Layer-by-layer Constructed Hyaluronic acid / Chitosan Multilayers as Antifouling and Fouling-release Coatings

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Abstract

Polyelectrolyte multilayers (PEMs) consisting of hyaluronic acid (HA) and chitosan (Ch) are extensively studied for biomedical applications and suppress bacterial and protein attachment. Here we prepared and tested HA/Ch PEMs as marine fouling-release coatings. PEMs were constructed by layer-by-layer assembly using spin coating. The multilayers were crosslinked for enhanced stability in the sea water environment by chemical and thermal treatment. Protein-repelling properties of the crosslinked multilayers were investigated by surface plasmon resonance spectroscopy (SPR). The marine antifouling and fouling-release properties were tested against the settlement of zoospores of the green alga Ulva linza and the subsequent development and removal of sporelings. With spin coating and thermal crosslinking, a thick yet homogeneous coating was obtained with antifouling properties against marine algal zoospores indicating the potential of these compounds for application in protective coatings.

Introduction

Marine biofouling, which involves the attachment of macromolecules, bacteria and marine organisms to surfaces, has a severe impact on the environment and generates massive costs for maintenance and cleaning of ships and other man-made structures immersed in the sea\textsuperscript{1,2}. Historically, biocidal self-polishing copolymer coatings have been applied to ships’ hulls, but these are increasingly being replaced by non-toxic alternatives\textsuperscript{3}. Novel coating concepts against
biofouling follow two strategies; non-fouling chemistries, which means marine organisms are deterred from settling in the first place, or fouling-release (FR) coatings that do not prevent attachment, but the bond between the attached organisms can be easily overcome and the organism removed by the shear force generated when a vessel moves through water. Modern Fouling-release technologies rely on silicone technology, amphiphilic polymers, or the incorporation of hydrophobic oils. Polyelectrolyte multilayer coatings are assembled from alternating exposure of surfaces to solutions with oppositely charged macromolecules. They can serve as model systems to test components for future integration into more advanced coating formulations. While polyelectrolyte multilayer coatings are well established in the biomedical context, for prevention of nonspecific protein adsorption (NSA) and for drug delivery, only few reports on their performance as marine antifouling coatings are published. Recently, low-fouling LBL polyethylene glycol (PEG) films on polydopamine coated substrates and a layer-by-layer constructed polyethylenimine-β-cyclodextrin (PEI-β-CD) and ferrocene-modified chitosan (Fc-CHT) via host-guest interaction showed promising marine antifouling (AF) properties.

In the search for novel chemical components with marine FR properties, natural biomacromolecules are frequently considered due to their abundance in nature and excellent biocompatibility. Polysaccharides grafted as monolayers show promising activity in marine AF assays. The polysaccharides hyaluronic acid and chondroitin sulfate and their trifluoroethylamine-modified hydrophobic counterparts were grafted on substrates and showed good resistance against the marine species Cobetia marina, Ulva linza and Navicula incerta. In the work of Ederth et al., galactoside-terminated alkanethiol self-assembled monolayers (SAMs) were identified as interesting components that resist protein adsorption and attachment of marine micro- and macrofoulers.
Among the different candidates, hyaluronic acid (HA) as a polyanion and chitosan (Ch) as a polycation appear to be particularly interesting to be explored for marine applications. Hyaluronic acid (Fig 1a) is a naturally occurring polysaccharide, consisting of alternating N-acetyl-β-D-glucosamine and β-D-glucuronic acid residues linked (1→3) and (1→4) respectively\(^{21}\). It is commonly employed in biomedical applications\(^{22,23}\) and it has also been evaluated as a potential antifouling coating\(^{18,24}\). HA and fluoride modified derivatives grafted as hydrophilic and amphiphilic thin film coatings showed good performance against the attachment of marine microorganisms\(^{18}\).

Chitosan (Fig 1b) consists of N-acetyl-β-D-glucosamine and β-D-glucosamine linked (1→4)\(^{21}\). It is obtained after partial N-deacetylation of chitin, a natural constituent of a number of organisms including arthropods and fungi. The antibacterial effect of Ch makes it promising as an antifouling coating component\(^{25,26}\). Recent studies showed that Ch kills bacteria through damaging their membranes\(^{27}\), which inspired research to use Ch to inhibit freshwater bacteria such as *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) if supplied as nanoparticles\(^{28,29}\), in solution\(^{30}\), or as thin film coating\(^{31}\). Constructed as pH-responsive and degradable coating, Ch suppressed the adhesion of the freshwater bacteria *S. aureus* and *E. coli*, and the marine alga *Amphora coffeaeformis*.\(^{32}\) Ch and its synthetic derivatives were used as additives in the matrix of commercial paints to enhance their antifouling performance. These antifouling coatings were immersed in the Mediterranean Sea for a static field tests in comparison to a standard antifoulant Diuron\(^{\circledR}\) and the results showed that on Ch-containing coatings there was less accumulation of barnacles and tubeworms than on the commercial controls\(^{33}\).
HA and Ch can be combined into a coating by layer-by-layer deposition on a wide range of substrates including glass\textsuperscript{34}. Such Multilayer coatings were proven to be protein resistant\textsuperscript{35} and reduced the attachment of mammalian fibroblast cells.\textsuperscript{35} As shown for chondrosarcoma cells, the compatibility of PEMs is influenced by their modulus\textsuperscript{36}. Particularly relevant for biomedical applications is their resistance against bacteria\textsuperscript{37}. Polyester fibers were protected by first grafting a Ch layer and subsequently an HA layer against the attachment of the clinically-relevant bacteria \textit{S. aureus} and \textit{Pseudomonas aeruginosa} (\textit{P. aeruginosa})\textsuperscript{38}. Also, commercial poly(ethylene terephthalate) (PET) films, a material widely used in medical implants equipped with a HA/Ch multilayer coating, showed good resistance against the attachment of \textit{E. coli}\textsuperscript{39}. 

