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***Langmuir* 2016**

DOI: http://dx.doi.org/10.1021/acs.langmuir.6b00839

Attachment of algal cells to zwitterionic self-assembled monolayers comprised of different anionic compounds

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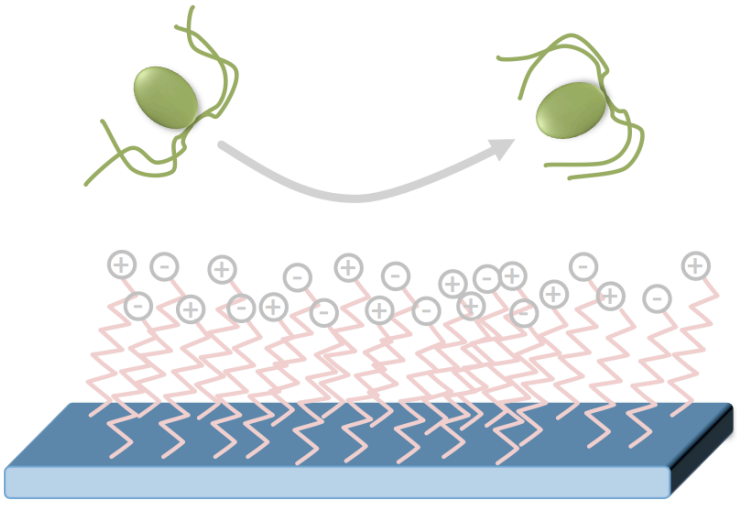
Keywords

Zwitterionic materials; self-assembled monolayers; biofouling; hydration, *Navicula incerta*, *Ulva linza*.

Abstract

The influence of zwitterionic self-assembled monolayers on settlement and removal of algae was studied. The monolayers were either constructed from zwitterionic thiols or from solutions of positively and negatively charged thiols. The cationic component was composed of quaternary ammonium terminated thiols and the anionic component contained sulfate or carboxylate termination. During assembly all surfaces showed a strong tendency for equilibration of the surface charge. Settlement and adhesion assays with zoospores of *Ulva linza* and the diatom *Navicula incerta*, and field tests of the initial surface colonization revealed the relevance of charge equilibration for the biological inertness of the prepared surfaces.

Graphical abstract



Introduction

Marine biofouling, the accumulation of biomass on man-made surfaces in contact with seawater, causes enormous economic and ecological damage and has at all times been a major problem for marine industries[1]. Coatings containing toxic ingredients that resist fouling can be replaced by fouling-release surfaces, which allow ships to self-clean while moving through the water[2-3]. While silicones and amphiphilic fluoropolymers are now established as commercial coatings, zwitterionic materials are promosing candidates and matter of intensive research[4]. Such materials show excellent protein and cell resistance as well as the ability to reduce settlement and adhesion strength of a range of biofilm forming organisms[4-6]. In many reports a charge neutrality at the interface provided the most reliable resistance of surfaces towards adhesion[5,7-9]. Some reports indicate that even a negative zeta potential can be suited to inhibit bacterial adhesion and zoospore settlement[9-13]. In turn, a positive charge rather caused high attachment of proteins and biofilm formers[5,9,13-14]. The stable hydration of the zwitterionic moieties was identified to be the key reason for their inert properties[15-16]. The importance of surface hydration in inert surface coatings is also well documented for ethylene glycol-based technologies[17].

To rapidly explore the potential of new surface chemistries, model surfaces such as self-assembled monolayers (SAMs) of alkanethiols on gold are frequently used[18-20]. Unlike polymeric films on surfaces, where factors such as grafting density, degree of crosslinking, or the chain lengths affect film properties, the characteristics of SAMs are mainly determined by their chemical termination. As the films are extremely thin, the major contribution to the elastic modulus comes from the substrate. Deposition from a mixture of oppositely charged thiols allows the synthesis of charge-balanced surfaces with a high degree of protein resistance[5,7]. The assembly of the mixtures of charged thiols is known to be charge driven across a wide ranges of surface compositions with a strong trend towards charge equilibrated surfaces[7,9,21-22]. In a previous study we evaluated the impact of mixed zwitterionic monolayers with different excess of charged moieties on marine fouling species, demonstrating that charge equilibration leads to the repellence of marine microorganisms[9].

In this work we investigate, how a variation of the anionic component alters fouling and compare the results against SAMs composed of the corresponding inherently zwitterionic compounds (Figure 1). The pure SAMs contained the positively charged trimethylammonium terminated thiol (TMAT) and two negatively charged thiols (sulfonate terminated (SAT) and carboxyl terminated (MUDA)), as well as mixtures of varying ratios of the positive and either one of the negative compounds. Additionally, two thiols with inherently mixed charges based on the analogue moieties as the pure compounds (sulfobetain (SB)[5] and carboxybetain (CB)), and a hydrophobic uncharged dodecanethiol (DDT) control were included (Figure 1). The obtained surfaces were characterized by spectroscopy and tested against colonization by two different algae, the diatom, *Navicula incerta*[23] and zoospores of the

green alga, *Ulva linza* [24]. In addition to the lab experiments, early biofilm formation was studied in field experiments at the Florida Institute of Technology (FIT) test site[25].

