**Effect of the dipole orientation in mixed, charge-equilibrated self-assembled monolayers
on protein adsorption and marine biofouling**

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(TOC Graphics)

Abstract

While zwitterionic interfaces are known for their excellent low-fouling properties, the underlying molecular principles are still under debate. In particular, the role of the zwitterion orientation at the interface has been discussed recently. To elucidate the effect of this parameter, self-assembled monolayers (SAMs) on gold were prepared from stoichiometric mixtures of oppositely charged alkyl thiols bearing either a quaternary ammonium or a carboxylate moiety. The alkyl chain length of the cationic component (11-mercaptoundecyl)-*N,N,N*-trimethylammonium (MUDTMA), which controls the distance of the positively charged end group from the substrate's surface, was kept constant. In contrast, the anionic component, and, correspondingly, the distance of the negatively charged carboxylate groups from the surface, was varied changing the alkyl chain length in the thiol molecules from seven (MOA) via 11 (MDDA) to 15 (MHDA). In this way, the charge neutrality of the coating was maintained, but the charged groups exposed at the interface to water were varied, and thus, the orientation of the dipoles in the SAMs was altered. In model biofouling studies, protein

adsorption, diatom accumulation and the settlement of zoospores were all affected by the altered charge distribution. This demonstrates the importance of the dipole orientation in mixed charged SAMs for their inertness to non-specific protein adsorption and the accumulation of marine organisms. Overall, biofouling was lowest when both the anionic and the cationic groups were placed at the same distance from the substrate's surface.

Introduction

The undesired growth of biomass on exposed surfaces, so-called biofouling, causes severe problems in several fields, such as biomedicine and the marine industries. Biofouling of implants can be life-threatening for patients, for example only 10 ng/cm² adsorbed fibrinogen can cause an implantable device failure.¹ Marine biofouling, on the other hand, is estimated to cost over 1 billion dollars annually.² Most contemporary marine protective coatings use biocides to minimize biofouling on for example ships' hulls.^{3–5} Although their action may be effective, they are often broad-spectrum in action. The impact of persistent biocides on the environment has necessitated legal action to ban or limit their use in marine coatings. If biocides become further restricted, the demand for biocompatible coatings that solely rely on low adsorption and easy release of fouling will greatly increase.^{6–9} Charge neutrality, steric repulsion and high hydration have been identified among the key properties that render coatings inert.^{10–12} Hence, zwitterion-containing polymers are usually relatively inert and promising ultra-low-fouling materials.^{13–16} Incorporated in different ways, e.g. in polymer brushes, or photo-crosslinkable polymer films, their low adsorption properties have been demonstrated against proteins,^{17–19} bacteria,^{10,20,21} and marine organisms.^{19,20,22} Self-assembled monolayers (SAMs) bearing zwitterionic groups have also shown excellent low-fouling properties.^{12,23} SAMs of mixed-charged components that show charge neutrality, i.e. a 1:1 mixture of cationic and anionic thiols, provided a high inertness against fouling organisms.²⁴ Such SAMs have shown a similar, or sometimes even enhanced antifouling activity compared to zwitterionic SAMs consisting of only one single compound.^{23,24} The mixed SAMs were, for instance, able to decrease protein adsorption compared to monolayers of sulfobetaines and phosphorylcholines, in particular when charge equilibration was achieved.¹⁸ In agreement, when investigating the adsorption of pepsin and lysozym on gradients formed from SAMs bearing a carboxylic and an ammonia group, the lowest overall protein adsorption was obtained for 1:1 mixtures of the charged groups].²⁵ Similarly, in studies on marine

antifouling properties, the performance of some mixed monolayers outperformed their linear zwitterionic sulfobetaine and carboxybetaine analogues.^{18,23} In addition to the chemical nature of the anionic or cationic groups, their positioning relative to each other was also found to be important. For instance, Vaisocherová et al demonstrated the importance of the positions of ammonium and the carboxylate groups in polycarboxybetaines.²⁶ While a separation of the cationic and the anionic group by one and two methylene groups strongly reduced human blood serum adsorption, the inertness was diminished for the analogous carboxybetaine with a propyl-spacer between the ionic groups.²⁶ Note that for such systems, the interpretation of the findings is complicated by the simultaneous increase of the pKa value of the carboxyl groups with increasing separation from the ammonium group, and by the markedly reduced chemical stability of ammoniopropionates that represent Mannich bases.²⁷ Moreover, the orientation of the anchoring of the zwitterionic moiety to the matrix has been discussed as an additional parameter.^{13,15,28–31} We recently revealed that the orientation in which sulfobetaines are anchored to the polymer backbone can indeed have a significant impact on the resistance of such polyzwitterion films against non-specific adsorption of proteins.^{19,28} Films of polysulfobetaines with a linearly anchored dipole orientation performed more effectively in antifouling coatings compared to a novel γ -shaped analogue, in which both ionic groups had the same distance to the polymeric backbone.

