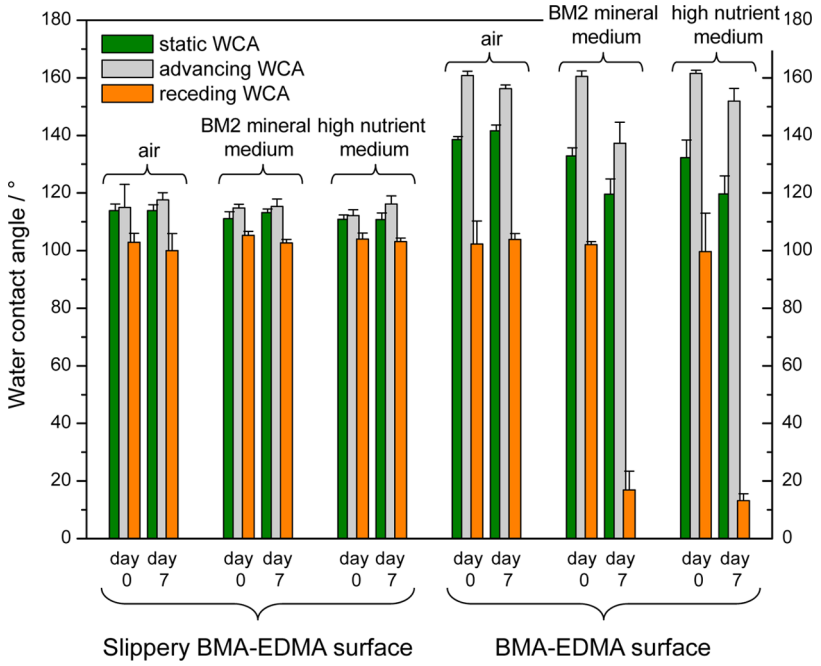

Figure 2. Water contact angles on the slippery BMA-EDMA surface and the non-infused porous BMA-EDMA surface before and after a 7 days incubation in BM2 mineral medium and in high nutrient medium. The reported water contact angle values are the average of the results from 4 individual measurement, and the error bars represent the standard deviations. According to the Student's t-test (two-sample assuming equal variances, $\alpha = 0.05$), the water contact angles (static, advancing, and receding) of the BMA-EDMA surfaces changed significantly ($P < 0.05$) after 7 days incubation either in BM2 mineral medium or in high nutrient medium.

were added to each well. The microtiter plate was incubated at 37 °C on a shaker at 100 rpm. To obtain homogeneous emulsions the PFPE concentration was limited to approximately 12.5%. After 1, 4, 8, and 22 h of incubation, aliquots of each bacterial suspension were removed and plated on MH agar (Merck, Darmstadt, Germany) by pour plate method. Plate counts (CFU mL^{-1}) were carried out after 18 h of incubation. Further characterization of the impact of PFPE liquid on bacteria was evaluated using the broth microdilution MIC (minimal inhibitory concentration) test according to CLSI (Clinical and Laboratory Standard Institute).

3. RESULTS AND DISCUSSION

Preparation and Imaging of the Slippery BMA-EDMA Surface. The slippery BMA-EDMA surface was prepared by applying the PFPE liquid on the porous BMA-EDMA surface synthesized according to a previously described method.[43,44] The PFPE liquid spread instantly and formed a fluid layer on top of the porous polymer film (Figure 1A). The X-ray phase contrast tomography technique was for the first time used to investigate the morphology of the slippery BMA-EDMA surface under water. The reconstructed phase-contrast images (Figure 1C) clearly show that the PFPE liquid filled the pores of the BMA-EDMA surface and an additional PFPE liquid layer was stabilized on top of the rough BMA-EDMA surface. The average thickness of the PFPE liquid layer is ~4-5 μm, as observed from the reconstructed X-ray propagation phase contrast tomography images.

