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Amphiphilic alginates for marine antifouling applications

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Abstract

Amphiphilic polymers are promising candidates for novel fouling release coatings for marine applications. We grafted amphiphilic alginates with fluorinated side chains to glass and silicon substrates and characterized the obtained films by contact angle goniometry, spectroscopic ellipsometry, XPS, and ATR-FTIR. The potential to inhibit protein attachment was tested against four different proteins and intermediate fluorine loadings showed the strongest reduction with respect to hydrophobic, aliphatic controls. A similar trend was observed in dynamic attachment experiments using *Navicula perminuta* diatoms and settlement experiment

with zoospores of the green algae *Ulva linza*. The results indicate that amphiphilic alginates are promising natural and renewable biomacromolecules that could be included in future protective coating technologies.

Introduction

Silicone paints are frequently applied to vessels as non-toxic marine protective coatings. They facilitate an easy removal of fouling organisms (fouling-release, FR) so that fast moving ships are capable to self-clean¹. Recently, coating research started to increasingly explore amphiphilic polymers²⁻⁴. In this class of polymers, hydrophobic and hydrophilic chains are arranged in close vicinity. While the exact mechanism by which such coatings reduce fouling is still under discussion, the presence of hydrophilic groups favors hydration and lowers the shear stress required to remove slimes^{1,5}. In particular the ability of an amphiphilic coating to reorganize under water, including segregation effects, seem to be advantageous for their performance. Recent innovations include the incorporation of amphiphilicity to SEBS polymers⁶, the use in PDMS-based polymers^{7,8}, incorporation into polyelectrolyte multilayers⁹, and the application of hyperbranched polymers that combine PEG and perfluorinated hydrocarbons^{10,11}. In most of the cases, the introduced amphiphilicity enhanced the fouling-release behavior in laboratory or in field studies. Amphiphilic coatings can form nano-domains which are currently hypothesized to contribute to the exceptional fouling-release properties¹⁰.

Since renewable materials are increasingly considered, polysaccharides offer a promising class of hydrophilic materials as alternatives to PEG. Besides their biocompatibility, they are capable of binding water tightly and bear numerous functional groups with potential for further chemical modification. Most importantly, they occur in nature and are fully biocompatible. Biomedical applications provided early examples of their antifouling capabilities¹²⁻¹⁴. For marine antifouling applications, self-assembled monolayer (SAM)-based

studies have demonstrated the importance of the structure of oligosaccharides to produce the most effective resistance to fouling organisms^{15,16}. The integration of additional hydrophobic or amphiphilic components into the polysaccharide hydrogel has been shown to increase **their** antifouling performance^{15,17,18}. In addition, polysaccharides, such as hyaluronans, chondroitins and alginates, showed promising performance as marine antifouling materials^{18–20}. A drawback of such coatings is that the carboxyl groups of the α -L-guluronate tend to bind bivalent cations within the polysaccharide matrix¹⁹, which can **be understood on the basis of the** “egg box”-gelation model²¹. Since this phenomenon results in changes within the film structure and reduces the water binding capability, some of the coatings show a decreased performance¹⁹. As blocking of the free carboxyl groups inhibits the binding of bivalent cations, different polysaccharides were post-modified with 2,2,2-trifluoroethylamine (TFEA) via EDC/NHS chemistry, which led to average fluorination degrees of 6-8% of the available carboxyl groups^{17,18}. Besides the inhibition of the gelation process, an amphiphilic character was obtained and the water contact angle of the inherently hydrophilic polysaccharides was shifted towards the Berg limit²². Such coatings show enhanced antifouling activity compared to the unmodified controls^{17,18}. In particular alginates are a very interesting class of biomacromolecules as they occur in the EPS of biofilms²³ and in the mucilage and cell walls of seaweeds²⁴. The industrial alginate production amounts to 40,000 tons per year²⁴ and provides a material that is **inherently abundant** in nature, renewable, biocompatible, and non-toxic.

