Biointerphases (2012) 7:26
DOI 10.1007/s13758-012-0026-x

ARTICLE

Microfluidic Assay to Quantify the Adhesion of Marine Bacteria

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Received: 2 December 2011 / Accepted: 28 February 2012 / Published online: 21 March 2012
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Abstract For both, environmental and medical applications, the quantification of bacterial adhesion is of major importance to understand and support the development of new materials. For marine applications, the demand is driven by the quest for improved fouling-release coatings. To determine the attachment strength of bacteria to coatings, a microfluidic adhesion assay has been developed which allows probing at which critical wall shear stress bacteria are removed from the surface. Besides the experimental setup and the optimization of the assay, we measured adhesion of the marine bacterium *Cobetia marina* on a series of differently terminated self-assembled monolayers. The results showed that the adhesion strength of *C. marina* changes with surface chemistry. The difference in critical shear stress needed to remove bacteria can vary by more than one order of magnitude if a hydrophobic material is compared to an inert chemistry such as polyethylene glycol.

1 Introduction

Biofouling, the colonization of submerged artificial or natural surfaces by undesired biological organisms, is a major problem for many marine industries resulting in both, environmental and economic penalties [1, 2]. As application of biocidal antifouling (AF) paints is increasingly being restricted, fouling-release (FR) coatings are currently considered as alternative. Such non-toxic alternatives appear attractive, as they seem to reduce fuel consumption compared to conventional ablative AF coatings [3–5]. Bacteria are among the first microorganisms to colonize submersed interfaces to form biofilms [1]. Both, bacteria and microalgae produce extracellular polymeric substances (EPS), which contain polysaccharides, lipopolysaccharides, proteins and nucleic acids [6]. Such substances mediate the initial adhesion to surface and constitute the matrix of the biofilms [7]. In some cases, marine bacteria influence subsequent colonization by invertebrates, algae [8] and tubeworms [9–11]. Understanding bacterial adhesion and optimization of coatings so that they can easily be cleaned are important to improve commercial fouling-release technologies.

In the past different techniques were used to quantify adhesion of biological material to surfaces: Atomic force microscopy (AFM) [7], spinning disk [12], hydrodynamic shear force assays such as a water jet apparatus [13], flow channels [14–16] or microfluidic channels [17, 18]. Most of these techniques are conventionally applied as laboratory assays. In field experiments, water jetting and grooming tools are used as techniques to assess cleanability of fouling-release coatings in real, mixed species environments [19–21]. Especially for laboratory tests, microfluidic assays have a number of advantages: they allow quantifying adhesion strength on relatively small sample areas and require only small amounts of bacteria. Experiments mostly only take some hours and the experiment can easily be parallelized. The main advantage of a microfluidic assay lies in the fact that typically ca. 400 cells can

This article is based on a poster presented at the 58th Symposium of the American Vacuum Society.

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simultaneously be investigated and the variation of the shear force across several orders of magnitude allows to record detachment of each single cell [12, 18, 22]. In contrast to many other approaches this means that the cell density is accurately known from the beginning, and as the field of view remains unchanged, the same initial seeding density is valid for the entire experiment. We recently described a microfluidic device which allows the measurement of cell-surface interaction [22]. Cells can be incubated in the channel for several hours after which they are removed by a stepwise increased flow. Using self assembled monolayers with different abilities to bind water we were able to detect that subtle changes in hydration strongly influence the adhesion strength of fibroblasts [22]. Furthermore this new assay revealed that cell removal from directed nanostructures depends on the flow direction [23] and that CD44+ leukemic cells attach to hyaluronans by a catch bond activated binding [24].

In this work we apply the microfluidic shear force assay to quantify the adhesion strength of the marine bacterium *Cobetia marina* on chemically different model surfaces. This bacterium is used as a model system for marine biofouling because it is frequently found in biofilms and influences secondary colonization by invertebrates and algae [3]. To demonstrate the applicability of the microfluidic assay, we used self-assembled monolayers as well-defined model surfaces. Self-assembled monolayers [25, 26] are highly useful tools to reproducibly prepare coatings and frequently applied to study response of marine biofouling organisms [14, 16, 27–32]. One major advantage is that the mechanical properties are determined by the substrate while physicochemical properties, such as wetting and hydration are determined by the thin organic film. The accumulation of *C. marina* on chemically differently terminated self-assembled monolayers (SAMs) revealed that surface properties change the amount of accumulated biomass [14, 29]. In this article we describe the effect of undecanethiol SAMs with $–CH_3$, $–NH_2$, $–OC_7F_{10}CF_3$ termination and polyethylene glycol (PEG) terminated SAMs on the adhesion strength of the marine bacteria *C. marina*. We chose these surfaces as they cover a large range of wettabilities with different inert properties as numerous recent studies revealed [14, 27, 29, 31, 33–38].