Stability of the Slippery BMA-EDMA Surface. The water contact angle (WCA) measurements of the slippery BMA-EDMA surface showed that static water contact angle decreased from ~133±3° on the BMA-EDMA surface to ~114±2° on the slippery BMA-EDMA surface. The WCA hysteresis at the same time dropped from ~58±4° on BMA-EDMA to only ~12±2° on the slippery BMA-EDMA confirming formation of a PFPE superficial layer on top of the porous BMA-EDMA that was visualized by X-ray tomography. The WCAs also agree with the values obtained on other slippery surfaces where the same PFPE fluid was used.[40,41] To test the stability of the slippery surface under the experimental conditions, we evaluated the water contact angle (WCA) changes during the incubation of the surface in both BM2 mineral medium and in high nutrient medium (1:4 BHI broth). The stability of the slippery BMA-EDMA surface was compared to the non-infused BMA-EDMA polymer surface. The slippery BMA-EDMA surfaces were incubated in sterile BM2 mineral medium and in sterile high nutrient medium in Petri dishes for 7 days. The sterile incubation medium was replaced every day to avoid bacterial contamination and subsequent growth. The water contact angles of the surfaces were measured before and after incubation periods. Figure 2 shows that WCAs on the slippery BMA-EDMA surface remained unchanged after the incubation. However, the static WCAs of the porous BMA-EDMA surface (without PFPE liquid infusion) decreased from 133±3° to 120±5° and the WCA hysteresis increased greatly from 58±4° to 120±6° after 7 days incubation in BM2 mineral medium. BMA-EDMA surfaces incubated 7 days in high nutrient medium also showed similar changes. The WCA changes of the BMA-EDMA surfaces may be attributed to a partial hydrolysis of the ester bonds on the surface and deposition of components from the medium. The deposition of different inorganic materials as well as lipoproteins on the BMA-EDMA surface incubated in high nutrient medium was proved using XPS and Raman Spectroscopy (see Supporting Information, Figures S1 and S2). The XPS data also confirm at least partial hydrolysis of ester bonds on the sample treated with high nutrient medium (see Supporting Information). The absence of changes of WCAs on the slippery BMA-EDMA surface indicates that the infusion of the porous BMA-EDMA surfaces with the PFPE fluid

improves its long-term stability in aqueous conditions. Stability experiments performed under flow conditions showed similar results (Supporting Information, Figure S3).

Biofilm Formation of *P. aeruginosa* Strain PA49 on the Slippery BMA-EDMA Surface. The bacterial adhesion on the slippery BMA-EDMA surfaces was studied directly on the test surfaces located in a plug flow biofilm reactor, through which the bacterial suspensions were continuously perfused by a peristaltic pump. The overnight cultures were diluted with medium to approximately 10^8 CFU mL^{-1} and used to start biofilm cultivation. Under flow conditions of 0.94 mL min^{-1} volumetric flow rate the surfaces were tested for 7 or 14 days. To quantify the biofilm formation, the surfaces were analyzed by fluorescence microscopy with subsequent software mediated data analyses. To investigate the bacterial adhesion, three surfaces: (i) slippery BMA-EDMA surfaces, (ii) BMA-EDMA (non-infused) surfaces, and (iii) glass slides, were incubated with different *P. aeruginosa* cultures in BM2 mineral medium and in high nutrient medium (1:4 BHI broth).

The bacterial adhesion experiments were started with the environmental *P. aeruginosa* strain PA49 on the slippery BMA-EDMA surfaces and on glass slides under the mentioned low flow conditions. After 7 days exposure period in BM2 mineral medium, DAPI and CTC staining analyses were performed to evaluate the total cell counts and respiratory active cell counts, respectively. Only a few respiring bacteria had adhered on the slippery BMA-EDMA surfaces, while massive biofilm coverage was observed on the glass slides. In both cases, loosely attached bacteria were removed by washing with sterile medium to discriminate between the attached and the planktonic bacteria. The fluorescence images are shown in Figure 3. The slippery

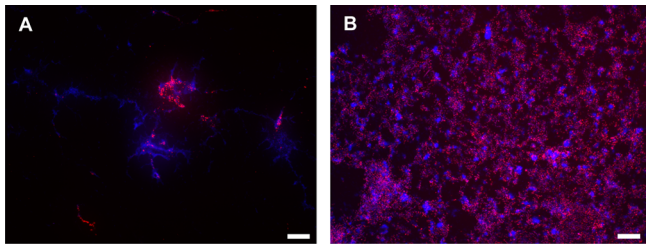

Figure 3. Fluorescence micrographs after 7 days surface exposure in BM2 mineral medium (flow rate: 0.94 mL min^{-1}) of *P. aeruginosa* strain PA49 stained with CTC (red) and DAPI (blue). (A) Slippery BMA-EDMA surface. (B) Glass slide. Scale bar 50 μm.