\textit{Fig 1: Molecular structure of the polysaccharides used to construct the PEMs: a) Anionic hyaluronic acid (HA) and b) cationic chitosan (Ch) c) amide formation used to crosslink HA and Ch.}
HA/Ch multilayers containing BMP2-loaded titanium nanotubes were also applied to medical devices as protection against pathogenic bacteria such as *S. aureus* and *E. coli*. Despite these promising results in the biomedical field, reports on the performance of HA/Ch multilayer coatings against marine biofouling organisms are still lacking.

For marine applications, the stability of the coatings deposited by electrostatically-driven layer-by-layer (LbL) is challenged by the high ion concentration, that causes HA/Ch multilayers to rapidly disassemble in the seawater environment. Thus a crosslinking step is necessary to enhance their stability. Crosslinking of HA/Ch was in the past achieved by thermal treatment and by chemical crosslinking using EDC/NHS, which in turn affected roughness and moduli of the PEMs. Such physicochemical properties are important as the resistance of coatings against marine foulers was previously found to depend on the surface morphology, its wettability, and the surface energy.

We prepared HA/Ch multilayers by dip and spin-coating, characterized their growth mode, and enhanced their stability by crosslinking using thermal and chemical treatment (chemistry schematically shown in fig 1c). The interactions between the obtained PEM coatings and proteins was investigated and their marine AF and FR properties were tested against settlement and removal of zoospores and sporelings of the green algae *Ulva linza*.

**Experimental Section**

**Chemicals:** 11-Amino-1-undecanethiol hydrochloride (AUDT), dodecanethiol (DDT), hydroxyl-hexa(ethylene-glycol)-undecanethiolate (EG6OH) and hydroxy-PEG2000-thiol (PEG) was purchased from ProChimia Surfaces (Sopot Poland). Acetone (HPLC Grade) was obtained from Alfa Aesar (Ward Hill, USA). Cyclohexane and chloroform were purchased from Fischer Chemicals (Hampton, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, USA) and used without further purification. Deionized water was purified with a
Milli-Q Plus system (Millipore, Schwalbach, Germany). Nexterion B clean room cleaned glass slides were purchased from Schott (Mainz, Germany). Gold substrates were purchased from Georg Albert (PVD-Beschichtungen, Silz, Germany). 0.5×PBS buffer was obtained from Fischer Scientific (Pittsburgh, USA) and used at pH 7.4.

**Construction of PEMs:** PEMs were constructed on gold-coated AUDT functionalized SPR chips, or (3-aminopropyl)trimethoxysilane (APTMS)-functionalized silicon wafers or glass slides. APTMS functionalization of surfaces followed previously developed protocols. In brief, the plasma-activated surfaces were put into a sealed dry flask under N₂ atmosphere with 5% APTMS solution in dry acetone and the reaction was carried out for 30 min under ultrasonication. The SPR chips were cleaned for 3 minutes in ethanol under sonication, then 24 hours immersion in 10 mM AUDT solution followed by 3 minutes rinsing in ethanol during sonication for AUDT deposition. Following previous literature reports, PEMs were constructed by either dip coating or spin coating. For dip coating (HA/Ch-DC), the substrate was first immersed in HA solution (1 mg/mL, pH 4.5, 0.15 M NaCl) for 3 minutes, with the substrate moving in a translational motion every 30 seconds, followed by washing in a 0.15 M NaCl pH 4.5 solution for 3 seconds with translational motion. The substrate was then immersed in Ch solution (1 mg/mL, pH 4.5, 0.15 M NaCl) with the same deposition and washing protocol. For the deposition by spin coating (HA/Ch-SC), the same HA and Ch solutions were used, MilliQ water was used in the washing step. The multilayers were constructed using a spin coater WS-650MZ-23NPP/LITE from Laurell Technologies Corporation (North Wales, USA). Substrates were initially spun at 1200 rpm for 10 s, then accelerated to 3000 rpm for 30 s. The procedure was repeated until the desired number of layer pairs of the (HA/Ch) films (typically i=7.5) were deposited. The multilayer construction started with HA and was terminated with HA.