2 Experimental

2.1 Preparation and Characterization of SAMs

Ethanol (p.a.) was purchased from Sigma-Aldrich (Munich, Germany). Deionized water was purified with a Milli-*Q* plus system (Millipore, Schwalbach, Germany), the final resistivity was ≥18 MΩcm. Nexterion® B glass slides (Schott, Mainz, Germany) were used as substrates for adhesion experiments and as substrates for deposition of gold films. Thin films of polycrystalline gold were prepared by thermal vapor deposition of 30 nm gold (99.99 % purity) onto Nexterion® glass slides predeposited with a 5 nm titanium adhesion layer. Evaporation was performed at a pressure of 2×10^{-7} mbar and a deposition rate of 0.5 nm s^{-1}, leading to a root-mean-square (rms) roughness of about 1 nm. The chemicals used for self-assembly were dodecanethiol (DDT, $HS–(CH_2)_{11}–CH_3$) and 11-amino-undecanethiol (AUDT, $HS–(CH_2)_{11}–NH_2$), purchased from Sigma-Aldrich. 11-(tridecafluorooctyloxy) undecanethiol (FUDT, $HS–(CH_2)_{11}–O–(CH_2)_2–(CF_2)_5–CF_3$) were retrieved from Prochimia, and Hydroxy-PEG2000-thiol (PEG, $HS–(CH_2)_2(OCH_2CH_2)_{44}OH$), was purchased from Rapp Polymere GmbH (Tuebingen, Germany). All chemicals were used as received without further purification. For the SAM formation the gold slides were first cleaned in an UV reactor for 2 h and then immersed into the corresponding 1 mM thiol solution in ethanol p.a. for 24 h, except for PEG where 48 h were required. Before and after immersion the samples were rinsed and sonicated for 3 min in ethanol p.a., and finally dried in a flow of nitrogen. The samples were stored under argon.

2.2 Surface Analysis

Successful assembly of the SAMs was verified by contact angle goniometry, spectral ellipsometry, and X-ray photoelectron spectroscopy (XPS). Sessile drop water contact angles were measured with a custom built goniometer under ambient conditions. Using digital images of the sessile droplet, the drop shape is modeled by the Young–Laplace equation and the contact angle at the interface is calculated. The contact angle was determined three times on each sample and the average is reported. SAM thickness measurements were performed with a M-44 multiple wavelength ellipsometer from J. A. Woollam Co., Inc. (Lincoln, NE). The organic film was modeled as a single Cauchy layer using the software WVASE from J. A. Woollam Co. The reported values are the average of three measurements. Film purity, composition, and thickness were analyzed by XPS using a Leybold-Heraeus MAX 200 X-ray photoelectron spectrometer with a magnesium anode as the X-ray source (Kα, 1253.6 eV).

2.3 Bacteria Culture

Cobetia marina [39] (DSM 4741), an aerobic, gram-negative bacterium, was obtained as dried culture from DSMZ ("Deutsche Sammlung von Mikroorganismen und Zellkulturen" GmbH, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and stored frozen in

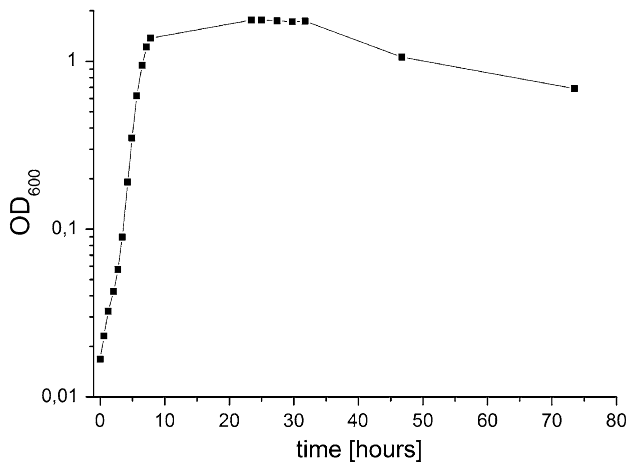

Fig. 1 Growth of *Cobetia marina* in MB as measured by the optical density at a wavelength of 600 nm (OD_{600})

stock aliquots in marine broth (MB) (2216, Difco, Augsburg, Germany) containing 20 % glycerol at −70 °C. MB and artificial sea water (ASW, Instant Ocean®) were prepared according to the manufacturer's instructions. Marine agar (MA) was prepared by the addition of 2 % Bacto agar (Difco) to MB. Bacteria from stored frozen stock aliquots were streaked onto MA plates. These stock cultures were stored at 4 °C for up to 3 weeks. For the experiments, a single colony from an agar plate was inoculated into 20 mL sterile MB and grown overnight while shaking on a vibrational table (65 rpm) at room temperature. Figure 1 shows the increase of optical density ($\lambda = 600$ nm) with time. After overnight culture (~14 h) the bacteria reached the stationary phase with an optical density of $OD_{600} > 1$.

Most assays described in literature prefer to work with bacteria in the log phase for adhesion experiments [40] as the results are most reliable. To bring bacteria into the log phase for our microfluidics experiments, the overnight culture was diluted 1:100 in MB and held in liquid culture for approximately 3 h. After this, the OD was frequently measured until the desired OD600 of 0.1 was reached. This suspension was harvested by centrifugation (Hettrich, Mikro 22 R at 10,000 rpm for 2 min), washed in sterile (0.45 μm filtered) ASW to remove any residual marine broth, and resuspended in ASW. Prior to use in the microfluidic experiment, the suspension was filtered through a 5 μm filter to remove larger bacterial aggregates. The number of bacteria in the suspension with an OD of 0.1 was 10^7 cells mL^{-1}as we determined by analysis of the number of colony forming units (CFU).

