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Section: Regenerative Medicine and Biomaterials

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Abstract

The effect of the activity of immobilized enzymes on the initial attachment of pathogenic bacteria commonly associated with nosocomial infections (*Pseudomonas aeruginosa* and *Staphylococcus epidermidis*) was investigated. The proteolytic enzymes, subtilisin A and the glycoside hydrolase cellulose, were covalently attached onto poly(ethylene-*alt*-maleic) anhydride copolymer films. A comparison between active and heat-inactivated surfaces showed that while the activity of immobilized cellulase reduced the attachment of *S. epidermidis* by 67%, it had no effect on the attachment of *P. aeruginosa*. Immobilized subtilisin A had opposite effects: the active enzyme had no effect on the attachment of *S. epidermidis* but reduced the attachment of *P. aeruginosa* by 44%. The results suggest that different biomolecules are involved in the initial steps of attachment of different bacteria, and that the development of broad-spectrum antifouling enzymatic coatings will need to involve the co-immobilization of enzymes.

Keywords: bioactive surfaces, biofilm, cellulase, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, subtilisin

Introduction

In general, the exposure of a surface to aqueous environments results on its colonization by organisms. This phenomenon, known as biofouling, has severe negative impacts in industry and in medicine, for example by clogging membranes and filters, or causing infections. The use of enzymes to control biofouling has been receiving increasing attention (Olsen et al. 2007; Kaplan 2009; Kristensen et al. 2008; Cordeiro and Werner in press 2011). Enzymes are currently available at affordable prices and are therefore viable for industrial use, and since they are biodegradable, they are also environmentally friendly. The most explored antifouling enzymatic modes of action include the degradation of cell wall components leading to cell lysis, and the degradation of extracellular polymer substances anchoring cells to surfaces (Cordeiro and Werner 2011).

Various laboratory-screening assays, studying the effects of enzyme formulations on the attachment of diverse foulers, identified the broad-spectrum serine protease subtilisin (the active component of Savinase, Alcalase and Esperase) as the most promising commercially available enzyme for antifouling strategies based on the degradation of adhesive biomolecules (Pettitt et al. 2004; Dobretsov et al. 2006; Leroy et al. 2008; Cordeiro and Werner 2011). Among the variety of enzymes tested to disperse biofilms, the glycoside hydrolase cellulase emerges as a common component of enzymatic solutions for removal and prevention of bacteria biofilm in industry. Studies showed that, although free cellulase affects *Pseudomonas aeruginosa* biofilm formation and mature biofilms (Loiselle and Anderson 2003), crosslinked cellulase only partially inhibited *P. aeruginosa* biofilm formation (Anderson et al. 2003).

Recently, we have developed a well-defined model system to investigate the influence of immobilized subtilisin A on the adhesion of major marine foulers (Tasso et al. 2009a; Tasso et al. 2009b). Our studies showed that the adhesion strength of *Ulva linza* spores and *Navicula perminuta* cells decreased in the presence of the active enzyme, and that the effect of the enzyme was significantly enhanced when the enzyme was surface-confined (Tasso et al. 2009b), revealing the important role of enzyme location at the surface-fouler interface on antifouling efficacy.

In this paper, we evaluate the impact of immobilized biofilm-degrading enzymes on the initial attachment of *P. aeruginosa* and *Staphylococcus epidermidis*, bacteria commonly associated with hospital-acquired infections. To achieve this purpose, subtilisin A and cellulase were covalently immobilized onto poly(ethylene-*alt*-maleic) anhydride copolymer films.

Materials and methods

Preparation of bioactive layers

The maleic anhydride (MA) copolymer films were prepared as described elsewhere (Pompe et al. 2003). Briefly, silicon wafers or glass slides were freshly oxidized in a mixture of aqueous solutions of NH_3 and H_2O_2 and thereafter surface-modified with 3-aminopropyltriethoxysilane (APTES) (Fluka, Germany) by immersing the freshly oxidized wafers in a solution of APTES in a 9:1 (v/v) mixture of 2-propanol and water followed by extensive rinsing with 2-propanol. Stable covalent binding was achieved by annealing at 120 °C for 1 h. Copolymer solutions of poly(octadecene-*alt*-maleic anhydride) (POMA) MW 30 000 - 50 000 (Polysciences Inc., USA) and poly(ethylene-*alt*-maleic anhydride) (PEMA) MW 125 000 (Aldrich, Germany) were prepared by dissolving POMA in THF at 0.08% (w/v), and PEMA at 0.15% (w/v) in THF/acetone (2:1 v/v). The copolymer solutions were spin-coated (Suess Microtec, Germany) onto the amino-functionalized wafers. Stable covalent binding of the polymer films was achieved by annealing at 120 °C to generate imide bonds with the aminosilane (Pompe et al. 2003). The annealed samples were extensively rinsed to wash out polymer excess. The properties of the films were investigated using a wide range of techniques, details can be found elsewhere (Pompe et al. 2003; 2005; Osaki and Werner 2003).

Reactive immobilization of enzymes onto MA copolymer films was achieved by exposing the copolymer layers to enzyme solutions of variable concentration, as described in (Cordeiro et al. 2011; Tasso et al. 2009a). Immediately prior to enzyme immobilization, the anhydride moieties of the copolymers were regenerated by annealing at 120 °C for 2 h. The cellulase solutions were prepared by dissolving cellulase in 50 mM sodium citrate buffer pH 4.0. Subtilisin A solutions were prepared by dissolving the enzyme in PBS modified by the addition of $CaCl_2$ and NaCl at 2 mM and 0.1 M, respectively; the pH of the solution was adjusted to 8.6 with NaOH (Tasso et al. 2009a). The immobilization was performed using in-house constructed immobilization chambers. After overnight exposure of the copolymer films to the enzyme solution the samples were rinsed 10 times with deionized water to remove unbound protein (Tasso et al. 2009a).