**Crosslinking of PEMs:** PEMs were thermally crosslinked (HA/Ch-SC-TC) in a vacuum drying oven (Heraeus vacutherm, Thermo Electron Corporation, Waltham, USA) for 6 hours at 180°C.
Chemical crosslinking (HA/Ch-SC-CC) was carried out through N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) chemistry. First, 400 mM EDC and 100 mM NHS were dissolved at pH 5.5 in an 0.15 M NaCl solution. The surfaces were then immersed into the solution for 12 hours, followed by 3 x 1 hour washing in 0.15 M NaCl solution. After washing, surfaces were dried in a N$_2$ flow.

**PEM stability in aqueous media:** PEM stability was investigated by immersing the multilayers for the desired time in MilliQ water, 0.5×PBS buffer, or salt water. To simulate the marine environment we used salt water that contains the major components (ion mass content > 50 ppm) of seawater$^{44}$. As only synthetic salts were used, we avoided adsorption of macromolecules that might form conditioning layers$^{45}$. The initial thickness and the one remaining after the different immersion steps were quantified by spectroscopic ellipsometry.

**Preparation of the reference coatings:** Self-assembled monolayers (SAMs) were coated on gold substrates which were cleaned under an ozone-generating UV lamp for 1h followed by ethanol rinsing and 3 min sonication in ethanol, before rinsing again with ethanol. The clean substrates were immersed into a 1 mM solution of DDT and EG6OH for 24h and 72h for PEG. After immersion, the substrates were rinsed with ethanol, sonicated for 3 min, dried under nitrogen and stored under an argon atmosphere. Octadecyltrichlorosilane (OTS) reference coatings were prepared by first cleaning the substrate in an oxygen plasma, then transferring the substrates into a desiccator and exposing them to a thorough purge with nitrogen. A 0.5 M OTS solution was prepared by adding the silane to a mixture of 75 vol% cyclohexane (≥99.98%) and 25 vol% chloroform (≥99.8%) at ≈5° C. The OTS mixture was added to the desiccator and sonicated for 30 min at 10°C. After the reaction, the surfaces were sonicated in cyclohexane and toluene for 3 min, and dried in a nitrogen flow.

**Spectroscopic ellipsometry:** PEM thickness was measured by spectroscopic ellipsometry with an M-2000 from J. A. Woollam Co. Inc (Lincoln, United States). Measurements were
performed at 3 incidence angles (65, 70 and 75°). The thickness of the dip coated multilayers was calculated with a nontransparent layer model using a β-spline to describe the wavelength-dependent refractive index and the absorption coefficient. The thickness of the spin coated multilayers was measured with a transparent single-layer model with a wavelength-dependent refractive index described by the Cauchy model (A=1.45, B=0.01). There were three measurements on each sample and the average value are reported.

**Water contact angle goniometry:** Static water contact angle measurements were carried out with a custom-built goniometer. Droplets of tridistilled water were dispensed on the surface then recorded by a CCD camera. The water droplet shape was fitted by Young’s equation to determine the contact angle. Three measurements were recorded on each sample and averages are reported.

**Atomic force microscopy (AFM):** Surface morphology was analyzed by atomic force microscopy (AFM) with a NanoWizard® AFM from JPK Instruments AG (Berlin, Germany). The measurements were carried out in tapping mode using an OTESPA-R3 (300 kHz) cantilever (JPK, Berlin, Germany). Surface roughness was measured by AFM and the RMS value was calculated with the following equation:

$$R_{RMS} = \sqrt[1]{\frac{1}{n} \sum_{i=1}^{n} |z_i|^2}$$

(1)

Where n is the number of data points and $z_i$ is the height deviation of the i-th point from the average height.

**Fourier transform infrared spectroscopy in attenuated total internal reflection geometry (ATR-FTIR):** PEMs as constructed and after crosslinking were characterized by ATR-FTIR spectroscopy using a TENSOR 27 FTIR from Bruker (Billerica, USA) equipped with a germanium ATR prism, with a resolution of 4 cm$^{-1}$. Each measurement comprised 250 scans.
The spectra were acquired with a liquid N\textsubscript{2} cooled mercury cadmium telluride (MCT) detector in the wavenumber range of 4000-600 cm\textsuperscript{-1}.

**Surface plasmon resonance (SPR) analysis:** SPR measurements were carried out using an SR7000DC from Reichert Technologies Life Science (Munich, Germany). PEMs were constructed on 12.5 mm × 12.5 mm glass slides. The slides were coated with a 50 Å titanium layer and a 600 Å gold layer and subsequently with a self-assembled monolayer of aminoundecanethiol before PEM construction. 1.515 refractive index matching fluid (Cargille, Thermo Fisher Scientific, Waltham, USA) was used to establish the optical contact between the prism of the SPR device and the SPR chip. Each SPR experiment started with PBS buffer flushing across the surface at a flow rate of 200 µL/min until a stable baseline was obtained. Subsequently, the PBS buffer flow rate was changed to 10 µL/min and the protein solution (1 mg/mL in 0.5× PBS) was injected into the system and incubated for 10 min. After the exposure phase, the flow was switched back to 200 µL/min PBS buffer to determine the irreversibly bound fraction of protein. The solutions of the proteins-fibrinogen, Bovine serum albumins (BSA), and lysozyme (Sigma Aldrich, St. Louis, USA)-were freshly prepared before each experiment. Each measurement was repeated three times and the averages are reported.