2.4 Microfluidic Bacteria Detachment Assay

Figure 2a shows the construction of the microfluidic device to study cell adhesion [22]. It consisted of a glass window (lid), the channel, and the coated surface. The channel itself was made of polydimethoxysiloxane (Sylgard 184, Dow Corning, Midland, MI) cast in a polished micro machined brass form and cured at 65 °C for 8 h. Window, channel, and sample of interest were mechanically held together by two disks connected by screws (Fig. 2a). The final channel dimensions after assembly were approximately 13 mm × 1 mm × 140 μm. The overall size is reduced compared to the setup we previously used to measure fibroblast adhesion [22] as higher shear forces are necessary to remove bacteria. The tubing inlet was connected to a reservoir containing ASW, onto which a nitrogen overpressure of 700 mbar was applied. The overpressure serves to avoid the formation of bubbles inside the channels and to reach higher maximum flow velocities. Four fully assembled channel systems were mounted on a base plate and placed on the motorized stage of

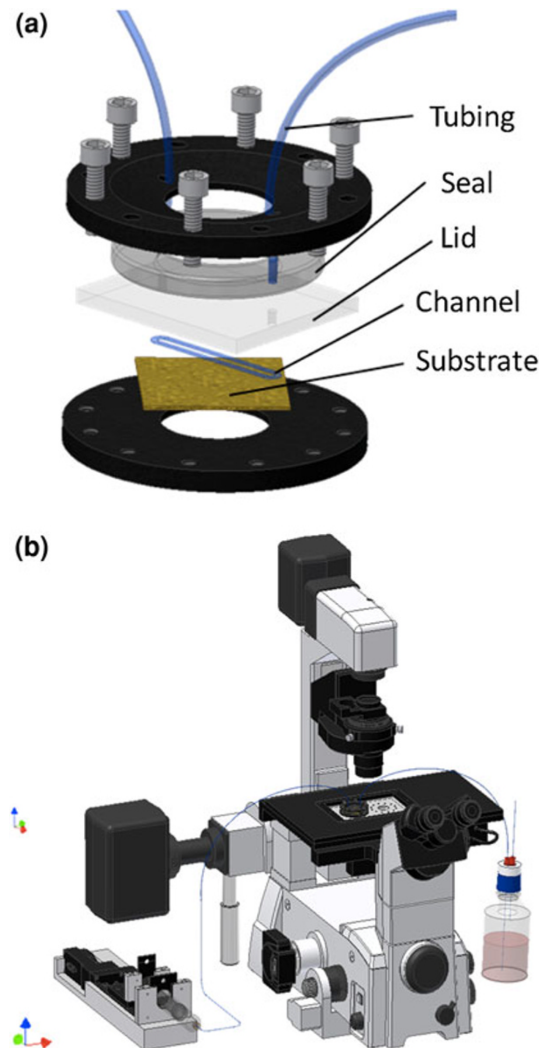

Fig. 2 Microfluidic setup **a** schematic representation of the microfluidic channel sandwich assembly, **b** microfluidic setup mounted under an inverse microscope [18]. **a** and **b** are reproduced from Ref. [18] with kind permission of the PCCP Owner Societies

an inverted microscope (Nikon TE-2000, Fig. 2b). Using microfluidic valves and connectors, each of the four systems were connected to a custom build, computer controlled syringe pump to aspire the medium. The pump is operated by a motorized, linear positioning stage. Prior to seeding the bacteria, the microfluidic channels were preconditioned with sterile ASW for 5 min. Then the suspension of *C. marina* (10^7 cells mL^{-1}) was injected into all four channels and bacteria were allowed to adhere for 2 h. After the incubation phase, the first channel was positioned under the microscope and only in this channel the flow rate was increased stepwise by 26 % every 5 s and detachment was followed via video microscopy with a 40× phase contrast objective (field of view of 256 µm by 192 µm, NA: 0.6). The detachment part of the assay took 4.5 min. After the detachment experiment in the first channel, the second, third, and fourth channel were positioned in the field of view of the microscope and investigated in the same way. The advantage of this procedure using four parallel channels was that four different surfaces could be investigated with the same batch of bacteria in the same physiological state. The wall shear stress τ created by a liquid flow has been calculated by Poiseuille's model [41] as shown in Eq. 1.

$$\tau = \frac{6Q\mu}{h^2 w} \qquad (1)$$

Q is the volumetric flow rate, μ the viscosity of the medium (for sea water $\sim 1 \times 10^{-3}$ kg m^{-1} s^{-1} at 20 °C [42]), and the channel's dimensions height h and width w. This model agrees well with more elaborate calculations [18].