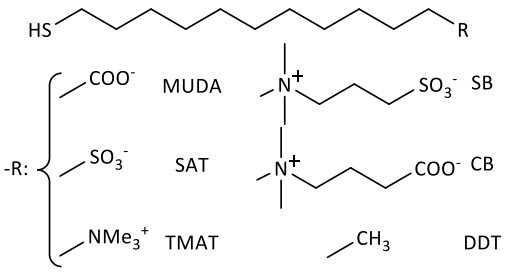


Figure 1. Structures and abbreviations of the alkanethiols used in this work. MUDA (carboxyl terminated thiol), SAT (sulfonate terminated thiol), TMAT (trimethylammonium terminated thiol), SB (sulfobetain), CB (carboxybetain), DDT (dodecanethiol).

Materials and Methods

Chemicals Ethanol *p.a.* was purchased from Sigma Aldrich (Germany). Thiols were obtained from Prochimia (Poland). All chemicals were used without further purification. Deionised water was further purified with a MilliQ® plus system (Millipore, Germany). Substrates (silicon wafers or NexterionB® float glass slides, Schott, Germany) coated with a 5 nm Ti adhesion layer and 30 nm Au were purchased from PVD Beschichtungen (Germany).

Self-assembled monolayers were prepared from 0.2 mM or 1 mM thiol solutions. In general, SAMs prepared out of the 0.2 mM solutions showed a better quality and reproducibility of sample quality. The pure thiols were dissolved in ethanol; 0.4 % NH_3 was added to the mercaptoundecanoic acid (MUDA) solutions. These stock solutions were mixed in the stated ratios for the preparation of mixed SAMs. Dodecanethiol (DDT), carboxybetaine- (CB) and sulfobetain (SB) thiols were used at a concentration of 1 mM in ethanol. For the field test a larger number of replicates was required and the TMAT and SAT samples were prepared from 1 mM solutions as described in a previous publication[9]. Prior to assembly, substrates were cleaned under an ozone generating UV lamp (90 min), ultrasonicated in ethanol *p.a*. (3 min), rinsed with ethanol and dried in a stream of N_2. The clean substrates were immersed in the different assembly solutions under ambient conditions for either 48 h (charged SAMs and the corresponding mixtures) or 24 h (DDT, SB, CB), subsequently rinsed with ethanol *p.a*, ultrasonicated in ethanol *p.a*. (3 min), rinsed with ethanol again and dried with N_2. All samples were stored under Ar until they were analyzed or used for the assays.

Static water contact angles were measured using a DSA100 goniometer (Krüss, Germany). After depositing a 3 µL droplet of MilliQ® water on the surface, the syringe was removed and the droplet shape recorded by a CCD camera. The shape was fitted using the software DSA3 to determine the static contact angle. All reported values are the average of measurements on at least three replicates, each investigated at three different positions. The given errors represent the standard deviation.

Film thicknesses were determined by spectral ellipsometry using an M44 instrument (J.A. Woollam, USA). The thickness of organic layers (SAMs, protein films) was measured by assuming a homogeneous film with a wavelength dependent refractive index described by the Cauchy model (A = 1.45, B = 0.01) using the WVase software.

X-ray photoelectron spectra (XPS) were recorded on a MAX200 (Leybold-Hereaus, Germany) equipped with a polychromatic Mg (K_α= 1253.6 eV) anode as X-ray source. Spectra were calibrated to the Au4f signal at 84 eV, peaks were fit applying a Voigt profile (Lorentz : Gauß ratio 4 : 1) and a background subtraction according to Shirley[26]. S 2p doublets were fit with a fixed peak splitting of 1.2 eV.

Protein adsorption assays were conducted following published protocols[27]. After pre-incubation of the samples in PBS (pH 7.4, ionic strength 165 mM) for 20 min on a shaker table (65 rpm), a 2 mg·mL^{-1} protein solution was added to achieve a final protein concentration of 1 mg·mL^{-1}. After another 30 min, the solutions were diluted with copious amounts of deionized water. Samples were gently rinsed with MilliQ® water while taking them through the water/air interface. Subsequently, they were dried in a stream of N_2 and the thickness of the adsorbed protein layer was determined by spectral ellipsometry.

Settlement and removal assays of zoospores of the green algae *Ulva linza* were conducted following published protocols[28]. Zoospores were collected from mature plants of *U. linza* growing on the Northumbrian coast, UK, as described previously[24]. Six replicates of each SAM were placed in separate compartments of a quadriPERM dish (Greiner Bio-One, Germany) and pre-incubated in filtered natural seawater (0.22 µm) 10 min prior to the assays. After adding a suspension of zoospores (10 mL; 1x10^6 spores·mL^{-1}) in 0.22 µm filtered natural seawater, samples were incubated for 45 min in darkness at 20 °C. Subsequently, they were gently washed to remove unsettled (*i.e.* motile) spores. Three replicate slides were fixed using 2.5 % glutaraldehyde in filtered seawater. On each of the replicate slides, the density of zoospores attached to the surface was counted in 30 fields of view (each FOV covers 0.15 mm²) using an AxioVision 4 image analysis system attached to a Zeiss Axioplan epifluorescence microscope[29]. Spores were visualized by the autofluorescence of chlorophyll. Removal of spores was achieved using a calibrated water channel, exposing the slides to a shear stress of 50 Pa after the 45 min incubation period[30]. Fixation was carried out as described above. The number of spores after exposure to flow was compared to the unexposed control slides. The statistical significance of the differences between the surfaces was determined by ANOVA analysis with post-hoc Tukey tests (significance level $p = 0.05$), comparing the number of cells in each FOV on the respective three replicates for the pre- and post-flow densities of cells separately.