**Ulva linza zoospore settlement assay:** Mature plants of *U. linza* were collected at Craster, UK (55°26′ N; 1°35′ W) and spores were released and isolated by previously published methods\textsuperscript{46}. For the settlement assay, the multilayer coated slides were immersed for 12 h in MilliQ water and 15 min in artificial seawater (ASW) (Tropic Marin\textregistered, Wartenberg, Germany) before analysis. A suspension of zoospores (10 ml; 1x10\textsuperscript{6} spores ml\textsuperscript{-1}) was added to individual compartments of quadriPERM\textsuperscript{®} dishes (Greiner Bio-One Ltd, Kremsmünster, Austria) containing the samples. After incubation for 45 minutes, in darkness, at room temperature, the slides were washed by passing back and forth 10 times through a beaker of seawater to remove unsettled (i.e. swimming) spores. Slides were fixed using 2.5% glutaraldehyde in ASW. The
density of zoospores attached to the surfaces was counted on each of the 3 replicate slides using Leica LAS-X image analysis system attached to a Zeiss Axioskop 2 fluorescence microscope. Spores were visualized by autofluorescence of chlorophyll. Counts were made for 30 fields of view (each 0.15 mm²) on each slide. The number of settled spores was subjected to a one-way ANOVA with Tukey test for statistical significance analysis. The significance threshold was set at $p < 0.05$.

**Ulva linza sporeling growth:** Spores were allowed to settle on the coating for 45 minutes and washed as described above. The spores were cultured using supplemented seawater medium for 7 days to produce sporelings (young plants) on 6 replicate slides of each treatment following previously published protocols. The sporeling growth medium was replenished every 48 hours. Sporeling biomass was determined in situ by quantifying the autofluorescence of the chlorophyll of the sporelings using a Tecan Spark fluorescence plate reader (Männedorf, Switzerland). The relative fluorescence unit (RFU) values were determined from the central region of each slide (2.5 cm x 2.0 cm²) as the mean of 70 fluorescence readings. The sporeling growth was determined on 6 replicate slides and averages were calculated. Error bars represent the standard error of the mean (SEM). Data were analyzed via one-way analysis of variance and post-hoc Tukey Test. The significance threshold was set at $p < 0.05$.

**Ulva linza sporeling removal assay:** The removal of sporelings from the samples was determined using a water jet system producing an impact pressure of 58 kPa. The fraction of sporelings remaining was determined using the same fluorescence plate reader as above. The percentage removal was calculated from readings taken before and after exposure to the shear stress. Data was processed with one-way analysis of variance and post-hoc Tukey test. The significance threshold was set at $p < 0.05$. 
Results and Discussion

Construction and crosslinking of polyelectrolyte multilayers

Fig 2: Growth kinetics of HA/Ch PEMs constructed by dip and spin coating. The thicknesses were determined by spectroscopic ellipsometry. Values are the average of three measurements on independent samples and error bars are the standard deviation of the three measurements.

The growth mechanisms of multilayers are of great importance since they affect the structure and distribution of the two polyelectrolytes within the multilayers. The reasons are three different processes that are involved in the growth of the PEMs: the adsorption of the polyelectrolyte, surface constrained complexation between the polyanion and the polycation that may involve mixing by diffusion, and the diffusion of the polyelectrolyte into the PEM film already present on the surface. Here, polyelectrolyte multilayers (PEMs) consisting of hyaluronic acid (HA) and chitosan (Ch) were assembled by layer-by-layer (LbL) deposition in two ways: dip coating (HA/Ch-DC) and spin coating (HA/Ch-SC). For the preparation by dip coating, the substrates were alternately immersed into the two polyelectrolyte solutions. Between the assembly steps, a washing step was used to remove loosely bound macromolecules. Both polymers were charged, which led to an electrostatically driven assembly process. For the preparation by spin coating, the polyelectrolyte solutions were applied to the surface and...
homogeneously distributed into a thin layer by spinning the sample. After each layer was deposited, a washing step was used to remove weakly bound polyelectrolytes\textsuperscript{49}. In both cases, the assembly process started with the aminosilane-coated surface on which the anionic HA was deposited. After deposition of a maximum of 15 layers (7.5 bilayers), an HA-terminated PEM was obtained. We decided on the HA termination as HA already showed good antifouling performance\textsuperscript{18,24} and a negative surface charge is in general found to be superior compared to positive charges\textsuperscript{50}.