3 Results and Discussion

The microfluidic detachment assay was capable of exerting well-defined shear forces in the range from 0.02 to 7,000 dyn/cm^2 (corresponding to 0.002–700 Pa). This allowed distinguishing weakly and strongly adhering bacteria. Even at high shear rates of 7,000 dyn/cm^2 the Reynolds number is in the order of 2,000, indicating a laminar flow even at highest flow rates. A typical experimental removal curve is shown in Fig. 3. In this case, removal of 300 bacteria was analyzed in the field of view. From this detachment curve, two characteristic values for bacterial adhesion can be derived: The adherent fraction and the critical shear stress τ_{50}. The adherent fraction of bacteria was calculated as the number of adherent bacteria after the first gentle flow was applied divided by the number of bacteria initially visible close to the surface. The critical shear stress needed to detach 50 % of the attached bacteria (τ_{50}) provided a measure how strongly the bacteria attached to the surface. The laminar shear stress was set into relation with the turbulent shear stress present at the surface of a moving ship 50 m downstream of the bow using calculations by Schultz et al. [43]

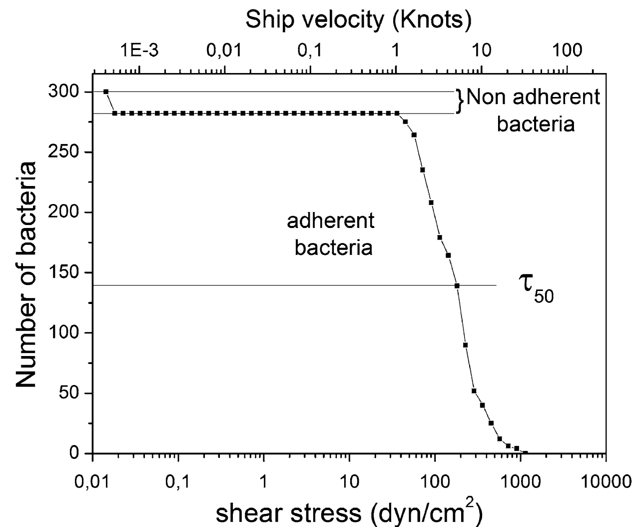

Fig. 3 Removal of bacteria from glass surfaces as function of the applied shear stress. The initial number of seeded bacteria was 300. The top *x*-axis shows at which ship velocity a comparable shear stress is reached according to calculations from Schultz et al. [43]. 10 dyn/cm^2 correspond to 1 Pa

which reveal that a wall shear stress of 560 dyn cm^{-2} are reached at a vessel velocity of $\sim$16 knots. These values are indicated at the top axis in Fig. 3 to give a rough idea of the range of shear forces used. However, this correlation needs to be used with some caution, as the flow situation at a ship hull is entirely different compared to the microfluidic experiment. Especially at low velocities deviations are likely, as a transition towards laminar conditions at the ship hull can be expected.

3.1 Influence of Medium and Incubation Time on the Adhesion Strength of Bacteria

For the experimental protocol, choice of the medium for the experiment and incubation time needed to be optimized. One consideration for the choice of medium is the potential formation of conditioning layers on the surfaces as they could mask the original surface chemistry and affect bacterial adhesion [44]. Therefore, dodecanethiol (DDT) SAMs were incubated either in artificial sea water (ASW) or in culture medium marine broth (MB) for 2 h. After exposure to the different waters, surfaces were analyzed by contact angle goniometry and spectral ellipsometry. Figure 4 shows that the thickness after immersion in MB was $\sim$13 Å and significantly thicker than for the sample incubated in ASW ($\sim$2 Å). Figure 5 shows that the wettability of the surface was barely influenced by thick conditioning layers formed in MB, while after immersion in ASW the surfaces became slightly more hydrophilic.

To understand if these adsorbed overlayers affect adhesion, removal curves of bacteria on pristine DDT

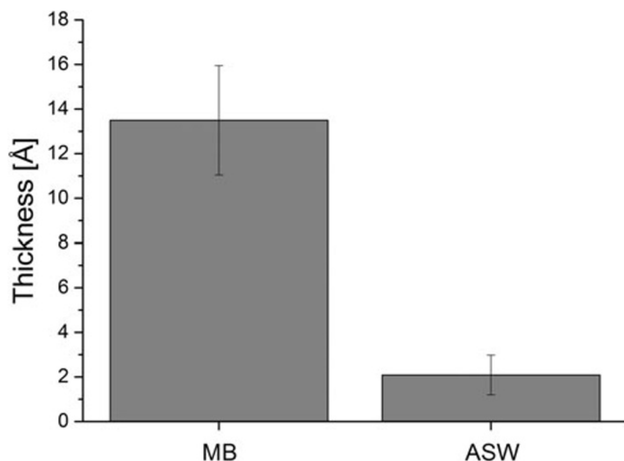

Fig. 4 Conditioning of a dodecanethiol (*DDT*) self-assembled monolayer by 2 h immersion into ASW and MB. The thickness of the additional overlayer was determined by spectral ellipsometry. *Error bars* are the standard error

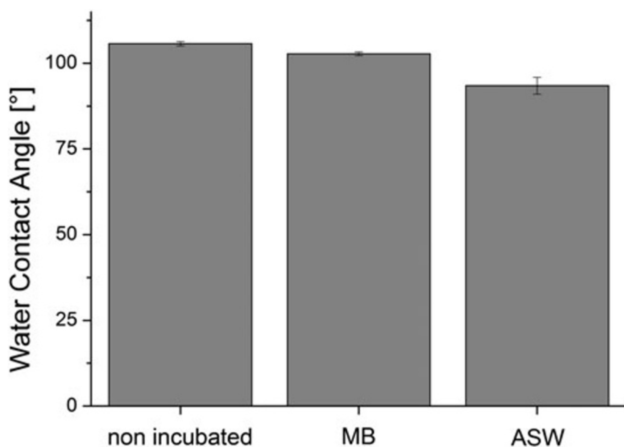

Fig. 5 Static water contact angle of DDT SAMs for the pristine surfaces, and those incubated for 2 h in ASW and MB. *Error bars* are the standard error

