Nanostructured biofunctional polymer coatings to prevent marine biofouling

Viele der Foulingeffekte im Meerwasser verursachenden Organismen nutzen Proteine bzw. Glycoproteine zu ihrer Verankerung an Oberflächen. Wir erkunden daher Strategien zur Vermeidung des Biofoulings im Meer durch die Immobilisierung von proteolytischen Enzymen, die Adhäsionsproteine hydrolytisch spalten. Hierzu wurde das Enzym Subtilisin A aus verschiedenen Lösungskonzentrationen kovalent an verschiedene Maleinsäureanhydridcopolymerfilme angekoppelt. Die resultierenden Enzymschichten wurden hinsichtlich Schichtdicke (Ellipsometrie) und Proteingehalt (Aminosäureanalyse) charakterisiert, die Aktivität der immobilisierten Enzyme durch den spektrophotometrischen Nachweis der Spaltung eines chromogenen Peptids erfasst. Die erhaltenen Ergebnisse belegen die Umsetzbarkeit des untersuchten Ansatzes: Es wurden stabile Enzymschichten mit definierter Zusammensetzung erhalten, deren Aktivität von der immobilisierten Enzymmenge sowie von der Charakteristik des Polymersubstrates beeinflusst ist. Die Wirkung der Enzymschichten auf die Anhaftung und Verankerung von zwei für das Biofouling im Meerwasser wichtigen Organismen, Ulva linza und Navicula perminuta, wurde untersucht. Die Enzymschichten zeigten dabei eine vielversprechende Verringerung der Besiedlung und Verankerung von Sporen der Ulva linza und eine reduzierte Adhäsion der Navicula-Zellen. Interessanterweise war die Antifoulingwirkung der Enzymschichten abhängig von den Eigenschaften der für die Anbindung der Enzyme genutzten Polymerbasisschichten.

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Introduction

All structures immersed in marine and freshwater environments (ships, pipelines, membrane filters, heat exchangers) are rapidly colonized by microorganisms, plants and animals [1]. This settlement and accumulation of organisms – designated as biofouling – generates high economical costs, as by clogging membranes and pipelines and by increasing the hydrodynamic drag on ship hulls resulting in loss of maneuverability and increase of fuel consumption [1, 2, 4, 7]. From the environmental perspective, biofouling creates unacceptable burdens due to the release of biocides from antifouling paints [1, 2, 4, 6, 8]. Additionally, the transport of foulers by ships results in the introduction of species into new geographical areas disturbing marine ecosystems [1-5]. Among the different approaches proposed for biofouling control, tribulytin (TBT) based paints have been most successful [4, 6-8]. However TBT has an adverse ecological impact, since it accumulates in non-target species causing malformations and other disorders leading to populations decline [8-11]. A global ban of the use of TBT is now effective worldwide and other antifouling biocides are facing increasing restrictions due to their negative environmental impact. As a result, novel solutions with no detrimental effect on the environment are desperately needed.
Many biofouling species, such as diatoms, algal spores and invertebrate larvae use proteins and glycoprotein polymers to attach to surfaces [12-14] leading to the idea of using enzymes able to hydrolyze adhesive proteins to prevent biofouling. Industry has been

using enzymes for centuries as in food processing, detergent formulation and drug synthesis [15] and they are now commercially available at low cost. Additionally, enzymes are biodegradable and are therefore expected to be environmentally friendly. Most studies that reported on the use of enzymes as antifouling agents are based on the release of enzymes and on commercial enzymes formulations containing large amount of stabilizers and preservatives that can contribute to antifouling making the contribution of the enzyme unclear [16].
We investigate the possibility of using immobilized proteolytic enzymes to prevent marine biofouling. For this purpose, maleic anhydride (MA) copolymers have been selected as immobilization platform since they can be deposited as thin films with a physicochemical profile determined by the choice of the co-monomer, adjustment of molecular weight and preparation conditions. The reactivity of the anhydride can be used to immobilize bioactive molecules by the spontaneous reaction of the anhydride group with amine (lysine) functions of the bioactive molecule. Due to its stability and promising antifouling properties [17], the proteolytic enzyme Subtilisin A was covalently immobilized onto maleic anhydride films and the resultant enzyme layer was comprehensively characterized concerning enzyme amount and activity. The enzyme-containing coatings were evaluated concerning their antifouling properties towards two major fouling species: the green algae *Ulva linza* and the diatom *Navicula perminuta.*

Thin film preparation and enzyme immobilization

Thin films of poly(octadecene-*alt*-maleic anhydride) (POMA) and poly(ethylene-*alt*-maleic anhydride) (PEMA) were produced by spin coating. Oxidized carriers (glass and silicon wafers) were surface-modified with an aminosilane prior to spin-coating in order to allow the covalent binding of the thin polymer film. More details can be found in [18]. The proteolytic enzyme Subtilisin A (Subtilisin Carlsberg) was covalently immobilized onto the copolymer films (Fig. 1) by exposing the films to enzyme solutions of variable concentration [19].