These studies illustrate that in addition to other molecular parameters, the molecular architecture also affects the fouling resistance of zwitterionic coatings. Still, they did not reveal which orientation of the zwitterion dipoles at the interface is the most effective to minimize fouling. The orientation of the sidechain zwitterionic groups with respect to the polymer backbone is chemically defined by the bond connectivity and its resulting molecular architecture. However, it is not evident that this intramolecular, ‘chemical’ positioning and orientation of the zwitterionic moieties translates directly into the average physical orientation of the zwitterions’ dipoles at the interface. This depends on the overall conformation of the system that is adapted to the specific situation and will also vary for different environments. Conformational and packing constraints allowing efficient backfolding or repositioning of the various ionic groups relative to the backbone could provoke a different effective dipole orientation at the interface than suggested by the molecular structure, or even might invert it. For instance, molecular simulations predicted that the phosphatidyl choline moiety in loosely packed SAMs assumes a conformation, which orients its dipole

nearly parallel to the surface. In other words, the positively and the negatively charged groups would be positioned at nearly the same average distance from the surface, although, according to the molecular structure, the ammonium group could be expected to be preferentially exposed at the interface.³²

To learn more about the effect of zwitterion dipole orientation at the interface, we designed a set of densely packed model SAMs on gold substrates from stoichiometric mixtures of oppositely charged alkyl thiols bearing either a quaternary ammonium or a carboxylate moiety. Such mixed ionic SAMs have been shown to behave as zwitterion mimics, performing equally well as SAMs made of truly zwitterionic thiols.^{18,23,33} While we kept the cationic component, (11-mercaptoundecyl)-*N,N,N*-trimethylammonium (**MUDTMA**), constant in the mixtures, anionic thiols of increasing length were employed, using either 8-mercaptooctanoic acid (**MOA**), 12-mercaptododecanoic acid (**MDDA**), or 16-mercaptohexadecanoic acid (**MHDA**) (figure 1). In this way, the distance of the positively charged end group from the substrate's surface was kept the same in all mixed SAMs, whereas the distance of the negatively charged carboxylate groups from the surface was varied, being either inferior (**MOA**), about equal (**MDDA**), or superior (**MHDA**) to the distance of the cationic groups. We used this set of SAMs to study whether, and if so to what extent, the terminating charge affects biofouling by investigating the non-specific protein adsorption and the retention of marine biofouling organisms. The mixed charged SAMs were characterized by spectroscopic ellipsometry, contact angle goniometry, and X-ray photoelectron spectroscopy. They were tested against non-specific adsorption of the model proteins fibrinogen and lysozyme, and against the attachment of two model marine fouling organisms, namely the diatom *Navicula perminuta* and zoospores of the green alga *Ulva linza*.

Figure 1. Structures and abbreviations of the thiols used to form the mixed self-assembled monolayers from top to bottom: (11-mercaptoundecyl)-*N,N,N*-trimethylammonium (**MUDTMA**), 12-mercaptododecanoic acid (**MDDA**), 16-mercaptohexadecanoic acid (**MHDA**) and, 8-mercaptooctanoic acid (**MOA**).

Materials and Methods

Sample preparation. Self-assembled monolayers were prepared on gold-coated glass slides following previously published protocols for mixed-charged monolayers.²³ The thiols were used as received (**MUDTMA**, **MDDA** and **MOA** from ProChimia surfaces; **MHDA** from Aldrich). All SAM samples were prepared from a 0.2 mM thiol solution in ethanol (p.a., Fisher chemicals). While **MUDTMA** was dissolved in pure ethanol, 0.4 vol% NH₃ (Sigma) was added to the carboxyl-terminated thiol stock solutions. When preparing solutions for mixed thiol assembly, the stock solutions were mixed in a 1:1 ratio. Prior to immersion into the thiol solutions, the slides were cleaned for 60 min under a UV lamp (Heraeus NIQ 40/18, 254 nm, 10 W, in atmosphere), rinsed with ethanol p.a., ultrasonicated (Triton Ultrasonic Cleaner, 50 W) for 3 min, rinsed with ethanol and dried in a N₂ stream. Clean gold-coated substrates were immersed into the thiol solutions for 48 h at room temperature, then rinsed with ethanol, ultrasonicated in ethanol for 3 min, and rinsed again in ethanol. The samples were stored under an Ar atmosphere until used for analysis or the bioassays.