SAMs were compared to the conditioned surfaces. Figure 6 shows the average detachment curves of four independent experiments, and Fig. 7 displays the average critical shear stresses (a) and average fractions of adherent bacteria (b). Both, for the different surfaces and the different repeats, the seeding density in the field of view had slight variations between 250 and 500 bacteria. For better comparability, the *y*-axis in the removal plots considers the adherent fraction of bacteria ("Bacteria fraction"). Such a representation allows direct comparison of the curves and to immediately spot the critical shear stress needed to remove 50 % of the adherent bacteria (τ_{50}). The corresponding numbers of adherent cells (corresponding to the adherent fraction of 1) are given in the figure caption as information about the absolute cell numbers counted. The detachment curve in Fig. 6 reveals that at shear forces of 40–200 dyn cm^{-2} the

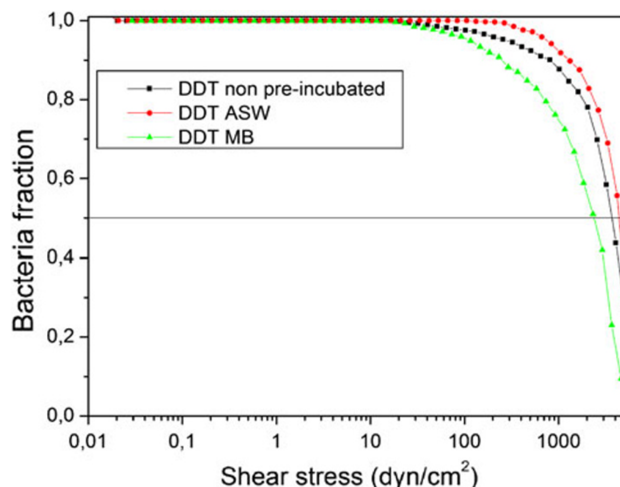

Fig. 6 Detachment of *Cobetia marina* from a pristine DDT SAM and from the same surface pre-incubated 2 h in different media (ASW, MB).The shown *curves* are averages of four experiments. Average numbers of adherent bacteria were: 425 (DDT), 307 (DDT/ASW), and 351 (DDT/MB)

adherent bacteria began to detach. The bar graphs in Fig. 7a show the critical shear stress τ_{50} needed to remove 50 % of the adherent bacteria. Within the error bar, adhesion was barely enhanced by pre-incubation of the surface in ASW. A pre-incubation in MB, however, reduced the attachment strength by 40 % (from 4,000 dyn/cm^2 to approximately 2,300 dyn/cm^2). From these results we concluded that incubation in MB leads to formation of a conditioning film on the surface, which affects bacterial adhesion much stronger than the thinner conditioning film formed after incubation in ASW.

To confirm that the active physiological status is maintained in ASW, the growth of bacteria after reaching the log phase was measured. The bacteria inoculated from agar plate in MB were allowed to grow to log phase using the protocol described in Sect. 2. When this point was reached, the bacteria were inoculated in ASW. The growth of the bacteria was followed during 2 h by measuring of the optical density at a wavelength of 600 nm. Figure 8 shows that bacteria continued growing in ASW during the course of the experiment despite the medium change. Comparing multiple assays in MB and in ASW (not shown) revealed that in general the performance of the assays in ASW was more reproducible compared to MB. Moreover, washing bacterial suspensions in ASW allows removal of excess extracellular polymeric substances (EPS) [45]. Consequently, ASW was used as medium for both, incubation and removal medium for our microfluidic assay.

As adhesion of bacteria is a time dependent process, one requirement of the assay was optimization of the incubation time. It was desirable to keep the incubation time short