This work focuses on a hydrophobic capping route for alginates leading to enhanced fluorine loadings. The capping reagent 2,2,3,3,3-pentafluoropropylamine (PFPA) was chosen instead of TFEA in order to obtain a higher fluorine loading after modification. To enhance the coupling efficiency, the coupling step was done in solution prior to the grafting step. **Alginic acid (AA)** based coatings with different degrees of PFPA modification were prepared,

characterized and studied with respect to their protein resistance as well as settlement and attachment of zoospores of the green alga, *Ulva linza*, and the diatom *Navicula perminuta*.

Experimental Section

Fluorination of alginic acid and preparation of polysaccharide coatings

Figure 1 Reaction scheme of polysaccharide modification and the immobilization reaction. a) NHS/EDC-mediated capping of AA-carboxylate groups with PFPA. **Stoichiometric** ratios of PFPE and the monosaccharide units in the coupling solution was varied between 25% and 75% of the functional groups. b) Immobilization reaction of (modified) AA onto an amine-terminated silane surface.

PFPA was purchased from Alpha Aesar (Germany). All other **chemicals** were purchased from Sigma-Aldrich (Germany). All chemicals were used without further purification. Deionized water was purified with a Siemens Water Technologies system. Si wafers (Siegert Wafer, Germany) and Nexterion B float glass slides (Schott, Germany) were used as substrates.

To functionalize the alginate (molecular weight 100 – 200 kDa, Sigma, Norway #71238) with PFPA, it was dissolved in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution (10 mM, pH 6-7) at a concentration of 1 mg/mL and activated via 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 50mM) and N-hydroxysuccinimide (NHS, 10mM) for 15 min. Subsequently, PFPA was added in different stoichiometric quantities (25%, 50%, 75%) to achieve the desired degree of modification of the carboxyl groups. The solution was stirred for 18h at room temperature. The amount of PFPA was adjusted to achieve theoretical degrees of modification between 25% and 75% of the available carboxylate groups (Figure 1a).

For the surface grafting step, the substrates were first coated with a layer of 3-aminopropyltrimethoxy silane (APTMS) following published protocols^{18,19}. The surfaces were cleaned in solvents of increasing polarity (toluol, ethyl acetate, ethanol, MilliQ water; 30sec each). The dried samples were activated in an O₂ plasma (O₂ pressure 0,4 mbar, 80 W, 22 kHz, miniFlecto-PC-MFC, GaLa Instrumente GmbH, Bad Schwalbach, Germany) and afterwards immersed into a 5% (v/v) APTMS solution in acetone and sonicated for 30 min under a nitrogen atmosphere. The surface coupling of the fluorinated AA polymer was performed by a reactivation of the remaining free carboxyl groups with EDC/NHS chemistry. After 15 min of activation, the APTMS-coated substrates were immersed into the polysaccharide solution on a shaker table at room temperature for 18 h. To terminate the reaction, the solution was diluted by an 8-fold excess of deionized water. The samples were kept on a shaker for another 2 days and the water was exchanged every 24 h. All samples were kept under MilliQ water until characterization and biological evaluation.

Spectroscopic ellipsometry

All film thicknesses were determined by spectroscopic ellipsometry (M-2000, J. A. Woollam, USA; CompleteEASE software package) operating in a wavelength range of 200–1000 nm at three different angles of incidence (65°, 70°, and 75°). The ellipsometry data were modeled as single organic layer on silicon and the wavelength depending refractive index was described by a Cauchy model with the parameters $A = 1.45$, $B = 0.01$ and $C = 0$. The presented data are the average of at least three different measurements on at least three different replicates. Error bars represent the standard deviation of the data.

Water contact angle goniometry

A custom-built goniometer was used to obtain the static water contact angle (CA). A droplet of MilliQ water was placed on the surface, monitored by a CCD-camera and afterwards analyzed regarding its shape by Young's equation. The data represent the average of three different replicates with three different spots measured on each replicate. Error bars represent the standard deviation.

ATR-FTIR

A Tensor 27 spectrometer (Bruker, Ettlingen, Germany) with an Ge-ATR cell and a liquid N₂-cooled MTC detector was used to obtain the ATR-FTIR spectra. Prior to the measurements, the spectrometer was purged with nitrogen for 30 min. As background the spectrum of the Ge-ATR crystal was used.