BMA-EDMA surfaces prevented up to 97.6% of *P. aeruginosa* PA49 biofilm formation compared to the glass slides over at least a 7 days period in BM2 mineral medium. The slippery BMA-EDMA surfaces demonstrated bacterial coverage of 1.8±0.8% of the area under investigation in three independent experiments. In contrast to these low coverage data, the glass slides exhibited suface coverages of 32.8±11.7% and a maximum value of 98.6±9.8% (Table 1A–C). Even a 14 days incubation period with an identical setup confirmed the previous results with a bacterial surface coverage of 8.2±4.8% on the slippery BMA-EDMA surfaces and 24.2±8.3% on the glass slides (Table 1D).

Biofilm Formation of Different Strains of *P. aeruginosa* on the Slippery BMA-EDMA Surface in High Nutrient Medium. To test the generality of the slippery BMA-EDMA surfaces, we studied the bacterial adhesion of different *P. aeruginosa* strains also in high nutrient medium (1:4 BHI broth)

Table 1. Comparison of the Bacterial Surface Coverage on the Slippery BMA-EDMA Surfaces and on Glass Slides with Environmental Wastewater *P. aeruginosa* Strain PA49 in BM2 Mineral Medium[a] for 7 Days (A–C) and 14 Days (D) Incubation Period[b]

	surface coverage in %	
	slippery BMA-EDMA surface	glass slide (control)
A	0.8 ± 0.5	33.0 ± 10.6
B	0.8 ± 0.3	32.6 ± 12.8
C	3.9 ± 1.6	98.6 ± 9.8
D	8.2 ± 4.8	24.2 ± 8.3

[a]Flow rate 0.94 mL min^{-1}. [b]Data from each independent experiment are listed.

with identical flow conditions. The bacterial adhesion of the laboratory reference strain *P. aeruginosa* UCBPP-PA14 (PA14) was compared to the environmental strains *P. aeruginosa* PA30, *P. aeruginosa* PA910, and *P. aeruginosa* PA49 (Table 2), previously isolated from wastewater effluents.[50]

Table 2. Comparison of the Bacterial Surface Coverage on the Slippery BMA-EDMA Surfaces, on the Glass Slides, and on the BMA-EDMA Polymer Surfaces of Different *P. aeruginosa* Strains[a] after 7 Days

	surface coverage in %		
bacterial type	slippery BMA-EDMA surface	glass slide (control)	BMA-EDMA surface
P. aeruginosa PA14	0.92 ± 0.33	2.14 ± 0.5	2.43 ± 0.89
P. aeruginosa PA30	1.95 ± 1.19	2.45 ± 0.16	86.27 ± 4.52
P. aeruginosa PA910	0.87 ± 0.25	2.28 ± 0.37	1.52 ± 0.15
P. aeruginosa PA49	11.96 ± 6.27	6.45 ± 1.87	36.34 ± 14.21

[a]1:4 BHI broth, flow rate 0.94 mL min^{-1}.