Figure 2 shows the increase in film thickness with increasing numbers of bilayers for the two deposition techniques. For dip coating, an exponential growth mode was found as the deposited film thickness per double layer increased with the number of dipping cycles. For the first bilayer (BL), a deposition rate of 8.7 nm/BL was obtained, which increased to 29.9 nm/BL for the 7\textsuperscript{th} bilayer. A total film thickness of 87 nm of HA/Ch-DC was measured after the 7.5 BLs were constructed. For the HA/Ch-SC PEMs constructed by spin coating, we observed a layer growth that was approximately linear. The average thickness increment per deposited bilayer was \( \approx 12 \) nm resulting in a total thickness of 57 nm for 7.5 deposited BL.
AFM was used to characterize the surface morphology and to determine the surface roughness by calculating the rms values (Fig 3 and Table 1). Spin-coated PEMs HA/Ch-SC showed a smoother morphology than the dip-coated HA/Ch-DCs. From the z-scale (colormap) (Fig 3) it becomes obvious that HA/Ch-SC PEMs showed a smoother morphology than HA/Ch-DC. The rms roughness of the spin-coated films was 1.9 nm, while the equivalent measure of the HA/Ch-DC PEMs was 23.8 nm. Thus, the roughness was 12x higher for the dipped multilayers even though they were only 1.5x thicker than the spin coated films.

HA/Ch-DC multilayers showed an exponential growth mode, which is usually caused by diffusion in and out of the polyelectrolyte multilayers. In the case of the HA/Ch system, Ch...
chains might be able to diffuse through the multilayer and form complexes with HA, which leads to their incorporation into the bulk of the multilayer. During the washing steps, such buried Ch molecules are not removed as they are hidden in the bulk and thus not accessible. The extent to which biomacromolecules are stabilized within the coating is determined by the electrostatic energy barrier of the outermost layer and the chemical potential of the free Ch chains within the multilayer. In the subsequent step of HA deposition, the negatively charged biomacromolecules form the outermost layer. Buried Ch chains in the multilayer could diffuse towards HA and form complexes which eventually result in thicker films\(^{37}\). During the preparation by spin coating, the number of accessible biomacromolecules is inherently limited due to the volume of the polyelectrolyte solution on the spinning substrate. This could limit the accumulation on the outermost layer and cause the observed rather linear growth mode. In particular the excellent homogeneity and smoothness of the surfaces obtained by spin coating led us to apply this preparation method for all the following resistance experiments.

<table>
<thead>
<tr>
<th>PEM preparation</th>
<th>Label</th>
<th>Coating thickness</th>
<th>Static water contact angle</th>
<th>RMS roughness</th>
</tr>
</thead>
<tbody>
<tr>
<td>After spin-coating</td>
<td>HA/Ch-SC</td>
<td>57 ± 3 nm</td>
<td>18° ± 3°</td>
<td>1.9 ± 0.1 nm</td>
</tr>
<tr>
<td>Thermally crosslinked</td>
<td>HA/Ch-SC-TC</td>
<td>41 ± 1 nm</td>
<td>73° ± 4°</td>
<td>2.0 ± 0.1 nm</td>
</tr>
<tr>
<td>Chemically crosslinked</td>
<td>HA/Ch-SC-CC</td>
<td>55 ± 5 nm</td>
<td>20° ± 5°</td>
<td>3.3 ± 0.1 nm</td>
</tr>
</tbody>
</table>

Table 1: PEM properties characterized before and after crosslinking: Label of the coatings, coating thickness as determined by spectroscopic ellipsometry (n=3) and static water contact angle (WCA) (n=3), in both cases the error represents the standard deviation. RMS roughness was measured with an AFM in air across a 10 \(\mu\text{m}\) x 10 \(\mu\text{m}\) area, the average height deviation was calculated with equation 1.

After construction by spin coating, the PEMs were crosslinked by amide formation between the carboxylate groups of the HA and the amine groups from the Ch. This reaction was either induced thermally or chemically. For thermal crosslinking (HA/Ch-SC-TC), the PEMs were heated to 180°C under vacuum for 6 hours. For chemical crosslinking (HA/Ch-SC-CC), the surfaces were immersed into an EDC/NHS solution for 12 hours. As shown in fig 3, also the
morphology of the crosslinked multilayers was studied by AFM and the rms roughness was determined (Table 1). Both crosslinking methods increased the roughness of the PEM coatings compared to HA/Ch-SC, the thermal method by 5% and chemical crosslinking by 74%. This is in accordance with previous studies\textsuperscript{36} which found that as constructed HA/Ch films were smooth and homogeneous, while the films that were chemically crosslinked with EDC/NHS chemistry showed an increased roughness. In this experiment, the difference between the as-constructed films and the crosslinked films was moderate, yet chemical crosslinking led to an increase in rms roughness of ~1.4 nm. On the contrary, thermal crosslinking caused less obvious morphological changes, though there was a slight increase in the rms roughness of 0.1 nm as compared to the non-crosslinked PEMs.

