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Entrapment of Subtilisin in Ceramic Sol–Gel Coating for Antifouling Applications

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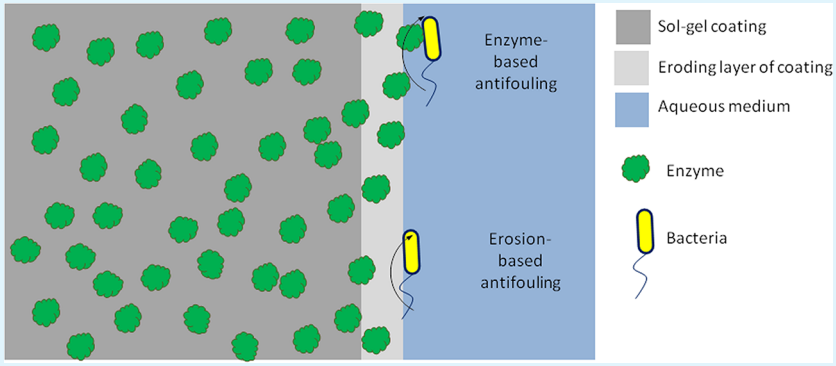

ABSTRACT: Enzymes with antifouling properties are of great interest in developing nontoxic antifouling coatings. A bottleneck in developing enzyme-based antifouling coatings is to immobilize the enzyme in a suitable coating matrix without compromising its activity and stability. Entrapment of enzymes in ceramics using the sol–gel method is known to have several advantages over other immobilization methods. The sol–gel method can be used to make robust coatings, and the aim of this study was to explore if sol–gel technology can be used to develop robust coatings harboring active enzymes for antifouling applications. We successfully entrapped a protease, subtilisin (Savinase, Novozymes), in a ceramic coating using a sol–gel method. The sol–gel formulation, when coated on a stainless steel surface, adhered strongly and cured at room temperature in less than 8 h. The resultant coating was smoother and less hydrophobic than stainless steel. Changes in the coating's surface structure, thickness and chemistry indicate that the coating undergoes gradual erosion in aqueous medium, which results in release of subtilisin. Subtilisin activity in the coating increased initially, and then gradually decreased. After 9 months, 13% of the initial enzyme activity remained. Compared to stainless steel, the sol–gel-coated surfaces with active subtilisin were able to reduce bacterial attachment of both Gram positive and Gram negative bacteria by 2 orders of magnitude. Together, our results demonstrate that the sol–gel method is a promising coating technology for entrapping active enzymes, presenting an interesting avenue for enzyme-based antifouling solutions.

KEYWORDS: *sol–gel, antifouling, savinase, subtilisin, biofilm, enzyme*

1. INTRODUCTION

Microbial fouling is a major problem in many industries, such as the biomedical,[1−3] marine,[4] and food processing and packing.[5] Traditional antifouling technologies are based on killing the fouling organisms by releasing biocides, such as TBT, copper, or zinc, from a coating.[6,7] Even though these coatings have limitations, they were able to reduce the fouling considerably.[8,9] Despite their efficiency in reducing most fouling organisms, the killing of nontarget organisms, pollution, and toxicity allegations[10−12] have opened up a new arena for nontoxic antifouling solutions.[13,14] Development of environmentally friendly antifouling coatings that do not release biocides is a hot topic in antifouling research (for a recent review, see Indrani Banerjee et.al., 2011[14]). Broadly classifying, there are two main strategies to achieve nontoxic antifouling materials. One is by modifying the surface properties of the material so that it prevents the fouling organisms from attaching firmly to the surface, and thereby facilitates easy release when the surface is exposed to flow. This strategy involves modifications of the physicochemical surface proper-

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ties, such as roughness, charge, and hydrophobicity.[15−18] The other strategy is to functionalize the surface with nontoxic antifouling compounds or biomolecules that interfere with cell adhesion or have a more specific or short-lived biocidal effect.[19−21]

Enzymes with antifouling properties have been proposed as a nontoxic alternative to traditional biocides.[22] A bottleneck in implementing enzymes for antifouling applications is to immobilize them in a suitable matrix without compromising their activity and stability. Two overall strategies have been used for immobilization: Covalent attachment and physical entrapment in polymers.[23] Covalent attachment often requires multistep reactions involving chemical modification of the biomolecules,[24] which might compromise their activity. Moreover, covalent attachment is possible only when suitable residues are available in the enzyme molecule. Physical entrapment, on the other hand, can be applied to all kinds of biomolecules, regardless of their chemical composition, as it does not involve chemical modification. The enzyme thus remains in its native conformation.[25]