X-ray photoelectron spectroscopy

To determine the derivatization efficiency of the polysaccharide films, X-ray photoelectron spectroscopy (XPS) was applied (electron spectrometer with a hemispheric analyzer Type CLAM2, VG Scientific, meanwhile Thermo Fischer Scientific, Waltham, Massachusetts,

USA). As X-ray source, a polychromatic aluminum anode ($K_{\alpha} = 1486$ eV) was used. The spectra were calibrated on the Si2p substrate signal at 103.4 eV and fitted with OriginPro2017G by applying a background subtraction according to Shirley²⁵ and a Voigt profile with a Lorentz to Gauß ratio of 4:1. To determine the elemental ratios between carbon (carbonic acid and amide contribution at 288 eV) and fluorine (689 eV), cross sections ($\sigma_{C1s} = 1.0$, $\sigma_{F1s} = 4.43$) from Scofield were used²⁶.

Coating stability test

The samples were immersed into salt water mixed together from the seven major ingredients of sea water²⁷ and filtered through a 0.45 μ m syringe filter for the desired time period. Afterwards, the surfaces were rinsed with MilliQ water to remove adsorbed salts and dried in a stream of nitrogen. The film thickness was determined by spectroscopic ellipsometry after each immersion step.

Protein adsorption assay

Table 1 Properties of proteins used for the adsorption experiments.

Protein	Molecular weight (kDa)	Net charge at pH 7.4	Protein concentration
Lysozyme	14.7	+	2 mg/mL
Pyruvate kinase (PK)	237	+	160 units/mL
Bovine serum albumin (BSA)	66.5	-	2 mg/mL
Fibrinogen	340	-	2 mg/mL

For the protein adsorption assays, Si wafers were used as substrates to provide the reflectivity needed for the spectroscopic ellipsometry experiments. Various proteins, which differ in

molecular weight and net charge at a pH of 7.4 (Table 1), were studied regarding their adsorption behavior. The selection of proteins and the procedure of the assay followed previously published protocols^{28,29}. The assay in its present form was developed in order to consider a certain range of protein charges and sizes. The attachment of these four proteins has so far been investigated on a large range of surface chemistries and allows now a comparative discussion. For this reason, non-resistant dodecanethiol (DDT purchased from Prochima, Spot, Poland) was assembled as SAM on gold-coated Si wafers and included in the assay as a non-resistant control. After a preincubation step in phosphate-buffered saline (PBS, 0.01M pH, 7.4) for 20 min, an equal volume of protein solution (2 mg/mL dissolved in PBS) was added and incubated for an additional 30 min on a shaking table. Subsequently, the samples were flooded with ample amounts of deionized water and carefully rinsed with MilliQ water when passing through the air/water interface to prevent the deposition of Langmuir films. After drying in a stream of nitrogen, the protein thickness was determined by spectroscopic ellipsometry. The adsorbed protein adlayers were modeled in the same way with the same parameters for the Cauchy model as the grafted polysaccharide films.

Settlement of zoospores of green algae

Zoospores were released from mature plants of *U. linza* and settled on the test surfaces following previously published protocols³⁰. Six replicates of each coating, as well as a hydrophobic *n*-octadecyltrichlorosilane (OTS) control were placed in individual compartments of quadriPERM dishes (Greiner Bio-One, Germany) and incubated in filtered artificial seawater (0.22 μ m) for 1 h prior the experiment. The OTS controls were chosen due to hydrophobic properties, low protein resistance, and typically high numbers of settled zoospores. After pre-incubation, the seawater was exchanged for a zoospore suspension (10 mL; 1×10^6 spores / mL) and again incubated for 45 min in darkness at 20°C. The slides were washed by passing back

and forth through a beaker of seawater for 10 times. Spores were then fixed in 2.5% glutaraldehyde solution in seawater. Using the autofluorescence of chlorophyll within the spores for visualization, the density of the attached spores was determined. Thirty fields of view (each 0.15 mm²) were acquired with fluorescence microscopy on each slide. Significant differences in spore settlement densities were determined using ANOVA and Tukey tests with a confidence value of $p < 0.05$.