Settlement and removal assays of *Navicula incerta* were conducted following published protocols. Diatoms in the log phase were washed three times with fresh medium (F/2) and the concentration of the suspension was adjusted to a chlorophyll content of 0.25 µg·mL^{-1}. Six replicates of each SAM were placed in separate compartments of a quadriPERM dish (Greiner Bio-One, Germany) and pre-incubated

for 10 min in filtered natural seawater (0.22 µm) prior to the assays. After adding a 10 mL suspension of the cells, samples were incubated for 2 h under ambient conditions. Subsequently, they were transferred into a container containing seawater without transferring them through the air-water-interface, where they were gently washed to remove unattached cells. Three samples were immediately fixed with 2.5 % glutaraldehyde and air-dried. On each of the replicate slides, the density of cells attached to the surface was counted in 30 fields of view (each FOV 0.15 mm²) using an AxioVision 4 image analysis system attached to a Zeiss Axioplan epifluorescence microscope[29]. Diatoms were visualised by the autofluorescence of chlorophyll. Removal of spores was measured using a calibrated water channel, exposing the three remaining slides to a 22 Pa shear stress after 2 h incubation and the washing procedure described above[30]. Fixation was carried out as described previously. The number of cells after exposure to flow was compared to the unexposed control slides. The statistical significance of the differences between the surfaces was determined by ANOVA analysis with post-hoc Tukey tests (significance level $p = 0.05$), comparing the number of cells in each FOV on the respective three replicates for the pre- and post-flow densities of cells separately.

Field tests were carried out in Florida (USA) at a test site near the Sebastian Inlet (27°53´59´´ N, 80°28´28´´ W). Three sets of samples were tested on three consecutive days, each containing two replicates of each test surface. Samples were immersed approximately 0.4 m below the water surface for 48 h; subsequently, they were fixed with 5 Vol % formaldehyde in filtered natural seawater (2 x 100 µm, 0.45 µm). After 1 h, this solution was gradually exchanged with distilled water. Subsequently, the samples were air-dried. The organism populations were analyzed by phase contrast microscopy (15× magnification, TE-2000-U, Nikon, Japan). On each sample, 60 FOVs (each 1340 µm × 1000 µm) were recorded and organisms larger than 10 µm were counted and identified according to the assignment of Zargiel *et al*[31]. The statistical significance of the differences between the surfaces was determined by ANOVA analysis with post hoc Tukey tests (significance level $p = 0.05$).

Results and discussion

Surface characterization

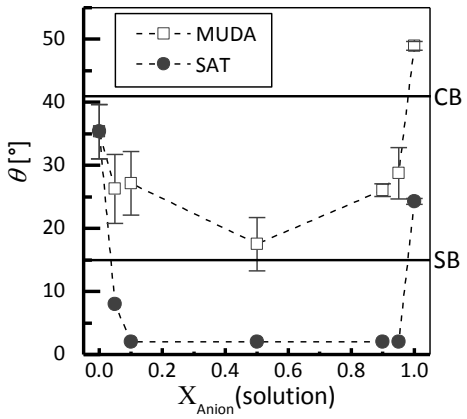


Figure 2. Static water contact angles *Θ* of pure and mixed SAMs for different mixture ratios X_{Anion} of the anionic compound (SAT or MUDA, 100% TMAT corresponds to $X_{Anion} = 0$). The solid horizontal lines show the contact angles of SAMs prepared from solutions of the zwitterionic thiols CB and SB. CA values are the average of three measurements on at least three samples; error bars show the standard deviation.

All SAMs were characterized by contact angle goniometry and spectral ellipsometry immediately after preparation. Figure 2 presents the static contact angles for different volume contents of SAT or MUDA in the assembly solution X_{Anion}. $X_{Anion} = 0$ corresponds to pure TMAT. Of the pure thiol SAMs, SAT was the most hydrophilic ($\Theta = 25° \pm 1°$), followed by TMAT ($\Theta = 35° \pm 4°$), while MUDA exhibited the highest CAs of $\Theta = 49° \pm 1°$. The mixed SAMs with both anionic compounds showed a higher hydrophilicity than both respective pure compounds. This decrease was more pronounced for the SAT-containing layers, which were completely wetted. The betaine SAMs showed a greater hydrophilicity than the pure thiol with the respective end-group (SAT for SB and MDUA for CB) but were more hydrophobic than the corresponding SAMs assembled from mixed solutions.

Table 1 summarizes the thicknesses *d* of the freshly prepared SAMs. Values of the pure compounds were in the expected range of 16 Å and in accordance with literature reports[5,32]. Mixed SAMs with SAT were thinner than the pure compounds. The betaine SB was thinner than expected for an ideally ordered densely packed monolayer (19 Å)[5], while CB met the expectation. All recorded values proved that no multilayers were formed, but indicated also, that ordering in the monolayers varied according to the head groups.

Table 1. Layer thicknesses d of pure and mixed SAMs dependent on the solution content of the anionic compound of SAT or MUDA (X=1 corresponds to 100 % anionic compound) and of DDT, SB and CB. All displayed values are the average of three measurements on at least three samples; the standard deviation was ± 2 Å at maximum.