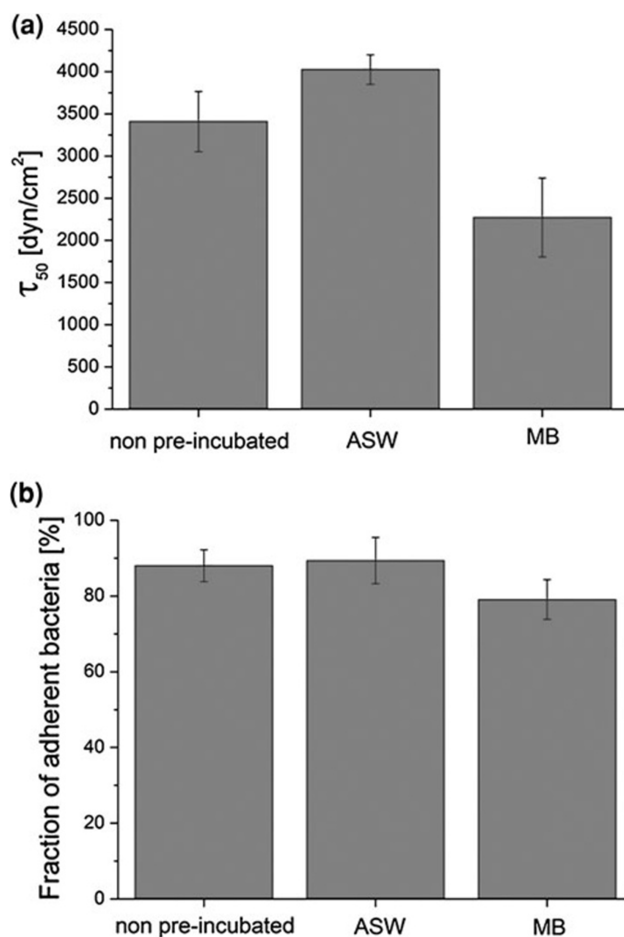

Fig. 7 Influence of surface conditioning on attachment of bacteria **a** average critical shear stress τ_{50} needed to detach 50 % of the adherent bacteria, **b** fraction of adherent bacteria. Pristine (non pre-incubated) DDT SAMs are compared to those pre-incubated 2 h in artificial sea water (*ASW*) and marine broth (*MB*). *Error bars* are the standard error

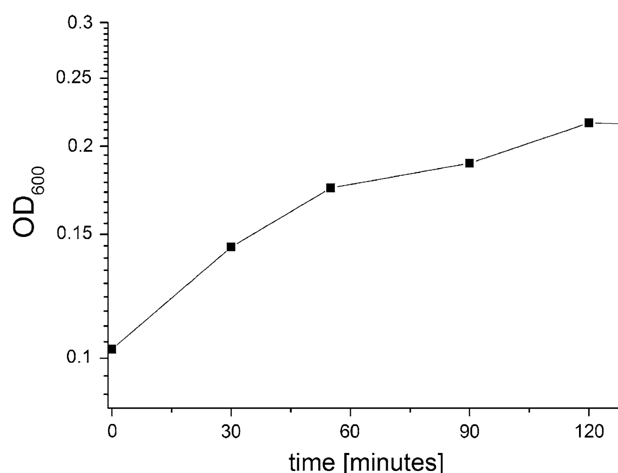

Fig. 8 Growth curve of *Cobetia marina* in ASW after reaching log phase in MB. Optical density was determined at a wavelength of 600 nm

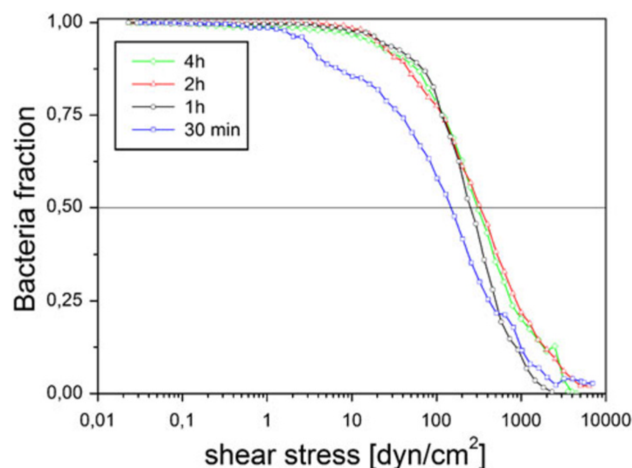

Fig. 9 Detachment of *Cobetia marina*from glass slides as function of incubation time. The *curves* are the average of four experiments. Initial numbers of adherent bacteria were: 228 (30 min), 234 (1 h), 391 (2 h), 394 (4 h)

in order to restrict the observations to adhesion of individual bacteria. On the other hand, the change in adhesion strength with time had to be as small as possible for maximum reproducibility. Thus, adhesion of *C. marina* was examined on glass slides for different incubation times. After 30 min, 1, 2, and 4 h incubation time in ASW, bacterial detachment was measured. Each experiment was repeated four times. The bacteria detachment curves, the average critical shear stress τ_{50} and the adherent bacteria fraction for the different incubation times ranging from 30 min to 4 h are summarized in Figs. 9 and 10. A trend towards stronger adhesion with increasing settlement time could be observed (Fig. 10a). The experiments showed furthermore that the ratio of attached bacteria barely depends on the incubation time and in all cases ~40 % of the bacteria adhere (Fig. 10b). This means that only a fraction of the bacteria was capable to adhere and this fraction attached rather quickly. In turn, complete establishment of thorough adhesion as indicated by the τ_{50} values occurred on a longer timescale and strengthened over time. Such time depending strengthening of the surface contact is in general known as the transition from a weak, temporary interaction of bacteria with surfaces to a permanent bonding as established by extracellular polymeric substances (EPS) [46]. As compromise for our lab assay we selected 2 h settlement time in ASW.