Static water contact angle goniometry. A custom-built goniometer equipped with a CCD camera was used to determine the static water contact angle. After dispensing one droplet ($\approx 50 \mu\text{L}$) of tridistilled water onto the surface, the shape was fitted using Young's equation to obtain the water contact angle.

Spectroscopic ellipsometry. The dry film thickness was determined by spectroscopic ellipsometry (M-2000, Woollam, USA, with CompleteEase software) measured at 65°, 70°, and 75° incidence angle. A transparent single-layer model with a wavelength-dependent refractive index described by the Cauchy model was used with the parameters $A=1.45$; $B=0.01$, $C=0$.

X-ray photoelectron spectroscopy (XPS): X-ray photoelectron spectroscopy measurements were carried out in an ultra-high vacuum (UHV) set-up equipped with a monochromatic Al K α X-ray source (1486.6 eV; anode operating at 14.5 kV and 30 mA) and a high resolution Gamdata-Scienta SES 2002 hemispherical analyzer. To reduce radiation damage to a minimum, the anode was preheated for 30 min prior to sample introduction into the measurement chamber. The base pressure in the measurement chamber was maintained at about 1×10^{-9} mbar during the measurements. The XP spectra were recorded in the fixed transmission mode with a pass energy of 200 eV. The energy regions for each element were each measured five times in succession with ten repetitions. Only the first five repetitions (25

scans per element) were averaged and analyzed due to the observed radiation damage in subsequent scans. The carbon peak was fitted using the CasaXPS limiting the peak positions and FWHMs. All spectra were calibrated using the C 1s C-C/C-H signal at 284.6 eV. Besides the aliphatic carbon contribution, the C-S peak from the thiol group was fitted at around 286.5 eV³⁴ for the pure carboxyl-terminated samples. For the pure trimethylammonium-terminated and the mixed samples the C-N peak was also fitted at around 286.5 eV³⁵ including inseparable C-S contributions at the same binding energy. The C-OOH peak was fitted at around 289 eV.²³

Nonspecific protein adsorption. The nonspecific adsorption of proteins on the surface was measured by two different methods following previously published protocols.^{19,36} The first method determined off-line the thickness increase of the organic film on the slides after 30 min of immersion in a protein solution by spectroscopic ellipsometry. The substrates were pre-immersed in 1.5 mL phosphate-buffered saline solution (PBS, Fisher scientific, pH 7.4) for 20 min on a shaker (60 rpm), before 1.5 mL of a 2 mg/mL protein stock solution was added to obtain a final concentration of the protein of 1 mg/mL for the experiment. The substrate was kept immersed for another 30 min in the 1 mg/mL solution before being gently washed with 300 mL of MilliQ-water while crossing the water/air interface. In the second method, protein adsorption was monitored in-line by using surface plasmon resonance spectroscopy (SRC7000DC, pump SR7500, autosampler SR7100, Reichert). Prior to protein injection, PBS buffer was injected with a flow rate of 200 μ L/min. Once a stable baseline was established, 100 μ L of protein solution (1 mg/mL, in PBS) was injected for 10 min at a flow rate of 10 μ L/min. Reversibly adsorbed protein was removed by injecting PBS for another 15 min at 10 μ L/min. The shift of the SPR signal before and after the protein injection phase indicated the amount of protein irreversibly bound to the surface.

Dynamic Attachment assay of *Navicula perminuta*. Dynamic microfluidic measurements of the accumulation of the diatoms were conducted following previously published protocols.^{37,38} A suspension with the concentration of 3.0×10^6 cells/mL in seawater (SW) was prepared from a continuous *Navicula perminuta* culture. IBIDI sticky slides 0.1 (IBIDI) were placed onto the samples coated with the self-assembled monolayers to form a microfluidic channel (150 μ m height). The suspension was pumped through the channel for 90 min exerting a constant wall shear stress of 0.16 Pa followed by a 10 min rinsing step with saltwater (SW, CaribSea, USA, pH 8.0) to remove non-settled diatoms. For quantification, 30 fields of

view from the central part of the channel were acquired by phase contrast microscopy (Nikon Eclipse TE2000-U, 10x phase contrast objective Nikon CFI Plan Fluor DLL NA 0.3, Nikon). Automatic diatom counting was conducted using the NIS Elements software (Nikon). Analysis of statistical significance was done by ANOVA with post-hoc Tukey test with a significance level of $p = 0.05$.