X (SAT or MUDA)	d [Å] (TMAT/SAT)	d [Å] (TMAT/MUDA)		d [Å]
0	15	15	DDT	11
0.05	13	13	SB	14
0.1	13	13	CB	18
0.5	14	12		
0.9	12	15		
0.95	14	15		
1	18	15		

The results of the XPS analysis of the prepared SAMs are shown in Figure 3. For clarity, only the 1 : 1 mixtures of the mixed SAMs (50 % SAT; 50 % MUDA) are represented. The single-component thiols TMAT and SAT carried a functional group, which gave rise to a distinct characteristic signal, marked in grey in the respective spectrum. The N 1s signal of the trimethylammonium in TMAT was observed at 402.5 eV (Figure 3A); the sulfonate of SAT showed a doublet at 168 eV in the S 2p spectra (Figure 3B) and the carboxyl group of MUDA was observed at 289 eV in the C 1s spectra. Mixtures of TMAT and the anionic thiols exhibited both characteristics for the pure compounds for mixing ratios between (1-99) % for SAT and (1-90) % for MUDA. The same applied to the betaine SAMs, for which both, the N 1s signal and an additional signal in the S 2p spectrum (for SB) or the C 1s spectrum (for CB), were detectable.

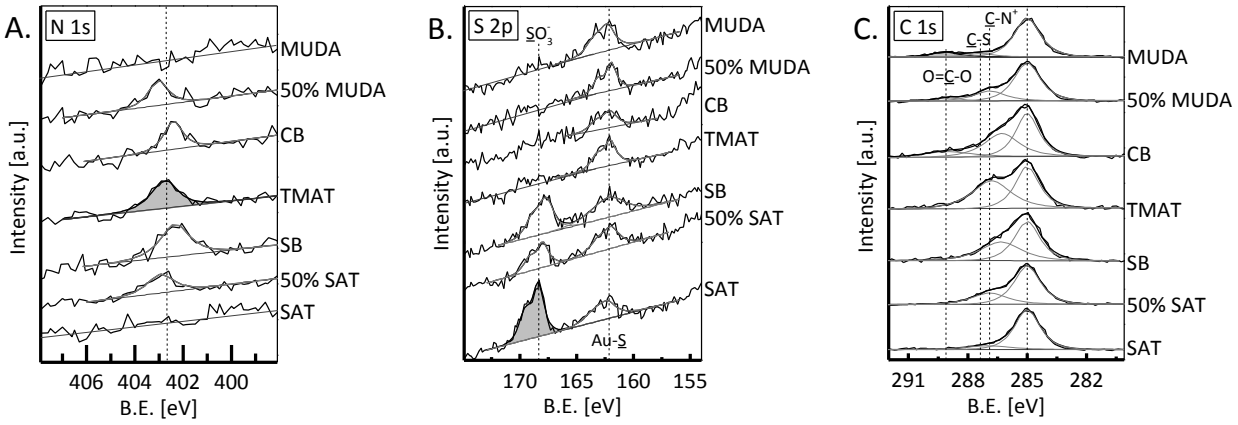


Figure 3. XP spectra of the N 1s (A), S 2p (B) and C 1s (C) binding energy regions auf the pure SAMs SAT, TMAT and MUDA, the 1-1 mixtures of TMAT with SAT (50 % SAT) or MUDA (50 % MUDA) and the betaine SAMs SB and CB. All C 1s signals were normalized to the intensity of the respective aliphatic signal at 285 eV.

The content of the anionic thiols in the assembled SAMs was quantified by the ratios of the photoelectron spectroscopy signals. In case of SAT-containing SAMs, the ratio of the two S 2p signals was representative for the content of the sulfonate-terminated thiol (Figure 4A). For MUDA-containing monolayers, the signal intensity of the carboxyl C 1s signal was compared to the one of a pure MUDA layer (Figure 4B). In agreement with previous reports[7], the surface composition was not governed by the composition of the solution, but by the interactions between the charged head groups. This interaction

produced equal amounts of both charges (1:1 ratio) on the surface. Only when one of the components was added in excess (>95%) a deviation favoring the species with higher solution concentration was observed. To confirm the stability of the prepared monolayers, their remaining thickness was evaluated after 4 d incubation is salt water. No significant change in film thickness was observed for this period. Thus, the stability in sea water was substantially higher than the duration of the biological assays.