Immobilization of biomolecules in robust silicates has been a focus of study for several years, and sol−gel materials have proven to be versatile hosts for active biomolecules.[26] The main advantage of using the sol−gel method for immobilizing enzymes is that the coatings retain a lot of water,[27] which enable the enzyme molecules to remain stable and active. Silicates in the form of sol−gel composites have been used to encapsulate a wide range of biomolecules, such as enzymes, catalytic antibodies, whole cells, etc.[28,29] So far, the sol−gel process has mainly been used for biosensor and biocatalytic applications.[30] Very few studies have explored the possibility of using sol−gel materials in enzyme-based antifouling applications.[31] The aim of this study was to develop a sol−gel-based coating for entrapping active enzymes with antifouling properties. We successfully encapsulated the serine protease, subtilisin (Savinase, Novozymes), and applied the sol−gel coating to stainless steel surfaces. The physical properties of the coating were analyzed, and the enzyme activity and ability of the coating to resist bacterial adhesion was measured over several months.

2. EXPERIMENTAL SECTION

2.1. Chemicals. Subtilisin was purchased as Savinase16.0L (P3111-Protease from *Bacillus* sp.) from Sigma-Aldrich, USA. Peptide Suc-Ala-Ala-Phe-pNA, used as a substrate for subtilisin, was bought from Bachem, Switzerland. N-propyltriethoxysilane (PTEO) was bought from Degussa, Germany, andhydrochloric acid 0.1 M was bought from Merck, Germany. N-methylaminopropyltrimethoxysilane, methyl-phenyldimethoxysilane (MPDMO), 3-glycidoxypropyltriethoxysilane (GLYEO), 3-aminopropyltriethoxysilane (AMEO), and 3-mercapto-propyltriethoxysilane (MTEO) were all purchased from ABCR Germany. 2-propanol was purchased from Merck, Germany and 20% Tickopur TR 13 (aq) was purchased from Monmouth Scientific, Somerset, UK. SYBR Green II and glycerol Reagent plus were purchased from Sigma-Aldrich, USA. All chemicals were used as received without further purification.

2.2. Savinase Doped Sol−Gel Coating Preparation. Glycerol (0.08 mL) was placed in a small vial. GLYEO (1.0 mL), PTEO (0.1 mL), MPDMO (0.1 mL), and MTEO (0.15 mL) was added and stirred for 5 min. To this solution, aqueous 0.1 M HCl(0.75 mL) was added. The resulting mixture (pH 4.0) was stirred for an additional 2 h at room temperature. From this base coating, 1 mL was transferred to a 1.5 mL microcentrifuge tube. For coatings containing subtilisin (≥16 U/ml), 0.2 mL enzyme solution was added and vortexed for 10 s at full speed. For the inactive enzyme control, subtilisin was denatured by heating to 90 °C for 30 min. Immediately before the enzyme coating was applied, N-methylaminopropyltrimethoxysilane (0.02 mL) was added and vortexed for 5 s at full speed.

2.3. Preparation of Enzyme-Coated Slides. The stainless steel (316b) slides (30 × 24 × 1 mm^3) were degreased with acetone, then submerged in 20% Tickopur TR 13 (aq.) overnight, and finally washed with deionized water and dried with compressed air. The slides were then primed with a standard primer (2% (AMEO) in 2-propanol) before applying the sol−gel coatings by spin coating for 8 s at 1000 rpm in a Polos MCD200-NPP apparatus (SPS-Europe B.V., Netherlands). The coating was cured at room temperature overnight. For spectrophotometric measurements, the coating was applied not to stainless steel but to glass slides (25 × 10 × 1 mm^3). These were manually coated with 10 μL of enzyme coating, to ensure an equivalent quantity of coating on each slide. The coating was cured at room temperature overnight.

2.4. Coating Thickness, Erosion, Hardness, and Adherence. The thickness of the coatings on stainless steel samples was measured with a Byko-Test 7500 F/NF thickness gage (Premier Colorscan Instruments Pvt. Ltd.., India). Thickness of sol−gel coatings on glass surfaces (used for the enzyme activity measurements) was measured using a digital micrometer. The coating thickness was determined by calculating the difference in thickness of glass surfaces with and with out sol−gel coating. The erosion rate was calculated by comparing the thickness of coatings with subtilisin on stainless steel before and after 104 days of incubation in deionized water. The coating hardness was measured by the ISO standard, pencil hardness (ISO, 15184) test. Adherence of the coating was evaluated by the ISO standard, cross hatch adherence (DIN EN ISO 2409) test.