Microfluidic diatom accumulation assay

Diatom culture and the microfluidic accumulation assay followed previously published protocols³¹. The diatom *Navicula perminuta* was used as a model organism. For the assay, the culture medium was exchanged for filtered seawater (FSW pH 8) and a total cell concentration of 2 million/mL was adjusted using OD₄₄₄. The microfluidic experiment was performed on coated Nexterion B glass slides with OTS as non-resistant control. IBIDI sticky slides 0.1 (IBIDI, Germany) were glued onto the samples and formed the channel system. For each coating, three accumulation assays were conducted at a constant wall shear stress of 0.18 Pa over 90 min. To remove any unattached diatoms, pure FSW was rinsed through the channels at the same speed as for the accumulation assay. Thirty fields of view (0.5478 mm²; 830 μ m x 665 μ m) in the middle of the channel were recorded with an inverted video microscope (Nikon Ti-E, Nikon Japan; 10-x phase contrast objective Nikon CFI Plan Fluor DLL NA 0.3, Nikon Japan). To account for slight variations in the physiological state of the diatoms, OTS was included as non-resistant control and the data obtained were normalized to the attachment on OTS (absolute diatom densities on OTS varied between 237 mm⁻² and 542 mm⁻²). The result presented is the average of three independent assays. Statistical analysis was carried out in the same as for the *Ulva* zoospore assay.

Results

Preparation and characterization of the amphiphilic polysaccharide coatings

Table 2 Coating chemistries analyzed in this work include the unmodified alginates (AA) and three PFPA modified ones (AA25PF, AA50PF, and AA75PF). The stoichiometric PFPA content in solution for the coupling step was chosen between 25% and 75% with respect to the available carboxylate groups. This resulted in a modification degree of the carboxylate groups which was determined by XPS (third column). The obtained film thickness and its wettability was determined by spectroscopic ellipsometry and water contact angle goniometry.

Chemistry	Stoichiometric PFPA content	Modification degree	Contact angle (°)	Ellipsometric thickness (nm)
AA	0 %	0%	17 ± 3	1.7 ± 0.3
AA25PF	25 %	9 %	28 ± 4	2.4 ± 0.4
AA50PF	50 %	18 %	34 ± 3	2.5 ± 0.4
AA75PF	75 %	28 %	47 ± 3	2.8 ± 0.5

The impact of the degree of capping of carboxylate groups in the AA polymer on the thickness of the grafted macromolecules and on their wettability was determined. The coupling was carried out in solution by pre-activating the AA by EDC/NHS and subsequently adding the PFPA. The molar ratio of the PFPA in the coupling solution with respect to the monosaccharide units in AA varied between 25% and 75% of the available carboxylate groups. Both, the unmodified AA as well as PFPA-modified AA were grafted onto an aminosilane functionalized surface after reactivation of the remaining carboxyl groups by EDC/NHS. The unmodified AA coatings showed a thickness of $1.7 \text{ nm} \pm 0.3 \text{ nm}$ and hydrophilic properties with a CAs of $17^\circ \pm 3^\circ$. Introduction of fluorinated compounds increased the thickness of the coatings with variations within the error bars of the measurements between 2.4 nm and 2.8 nm (Table 2). Decisive differences were observed for the wettability of the different coatings. An increasing

load of fluorine-rich moieties increased the water contact angles. Coatings with the highest fluorine load reached a water contact angle of $47^{\circ} \pm 3^{\circ}$, which is an increase of 30° compared to the pristine AA (Table 2).

Figure 2 ATR-FTIR spectra of an APTMS film, an unmodified AA coating and three modified AA films with increasing amount of PFPA. The intensity of the spectra are normalized to the Si-O vibration at 1230 cm^{-1} .

In order to prove the PFPE modification and the successful grafting to the substrates, ATR-FTIR spectra were recorded. As shown in Figure 2, all coatings showed a distinct vibration mode at 1658 cm^{-1} that can be assigned to the deformation vibration of the terminal NH_2 groups.^{32,33} After grafting of AA to the APTMS coated substrates, the vibration at 1658 cm^{-1} is strongly enhanced and originates from the $\text{C}=\text{O}$ stretching vibration dominated by the newly formed amide bond. Additionally, the vibration at 1740 cm^{-1} can be assigned to the six membered lactone ring of AA.³³ In the PFPA modified layers, further amide bonds were formed, so that the amide I band at 1658 cm^{-1} becomes more intense with increasing amounts of PFPA. The same is true for the less intense amid II band at 1545 cm^{-1} .³² The 1460 cm^{-1} mode

can be found in all spectra and might originate from the CH₂ bending vibration from the underling APTMS layer and the coupled PFPA.³³

Figure 3 F1s photoelectron spectra of a plasma-treated silicon wafer, an unmodified AA film and three modified AA films with increasing PFPA content.