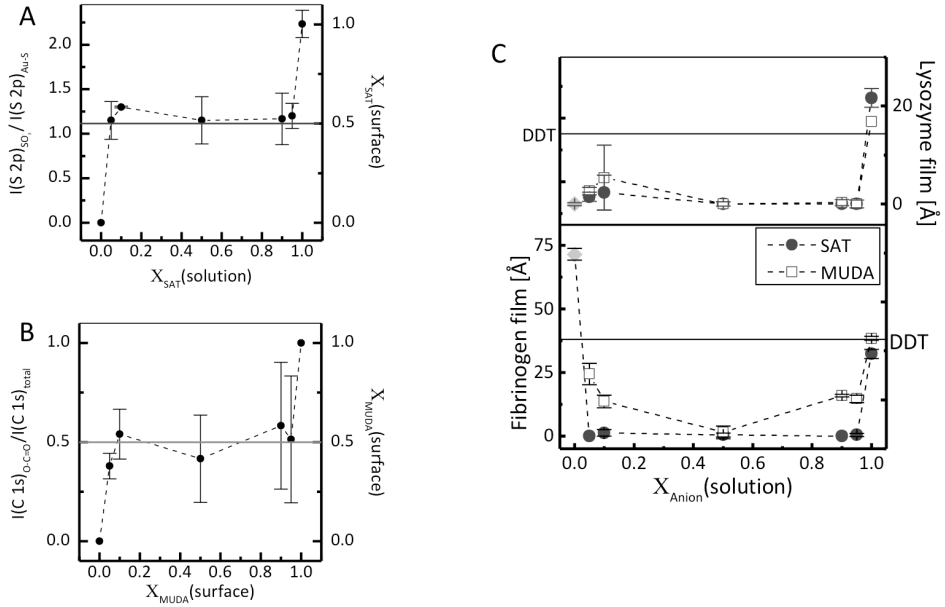


Figure 4. Content of anionic compound in the assembled SAM for different solution contents X (pure TMAT solutions corresponds to *X* = 0) and their resistance towards protein adsorption. A) Mixed SAT SAMs, SAT content determined by the ratio of the two S 2p signals. B) Mixed MUDA SAMs, MUDA content determined by the ratio of the O-C=O signal intensity compared to the overall C 1s signal intensity, relative to the ratio in the pure MUDA. C) Thickness of adsorbed fibrinogen (lower panel) and lysozyme (upper panel) dependent on the fraction of anionic compound (SAT and MUDA, pure TMAT corresponds to *X* = 0 and is represented by a grey diamond) in solution, compared to the hydrophobic DDT control. All XPS peak ratios are the average of at least three (SAT, A) or two (MUDA, B) replicates. Protein data in C are the average of data obtained for at least three replicates. Error bars represent the standard deviation.

Protein adsorption

The resistance of the monolayers against the adsorption of two oppositely charged model proteins, fibrinogen and lysozyme, was evaluated. Figure 4C summarizes the adsorbed protein layer determined by spectral ellipsometry after incubation of the samples in a protein solution. The highest amount of negatively charged fibrinogen adsorbed to the positively charged TMAT, while the positively charged lysozyme preferentially adsorbed to the negatively charged SAT and MUDA. Therefore, dominance of charge in the interactions between protein and charged surfaces was apparent. On the mixed SAT samples, adsorption of both proteins was fully suppressed. The observations are in good agreement with earlier reports of Chen et al.[7] on mixed, charged SAMs and of Ekblad et al.[8] on charge gradient surfaces. Protein resistance was enhanced in the present experiment compared to that in a previous study, where SAT- and TMAT-containing SAMs were prepared from more concentrated solutions (1 mM instead of 0.2 mM in the present study)[9]. Based on the XPS analysis above, the protein resistance showed that the equilibrated charge distribution at the surface was a key factor in the creation of

protein resistant properties. Deviation from a 1:1 composition at excess concentrations of one of the components in solution leads to a loss of the inert properties.

Attachment and removal of Navicula incerta

Cell densities of the diatom *N. incerta* after settlement and after removal assays are shown in Figure 5. The highest number of attached cells after gentle washing was found on the trimethylammonium terminated TMAT. On carboxyl-containing SAMs (MUDA, MUDA mixtures, and the betaine CB), cell numbers were comparable to that on TMAT, while being substantially reduced on sulfonate containing surfaces (mixed SATs and SB, ANOVA, Tukey's pairwise comparison, $p < 0.01$). After exposure to a calibrated water channel of 22 Pa, a significant amount of diatoms was removed from all test surfaces except for the hydrophobic DDT control, on which pre- and post-flow cell numbers were identical. The composition of the mixed assembling solutions only influenced post-flow cell densities in the case of the 95 % SAT samples, on which the remaining spore density was higher than on the other substrates and between the other mixed SAT surfaces and pure SAT. The anionic component was very important and sulfonate-containing SAMs showed, in general, a strongly reduced settlement and enhanced removal as compared to the carboxylate component.

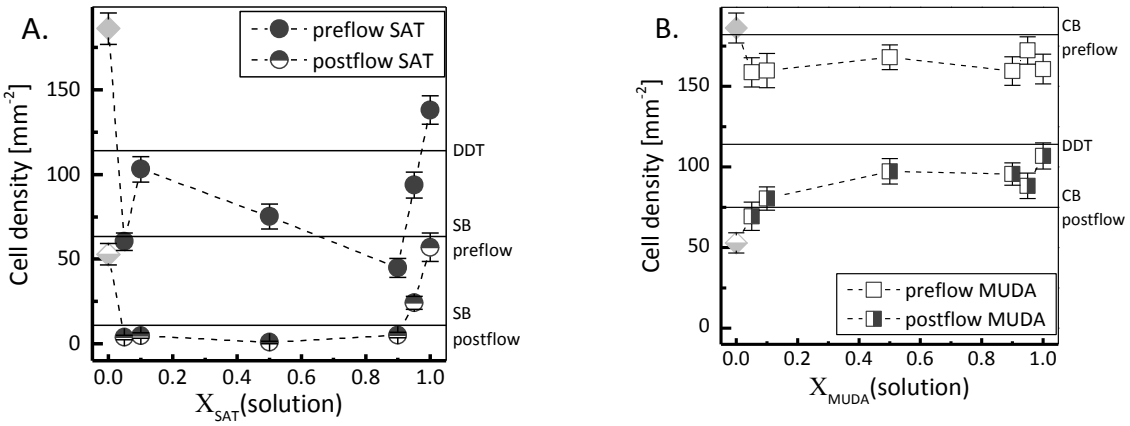


Figure 5. Density of attached cells of *N. incerta* after 2 h incubation before and after exposure to a 22 Pa shear stress in dependence of the fraction of anionic compound in solution (A. SAT and B. MUDA, pure TMAT corresponds to *X* = 0 and is indicated by diamond shaped symbols), compared to the hydrophobic DDT control and the betaine SAMs SB and CB. Each point shows the mean from 90 counts, 30 of each replicate slide; error bars show the 95 % confidence limits.