2.5. Surface Characterization Using Atomic Force Microscopy. The surface roughness of stainless steel and sol−gel coatings was measured by atomic force microscopy (AFM) before and after incubation in deionized water or PBS for 2 h. AFM images were obtained using a NanoWizard II AFM (JPK Instruments, Germany) in intermittent contact mode at room temperature under ambient conditions with a scan rate of 0.6 Hz, using silicon cantilevers (OMCL-AC160TS, Olympus, Japan) (k = 12 and 103 N/m, f = 200 and 400 kHz, tip radius = 10 nm). Surface roughness (Ra and Rz values) expressed in nanometers (nm) was determined from 10 × 10 μm images using Scanning Probe Image Processor (SPIP-version-5.1.0) software, after flattening of the images using the JPK image processing software. Three areas were randomly chosen and imaged on three different samples for each surface type. Each was treated as individual replicate in calculating the mean and standard deviation, because of the fact that the variation within samples was larger than between samples (one way ANOVA). Difference in the roughness values between sol−gel coatings were analyzed by one way analysis of variance. Roughness differences between individual sol−gel coatings and stainless steel was analyzed by Student's t test.

2.6. Determination of Surface Hydrophobicity. The surface hydrophobicity was determined as the function of water contact angle, using a Kruss DSA 100 contact angle measuring system under ambient conditions. Contact angle measurements were made before and after incubating the coatings in water or PBS for 2 h. Measurements were done with deionized water. Contact angles were calculated from the images of the water droplet on the surface with ImageJ software using the DropSnake plug-in (National Institutes of Health, Bethesda, MD).[32] Three measurements were taken from different locations on three different samples for each type of surface. Statistical analyses were done as described for the roughness measurements.

2.7. Surface Characterization Using X-ray Photoelectron Spectroscopy (XPS). XPS spectra were recorded using a Kratos Axis UltraDLD instrument (Kratos Ltd., Telford, UK) equipped with a monochromated aluminum anode (Al kα 1486 eV) operating at 150 W power (15 kV and 10 mA) with 20 eV pass energy for all survey spectra. Charge neutralization was used to neutralize any positive charge build-up during measurements on electrically nonconducting surfaces. A hybrid lens mode was employed during analysis (electrostatic and magnetic). XPS spectra of the coatings were recorded at three different spots on each sample. Relative atomic

percentages were calculated from the average of three spectra in each spot. The takeoff angle with respect to normal to the surface was 0° for all measurements.The measured binding energy positions were charge corrected with reference to 285.0 eV, corresponding to the C−C/C-H species. Quantification was conducted using CasaXPS software. A linear background was used for all spectra. The relative atomic percentage of nitrogen enables quantitative estimation of the amount of protein present on a given surface[33] and in this report the relative atomic percentage of nitrogen was used to estimate the amount of subtilisin within top 10 nm (analysis depth) of the sol−gel coatings.

2.8. Measuring the Activity of Subtilisin with Suc-Ala-Ala-Phe-pNA. The enzymatic activity of sol−gel coatings containing subtilisin was determined using the colorimetric method. Enzyme activity was determined after storing the slides in water for different lengths of time. Coated glass slides (24 pieces) were placed on the bottom of a beaker containing 1 L of demineralized water, which was replaced every week with fresh water. The beaker was left at room temperature, and three samples were removed for enzyme activity measurements after 20 h followed by 7, 30, 60, 90, 150, 210, and 270 days. Prior to activity determination, each sample was washed with 3 mL of deionized water and dried to exclude excess water. The three samples were placed in separate vials, with the coating facing up. To each vial a fixed concentration of substrate Suc-Ala-Ala-Phe-pNA (2 mL, 0.2 mM in PBS buffer pH 7.4, 0.01 M) was added. After 10, 20, 30, 40, 50, and 60 min at room temperature, the total volume of the vial was transferred to a cuvette, and the absorbance (λ = 405 nm) was measured, using a Shimadzo UV-1201 spectrophotometer. After each measurement the volume was returned to the vial for continuation of the experiment.