Additionally to the IR spectroscopy, XPS was used to chemically characterize the modified AA coatings. The successful coupling of PFPA was monitored using the F1s signal at 689 eV, which increased with increasing solution concentration of PFPA during the coupling reaction (Figure 3). To determine the fraction of PFPA modified carboxyl groups, the ratio between the F1s signal at 689 eV (Figure 3) and the carbonyl contribution to the C1s signal at 288 eV (supplementary figure 1) were calculated considering of the respective elemental cross sections by Scofield²⁶. The fluorination reaction resulted in 9.3%, 18.3% and 28.3% of PFPA modified carboxylic acid groups. Since the derivatization was performed prior to the grafting step, a uniform derivatization throughout the polysaccharide macromolecules can be assumed.

Stability of the polysaccharide coatings

Figure 4 Relative thickness change of polysaccharide coatings with different degree of PFPA modification after incubation in salt water. Thicknesses were determined by spectroscopic ellipsometry. Error bars represent the standard deviation (n=9).

To investigate the coating stability in media with high salinity, the surfaces were incubated in seawater for 14 days. The thickness change was measured by spectroscopic ellipsometry after different immersion times. Figure 4 shows that the overall thickness change for all four coatings varied by less than 20 % over the course of the two weeks. The unmodified AA coating was the most stable one since it lost only ≈ 1 % of its thickness during the incubation. The PFPA-modified coatings lost slightly more material, with the AA75PF coating losing the most (20 %). The stability in MilliQ water (data not shown here) was higher and variations for all coatings were less than 17 %. Over the short-term experiments of 90 min for the diatom accumulation and 45 min for the spore settlement, we are confident that all coatings were stable.

Figure 5 Protein adsorption on polysaccharide coatings with different degree of PFPA modification. Adlayer thicknesses were determined by spectroscopic ellipsometry. Error bars represent the standard deviation.

The surfaces were tested against the adsorption of different proteins, which varied in size and net charge at a pH of 7.4 (see Table 1). Figure 5 shows that all of the coatings reduced the adsorption of the proteins with respect to the DDT control significantly ($p < 0.05$). Most of the fluorinated coatings reduced the adsorption to values below the ones found on the unmodified AA coating. This was particularly pronounced for the adsorption of pyruvate kinase and fibrinogen, which was significantly lower ($p < 0.05$) on the PFPA-modified surfaces compared to AA. Comparing all PFPA-containing surfaces, a low to medium degree of modification led to the most efficient reduction of protein adsorption. For the highest degrees of fluorination, the protein adsorption was found to increase. In the case of lysozyme, the protein film thickness became even higher than on the pristine AA coating.

Settlement of zoospores of the green algae *Ulva linza*

Figure 6 *U. linza* spore density after the 45 min settlement assay. Each point is the average of 180 counts on six replicate slides. Error bars represent the 95% confidence limits.

Spores of the green algae *U. linza* are able to explore and select surfaces suitable for settlement^{34,35}. Figure 6 shows the density of zoospores on the polysaccharide coatings after the 45-min settlement assay. Spore numbers were significantly ($p < 0.05$) reduced on the AA, AA25PF, and AA50PF coatings with respect to OTS. On the AA75PF coatings that contained the highest PFPA content, spore density was significantly ($p < 0.05$) enhanced when compared to the hydrophobic control (Figure 6). The lowest spore density was observed at the lowest degree of PFPA capping (AA25PF). The result compares well with the protein adsorption data, in which a low to medium degree of PFPA modification showed the greatest inhibition of protein attachment.

297 **Dynamic** diatom accumulation assay

298
 299 **Figure 7** Diatom density relative to the non-resistant OTS control after the dynamic attachment
 300 experiment. The dynamic assay was performed at 0.18 Pa for 90 min. OTS was included as
 301 non-resistant control. Data represent the average of 30 fields of view on three replicates per
 302 chemistry. Error bars are the standard error.