The percentage of *N. incerta* remaining on the substrates after flow was representative of their adhesion strength on the test surface. In Figure 6 the fractions of remaining cells were plotted against the static water contact angle of the surfaces. For clarity, only the 1 : 1 mixtures of the mixed SAMs were included. As general trend the fraction of remaining cells, representing how strongly the diatoms were attached, increased with the water contact angle. This finding is in agreement with literature regarding high initial attachment[23,33] and strong adhesion[28] of diatoms on hydrophobic surfaces, such as an uncharged DDT SAM. A correlation between the charge (positive or negative or charge carrying moiety) and the strength of adhesion was not evident (results not shown).

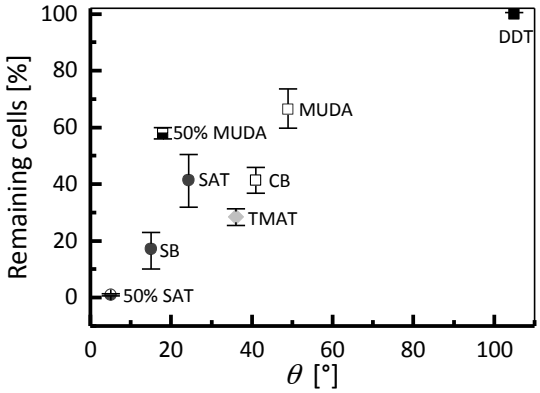


Figure 6. Percentage of remaining cells of *N. incerta* after exposure to a shear force of 22 Pa increased with the static contact angles of the respective samples. Each point shows the mean from 90 counts, 30 of each from 3 replicate slide; error bars show the 95 % confidence limits.

Adhesion behavior and ease of removal of zoospores of Ulva linza

Figure 7 summarizes the results of settlement and removal experiments with zoospores of *U. linza*. The highest number of settled spores was observed on the positively charged TMAT. Compared to the hydrophobic control, for which a preference of the spores is well documented[20,28], the spore density was nearly twice as high. The positive charge might be the reason for the high settlement density, as high settlement of the spores on peptide surfaces exhibiting a positive net charge has been reported previously[34]. The negatively charged SAMs, SAT and MUDA, reduced the spore density significantly (ANOVA, Tukey pairwise comparison, $p < 0.01$). The repulsive influence of negative charges might be related to the negative zeta potential of the *U. linza* zoospores[11]. On the mixed SAMs, settlement differed remarkably depending on the anionic compound used: On MUDA-containing surfaces, spore settlement was rather low and even small solution concentrations of MUDA (10%) caused low settlement. In contrast, on the highly hydrophilic SAMs containing SAT, the settlement density was comparable to pure TMAT and was higher than on DDT. It required solutions with 90% SAT to cause a decrease in spore settlement. On both betaine SAMs (SB, CB), cell numbers were as low as on the pure anionic compounds.

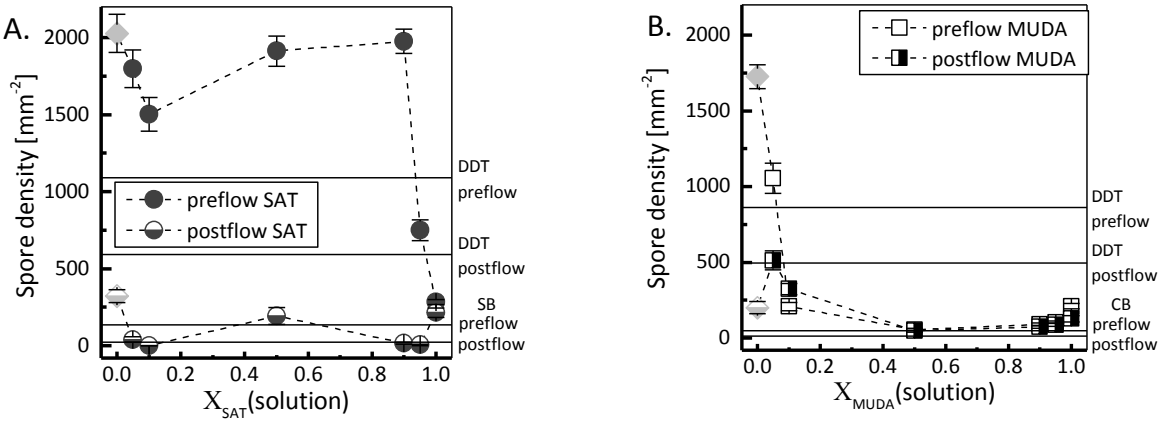


Figure 7. Density of attached spores of *U. linza* after 45 min incubation before and after exposure to a 50 Pa shear stress in dependence of the fraction of anionic compound in solution (A. SAT and B. MUDA, pure TMAT corresponds to *X* = 0 and is

indicated by diamond shaped symbols), compared to the hydrophobic DDT control and the betaine SAMs SB and CB. Each point shows the mean from 90 counts, 30 of each replicate slide (n = 3); error bars show the 95 % confidence limits.

To evaluate the adhesion strength of the spores, they were exposed to a shear force of 50 Pa in a calibrated water channel. Since the spore densities on MUDA-containing samples were already low after initial settlement, the percentage of remaining cells (as provided for the *N. incerta* experiments) would be misleading. Therefore, the number of spores remaining on the different surfaces after exposure to the water flow were plotted against the static water contact angles (Figure 8). The diagram shows as a general trend an increased number of remaining spores at higher contact angles, indicating that spores were stronger attached to hydrophobic surfaces. The highest spore density remained on the hydrophobic control, the lowest on the most hydrophilic zwitterionic 90 % SAT SAM.