2.9. Short Time Bacterial Adhesion Assay. *Staphylococcus xylosus* DSM 20266 (Braunschweig, Germany) (Gram positive) and *Pseudomonas fluorescence* (Gram negative) were used as test organisms. *S. xylosus* was routinely grown in 1% tryptic soy broth at 30 °C, whereas *P. fluorescence* was cultivated in 3% TSB at 30 °C. Overnight cultures of both strains were prepared by inoculating from a primary inoculum which was grown until a late exponential phase. Cells were harvested at OD_{600} 0.8−1.0 by centrifugation at 5000 rpm for 10 min. Cell suspensions were prepared by washing the harvested cells twice and resuspending them in (PBS). The final cell density was adjusted to OD_{600} 0.05−0.07.

Test surfaces (30 mm × 24 mm) were incubated in a 6 well plate containing bacterial suspension (prepared as described above) and incubated with shaking (120 rpm) for 2 h at room temperature. Surfaces were recovered and nonadhered bacteria were gently removed by dipping the surfaces in sterile PBS three times. The remaining bacteria were stained with 20 μL of 20 × SYBR Green II RNA stain (2 μL ml^{-1} of 10 000 x SYBR Green II stock), covered with glass coverslip, and sealed with nail polish to avoid evaporation. Slides were kept in the dark at 4 °C until quantification of adherent bacteria.

Quantification of bacteria was performed using a Zeiss Axiovert 200 M epifluorescence microscope (Carl Zeiss GmgH, Jena, Germany) equipped with Zeiss filterset 10 and 63× or 100× oil immersion objectives. Cells were counted in 190 μm^2 or 120 μm^2 grids (depending on the magnification used) in random positions on the slide until a minimum of 1000 cells had been counted on each sample.

3. RESULTS

3.1. Properties of Sol−Gel Coating and the Effects of Enzyme Entrapment. The sol−gel coating designed to host active enzymes was prepared with carefully chosen alkoxysilane precursors (Table 1). The ratio for the individual components was optimized to ensure that the coating had appropriate porosity, retained enzyme activity, and could be cured at room temperature in less than 8 h. The resulting coating was visually smooth, transparent with an average coating thickness of approximately 5 μm (single layer, data not shown). Coatings on glass used for long-term incubations were 22 ± 1.7 μm. Cross hatch tests were performed to determine the adhesion strength of the coatings. Sol−gel coatings exhibited strong adherence to stainless steel (Table 2). The coating was less hydrophobic than SS, and 1 μm circular pores in the coating contributed to a relatively high surface roughness (Table 2, Figure 1).

Table 1. Composition of the Sol−Gel Coating

coating ingredients	quantity (%)
glycerol	3
GLYEO	37.6
MTEO	5.6
MPDMO	3.8
PTEO	3.8
methylpropylamine silane	1.5
savinase	16.5

Table 2.

coating	hardness[a]	cross hatch test[b]	contact angle (deg)	roughness (Ra, nm)
sol−gel	4H	0	70.1 ± 1.5	15.3 ± 4.2
sol−gel + subtilisin	3H	0	52.9 ± 1.0	6.1 ± 1.4
sol−gel + denatured subtilisin	NA	NA	50.5 ± 4.1	1.8 ± 0.04
stainless steel	NA	NA	80.6 ± 3.1	24.5 ± 10.5

[a]Pencil hardness, ISO 15184. [b]Cross hatch adherence test DIN EN ISO 2409; rating 0−5, rating 0 equals <5% damage of the coating, rating 5 equals ≥65% damage of the coating.

Addition of enzyme up to 20% (v/v) did not compromise the coating properties with respect to curing time, thickness, and adhesion to the substrate (data not shown). However, coatings with enzyme were less hard and the surface properties changed slightly, as they became more hydrophilic and smooth (Table 2). The circular pores observed in coatings without enzyme were less pronounced in coatings harboring the enzyme (Figure 1).

3.2. Long-Term Enzyme Activity in Sol−Gel Coatings and Coating Erosion. The enzyme activity of the sol−gel coating decreased by approximately 1 order of magnitude within the first 2 h of incubation in PBS or demineralized water (Figure 2), presumably because of leaching of nonimmobilized enzyme. During this short incubation, the coating topography changed, which indicates erosion of the coating surface (Figure 1a, d). Coating thickness measurements before and after incubation in water showed that the thickness of the coating was reduced by 3.8 ± 0.3 μm in 104 days, corresponding to an erosion rate of approximately 36 nm per day. Coatings without enzymes contained porous structures, and these pores became larger during the incubation. Coatings with enzymes had a more smooth surface topography without pores. On these surfaces, aggregates of up to a few 100 nm in diameter appeared after the 2 h incubation (Figure 1b, e). We hypothesize that these structures were aggregated enzymes appearing at the surface as the coating gradually eroded. This hypothesis was supported by an apparent increase in the amount of enzyme on the coating surface, determined by the relative atomic % N measured by XPS (Table 3).