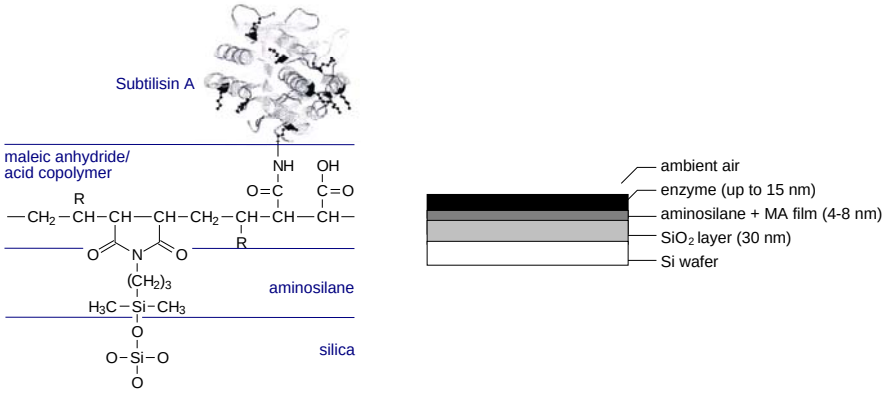

Fig. 1:
Schematic representation of the enzyme layered coatings (left) and model used for ellipsometry measurements (right).
(R = H for PEMA, R = $(CH_2)_{15}CH_3$ for POMA)

Characterization of enzyme coatings

The immobilized enzyme layer thickness was determined by ellipsometry assuming an optical five layer model system (Fig. 1).
The amount of immobilized enzyme was quantified via amino acid analysis (using high performance liquid chromatography (HPLC)). Details on the experimental procedure and numerical analysis of the amino acid distribution can be found in [20].
The evaluation of the immobilized enzyme activity was performed by detection of the product resultant from the cleavage of N-Succi-

nyl-Ala-Ala-Pro-Phe-pNitroanilide by spectroscopy. Subtilisin A cleaves the substrate into peptides and *p*-nitroaniline (pNa), the formation of which was followed by absorbance measurements at 405 nm.

Marine biofouling assays

Zoospores were released from reproductive thalli of the green macroalga *Ulva linza* in artificial sea water (ASW) and were prepared for assays as described in [21]. Settlement and adhesion assays followed the principles outlined in [22]. Samples were incubated with zoospores for 45 min in darkness, rinsed to remove non-settled spores and preserved with glutaraldehyde to determine the number of settled spores [23]. To evaluate adhesion strength, substrates were further incubated in ASW for 90 min after spore settlement, after which they were exposed to an impact pressure of 34 kPa using the water jet apparatus [22]. The density of spores was determined by an image analysis system attached to an epi-fluorescence microscope via a video camera [23].
Cultures of the diatom *Navicula perminuta* were grown in F2 medium at 18 °C with 16 h : 8 h, light : dark cycle. Cells were allowed to settle and adhere to substrates for 2 h at 18 °C in an illuminated environment. Substrates were then washed to remove non-attached cells, incubated in ASW for 3 h and subsequently exposed to a 35 Pa wall shear stress in the flow channel device [24]. Quantification of diatom cells followed a similar procedure as for the *Ulva* zoospores assay [23].

Properties of the enzyme coatings

The evaluation of the amount of enzyme immobilized onto the MA copolymer coatings was performed by determining the enzyme layer thickness by ellipsometry (Fig. 2 left) and by quantifying the amount of immobilized enzyme by amino acid analysis (using HPLC) (Fig. 2 right) [19].