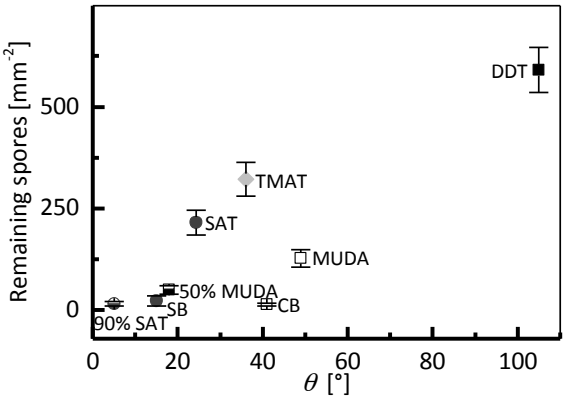


Figure 8. Remaining spores of *U. linza* after exposure to a 50 Pa shear stress correlated with the static contact angles of the respective samples. Each point represents the average of 90 analyzed fields of view, 30 on each replicate slide (n = 3); error bars show the 95 % confidence limits.

Two conclusions can be drawn from the data: Firstly, the initial settlement of the motile zoospores was highly sensitive to the exact chemical termination of the SAMs. They exhibited a preference for the positively charged trimethylammonium group, even when the moiety was present in combination with the sulfonate group. In contrast the presence of a carboxyl group prevented adhesion, even when combined with the attractive ammonium group. Secondly, adhesion on the initially attractive zwitterionic sulfonate-surfaces was weakened significantly. The findings support the earlier notion of Zhang et al.[35] that sulfate-containing methacrylates have the ability to reduce settlement and enhance removal of zoospores and sporelings of green algae.

Field test

Under real marine conditions, a large variety of organisms are present and synergetic effects between species are well known[36-37]. To correlate the laboratory data with initial settlement in the real ocean environment, field tests were carried out with the SAT/TMAT- SAM system. Test samples were immersed in the ocean for 24 h (one set) and 48 h (in three sets on subsequent days) and the settled organisms were identified by optical microscopy. The percentage distributions of organisms on the different chemistries after 48 h are compared in Figure 9. Pennate diatoms of the genera *Navicula*,

Nitzschia, *Gyrosigma* and *Progonoi* and centric diatoms (*Coscinodiscus*, *Cocconeis* and *Triceratium*) were counted in two groups. Diatoms from the genera *Amphora*, *Petrichous* and *Cylindrotheka Cylindotheka* were individually counted. The first group was the most abundant on all inspected coatings, forming ~ 60 % of the population on the surface. Between the different surface chemistries, no differences in the occurrence of the organisms could be identified. This was in line with previous literature reports in which wettability of the coatings had no impact on the diversity of colonizing species[38].

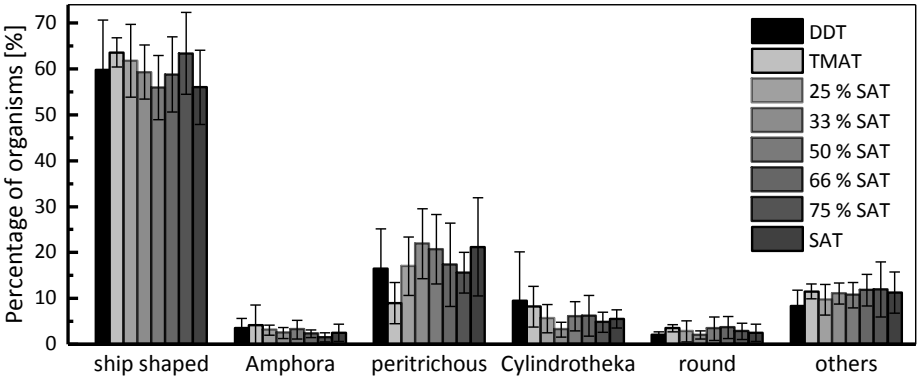


Figure 9. Occurrence of organisms after 48 h immersion at the FIT test site on samples with increasing SAT content in the assembling solution, compared to a hydrophobic DDT control. Each point shows the average of 60 FOVs on each of the 6 replicates. Error bars show the SEM.

The numbers of attached organisms were also quantified (Figure 10). The highest densities were found on the hydrophobic DDT, which was again included as a hydrophobic non-charged control. After 24 h immersion, no significant differences could be found on the charged test substrates ($p>0.05$), whereas a longer immersion time revealed variations in settlement on the different chemistries. On both pure charged compounds (SAT and TMAT), the density of organisms was significantly reduced compared to the DDT control ($p<0.05$). More organisms attached on the positively charged TMAT than on the negatively charged SAT, albeit this was statistically insignificant ($p>0.05$). On the mixed samples, a significant reduction in settlement could be observed as compared to DDT and TMAT ($p<0.05$). Compared to SAT the reduction was only statistically significant with respect to of 25%, 66% and 75% SAT ($p<0.05$). The lowest settlement on the 75 % SAT surface was in good agreement with a low attachment strength of diatoms found in a recent study[9].