![ATR-FTIR spectra of a) pure HA powder, b) HA/Ch-SC PEMs as constructed c) HA/Ch-SC-TC which were thermally crosslinked under vacuum at 180°C for 6 hours, and d) HA/Ch-SC-CC which were chemically crosslinked using 400 mM EDC and 100 mM NHS for 12 hours.](image)

The amide bond formation during crosslinking was confirmed for both crosslinking methods using ATR-FTIR spectroscopy (Fig 4), revealing a decrease of the carboxylate peaks at 1407 cm\(^{-1}\) and 1605 cm\(^{-1}\) and an increase of the amide I peak at 1651 cm\(^{-1}\) and the amide II signal at 1556 cm\(^{-1}\)\textsuperscript{51}. The amide peak intensity of HA/Ch-SC-CC was slightly larger than that of the thermally crosslinked HA/Ch-SC-TC, indicating a higher degree of crosslinking of the
chemically crosslinked PEM. The peak at 1075 cm\(^{-1}\) originated from the pyranose ring vibrations of the saccharides\(^{52}\), the mode at 1374 cm\(^{-1}\) was due to OH in-plane bending vibrations\(^{52}\), and 1154 cm\(^{-1}\) caused by the C-O-C ether stretching vibrations\(^{52}\). While the latter were still detected after chemical crosslinking, the signal was slightly reduced after thermal crosslinking, indicating a potential chemical change at the ether positions due to the elevated temperature.

The method of crosslinking substantially affected the wettability of the surface (Table 1). Chemical crosslinking caused a minor increase of the contact angle from 18\(^\circ\) (as constructed) to 20\(^\circ\), albeit the change was within the error of the measurement. HA/Ch-SC-TC multilayers had rather high contact angles of 73\(^\circ\), thus thermal treatment led to increased hydrophobicity of the coatings. Despite the similar chemistry, HA/Ch-SC-TC and HA/Ch-SC-CC differed strongly in wettability. As shown in figure 5, the crosslinked multilayers were stable in MiliQ water, PBS buffer and salt water. For chemically crosslinked multilayers, a minimum of 94\% of the initial thickness remained after 7 days immersion in salt water. In the case of HA/Ch-SC-TC, a thickness decrease of around 20\% was observed in all three media. All coatings were stable enough for the protein adsorption assays and for the Ulva linza adhesion tests.

![Fig 5: Stability of crosslinked HA/Ch-SC-TC and HA/Ch-SC-CC PEMs prepared by spin-coating in (a) MiliQ (b) PBS (pH=7.4) and (c) salt water (SW). Thermal crosslinking was achieved under vacuum at 180\(^\circ\)C for 6h. Chemical crosslinking was carried out by immersion of the multilayers in 400 mM EDC 100 mM NHS solution for 12 h.](image-url)
Protein resistance of the PEM coatings

![Graph showing protein adsorption on HA/Ch-SC PEMs studied by SPR. Three proteins were tested: fibrinogen, BSA, and lysozyme.](image)

**Fig 6: Protein adsorption on HA/Ch-SC PEMs studied by SPR.** Three proteins were tested. Fibrinogen and BSA were negatively charged, lysozyme was positively charged at the pH of the buffer. Fibrinogen had a molecular mass of 340 kDa, BSA 69 kDa and lysozyme 14 kDa. Self-assembled monolayers of hexa(ethylene glycol) (EG6OH) and 1-dodecanethiol (DDT) were included as control surfaces. PEMs were chemically crosslinked using 400 mM EDC and 100 mM NHS for 12 hours or thermally under vacuum at 180°C for 6 hours. All PEMs were terminated with HA. Bargraphs show the amount of irreversibly attached protein on HA/Ch-SC-TC and HA/Ch-SC-CC PEMs and the references surfaces. The irreversibly attached amount of protein was quantified via the change of sensor response units between the start of the assay and the end of the rinsing phase. Reported values are the average of three measurements and error bars are the standard deviation. The inset shows a magnification of surfaces with very low protein adsorption.

After construction of the PEMs, their protein resistance was investigated by SPR (SPR curves are shown in the supplementary figure S1). Three proteins were used, fibrinogen, BSA, and lysozyme. They were chosen as they have different molecular weight and net charges at the pH of the buffer solution^{53}. Two were relatively small proteins with negative net charge (BSA, 69 kDa) and positive net charge (lysozyme, 14 kDa) and one was considerably larger (fibrinogen, 340 kDa) with a negative charge. Figure 6 shows the irreversibly attached amount of the three tested proteins on the PEMs. Self-assembled monolayers terminated by hexa(ethylene glycol) (EG6OH) were included as positive and 1-dodecanethiol (DDT) as negative controls. For the measurement, first PBS buffer was flushed across the surfaces at a flow rate of 200 μL/min until a stable baseline was obtained, then the protein solution was injected over 10 min. After
the injection phase, a flow of buffer was applied to rinse off non-adherent proteins. The change of sensor response units between the start of the assay and the end of the rinsing phase was defined as irreversibly attached amount of proteins.