Zeta potential measurement. *Navicula perminuta* (10^6 cells/mL) were suspended into a mixture of SW and MilliQ (1:3). The zeta potential was measured at 25°C with a Malvern Instruments Zetasizer nano series, using the DTS1070 cell. For each of three different freshly prepared diatom suspensions, the zeta potential was determined 6 times.

Settlement and removal of zoospores of *Ulva linza*. Zoospores were obtained from mature plants of *Ulva linza* collected from Craster, U.K. Following the method of Callow et al,³⁹ a suspension of zoospores (10 mL; 1×10^6 spores mL⁻¹) was added to individual compartments of quadriPERM® dishes (Sarstedt Ltd.) containing 6 replicates of each sample. After 45 min in the dark at 20°C, the slides were washed by passing backwards and forwards 10 times through a beaker of 0.22 µm filtered artificial seawater (ASW, Tropic Marin® salts) to remove unsettled (i.e. swimming) spores. Slides were fixed using 2.5% glutaraldehyde (Thermo Fisher Scientific, USA) in ASW. The density of zoospores attached to the surface was counted on each slide using an image analysis system attached to a fluorescence microscope (Zeiss Axioscope 2 plus). Spores were visualized using the autofluorescence of chlorophyll (excitation and emission: 546 and 590 nm). Thirty fields of view (each 0.15 mm²) were recorded and analyzed on each slide. Zoospore removal was determined using another three slides coated with the SAM and zoospores settled for 45 min by the method above. Slides were exposed to a shear stress of 52 Pa in a specially designed turbulent flow water channel.⁴⁰ Slides were then fixed. The number of spores remaining attached was counted and compared to the number on the control slides after settlement.

Results and Discussion

Surface characterization

Table 1 SAM film structure and thicknesses as determined by spectroscopic ellipsometry and static water contact angle (WCA) of the self-assembled monolayers. Reported values are the average of 20 replicate slides ± standard deviation.

Thiol compounds	Distribution of charges at the interface	SAM thickness [nm]	Water contact angle [°]
MUDTMA		1.4 ± 0.1	31.0 ± 0.9
MUDTMA + MOA		1.3 ± 0.2	24.0 ± 0.6
MUDTMA + MDDA		1.5 ± 0.2	17.9 ± 0.7
MUDTMA + MHDA		1.7 ± 0.2	25.4 ± 0.9
MDDA		1.5 ± 0.3	28.6 ± 1.0

Mixed charged SAMs were obtained by immersing the gold-coated samples into a 1:1 mixture of the corresponding cationic and anionic thiol solutions. To verify that the samples had the desired characteristics, the SAMs were examined by spectroscopic ellipsometry to measure their thickness, and by water contact angle goniometry to determine their wettability (Table 1). The layer thicknesses of the charged and the charge-equilibrated self-assembled monolayers were all between 1.4 and 1.5 nm. The values for the positively charged references and the mixed charged SAM of **MDDA+MUDTMA** correspond to literature values.^{18,23} The increasing layer thickness of the mixed SAMs containing MOA, MDDA or MHDA correlates with the increasing aliphatic chain length of the anionic thiols, ranging from 1.3 nm (**MOA+MUDTMA**) to 1.7 nm (**MHDA+MUDTMA**). The charged SAMs had a static water contact angle (WCA) of 31° (**MUDTMA**) and 28.6° (**MDDA**). In comparison to the charged references, all zwitterionic mixed charged surfaces reduced the contact angles. Within the mixed charged surfaces, the SAM consisting of **MDDA+MUDTMA** was somewhat more hydrophilic (18°) compared to monolayers of **MOA+MUDTMA** (24°) and **MHDA+MUDTMA** (25°). Accordingly, charge-equilibrated surfaces with an equilibrated distribution of charges

within a single plane seemed to enhance their interaction with water (e.g. by hydrogen bonds) compared to the surfaces of SAMs with a charged terminal group.