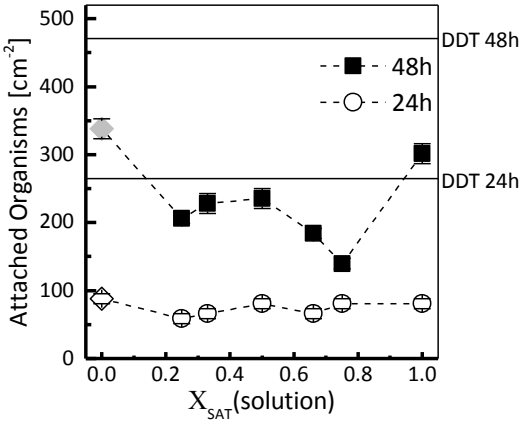


Figure 10. Total numbers of attached organisms after 24 h and 48 h static immersion at the Sebastian test site of the FIT in dependence of the fraction of SAT (pure TMAT corresponds to *X* = 0 and is indicated by the grey diamond shape) compared to the hydrophobic DDT control. Each point shows the average of 60 FOVS on each of the 2 replicates for 24 h and 6 replicates for 48 h. Error bars show the SEM.

Summary and conclusion

Charged SAMs with different anionic components, their mixtures, and respective betaines were prepared and characterized. The assembly process of the mixtures of thiols with oppositely charged moieties was demonstrated to be charge driven as expected from the literature[7,9,22]. Deviations from the ideal 1 : 1 composition of the resulting SAMs were only detectable for the SAMs prepared from assembling solutions with > 95 % content of one of the thiol compounds. All mixed SAMs exhibited a higher hydrophilicity than monolayers formed from the single components. Protein adsorption on the mixed charged SAMs was in all cases low. Excess charge led to enhanced protein adsorption with pronounced adhesion of proteins that carried the opposite charge to the SAM.

The adhesion of two model algae, *Navicula incerta* and zoospores of *Ulva linza*, was enhanced on the positively charged TMAT SAMs and was even higher than on the uncharged, hydrophobic SAM DDT. A negative charge excess on the surface caused an increase in the number of attached diatoms, while settlement density of zoospore was reduced. The anionic component played an important role in attachment on the mixed surfaces, having contrasting effects on the two algae. A reduction of the initially adherent microorganisms was caused by SAT-containing SAMs in the case of *N. incerta*, and by MUDA-containing monolayers for zoospores of *U. linza*. The respective betaines SB and CB, which carried the same charged moieties as the mixed SAMs (either sulfonate in SB or carboxylate in CB), showed the same trend as the corresponding mixed SAMs. The only exception was the settlement density of the spores of *U. linza* on SB, which was in the range of pure SAT.

Both single charged SAMs (positive and negative) caused a decrease in the numbers of adherent organisms in the field studies (48 h exposure) compared to the DDT control. The mixed charged surfaces showed an even more pronounced reduction in settlement. As a general trend we can say that accumulation of biofilm formers is stronger on the positively charged surfaces than on the negatively charged surface. In this process the adhesive and the extracellular polymeric substances (EPS) used by the organisms might be relevant. These substances and most of the cells themselves are known to be negatively charged[10-11,39-40]. A classical electrostatic repulsion between the negatively charged adhesive or EPS and the surface would be expected as it was shown by the protein resistance data. However, the typical Debye length in seawater is below one nanometer. This implies that any electrostatic contribution can only be exerted if molecules are in very close contact. It is likely that other effects, such as hydration and hydrogen bonding additionally guide the observed trends.

On this basis it is surprising that protein resistance and some of the settlement data showed similar trends and excess charges in nearly all cases increased attachment compared to charge-

equilibrated surfaces. The heterogeneous response of the organisms to the negatively charged surfaces in laboratory conditions and in the field is puzzling and might be connected with the attachment mechanism: cells of *N. incerta* cannot swim actively and are moved passively by currents and gravity to contact a surface. Upon doing so, the EPS materials used as motility polymers and for attachment, are the materials that undergo interaction with the surface[23]. In general, the initial attachment of diatoms is greater on hydrophobic than on hydrophilic surfaces[33]. The results on the zwitterionic SAMs showed an agreeing trend of higher settlement with increasing hydrophobicity. In contrast to the diatoms, motile zoospores of *U. linza* are not surrounded by a rigid framework or a cell wall; their outermost layer is a lipoprotein membrane. A strong interaction of this membrane with cationic surfaces was already proposed in the context of the adhesion to surfaces with cationic oligopeptides[14]. Such an interaction is likely to be the reason for the high sensitivity of the spores for the SAM terminations: The highest affinity was observed for an excess of the positively charged components, while amongst the negatively charged moieties tested, the carboxyl groups caused the greatest reduction in the initial settlement. The fact that the well hydrated zwitterionic surface is relatively resistant against settlement agrees with our previous report on the relevance of hydration of ethylene glycol surfaces for the prevention of settlement[17].

It has already been documented that the attachment characteristics of organisms are different on betaines with sulfonate or carboxyl moieties[4]. Also in this work preference for the different anionic components varies with species. This raises the question why the different zwitterionic SAMs caused different responses. Sulfonates are classified as chaotropic, while the interactions of the carboxyl group carried more characteristics of water structuring kosmotropes[41]. Also a theoretical evaluation of the water structure around polymeric sulfo- and carboxybetaines revealed differences between molecules that are structurally closely related[42-43]. Obviously not only electrostatic contributions, but also the properties of the water film surrounding the highly hydrated zwitterionic surfaces as well as hydrogen bonding are likely to be involved, not only in protein adhesion, but also in interactions with biofouling organisms. As conclusion, zwitterionic compounds have great potential to be applied into the next generation of non-toxic fouling release coatings, but fine tuning of the anionic components and the precise molecular architecture of the charged groups seems to be the key for a successful coating technology.

Acknowledgements

The authors acknowledge support from the Office of Naval Research [N000141210498 and N000141512324] (SB and AR), [N00014-13-1-0634] (ASC, JAF and SCF), and the cluster of excellence RESOLV (EXC 1069).

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