In accordance with experiments using BM2 mineral medium, only single cells or microcolonies of the reference strain *P. aeruginosa* PA14 were observed on the slippery BMA-EDMA surfaces when high nutrient medium (1:4 BHI broth) was used for the 7 days exposure period (Table 2). These results confirmed the observation from Epstein et al.[41] The *P. aeruginosa* environmental strains PA30 and PA910 showed similar results to the *P. aeruginosa* strain PA14 on the slippery BMA-EDMA surfaces. The multiresistant environmental *P. aeruginosa* strain PA49 exhibited, however, an increased colonization of the slippery BMA-EDMA surfaces: 11.96±6.27% (Table 2), which was about 13 times more than the coverage values measured for the reference strain *P. aeruginosa* PA14 and almost two times more than PA49 occupied the reference glass slide. These results clearly show that the biofilm formation of *P. aeruginosa* on the slippery BMA-EDMA surface was strain dependent. Much higher bacterial coverages were found on BMA-EDMA (non-infused) surfaces and glass slides (Table 2). We observed that the bacterial coverages on glass control surfaces were much lower in high nutrient medium compared with those in the low nutrient BM2 mineral medium (Table 1). We speculate that these differences in the glass controls might be because that the medium dependent surface conditioning changed the adhesion behavior of the *P. aeruginosa* strains under investigations. Therefore, it is more meaningful to compare separately the different *P. aeruginosa* strains and their biofilm formation on each surface to exclude media dependent side effects.

The good stability of the slippery BMA-EDMA surface in high nutrient medium (Figure 2) indicate that the effect of the culture medium on the PA49 biofilm formation on the slippery BMA-EDMA surface is not due to the instability of the slippery BMA-EDMA surface.

Toxicity Test of the PFPE Liquid. To demonstrate that the prevention of the biofilm adhesion on the slippery BMA-EDMA surface was not caused by toxicity of the PFPE liquid, the impact of PFPE liquid on the bacterial growth was tested. The toxicity and MIC tests with PFPE liquid showed that the PFPE liquid itself did not have an antimicrobial effect. In BM2 mineral medium with up to 12.5% PFPE liquid, the bacteria showed the same growth kinetics as in pure BM2 mineral medium. Up to 4 h no increase in CFU mL^{-1} was observed. After 22 h in all samples bacterial concentrations of $\sim 3.8 \times 10^8 \pm 8.3 \times 10^7$ CFU mL^{-1} were reached (Table 3).

Table 3. Growth of *P. aeruginosa* Strains PA14, PA30, PA910, and PA49 Was Monitored in BM2 mineral Medium with up to 12.5% PFPE Liquid[a]

	average CFU mL^{-1} (PA14, PA30, PA910, PA49)	
PFPE liquid	8 h incubation	22 h incubation
0.0 %	$6.4 \times 10^5 \pm 5.9 \times 10^5$	$3.2 \times 10^8 \pm 5.4 \times 10^7$
0.4 %	$5.7 \times 10^5 \pm 4.6 \times 10^5$	$2.7 \times 10^8 \pm 1.4 \times 10^8$
0.8 %	$3.8 \times 10^5 \pm 1.3 \times 10^5$	$5.2 \times 10^8 \pm 2.8 \times 10^7$
1.6 %	$7.1 \times 10^5 \pm 2.6 \times 10^5$	$4.6 \times 10^8 \pm 2.3 \times 10^8$
3.1 %	$5.9 \times 10^5 \pm 3.2 \times 10^5$	$3.8 \times 10^8 \pm 2.1 \times 10^8$
6.3 %	$6.8 \times 10^5 \pm 4.3 \times 10^5$	$3.0 \times 10^8 \pm 1.4 \times 10^8$
12.5 %	$5.5 \times 10^5 \pm 4.7 \times 10^5$	$3.9 \times 10^8 \pm 2.2 \times 10^7$

[a]The colony-forming units (CFU) per milliliter were determined after 1, 4, 8, and 22 h. No increase in CFU mL^{-1} were detected until 4 h of incubation. Standard deviations are given.

Clonal Diversity of the Environmental *P. aeruginosa* Strains. *P. aeruginosa* is known to exhibit a high flexibility in growth and adaptation to adverse conditions due their variable accessory genome, as mentioned before. Therefore, to investigate whether the difference in the observed clone behavior on the slippery BMA-EDMA surface is also reflected in the resistance to antibiotics, the *P. aeruginosa* clones were tested with several of the most commonly therapeutically used antibiotics. The antibiogram results verified the clonal diversity of the environmental isolates (Table 4). While the *P. aeruginosa* PA30 and PA14 strains were antibiotic susceptible against the mentioned antibiotics, the two other *P. aeruginosa* strains were multiple resistant, whereas PA49 exhibited the highest antibiotic resistance against clinically relevant antibiotics.