During the subsequent weeks, the enzyme activity increased and peaked after 1 month incubation in water. It then decreased gradually, reaching 13% of its initial activity (at 2h) after 9 months incubation (Figure 2). When stored dry, the enzyme activity was even more stable, and approximately 78%

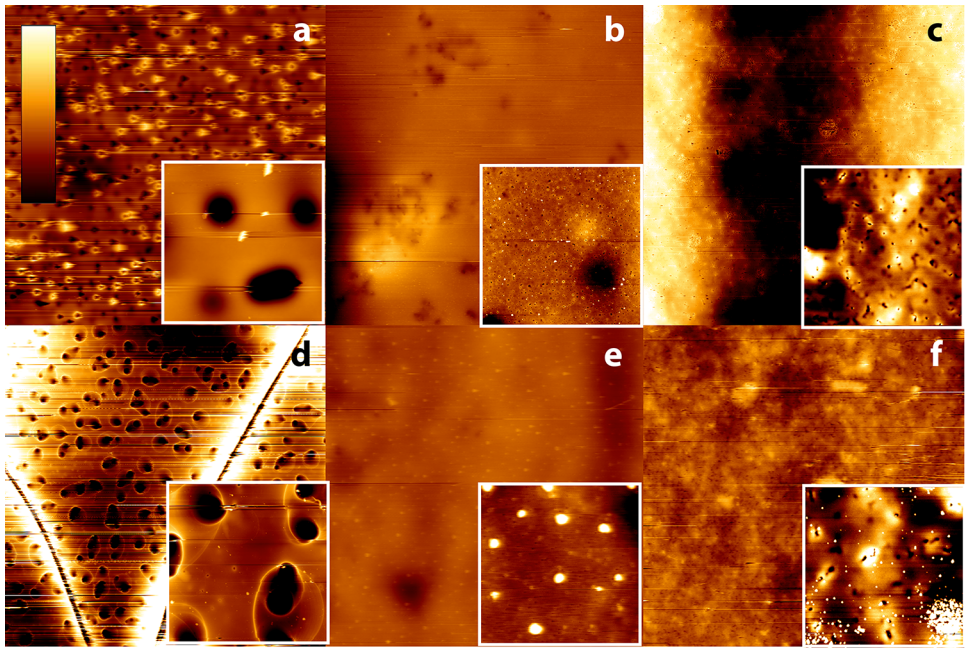

Figure 1. AFM images of sol–gel coatings (A, D) without enzyme, (B, E) with denatured enzyme, and (C, F) with active enzyme. Images were acquired (A–C) before and (D–F) after incubation in PBS for 2 h. Large images are 100 × 100 μm (height scale 200 nm) and inset images are 10 × 10 μm (height scale 0 to 20 μm).

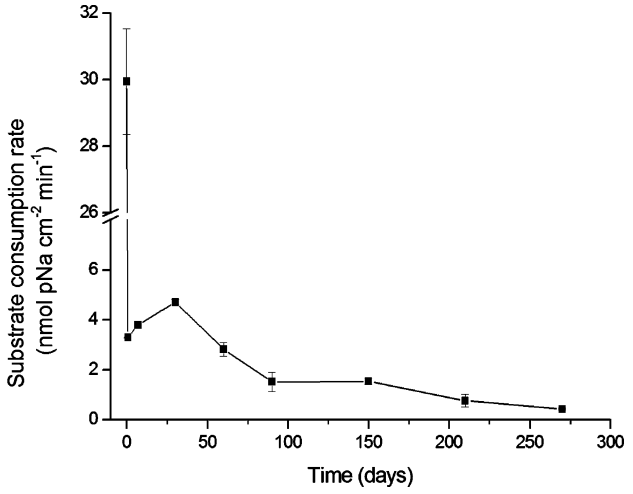

Figure 2. Enzyme activity of coatings incubated in PBS for up to 270 days. Activity is expressed as the rate of substrate consumption per cm^2 surface area. Error bars = s.d. (n = 3).

Table 3. Relative Atomic Percentage of Nitrogen (N %) on Surfaces Measured Using XPS. The N% (average ± standard deviation, n = 3) is Proportional to the Amount of Enzyme Present on Surface

	N% start	N% after incubation in PBS
sol–gel	0	0
sol–gel + subtilisin	0.7 ± 0.2	1.3 ± 0.3[a]

[a]Significant difference between surfaces analyzed before and after incubation in PBS for 2 h (t test, p < 0.05).

of the initial activity of the enzyme was retained by the coating after 25 months (the pNa hydrolysis rate decreased from 1.22 to 0.95 nmol cm^{-2} min^{-1}).