Figure 2. XPS spectra of the C 1s region of **MOA+MUDTMA** in comparison with pure **MOA** and **MUDTMA** (left), **MDDA+MUDTMA** in comparison with pure **MDDA** and **MUDTMA** (middle), and **MHDA+MUDTMA** in comparison with **MHDA** and **MUDTMA** (right). All C-C 1s signals were normalized to the aliphatic signal at 284.6 eV.

X-ray photoelectron spectroscopy XPS was used to analyze the chemical composition of the pure and the mixed SAMs (figure 2). For the carbon C 1s core level, differently bound carbon moieties can be distinguished. While C 1s signals of aliphatic carbons are at 284.6 eV, carbon of a carboxyl group that is bound to oxygen atoms is shifted to 289.0 eV,²³ and carbon next to nitrogen or sulfur to 286.5 eV.³⁵ For all carboxyl-group containing SAMs (**MOA**, **MDDA**, **MHDA**) the carbon bound to oxygen appeared at approximately 289.0 eV, while for the quaternary ammonium compound (**MUDTMA**), a peak of the carbon atoms adjacent to the nitrogen at 286.5 eV can be detected including inseparable C-S contributions. The intensities of the COOH signal at 289.0 eV as well as the C-S signal at 286.5 eV of the pure carboxylic acids decreased with increasing chain length as expected as the aliphatic peak was used for normalization. However, the COOH signal of the mixed SAM coatings followed a different trend: the lowest relative intensity was observed for **MDDA+MUDTMA**. While the decrease of signal intensity from **MOA+MUDTMA** to **MDDA+MUDTMA** could be attributed to the increased carbon spacer length, the increase in intensity from **MDDA+MUDTMA** to **MHDA+MUDTMA** could be explained by the exposure of the carboxylic group at the interface. The combined C-N/C-S signals at 286.5 eV visible in all mixed SAMs were larger than the pure C-S signal of the corresponding pure carboxylic SAM of **MDDA**, and their normalized intensity declined upon increased chain lengths of the carboxylic acid. The trend that the relative increase of the C-N/C-S signals over the pure C-S signal for the three mixed SAMs became smaller with

increasing chain length might have had an additional contribution from the positioning of the ammonia group within the SAM. While the nitrogen is exposed at the interface in the **MOA+MUDTMA** coating (in analogy to the increased relative intensity of the COOH signal in the case of **MHDA+MUDTMA**), it is most likely buried in the **MHDA+MUDTMA** film, which would reduce the peak intensity additionally. Due to this complicated steric situation and the different spacer lengths, it was not possible to quantify the ratio of **MUDTMA** to the various carboxylic acids within the respective SAMs. Nevertheless, the data clearly show qualitatively the presence of both anionic and cationic species in the mixed assembled SAMs.

Protein Adsorption

The resistance of the SAMs against non-specific adsorption of proteins was evaluated by spectroscopic ellipsometry, and in-situ by surface plasmon resonance spectroscopy (SPR). The organic layer thickness after 30 min of incubation in a protein solution was determined by spectroscopic ellipsometry to allow the comparison to previously published data on the protein resistance of mixed charged SAMs.²³ Additionally, SPR in-situ experiments were used to compare the shift in plasmon resonance before and after protein exposure to determine the amount of irreversibly bound protein. Two model proteins, which differed in size and net-charge, were used. The nonspecific adsorption at pH 7.4 of the slightly negatively charged fibrinogen (molar mass 340 kDa, pI 5.5⁴¹) was compared to the nonspecific adsorption of the positively charged lysozyme (molar mass 14 kDa, pI 10.9⁴²).

Figure 3. Irreversible adsorption of fibrinogen and lysozyme on the mixed SAMs as determined by (A) SPR and (B) spectroscopic ellipsometry. Reported values are the average of 3 measurements on 3 replicate slides. Error bars are the standard deviation ($n=3$).

The protein adsorption followed the same general trend in both protein assays. As expected, the highest fibrinogen adsorption was found on the positively charged trimethylammonium-terminated monolayers of **MUDTMA**, which is known to rapidly accumulate most proteins.^{23,24} In the ellipsometry assay, a protein adlayer of 5.5 nm was determined, while a density of 284 ng/cm² protein was adsorbed in the SPR assay (figure 3). In contrast, the adsorption of the positively charged lysozyme on the cationic SAMs of **MUDTMA** was very low. Adsorption of lysozyme was highest on the anionic monolayer of **MDDA** (2 nm, 71 ng/cm²). Again, this finding was expected since the SAM surface and the protein have opposite charges. The lower adlayer thickness and the different magnitude of the SPR-shift of adsorbed lysozyme (71 ng/cm² **MDDA**) compared to fibrinogen (233 ng/cm² **MDDA**) can be explained by the size difference of the proteins. While lysozyme has a size of 4 nm x 3 nm x 3 nm, fibrinogen with a size of 47 nm x 5 nm x 5 nm is much larger.⁴¹