3.2 Adhesion of *Cobetia marina* to SAMs with Different Chemical Termination

As a first application of the microfluidic setup, we investigated SAMs with different chemical termination and quantified bacterial adhesion strength using the above-

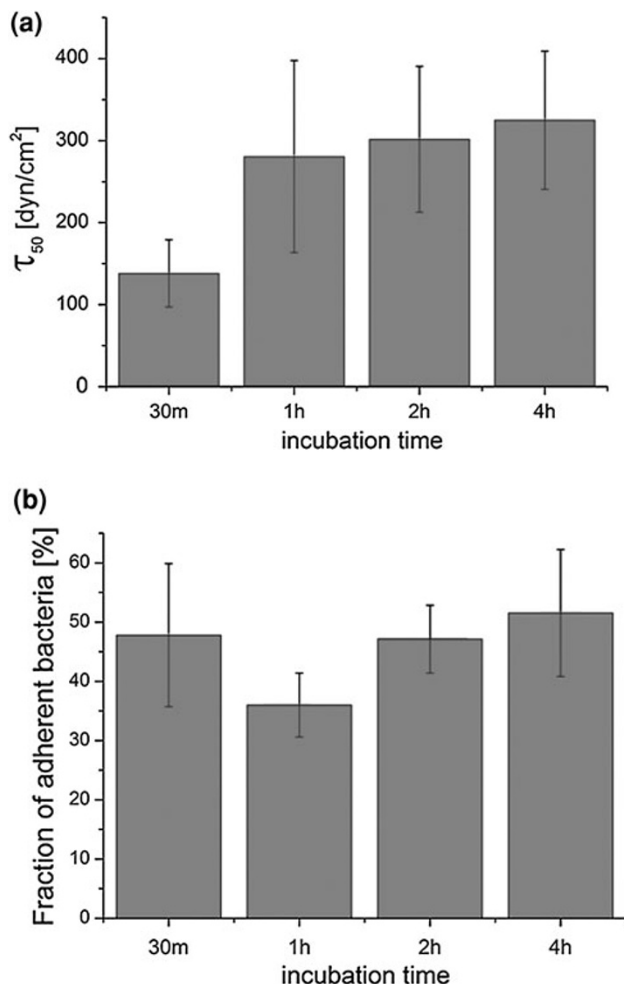

Fig. 10 **a** Average critical shear stress τ_{50} needed to detach 50 % of the adherent bacteria. **b** Fraction of adherent bacteria settled on glass slides for different incubation times. *Error bars* are the standard error

Table 1 Properties of the different self assembled monolayers: water contact angle and film thickness as determined by spectral ellipsometry

Surface	Contact angle/°	Thickness ellipsometry/Å
FUDT	113 ± 2	16 ± 1
DDT	106 ± 1	13 ± 1
AUDT	54 ± 1	16 ± 1
PEG	30 ± 1	30 ± 2

The reported values are the averages of three measurements

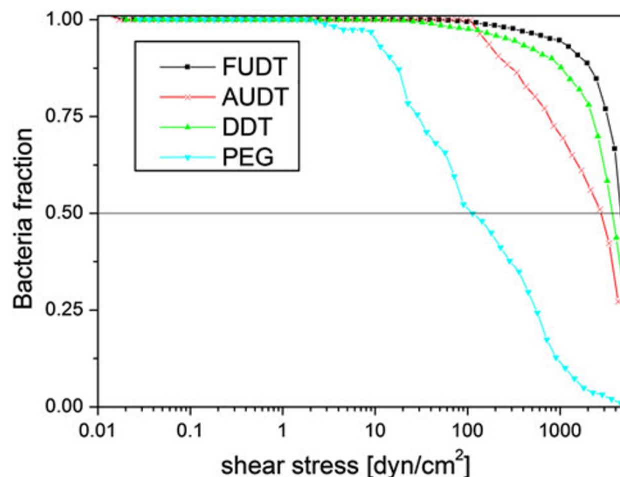

Fig. 11 *Cobetia marina* detachment curvesfrom SAMs with different chemical termination. *Curves* are average of four experiments. Initial number of adherent bacteria: 490 (FUDT), 425 (DDT), 469 (AUDT), and 268 (PEG)

derived experimental parameters. The coatings differed especially in their wettability as it is shown in Table 1. DDT and FUDT SAMs were hydrophobic (water contact angle of 106° and 113° respectively). AUDT SAM presented an intermediate wettability with a contact angle of 54° and PEG SAMs were hydrophilic with a contact angle of 30°. As also shown in Table 1, all SAMs have a similar thickness, except PEG, which is slightly thicker.

The assay has been carried out four times for each surface. Figure 11 shows the mean detachment curves from four experiments and Fig. 12 the average critical shear stress (a) and fraction of adherent bacteria (b). The detachment curve in Fig. 11 revealed that at shear forces of 2 dyn cm^{-2} the adherent bacteria began to detach from PEG. For the other SAMs the first bacteria started to detach at higher shear forces of 100 dyn cm^{-2}. Figure 12 shows that the chemical termination of the SAMs influenced especially bacterial adhesion strength (Fig. 12a) and to a lesser degree the fraction of cells that adhered to the surfaces (Fig. 12b). This is an important observation as it implies that the selection of the surface and the commitment of the bacteria to adhere were less affected by the surface chemistry compared to the adhesion strength. Especially in the case of PEG2000-OH, the fraction of adherent bacteria and the attachment strength were substantially reduced. The critical shear stress needed to dislodge bacteria from PEG-coated surfaces is only 5 % of that needed for removal from the other SAMs. This supports the general notion that hydrophilic, highly hydrated ethylene glycol surfaces have good short term resistance and the ability to reduce attachment of marine biofoulers [37, 45, 47, 48]. The general trend that with increasing hydrophilicity adhesion strength was reduced followed the general description of the Baier curve that hydrophilic coatings with elevated surface energy are less prone to biomass accumulation [33]. In general, water contact angles below the Berg limit of 65° lead to a situation where binding strength of water to the coating is of similar order