3.3. Bacterial Retention on Sol–Gel Enzyme Coatings.

In comparison to stainless steel, the sol–gel-coated surfaces retained approximately 1 order of magnitude fewer bacteria (p < 0.005). This was the case for both the Gram positive (*S. xylosus*) and Gram negative (*P. fluorescence*) bacteria tested (Figure 3). A further reduction in bacterial retention was observed upon incorporation of active enzymes in the sol–gel coating (p < 0.005) (Figure 3). Incorporation of denatured enzyme did not have a significant effect on bacterial retention

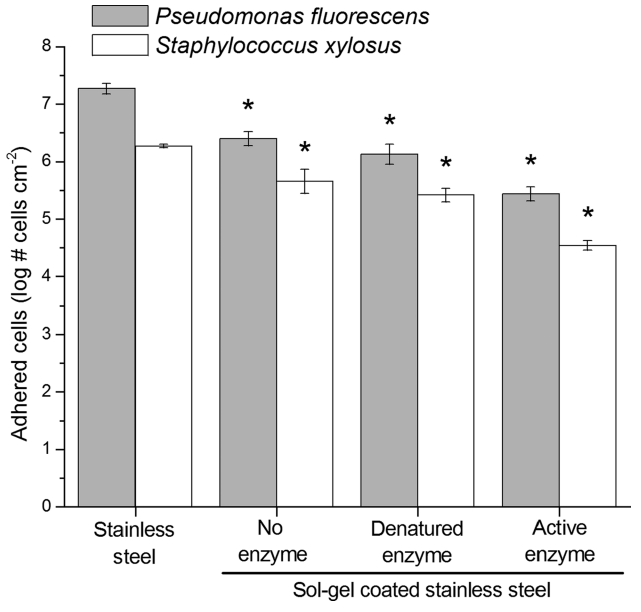

Figure 3. *S. xylosus* and *P. fluorescence* incubated for 2 h in PBS with sol–gel coatings and stainless steel control. Fluorescently stained cells on the surface were manually counted under an epifluorescence microscope. Error bars indicates the standard deviation (n = 3). *p < 0.005.

(p = 0.15 and 0.09 for *S. xylosus* and *P. fluorescence* respectively), confirming the functionality of the active enzyme toward bacterial attachment.

4. DISCUSSION

Enzymes are attractive nontoxic alternatives for antifouling applications. A number of different enzymes belonging to the oxidoreductases, transferases, hydrolases, and lyases have previously been studied for their antifouling properties.[22,34,35] Proteases are known to have antibiofilm properties against both Gram positive[36] and Gram negative[37] bacteria, in which the serine proteases appear particularly effective.[37−40] We therefore chose to use a commercial formulation of the serine protease subtilisin (Savinase16.0L from Novozymes Corp.), which is produced by *Bacillus* sp.

Immobilizing the enzyme in a coating matrix without compromising its stability and activity is a bottleneck in achieving enzyme-functionalized antifouling coatings. The choice of immobilization method is an important parameter. Broadly classified, enzyme immobilization techniques fall under two categories; chemical modification of the polypeptide by binding to an insoluble molecule, and physical entrapment of the enzyme in an inert matrix.[23] Immobilization by chemical modification often suffers from losing the native state of the enzyme, and as a result, the catalytic properties are compromised.[24] Physical entrapment, on the other hand, can preserve the nativity while also providing the essential aqueous microenvironment to the immobilized enzyme.[25] Silicate glasses prepared by the sol−gel method were demonstrated to host such an environment for the entrapped biomolecules.[41,42] Entrapment in synthetic polymers, such as polyurethane[43] and vinyl polymer,[44] has also been reported. Physical entrapment via sol−gel process is relatively simple, and it is advantageous for entrapment of enzymes because it is chemically inert, has tunable porosity, and cures at room temperature. Furthermore, sol−gel coatings are optically transparent, mechanically stable, and have negligible swelling behavior.[27,30,45−47]

Subtilisin has previously been immobilized for antifouling/antibacterial applications by chemical modification. Covalent attachment of subtilisin to maleic anhydride copolymer thin films was shown to have antifouling properties in the marine environment.[21,48] In an another study, polycaprolactam cross-linked with subtilisin was developed for food packaging applications.[49] Subtilisin was also entrapped in sol−gel matrices for biocatalytic applications, and the entrapped enzyme was sufficiently stable to allow reuse of the catalyst for several cycles.[50−52] We present the first attempt to immobilize subtilisin by physical entrapment using the sol−gel method for antifouling applications.