Table 4. Antibiogram of *P. aeruginosa* Strains Enriched from Wastewater Compartments of a German City[a]

bacterial type	GM	CIP	IPM	CAZ	AN	AZ	PT
P. aeruginosa PA14	S	S	S	S	S	S	S
P. aeruginosa PA30	S	S	S	S	S	S	S
P. aeruginosa PA910	R	R	R	R	S	R	I
P. aeruginosa PA49	R	R	R	R	R	R	R

[a]Agar diffusion testing for gentamicin (GM; 10 μg/disc), ciprofloxacin (CIP; 5 μg/disc), imipenem (IPM; 10 μg/disc), ceftazidime (CAZ; 30 μg/disc), amikacin (AN 30 μg/disc), azlocillin (AZ 75 μg/disc), and piperacillin/tazobactam (PT 100/10 μg) resistance; S: susceptible; R: resistant; I: intermediate.

Genotypic differences among the *P. aeruginosa* strains were demonstrated using RAPD-PCR to show their clonal diversity, too. Here, four different oligonucleotide primers (10mer) randomly matching within the genomes of *P. aeruginosa* strains were applied to generate polymorphic DNA fingerprint pattern of the different isolates. The RAPD-PCR DNA fragment pattern generated from the genomes of the *P. aeruginosa* strains PA14, PA30, PA910, and PA49 is shown in Figure 4.

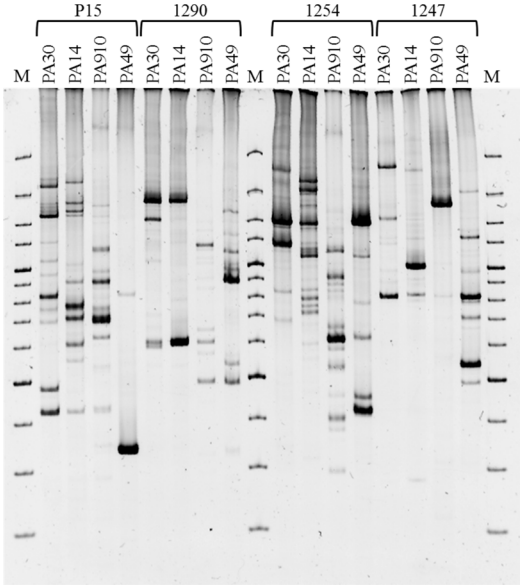

Figure 4. RAPD-PCR fingerprints of *P. aeruginosa* strains PA30, PA14, PA910, and PA49 for each primer P15, 1290, 1254, and 1247. M: molecular weight size marker (GeneRuler 100 bp Plus DNA Ladder, Thermo Scientific, Schwerte, Germany).

Differences could be observed in the number and size of randomly amplified DNA fragments for each primer among the four different *P. aeruginosa* strains. The similarities among the generated DNA pattern were calculated (Table 5) using the index of similarity, where 1 indicates a 100% and 0 no similarity between two samples. A high genomic diversity became obvious among the different *P. aeruginosa* strains exhibiting similarity indices of about 0.5 and lower. The multiresistant wastewater isolate *P. aeruginosa* PA49, which showed a quite different biofilm potential on slippery BMA-EDMA surfaces, especially under high nutrient conditions, showed a very low similarity to the PA14 laboratory reference, when primer P15 was used. Intermediate similarities were found when other primers were applied for RAPD-PCR.

Comparing genomic analyses revealed that the *P. aeruginosa* genome is a mosaic consisting of a relatively conserved core genome and a variable accessory genome. In contrast to the core genome, the accessory genome encompasses genes that are found in some *P. aeruginosa* strains but not others. Genetic elements within the accessory genome may encode properties that contribute to the niche-based adaptation of the particular strains that harbor them.[45] Regarding the presented results of our study, differences in adhesion properties among the used *P. aeruginosa* strains might result from the plasticity of the accessory genomes. In case of the environmental *P. aeruginosa* strain PA49, being a multiresistant wastewater isolate, a completely different response in biofilm formation on the slippery BMA-EDMA surfaces was found. In high nutrient