303 In contrast to zoospores, diatoms like *N. perminuta* are non-motile in the water column and
 304 rely on currents and gravity to reach a surface^{36,37}. A microfluidic diatom test was used to probe
 305 the initial attachment of diatoms to the surface³¹. As previously reported, the initial contact of
 306 the diatoms is challenged by the presence of a 0.18 Pa wall shear stress which makes the assay
 307 perceptive for differences in initial attachment strength³¹. For all surfaces, a significant
 308 reduction in diatom accumulation was observed as compared to the non-resistant OTS control
 309 ($p < 0.05$). Significant differences in diatom attachment were identified between the AA- and
 310 PFPA-modified surfaces ($p < 0.05$). Within the fluorinated set, significant differences were
 311 observed between AA75PF and AA25PF as well as between AA75PF and AA50PF. The
 312 AA25PF and AA50PF modifications were not significantly different. In line with the zoospore

settlement assay, the highest reduction in attachment was found for the intermediate degrees of PFPA modification (AA25PF and AA50PF).

Discussion

A range of PFPA modified AA coatings was prepared and characterized. The efficiency of the modification was determined by XPS and three different PFPA molar ratios were added for the derivatization reaction that resulted in 9%, 18% and 28% functionalization of the carboxylic acid groups. This exceeds the capping efficiency from previous studies which was up to 8%^{17,18}. In addition, the fluorine content per capping group was increased by $\approx 70\%$ as PFPA was used instead of TFEA. Thus, the maximum fluorine content of the amphiphilic polysaccharide films was increased by $\approx 500\%$ as compared to our previous work^{17,18}.

While the grafting density was barely influenced by the derivatization, a shift (by up to 30°) towards larger water contact angles was observed with increasing PFPA loading. As the thickness of the grafted polysaccharide coatings did not change for the different degrees of functionalization, sufficiently free carboxylic acid groups were available for the grafting reaction. With contact angles of up to 47° , a wettability slightly below the Berg limit of 65° ^{22,38} was achieved. Previous experiments with ethylene glycols with different chemical termination showed a high resistance to proteins and marine fouling organisms in this wettability range³⁹.

All coatings were able to reduce the adsorption of the four selected model proteins compared to the hydrophobic DDT control surfaces. This result is in good agreement with previous protein adsorption experiments that used a post-grafting modification approach. The results show that low protein adsorption occurred for the intermediate loadings with 9 % and 18 % capping efficiency. At the highest loadings, and therefore the most hydrophobic surfaces, we found an adverse effect and an increase in the amount of adsorbed protein. It is well known from literature that fluorinated surfaces readily facilitate protein adsorption⁴⁰, surface conditioning²⁹, and

attachment of diatoms⁴¹ as they facilitate hydrophobic van-der Waals interactions. A balanced amphiphilicity with limited possibilities for hydrophobic interactions seems to be the ideally suited for optimized resistance. Also for the attachment of marine fouling organisms, the intermediate PFPA loadings reduced the attachment density of zoospores of *Ulva linza* and cells of the diatom *Navicula perminuta* to very low values. The observed reduction supports literature reports in which the amphiphilic concept has proven to be an effective way to enhance the fouling-release properties of surfaces⁴. The range of hydrophilic materials in such technologies can clearly be extended towards further hydrophilic biomacromolecules in particular from renewable sources.

Conclusion

Using a derivatization of hydrophilic alginates with fluorinated substituents provides a route to amphiphilic polysaccharides that show promising antifouling properties. Compared to recent publications we increased the fluorine loading per capping molecule and enhanced the capping efficiency for optimized amphiphilicity. Indeed, an optimized fluorine loading was detected that minimized attachment strength of diatoms and settlement of zoospores of the green algae *Ulva linza*. The data suggests that amphiphilic polysaccharides could be useful building blocks for modern fouling-release coatings as a natural, renewable biomaterial of maritime origin with excellent biocompatibility. It can be expected that such materials find their application in the next generation of environmentally benign coating technologies.

Associated Content

List of Supporting Information

- XPS C1s spectra for AA, AA25PF, AA50PF and AA75PF

362

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365

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