The overall challenge for developing coatings that contain active enzymes is to adapt the coating formulation to avoid denaturation of the enzyme, while also ensuring that the general properties of the final coating are not compromised. In formulation of sol−gel coatings, the formation of alcohol during hydrolysis of organoalkoxysilane precursors could denature the enzyme. Savinase has a relatively high tolerance toward organic solvents. Therefore, we did not take any extra precautions on this issue. However, the precursors themselves may also affect the enzyme. We initially tested the activity of Savinase in different mixtures of organo-functionalized silane precursors that are frequently used in formulation of sol−gel coatings. The primary aminosilane AMEO inactivated the enzyme, whereas MTEO and PTEO did not (data not shown). It was beyond the scope of this paper to further investigate why comparable precursors inactivated subtilisin to different extent, and we therefore proceeded with the coating formulation with the latter two plus additional precursors: GLYEO and MPDMO. Formulations of sol−gel coatings with several combinations and ratios of these precursors were evaluated in terms of (1) curing time for the coating, (2) coating appearance, and (3) enzyme activity. MPDMO and PTEO are mono- and difunctionalized precursors with hydrophobic nonreactive organic side groups. Inclusion of these precursors is known to significantly influence the porosity of the coating.[42] The initial curing time for the developed coating matrix was 36 h. Several strategies were tested to reduce the curing time. Addition of NaCl was attempted, but although it reduced the curing time, it had adverse effects on the coating properties (data not shown). However, addition of the sondary amine, methylpropylamine silane reduced the curing time dramatically, and the final curing time was thus only 8 h.

The optimal pH for subtilisin is between 7 and 8, but it remains active to pH 5.5 (data not shown). One of the operational factors in the sol−gel coating preparation is hydrolysis of the precursors. Often, this occurs at either high or low pH. The hydrolysis is controlled by the concentration of the acid or base catalyst. Acid-catalyzed hydrolysis is faster than base-catalyzed hydrolysis, and HCl is therefore often used. To avoid denaturation of subtilisin, hydrolysis was performed at pH 3−5, prior to addition of the enzyme. The pH was controlled by adjusting the ratio of alkoxysilane precursors to HCl. The subsequent addition of Savinase resulted in an increase of the pH to approximately 7.

Shrinkage during drying and aging causes a buildup of pressure in the pores and cavities within the coating. This phenomenon might lead to loss of enzyme activity and reduced accessibility of the substrate. Glycerol does not cause enzyme denaturation and can be added to facilitate a more controlled drying process. The maximum concentration of glycerol that could be added without compromising the coating properties was 8 vol%. Higher concentrations led to reduced adhesion of the coating to the substrate, even though a primer was used (data not shown).

Subtilisin does not kill the bacteria, but prevents them from forming a biofilm through degradation of adhesive cell surface proteins. Exposure of the enzymes on the coating surface is an important parameter in maintaining the antifouling properties of the coating.[53] There are two ways in which the enzyme could be exposed; (1) by controlling the porosity of the coating to allow leaching of enzyme from within the coating[54] and/or 2) by gradual erosion of the coating matrix, leading to release of entrapped enzyme in the eroding layer.[55] In our coating, the surface microstructures of the enzyme-free coating changed after incubation in water or PBS (Figure 1a, d). This led us to speculate that the coating was undergoing gradual erosion. The coating containing enzyme did not exhibit such microstructures, but, similar erosion was assumed to be taking place, and indeed, the erosion rate calculated after 104 days of incubation was 36 nm per day. Furthermore, globular structures ranging from a few hundreds of nanometers appeared on the surface after incubation in PBS (Figure 1f (inset)), indicating that the coating surface was undergoing some form of change. Incubation in PBS also led to an increase in the relative atomic percentage of nitrogen (Table 3), suggesting that the enzyme was appearing on the surface of the coating. Hence the

aggregates appearing after incubation in PBS were likely to be the exposed enzyme. Together, these data suggest that the coating surface gradually erodes, facilitating exposure of enzymes on the surface. However, release of enzymes by diffusion could not be ruled out.