Table 5. Comparison of RAPD-PCR Fingerprints with the Index of Similarity[a]

	P15				1290				1254				1247			
	PA14	PA30	PA910	PA49	PA14	PA30	PA910	PA49	PA14	PA30	PA910	PA49	PA14	PA30	PA910	PA49
PA14	1.00				1.00				1.00				1.00			
PA30	0.48	1.00			0.33	1.00			0.40	1.00			0.46	1.00		
PA910	0.52	0.46	1.00		0.14	0.13	1.00		0.22	0.11	1.00		0.25	0.22	1.00	
PA49	0.00	0.12	0.13	1.00	0.15	0.27	0.35	1.00	0.2	0.17	0.15	1.00	0.25	0.24	0.17	1.00

[a]A calculated value of 1 represents a 100% similarity between two samples, whereas a value of 0 indicates no similarity between two samples on genomic level.

medium a biofilm development was not prevented by the specific surface modification. Therefore, we speculated that specific genes of the accessory genome might be induced by media components forming a strong conditioning film at the slippery interface making it attractive for *P. aeruginosa* PA49 colonization.

Effect of Components in the Bacterial Culture Medium on the Biofilm Formation of *P. aeruginosa* on the Surface. To analyze the effect of the components of the high nutrient medium on the bacterial adhesion, the slippery BMA-EDMA surfaces were preincubated for 48 h in high nutrient medium (1:4 BHI broth) and subsequently used for the bacterial adhesion assay for 7 days in BM2 mineral medium. The preincubated slippery BMA-EDMA surfaces were only slightly covered with respiring, CTC active bacteria. Based on these results, no differences of the bacterial surface coverage between the slippery BMA-EDMA and the preincubated slippery BMA-EDMA surfaces were observed. The preincubated slippery BMA-EDMA surfaces exhibited a biofilm coverage of 0.84±0.26% of the surface, the slippery BMA-EDMA surfaces without 48 h of preincubation showed a coverage of 1.3±0.18%, and the glass slides three fold higher coverage (6.6±1.38%). These results obtained no evidence for the formation of a conditioning film of high nutrient media components. Furthermore, it is already published that *P. aeruginosa* and next of kin species are able to secrete extracellular polymeric substances (EPS) forming a conditioning film at the surface making it attractive for colonization.[51] It has to be still analyzed if the EPS fraction of *P. aeruginosa* strain PA49 differs from the other strains under investigation.

4. CONCLUSION

In this study we demonstrated a novel hydrophobic liquid-infused porous polymer surface based on a monolithic poly(butyl methacrylate-co-ethylene dimethacrylate) (slippery BMA-EDMA surface) with slippery properties and long-term stability in aqueous environments. We have shown that the slippery BMA-EDMA surface prevents biofilm formation of different strains of opportunistic pathogen *P. aeruginosa* up to 7 days in low nutrient medium. In contrast, in high nutrient medium, the biofilm formation on the slippery BMA-EDMA surface was highly strain dependent. The slippery surfaces exhibited antifouling properties against laboratory reference clones of *P. aeruginosa* and most of the environmental *P. aeruginosa* strains studied. However, the antibiotic multiresistant *P. aeruginosa* isolate PA49 was able to form dense biofilms on the slippery BMA-EDMA surface in presence of high nutrient medium.

Although the results confirmed the superior antibacterial behavior of the slippery BMA-EDMA surface, they, at the same time, point to a limitation in applications of such surfaces. The difference in biofilm formation between the laboratory reference strains and the environmental *P. aeruginosa* strains was attributed to their specific genotypic background. Finally, our results emphasize the importance of comparing different bacterial strains (both wild type and laboratory strains) as well as different experimental conditions when evaluating antibacterial properties of novel surface coatings.

■ ASSOCIATED CONTENT

Supporting Information

Raman and XPS spectrum of the BMA-EDMA surface before and after 7 days incubation in BM2 mineral medium or in high nutrient medium. Water contact angles on the slippery BMA-EDMA surface and the non-infused porous BMA-EDMA surface before and after 7 days incubation in BM2 mineral medium flow condition. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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