HA/Ch-SC-TC PEMs showed high, irreversible attachment of the negatively charged proteins fibrinogen, (3010 µRIU) and BSA (927 µRIU), which was nearly as high (88% and 91%) as on the hydrophobic DDT controls (3425 µRIU for fibrinogen and 1019 µRIU for BSA). Even larger amounts of the positively charged lysozyme accumulated on the PEMs as compared to the DDT controls. In the case of HA/Ch-SC-CC PEMs, the multilayers showed a high resistance towards fibrinogen (30 µRIU and 1% compared to DDT) and BSA (37 µRIU and 4% compared to DDT) comparable to the protein resistant positive control EG6OH (12 µRIU and less than 1% compared to DDT for fibrinogen, 27 µRIU and 3% compared to DDT for BSA). In the case of lysozyme, the resistance of the chemically crosslinked PEMs was worse, and the amount of adsorbed protein was larger than on the DDT control and on the thermally crosslinked PEMs. The strong attachment of lysozyme on both PEMs could be caused by attractive electrostatic forces between unreacted negative charges of the HA-terminated PEMs and the positively-charged protein. In recent publications, monolayers of HA were tested against the same proteins. Against fibrinogen and BSA, the grafted macromolecules showed excellent protein resistance, while lysozyme attachment was only reduced to 50% compared to the DDT control. Chitosan in contrast tends to enhance protein adsorption and cell attachment when grafted by silane chemistry. It was speculated that this is due to the positive charge under buffer and physiological conditions.

The high resistance of HA/Ch-SC-CC towards the negatively charged proteins correlates with the hydrophilic properties of the obtained films (WCA 20°) (Table 1). Thermally crosslinked PEMs were more hydrophobic (WCA 73°) and in general less resistant against protein attachment, which is in good agreement with the general criterion of the Berg limit.
case of chemically crosslinked PEMs against NSA of the positively charged lysozyme, the repulsive nature of the hydrated, hydrophilic surfaces seemed to be overcompensated by electrostatic interactions.

**Zoospore settlement and adhesion assay**

![Graph showing spore density on different surfaces](image)

*Fig 7: Density of settled zoospores on HA/Ch-SC PEMs (45 minutes) assay. Each point is the average from 90 counts on 3 replicate slides. Hydroxyl-PEG2000-thiol (PEG) and octadecyltrichlorosilane (OTS) were included as positive and negative references. Glass and polydimethylsiloxane (PDMS) were also included as reference surfaces. Error bars show 95% confidence limits. Statistical significance of data was analyzed according to a one-way ANOVA with post-hoc Tukey test (p<0.05). Statistically significantly different values are marked by the brackets with the asterisk.*

The antifouling and the fouling-release properties of HA/Ch-SC-CC and HA/Ch-SC-TC were also tested in a zoospore settlement assay and a sporeling growth and removal assay, respectively. Samples were immersed into a suspension of the zoospores and the number of spores settled on the surfaces after 45 minutes was counted (Fig 7). Hydroxy-PEG2000-thiol (PEG) and octadecyltrichlorosilane (OTS) monolayers were included as positive and negative references, respectively. Glass and polydimethylsiloxane (PDMS) were also included as reference surfaces. Settlement on glass (M=990 mm$^{-2}$, SD=102), on the chemically crosslinked PEMs (M=855 mm$^{-2}$, SD=51), on PDMS (M=785 mm$^{-2}$, SD=68), and on the hydrophobic OTS
(M=74, SD=29) was relatively high. On PEG, the positive reference, settlement was strongly reduced (M=257, SD=38). Even lower settlement was found on the thermally crosslinked PEMs (M=99.6, SD=9.4).

To test if the observed differences are statistically significant, an ANOVA with post-hoc Tukey test was applied (p<0.05). Although HA/Ch-SC-CC are hydrophilic and resistant against non-specific adsorption of proteins, the number of Ulva linza zoospores that settled on the surfaces was unexpectedly high. The thermally crosslinked PEMs had significantly lower settlement than all other surfaces including the PEG positive reference. The good antifouling performance of thermally crosslinked PEMs contrasts with its poor resistance to non-specific protein adsorption.

**Fig 8**: a) The biomass of sporelings on HA/Ch PEMs after 7 days incubation before (grey bars) and after (black bars) exposure to a water jet with 58 kPa impact pressure. Each point is the average biomass on 6 replicate slides measured using a fluorescence plate reader (RFU: relative fluorescence units). The biomass on thermally crosslinked PEMs were lower than on chemically crosslinked PEMs, both before and after flow. Statistically significant differences are indicated by the stars according to one-way analysis of variance and post-hoc Tukey test (p<0.05). Error bars represent the standard error of the mean (SEM). b) Percentage removal of 8-day-old sporelings from HA/Ch PEMs due to an impact pressure of 58 kPa. Each point is the average removal of biomass from 6 replicate slides measured using a fluorescence plate reader. Error bars show the standard error of the mean derived from arc-sine transformed data. Statistically significantly different numbers are marked by the brackets with the asterisk (ANOVA with post-hoc Tukey test, p<0.05).
HA/Ch PEMs were also tested in a sporeling assay, in which the coatings were subjected to a 45 minutes zoospore settlement assay and subsequently cultured in supplemented seawater medium for 7 days to allow the young plants to develop on the surface. The sporeling removal assay measured the relative strength of attachment of the sporelings by exposing the samples to a 58 kPa impact pressure from a water jet. Fig 8a shows the biomass formed on the 6 replicates of each coating and reference surface after 7 day’s growth. Biomass formation was similar on all coatings (grey bars). The only statistically significant reduction confirmed by post-hoc Tukey test was that there was lower biomass accumulation on the thermally crosslinked multilayers as compared to the chemically crosslinked multilayers (Fig 8a, grey bars).