We observed an initial drop in the enzyme activity of the freshly made coating during the first two h of incubation in water or PBS (Figure 2). This initial drop in enzyme activity (within two h) is probably caused by washing out unbound enzyme from the coating surface. All experiments were therefore carried out after an initial 2 h incubation of the samples to remove unbound enzyme, and the initial activity measurements were performed after this treatment. Enzyme activity subsequently increased over the following 30 days of incubation, followed by a slow gradual decrease over several months (Figure 2). Because we observed signs of erosion on the surface of enzyme-free sol−gel coatings, we interpret the gradual decrease in enzyme activity as an indication of a gradual erosion of the coating, leading to a continuous release of enzyme. Interestingly, the decrease in enzyme activity was much slower in coatings that had been stored dry. This is an important result because it demonstrates the feasibility of long-term storage of coated products before they are taken into use.

Gradual erosion can itself contribute to the antifouling properties of a coating. The 10-fold decrease in bacterial retention observed in enzyme-free sol−gel-coated stainless steel could thus be due to a combination of the lower surface roughness and the gradual erosion of the sol−gel. Erosion or self-polishing is a property that is frequently used in antifouling paints for marine applications.[13] The water movement across the surface is important for the functionality of such paints, and our coatings could therefore also be tested under flow to investigate how water movement affects erosion, enzyme release, and the effect of both these parameters on the antifouling properties of the surface. However, this is beyond the scope of the current study.

Although the release of enzyme is required for the functionality of the coating, rapid leaching might leave the coating less functional over time.[53] The biocide release property of the traditional marine antifouling paints (e.g., TBT-based antifouling paints) lasts for several years. To the best of our knowledge, previously described enzyme-based antifouling formulations could not achieve such long-term enzyme release properties. However, attempts have been made to prolong the duration of enzyme release from coatings. A hydrogen peroxide release-based antifouling system was shown to have continuous release of hydrogen peroxide over a period of 3 months.[56] In another study, the half-life of protease immobilized in silicates was calculated as 358 days, while the laboratory measurements were done up to 7 days only.[19] With reference to the use of subtilisin for antifouling and antibacterial applications, covalent attachment of the enzyme to maleic anhydride copolymer films were shown to be active for 24 h[21] and 48 h[48] in marine environment. Also, subtilisin cross-linked to polycaprolactam, developed for food packaging applications, was shown to have a minimal loss of activity after 56 days.[49] We have measured the coating activity up to 9 months, and showed that half of the initial activity was still retained after 5 months, and 12% activity even remained at the end of the study. Being first of its kind, entrapping savinase in a sol−gel matrix for antifouling application, we could not compare our results with other existing studies in terms of long-term coating activity. We recognize that the activity of the subtilisin in the sol−gel coating could potentially be prolonged by controlling coating's erosion rate, the amount of entrapped enzyme, and the thickness of the coating. These topics could be the focus of further studies for optimizing the application of sol−gel coatings for particular enzymes .

The subtilisin containing sol−gel coating was tested for its antifouling potential against biofilm forming Gram positive and Gram negative bacteria. The incubation with approximately 10^6 bacteria per milliliter is a representation of the amount of bacteria in the marine environment (Lewin, 1974). In both cases, the sol−gel coating with subtilisin retained 2 orders of magnitude fewer bacteria when compared to stainless steel ($p < 0.005$) and 1 order of magnitude fewer bacteria when compared to sol−gel coating ($p < 0.005$) (Figure 3), demonstrating that the sol−gel coating alone possessed antifouling properties (probably through erosion) and that encapsulation of enzymes enhanced the antifouling effect. No effect was obtained when entrapping denatured enzyme ($p = 0.15$ and 0.09 for *S. xylosus* and *P. fluorescence*, respectively). The laboratory tests were done under static conditions, and for a short period of time. Testing the coatings against bacteria in dynamic flow conditions and for longer incubation times would be interesting. Subtilisin can also resist attachment of macrofouling organisms,[48] and it would therefore also be highly interesting to investigate the effectiveness of sol−gel coatings with immobilized subtilisin to prevent macrofouling in marine environments.

5. CONCLUSIONS

In conclusion, a ceramic coating based on sol−gel technology was designed and fabricated from inorganic and organic precursors. A serine protease, subtilisin (Savinase), was physically entrapped in the coating. The coating retained the enzyme activity for a long time (9 months). The coating with subtilisin was able to resist the initial colonization of biofilm forming Gram positive and Gram negative bacteria. Our results strongly suggests that sol−gel-based inorganic/organic hybrid coatings are promising technology for the immobilization of enzymes and have a great potential for antifouling applications.

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Notes

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