All mixed charged SAMs showed markedly reduced fibrinogen fouling compared to the net charged SAMs, as well as markedly reduced lysozyme fouling compared to the negatively charged SAM of **MDDA**. The lowest protein adsorption was found on the mixed charged monolayers made from the thiols that have the same aliphatic chain lengths, namely **MDDA+MUDTMA**. This observation indicates that the anti-fouling properties of mixed-charged SAMs are not solely characterized by electrostatic repulsion between the exposed charge and the protein.

The results of the SPR assays are statistically much more significant than the results of the assay utilizing spectroscopic ellipsometry. An ANOVA with post-hoc Tukey test with a significance level of $p < 0.05$ indicates that the SPR results allow a significant distinction between all surfaces for lysozyme, and for all surfaces except **MOA+MUDTMA** and **MDDA+MUDTMA** for fibrinogen. The ellipsometric assay only allows for a significant discrimination between the lysozyme adsorption on **MDDA** and all other surfaces and the fibrinogen adsorption on **MUDTMA** compared to all other surfaces. The differences between the other non-mentioned surfaces are not statistically significant on a level $p < 0.05$. The in-situ SPR experiments are in this case superior to the assay with spectroscopic ellipsometry due to the higher significance of the results gained.

Dynamic accumulation of *Navicula perminuta* in a microfluidic assay

Figure 4. Diatom attachment on the self-assembled monolayers, normalized to attachment on pure MUDTMA SAMs. The microfluidic assay was performed over 90 min at a wall shear stress of 0.16 Pa. Reported values are the average of 3 measurements on 3 replicate slides. Error bars are the standard deviation ($n=3$).

The relative diatom accumulation on the various SAMs is shown in figure 4. In line with the protein adsorption assay, a high number of diatoms attached on MUDTMA. The monolayer **MOA+MUDTMA** revealed the weakest antifouling performance. This contrasts with the protein adsorption assays in which the monolayer of **MOA+MUDTMA** presented the second-best performance against the nonspecific adsorption of both proteins that were tested. In the case of diatoms, this specific mixed SAM, in which the cationic groups are exposed at the interface, performed rather poorly and exhibited high diatom densities similar to those on the **MUDTMA** SAMs. The **MDDA+MUDTMA** SAM provided the best fouling protection against the diatoms as it had against the proteins. The poor performance of both the cationic monolayer and the mixed **MOA+MUDTMA** monolayer suggests that an excess positive charge directly at the interface can favor diatom accumulation despite the net neutral charge of the layer. **MOA+MUDTMA** and **MUDTMA** can be discriminated from all other chemistries on a significance level of 5% in an ANOVA with post-hoc Tukey test, but cannot from each other. Also statistically significant is the difference between diatom accumulation on

MDDA+MUDTMA and **MHDA+MUDTMA**, while the differences between the other mixed SAMs were not statistically significant ($p < 0.05$).

Zeta potential of diatoms

To understand the adhesion behavior of diatoms on charged SAMs, zeta potential measurements were carried out in order to establish the surface charge of diatoms using the Smoluchowski approximation. To avoid corrosion of the gold electrodes during the measurements and to reduce the conductivity, the potential measurements were carried out in diluted sea water at a pH of 8.0. The zeta potential (ζ) of the diatom *Navicula perminuta* was found to be -11.5 ± 0.3 mV.

Settlement and removal of zoospores of *Ulva linza*

In addition to diatom accumulation, the settlement and removal of zoospores of the green alga *Ulva linza* was tested. While diatoms settle on surfaces as a result of passive encounters, the zoospores of *U. linza* actively explore surfaces and settle on favorable ones. The results of the *U. linza* zoospore settlement and removal assays are shown in figure 5. Again, zoospore settlement was highest on the cationic **MUDTMA** SAM and the mixed charged **MOA+MUDTMA** SAM, in which the cationic groups are exposed at the interface due to the short alkyl chains of the anionic thiol. The best protection against spore settlement was provided by the anionic **MHDA** SAM, and the mixed charged monolayer of **MHDA+MUDTMA** (4% compared to MUDTMA). The mixed SAM was particularly effective in which the anionic groups should dominate the interface due to the longer alkyl chains of **MHDA** compared to **MUDTMA**. Zoospore settlement was also reduced on mixed SAMs of **MDDA+MUDTMA** (11% compared to MUDTMA), in which the cationic and the anionic groups were equally distant from the surface and thus both similarly exposed at the interface. However, the protective effect was weaker than for the SAMs of **MHDA** or **MHDA+MUDTMA**. Before the shear stress was applied, the *U. linza* settlement on all surfaces was significantly different ($p < 0.05$).