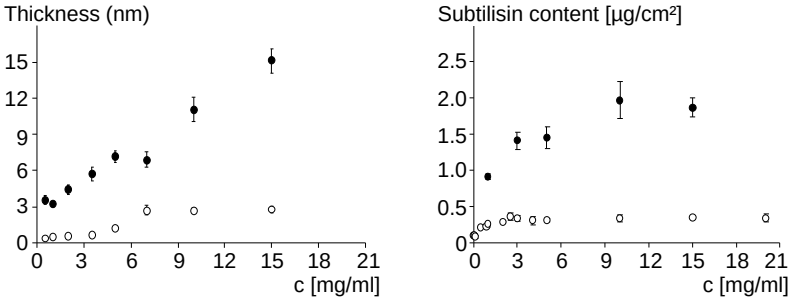

Fig. 2:
Layer thickness (left) and content (right) of Subtilisin A immobilized onto MA copolymer films (● PEMA; ○ POMA) in dependence of enzyme concentration in solution (c) (error bars = ± standard deviation)

The ellipsometry and HPLC data for both coatings correlate well. The hydrophobic POMA copolymer coating shows a compact non-swelling interface. At low enzyme concentrations, the immobilized enzyme is likely to undergo conformational changes; the enzyme spreads to increase the contact area between coating and hydrophobic moieties of the enzyme. The surface is saturated at enzyme concentrations in solution of about 6 $mg \cdot ml^{-1}$ – corresponding to an immobilized amount of 0.35 $\mu g \cdot cm^{-2}$. The ellipsometry results point to the formation of an enzyme monolayer onto this film (Fig. 2). The enzyme immobilization results on the PEMA copolymer coating show no saturation up to the maximum enzyme concentration in solution investigated. The hydrophilic, strongly swelling PEMA film allows for the penetration of the enzyme into the copolymer layer resulting in higher amounts of immobilized

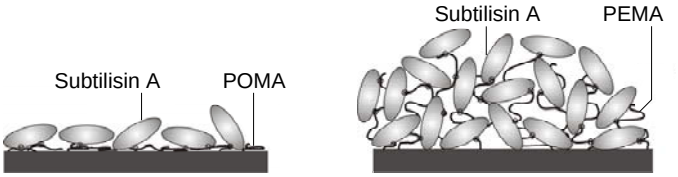

Fig. 3:
Schematic illustration representing the immobilization of Subtilisin A onto POMA and PEMA copolymer thin films

enzyme within the interfacial volume phase (about 5 times more than onto POMA). A schematic illustration depicting the covalent immobilization of Subtilisin A onto POMA and PEMA copolymer films is presented in Fig. 3.

The enzymatic activity of the bioactive coatings was determined by following the progress curve of the catalysis of N-Succinyl-Ala-Ala-Pro-Phe-pNa. The slope of the initial linear progress curve corresponds to the initial reaction rate (initial activity). The amount of substrate cleaved per unit time normalized to the amount of immobilized enzyme (specific activity) for MA coatings containing different amounts of enzyme is presented in Fig. 4.

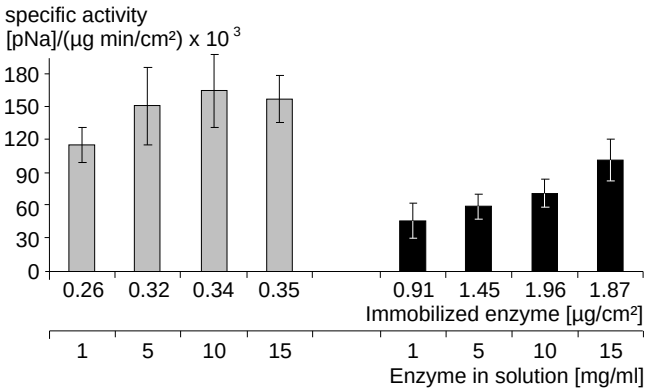

Fig. 4:
Specific activity (product formed ([pNa]) per unit of time per weight of enzyme per area) for PEMA (■) and POMA (□) copolymer films containing different enzyme amounts (error bars = ± standard deviation)

Although fewer enzyme can be immobilized onto the hydrophobic POMA coating, a higher specific activity is observed as compared to PEMA-enzyme-containing films. The inclusion of the enzyme in the 3D polymer layer and the strong acidic characteristics of the hydrolyzed PEMA will both limit substrate accessibility and possibly trigger enzyme conformational changes – which is believed to limit the substrate conversion [19].