After exposure to an impinging water jet, less biomass remained on HA/Ch-SC-TC than on HA/Ch-SC-CC (black bars in fig 8a), this difference was statistically significant according to one-way analysis of variance and post-hoc Tukey test (P<0.05). From the pre- and post-flow biomass values, the percentage removal can be calculated. A higher percentage was removed from the thermally crosslinked multilayers (40%) than from the chemically crosslinked ones (21%) (Fig 8b). Neither of the PEMs were able to outperform the PDMS reference coatings (75%) (differences were statistically significant, p<0.05).

In both, protein resistance and U. linza attachment and removal experiments, the results strongly depended on the method of crosslinking. Due to the rapid degradation of the non-crosslinked polyelectrolyte multilayers in salt water, they unfortunately cannot be tested in the marine environment without crosslinking and a certain stabilization is mandatory. The disadvantage of crosslinking is that it was generally observed in the past that enhanced crosslinking rather reinforces attachment, e.g. of mammalian cells. In previous publications it was also found that chemical crosslinking led to a reduction in resistance of the PEMs to cell attachment as compared to non-cross linked PEMs. Multilayers consisting of poly(L-lysine)/hyaluronan (PLL/HA) and HA/Ch PEMs were found to be more favorable for chondrosarcoma cells to
spread and adhere after they were chemically crosslinked by EDC/NHS chemistry, which was speculated to be due to the increased film stiffness\textsuperscript{36}. An explanation for the reduced resistance could be the alteration of the chain arrangements of the polymers during the crosslinking process, which might lead to more compact and smoother multilayers as observed by Muzzio et al.\textsuperscript{42} Thus, if we intend to create inert coatings, a reduction of the degree of crosslinking to a minimum could be advantageous.

The experiments on NSA of proteins in this work supports the promising low-fouling potential of the investigated HA/Ch PEMs. Interestingly, the protein resistance is particularly low on the chemically crosslinked PEMs which have more hydrophilic properties and a slightly larger roughness. According to the Baier curve\textsuperscript{56}, this behavior is expected and matches previously observed trends.\textsuperscript{57,58}

In the \textit{U.linza} experiments we found a general trend that the thermally crosslinked polyelectrolyte multilayers do show better settlement inhibiting and fouling-release properties than the chemically crosslinked ones. In this case, contact angles around 70° and smoother surfaces seem to be more efficient in reducing the attachment of \textit{U.linza} zoospores and sporelings. In particular for the sporeling removal, the roughness could be a decisive factor. Compared to previous publications, the results were rather unexpected as several reports describe that settlement and adhesion of \textit{Ulva linza} zoospores are lower on hydrophilic surfaces\textsuperscript{59,43}. Obviously, the investigated system belongs to the few results for which a certain hydrophobicity enhances the antifouling performance.\textsuperscript{60} Thus, for PEMs for marine applications, the crosslinking mechanism plays an important role and has to be carefully selected.
Summary and conclusions

Thick, yet homogeneous polyelectrolyte multilayers consisting of natural occurring and biocompatible hyaluronic acid and chitosan were constructed and crosslinked as marine antifouling coatings. Dip coating and spin coating methods for construction were compared and spin coating was found to yield smoother multilayer films. The multilayers were then crosslinked for enhanced stability. While both, chemical and thermal crosslinking reinforced the coating against degradation in seawater, they affected the coating properties in a different way. Chemical crosslinking generated hydrophilic coatings with higher roughness whereas thermal crosslinking produced hydrophobic coatings with lower roughness. The hydrophilicity of the resulting coatings was highly relevant for their protein resistance and chemically crosslinked, hydrophilic multilayers showed superior resistance as compared to the thermally crosslinked multilayers. While the single component HA is rather protein resistant in literature reports and the positively charged Ch enhances protein attachment, the combination of both with optimized crosslinking seems to provide a coating with superior protein-resistance. In settlement tests against zoospores of the green alga Ulva linza, thermally crosslinked multilayers outperformed chemically crosslinked multilayers in both zoospore settlement and sporeling removal assays. The result was unexpected since Ulva linza zoospores were reported to settle less readily on hydrophilic surfaces. The results highlight the potential of the investigated renewable, biocompatible, and biodegradable biopolymers and suggest their application in future antifouling formulations. In order to use the multilayers directly as coatings, the crosslinking has to be further optimized.

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References


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(61) See supplementary material at [http://dx.doi.org/10.1116/1.5110887](http://dx.doi.org/10.1116/1.5110887) for surface plasmon resonance spectroscopy (SPR) results of HA/Ch PEMs protein assay.