The attachment strength of the zoospores of *U. linza* to the different SAMs was tested by exposure of the fouled samples to a turbulent water flow at a wall shear stress of ≈ 52 Pa. The higher the fraction of algae removed, the lower was the attachment strength to the surface. The results displayed in figure 5B show that the binding strength was lowest on the mixed SAM of **MDDA+MUDTMA**, from which 63% of the settled zoospores were removed. 0% and

14% removal of spores was determined for the monolayers with the lowest spore density, **MDDA** and **MHDA+MUDTMA**, respectively. As in the other fouling experiments described above, **MHDA+MUDTMA** SAMs offered the best protection and from algal spores. Whilst the mixed monolayer of **MDDA+MUDTMA** was the best performing SAM in the protein resistance assays and in the diatom accumulation experiments; its resistance against the settlement of zoospores was only moderate and even weaker than that of the negatively charged monolayer of **MDDA** before a shear stress was applied. However, a high fraction of the zoospores adhered only weakly on the mixed **MDDA+MUDTMA** layer and could be easily removed by shear in contrast to the other SAMs studied. After the shear-induced removal, the difference between **MDDA+MUDTMA** and **MDDA** was no more statistically significant, while all other surfaces were significantly different.

Figure 5. Settlement of zoospores of *Ulva linza* on the different SAMs. A: Density of settled zoospores on the charged and mixed charged monolayers after 45 min of settlement (gray), and after exposure to a shear stress of 52 Pa (blue). Reported values are the average of 90 measurements on 3 replicate slides. Error bars indicate the standard deviation (n=90). B: Efficiency of spore removal from the monolayers after applying a shear stress of 52 Pa. Reported values are the average of 90 measurements on 3 replicate slides. Error Bars indicate the standard deviation (n=90).

To sum up, a set of zwitterionic SAMs with overall charge neutrality, but varying distance of the positively/negatively charged groups from the surface and thus at the interface to the water, was developed. Prior to the biological assays, the SAMs were characterized via contact angle goniometry, spectroscopic ellipsometry, and XPS. The relative thickness of the mixed monolayers correlated with the chain length of the thiols, increasing with the number of methylene units in the anionic thiols. The highest wettability was found on the mixed charged monolayer with equal chain length.

The relative positioning of the anionic and cationic groups in mixed charged SAMs with respect to the interface had a significant impact on their antifouling ability. All mixed charged SAMs reduced the non-specific protein adsorption compared to the cationic **MUDTMA** and anionic **MDDA** monolayer. The observation that any surface excess charge in the SAM enhanced attachment of proteins is in line with previous reports by Ekblad et al,⁴³ who obtained a higher protein adsorption on the charged end positions within a charged polymer gradient, and by Holmlin et al,¹⁸ who observed a significant reduction of non-specific adsorption on mixed charged SAMs compared to the corresponding single component charged monolayers. Fibrinogen adsorption was highest on the cationic SAM of **MUDTMA**; this may be expected due to the opposite net charges of the protein and the coating. Within the tested mixed monolayers, **MHDA+MUDTMA** was the least effective in repelling both proteins, lysozyme and fibrinogen.

The mixed SAM of **MOA+MUDTMA** displayed the weakest performance against both marine fouler species. This is likely due to the terminating cationic quaternary ammonium group, as both of the tested marine organisms are characterized by a negative zeta potential (*Ulva linza* = -19.3 mV).⁴⁴ The strong influence of the terminally charged groups located at the interface and the zeta potential is of fundamental interest, as the high salinity of saltwater results in a very small Debye length (less than 0.3 nm).⁴⁵ Despite this low Debye length, the influence of charged surfaces in saltwater on fouling has been demonstrated in several other studies. Schilp et al⁴⁶ revealed that zoospores of *Ulva linza* have a reduced tendency to settle on negatively charged surfaces,⁴⁷ which is in line with the low spore density found on SAMs of **MDDA** in this work.