Settlement and adhesion of the green algae *Ulva linza*

The amount of *Ulva* zoospores onto PEMA coatings containing no (PEMA), active (PEMA A) and denatured (PEMA D) enzyme after settlement and after exposure to an impact pressure of 34 kPa using the water jet is presented in Fig. 5.

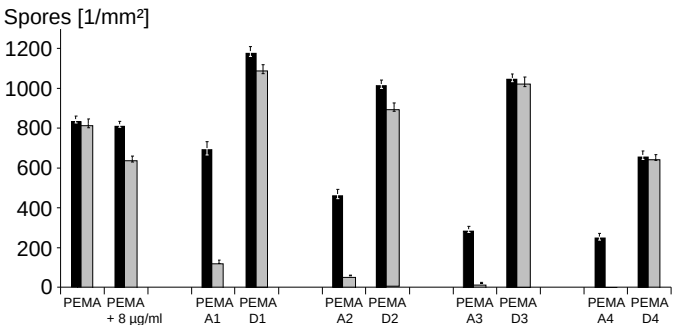

Fig. 5:
Density of Ulva spores onto PEMA, PEMA containing variable amounts of (A) active and (D) denatured enzyme after settlement (■) and after exposure to an impact pressure of 34 kPa using the water jet (□). PEMA + 8 µg·ml^{-1} = PEMA coating exposed to a solution of 8 µg·ml^{-1}Subtilisin A for 90 min after spore settlement. Concentration of enzyme used for immobilization onto coatings:
A1 and D1 = 3 mg·ml^{-1};
A2 and D2 = 10 mg·ml^{-1};
A3 and D3 = 20 mg·ml^{-1};
A4 and D4 = 30 mg·ml^{-1}.
The immobilized active enzyme was denatured by incubation of the coatings at 90 °C for 45 min.
(N = 90, error bars = ± 2 x standard error)

The results show a clear dependence of spore settlement and spore removal with enzymatic activity and amount of immobilized enzyme. The density of spores settled onto the active coatings

decreases with increased amount of immobilized enzyme and activity. All denatured coatings showed higher spore settlement than their active-coating counterparts. The number of settled spores onto the denatured coatings decreased with increasing the amount of immobilized enzyme, probably reflecting the changes in surface properties by increasing enzyme loading. The ease of spore removal, a proxy measure of spore adhesion strength, increased with increasing enzymatic activity, while a very low spore removal was observed for all denatured controls. This validates the hypothesis that immobilized Subtilisin A is effectively able to degrade the adhesive proteins secreted by the algae *Ulva linza* [25].
A higher antifouling efficiency was observed for the enzyme immobilized onto PEMA coatings (PEMA A3) when compared with equivalent amount of enzyme in solution (PEMA + 8 $\mu g \cdot ml^{-1}$). PEMA A3 has an immobilized enzyme amount comparable to an enzyme concentration in solution of 8 $\mu g \cdot ml^{-1}$, but the percentage of removal differed markedly – 93 % removal for the immobilized enzyme as compared to 21 % removal for the free enzyme The immobilization of enzymes increases stability guarantying a long-term degrading ability and on the other hand ensures that the enzyme is at the right place to degrade the secreted adhesives.
The influence of the intrinsic properties of the polymer precoating used for immobilization on the antifouling properties of the enzyme coatings was investigated by comparing POMA and PEMA based enzyme films with similar initial activities (Fig. 6).

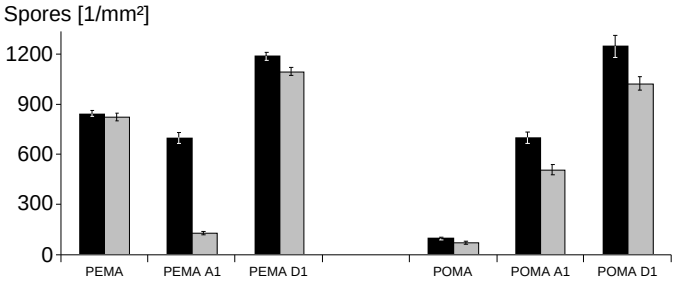

Fig. 6:
Density of Ulva spores onto PEMA and POMA based coatings after settlement (■) and after exposure to an impact pressure of 34 kPa using the water jet (□). PEMA; POMA = base coatings with no enzyme.
A = active/native enzyme;
D = denatured enzyme (exposure of equivalent coating A to 90 °C for 45 min). Concentration of enzyme used for immobilization onto coatings: PEMA A1 and D1 = 3 $mg \cdot ml^{-1}$; POMA A1 and D1 = 7 $mg \cdot ml^{-1}$. Initial enzymatic activity of coating PEMA A 1 is similar to coating POMA A1.
(N =90, error bars = ± 2 x standard error)