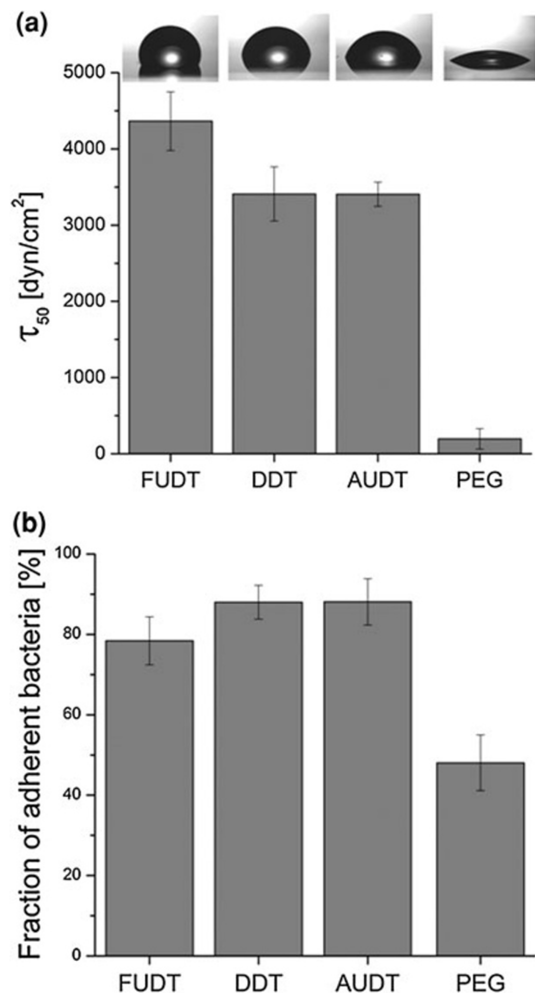

Fig. 12 Bacteria attachment to DDT, FUDT, AUDT, and PEG SAMs. The SAMs vary in water contact angle. **a** Average critical shear stresses needed for removal of 50 % of the adherent bacteria. **b** Fraction of bacteria which were found to be adhered to the surfaces after 2 h incubation in ASW. *Error bars* are the standard error

as the self-association energy in water [36, 49]. Our experiments suggest that rather the adhesion strength was affected by the different wettability and to a lesser extend the fraction of bacteria that committed to settle on the surface.

Especially the major difference in attachment strength of bacteria on the hydrophobic surfaces ($\approx$3,500–4,000 dyn/cm^2) and on PEG coated surfaces ($\approx$200 dyn/cm^2) by more than one order of magnitude showed that the microfluidic detachment assay was capable of discriminating the adhesion strength of bacteria to surfaces and thus to correlate surface properties with their ability to reduce bacterial attachment strength. In the future, we intend to apply this technique to test different coatings in order to find optimized surface compositions and properties, which are able to minimize bacterial adhesion strength.

4 Conclusion

We established a microfuidic assay to quantify adhesion strength of bacteria. The total duration of an experiment using four channels was less than 3 h, which allowed multiple experiments per day and thus a high sample throughout. Also the assay only required small glass chips (dimensions of 25 $\times$ 25 mm^2). As the fluidic environment was well controlled, quantitative data on the attachment strength of $\approx$400 cells could be probed simultaneously. Most importantly, detachment of single, individual cells was observed and thus the shear stress needed for their removal was obtained for each single bacterium in the field of view (256 by 192 μm^2). The assay covers six orders of magnitude of wall shear stresses and the situation in the microchannels was correlated with the turbulent hydrodynamic shear acting on the hull of a vessel cruising through the ocean. From detachment curves, both the adherent fraction and the critical shear stress for removal of 50 % of the adherent cells can be obtained. The decisive experimental parameters such as incubation time and medium were optimized for the biofouling marine bacterium *C. marina*. SAMs with different chemical termination were investigated towards their influence on both, fraction of attached cells and adhesion strength. The assay discriminated well between bacteria which adhere on hydrophobic SAMs and resistant PEG coatings and showed that the critical shear stress needed for bacterial removal differed by more than one order of magnitude. Thus the assay is a sensitive tool for the quantification of bacteria-surface interaction and capable to accurately discriminate the fouling-release potential of surfaces.

Acknowledgments The article was written following the award of the Biointerfaces division poster price to M.P.A.S. sponsored by Biointerphases at the 58th AVS International Conference and Exhibition held in Nashville, TN. The authors thank the Office of Naval Research (Grant number N00014-08-1-1116) and the Helmholtz program Biointerfaces for support of this work. The stimulating discussions with M. Grunze are kindly acknowledged. We thank G. Albert for preparation of the gold coated glass slides. We are gratefully indebted to L. Ista and G. Lopez (University of New Mexico, NM, USA) for their kind introduction into bacteria culture. We also acknowledge helpful advices from John Finlay and Maureen Callow (University of Birmingham, UK).

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