The overall observed trends in protein adsorption and the accumulation of zoospores between the mixed charged **MDDA+MUDTMA** and the charged monolayers **MDDA** and **MUDTMA** are largely in line with previously published results from Bauer et al,²³ where only a small difference with a slightly higher spore density of the zoospores was found on mixed SAMs of **MDDA+MUDTMA** compared to SAMs made only from **MDDA**.

The combined results reveal that the appropriate spatial arrangement of the anionic and cationic charges is also important in addition to the formal charge equilibrium within a zwitterionic coating. The possibility of the negatively and positively charged groups coming close together at the interface, i.e., to be placed at the same distance from the support's

surface, is crucial for the effectiveness of zwitterionic coatings. Our experiments show that even though formally the positive and negative charges were equilibrated within the coating, the forced separation into cationic and anionic sublayers adversely affected the fouling inertness of the film. As might be expected, the preferential positioning of the positive charges directly at the interface resulted in the weakest fouling resistance, except for the rare scenario in which the foulant species itself was strongly positively charged. Overall, the best antifouling performance was achieved when charge equilibrium was molecularly 'imprinted' right at the interface.

While densely packed self-assembled monolayers are most useful for model studies, for practical reasons it is difficult to imagine the use of zwitterionic SAMs for other than sensor applications.¹³ Therefore, as a more widely applicable alternative, coatings based on zwitterionic polymers are currently intensely explored for fouling protection.^{10,13,16,47} Characteristically, their reported antifouling behaviors lack a clear correlation with the molecular structure up to now, that would enable their rational design.^{13,16} The results of this study raise the interesting question of how the charge distribution within SAM films can be implemented in new designs of polyzwitterions in order to improve their antifouling performance systematically. In fact, apart from the known effect of the bonding distance separating the cationic and the anionic groups in the zwitterionic moieties on fouling resistance,²⁶ there are only a few reports that demonstrated the effects of specific orientations of the anchoring of the zwitterionic moieties within the polymers' molecular structure.^{19,28,30,48} Unfortunately, such polymer films (most often representing hydrogels) have the possibility of conformational rearrangements and overall low steric constraints. Thus, it is by no means clear (yet) how a predefined chemical orientation of the zwitterionic units within the macromolecules determines their molecular orientation at the aqueous interface, and how the formation of an even locally charge-balanced interface could be favored by an appropriate chemical design.

Summary & Conclusion

Equilibrated mixed charged thiol monolayers (SAMs) that mimic zwitterionic systems were prepared and characterized. Their ability to prevent biofouling was evaluated for a set of model proteins and marine fouling organisms in laboratory assays and compared to reference

SAMs made exclusively of either cationic or anionic thiols. The aliphatic chain length of the cationic component was kept constant in the mixed charged SAMs, while the length of the anionic component was varied, to be shorter, equal, or longer than the cationic thiol's chain. Even though the positive and negative charges were equilibrated in all these mixed charged SAMs, the relative positioning of the cationic and anionic groups within the SAM had a major impact on their fouling inertness. Overall, the mixed charged SAMs were less prone to fouling than the charged references. However, when using mixed thiols of differing chain lengths, the forced separation into cationic and anionic sublayers adversely affected the fouling inertness of these SAMs compared to ones made of thiols of equal chain length, which showed the best antifouling performance. Notwithstanding the generally superior performance of the mixed charged SAMs, results depend also on the species of fouling organism. In specific scenarios, electrostatic repulsion can prevent the fouling of charged SAMs by fouling organisms that have an identical charge. The results underline the importance of equilibrated charges in a zwitterionic system. Still, beyond this requirement, an appropriate spatial arrangement of the cationic and anionic groups towards the interface is crucial as well. For practical polyelectrolyte coatings rather than model SAMs, the specific incorporation of the zwitterionic side moieties, as well as their steric freedom (allowing reorientation) are of the utmost importance for spatial charge equilibration, and consequently, for the high inertness of polymeric antifouling coatings.

Acknowledgements

The authors are grateful to the Deutsche Forschungsgemeinschaft (DFG), grants LA 611/14-1, LA 611/14-2, RO 2524/4-1, and RO 2524/4-2, the Office of Naval Research (ONR), grant N00014-16-1-2979 and N0014-20-12244 (AR), N00014-16-1-2988 (ASC) and N00014-16-1-3125 (ASC and JAF), and the Deutsche Forschungsgemeinschaft (DFG) – GRK2376 / 331085229 for providing financial support.

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