The settlement of *Ulva* spores was observed to be higher onto the hydrophilic PEMA coating than onto the hydrophobic POMA. The number of settled spores onto the coatings with similar enzymatic activities was found to be equivalent; however, the corresponding percentages of removal were substantially different (lower adhesion strength of spores to PEMA A1 as compared to POMA A1), highlighting the fact that the properties of the base coating are playing an important role [25].

Adhesion of the diatom *Navicula perminuta*

The effect of enzymes onto the settlement of *Navicula perminuta* was not examined since *Navicula* cells are unable to actively seek for a surface for attachment [12]. The adhesion strength of *Navicula* cells to PEMA coatings containing no (PEMA), active (PEMA A) and denatured (PEMA D) enzyme was investigated by exposing the coatings to a wall shear stress of 35 Pa in the flow channel (Fig. 7).
The percentages of removal obtained for the denatured controls were independent of the amount of immobilized enzyme (ca. 63 % for all denatured controls), while the percentages of removal from active enzyme-containing coatings increased with increasing the amount of immobilized enzyme and enzymatic activity. This observation supports that the adhesive secreted by *Navicula* cells

is constituted by a proteinaceous component susceptible to degradation by the enzyme [13, 17]. Similarly to the *Ulva* assays, a higher antifouling efficiency was observed for the immobilized enzyme (89 % cell removal for coating PEMA A3) as compared with the free enzyme (77 % cell removal for free enzyme (PEMA + 8 $\mu g \cdot ml^{-1}$)) [25].

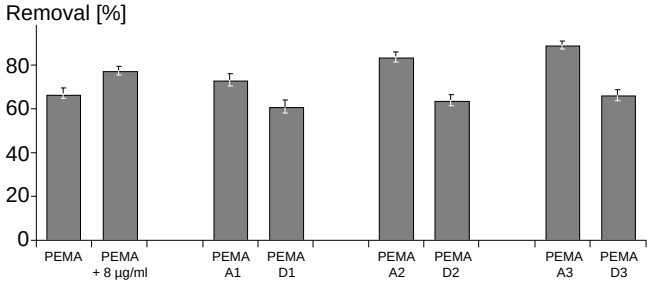

Fig.7:
Percentage of removal of Navicula cells from PEMA and PEMA coatings containing active (A) and denatured (D) enzyme after exposure to a wall shear stress of 35 Pa in the flow channel. Concentration of enzyme used for immobilization:
A1 and D1 = 3 $mg \cdot ml^{-1}$;
A2 and D2 = 10 $mg \cdot ml^{-1}$;
A3 and D3 = 20 $mg \cdot ml^{-1}$
PEMA + 8 mg/ml = PEMA coating exposed to a solution of 8 $\mu g \cdot ml^{-1}$ Subtilisin A for 3h after settlement (N = 90; error bars = ± 2 x standard error)

Conclusion

The proteolytic enzyme Subtilisin A was successfully immobilized onto maleic anhydride copolymer coatings. Determination of the enzyme layer thickness and immobilized enzyme amount revealed that the hydrophilic, strongly swelling PEMA film allows for the penetration of the enzyme into the layer resulting in higher amounts of immobilized enzyme. Although less enzyme is immobilized onto POMA copolymers films, a higher activity per immobilized enzyme was observed on this copolymer layer when compared with PEMA-enzyme containing films. This may be explained by the limited accessibility and/or enhanced structural alteration of the enzyme in the 3D structure of the PEMA layer.
Experiments with two major marine fouling species showed that Subtilisin A immobilized onto PEMA copolymer films decreases the settlement of *Ulva linza* zoospores and weakens the anchorage of both *Ulva* spores and *Navicula perminuta* cells. The choice of the copolymer influenced the effect of the immobilized enzyme – the hydrophilic PEMA coatings produced a higher antifouling efficiency than the hydrophobic POMA layers. These observations validate the hypothesis that immobilized proteolytic enzymes are efficiently able to degrade adhesives secreted by the tested marine species and can therefore be used as antifouling agents.

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