Characterization of bioactive layers

The immobilization and characterization of subtilisin A onto MA copolymer films are described in detail elsewhere (Tasso et al. 2009a; 2009b).

The dry thickness of the cellulase layers prepared onto MA copolymer films were determined by single-wavelength ellipsometry (Sentech, Germany) at an angle of incidence of 70° at 632.8 nm. Upon determination of

the thickness of all layers below the protein layer, a five-model approximation (Si/SiO_2/silane + MA film/protein/air) was used to determine the thickness of the protein layer. The refractive index for the MA copolymer film was taken from Osaki and Werner (2003). The refractive index of the protein layers was maintained fixed for all protein-containing surfaces at 1.465 (Fischer et al. 2010). All ellipsometry measurements were carried out at 20 °C and 55% air humidity.

The activity of immobilized cellulase was determined through the detection of released reducing sugars from carboxymethylcellulose (CMC) using 3,5-dinitrosalicyclic acid (DNS) adapting the procedures described in (Ghose 1987). Briefly, substrate solutions consisting of 2% w/v CMC in 0.05 M sodium citrate buffer pH 4.8 were prepared by stirring the solution overnight at room temperature and subsequently stored at 4 °C. Activity assays were performed by pre-incubation of the CMC substrate at 50 °C and by immersion of the immobilization chambers in a water bath kept at 50 °C. Upon temperature equilibration, the substrate solution was added at constant volume to each surface. At intervals, 50 µl was extracted and added to respective wells in PCR plates already containing 150 µl DNS reagent. (DNS reagent was prepared by dissolving 0.5 g 3,5-dinitrosalicyclic acid in 20 ml 1 M NaOH and 15 ml distilled water after which 15 g potassium sodium tartrate was added and the solution was made up to 50 ml with distilled water.) Using a thermal cycler, the PCR plates were immediately heated up to 99 °C, kept at this temperature for 5 min and then cooled down to room temperature. Aliquots were extracted, transferred to microtiter plates and the volume made up to 200 µl with distilled water. Absorbance was measured at 540 nm. Blanks consisting of the substrate solutions not contacting active enzyme were used. A calibration curve using glucose was prepared to allow for the conversion of the measured absorbance to the equivalent concentration of released glucose.

Experiments with bacteria

The antifouling potential of the activity of immobilized enzymes was investigated using biofilms of single bacterial species (*Pseudomonas aeruginosa* (DSMZ 939; ATCC 15442) and *Staphylococcus epidermidis* (DSMZ 1798; ATCC 12228)). The bacterial were kept on agar plates (TSBY and LB agar for *S. epidermidis* and *P. aeruginosa*, respectively) at 2 °C in the dark.

Test samples consisting of PEMA copolymer films decorated with active enzyme were compared with control samples consisting of PEMA copolymer films exposed overnight to enzyme buffer (i.e. polymer coatings not containing enzyme), and with control samples consisting of PEMA copolymer films containing heat-denatured enzyme. The heat-denatured subtilisin A controls were prepared by heating immobilized subtilisin A layers to 120 °C for 45 min as described in (Tasso et al. 2009b). Inactivated cellulase layers were prepared by

autoclaving correspondent active layers for 20 min at 120 °C. Immediately after enzyme immobilization, test and control samples (in triplicate) were incubated with bacteria suspensions of approximately 10^6 c.f.u./ml at 37 °C for 24 h (humidity >90%) under static conditions using sterile in-house constructed chambers (similar to those used for enzyme immobilization). Suspensions of *S. epidermidis* and *P. aeruginosa* were prepared in 5% TSBY medium for *S. epidermidis*, and in 10% LB medium for *P. aeruginosa*. Biofilm growth conditions were optimized with respect to bacterial cell and media concentrations, as well as incubation times for each strain, to ensure reproducible and homogenous biofilm formation (data not shown). After incubation, the slides were removed from the chambers and washed three times to remove loosely bound bacteria by immersion in 50 ml saline solutions in Falcon conical centrifuge tubes. Samples were then transferred to tubes containing 50 ml saline and submitted to 1 min vortexing 5 min ultrasonication (VWR ultrasonication cleaner USC300D, power level 5) and 1 min vortexing. The cells recovered from the surfaces after washing and ultrasonication (i.e. sessile bacteria) were plated in serial dilutions for conventional viable cell count analysis. Biological data were tested towards significance by pairwise comparisons within the analysis applying Students t-Test.

Results and discussion

Bioactive layers

The strategy used to graft PEMA and POMA copolymer layers onto SiO_2 surfaces, and to covalently immobilize the enzymes, is outlined in Figure 1. For the covalent binding of MA copolymers, primary amino functionalities were introduced on the glass surfaces by binding an APTES precursor layer. Upon elevation of temperature, the introduced primary amines reacted with the anhydride group of the MA copolymers forming imide bonds. Subtilisin A and cellulase were then covalently immobilized onto PEMA and POMA copolymer films through the spontaneous reaction of free, non-reacted anhydride groups with primary amines of the enzymes. The variation of the concentration of the immobilization solutions further allowed obtaining enzyme-decorated films of distinct properties.

INSERT FIGURE 1

MA copolymer films covered with subtilisin A, were extensively characterized concerning immobilized amount (determined by ellipsometry, amino acid analysis using HPLC and confocal laser scanning microscopy using fluorescently labeled enzyme) and activity (determined by following the degradation of Suc-AAPF-pNA by absorbance spectroscopy at 405 nm). Further details can be found in Tasso et al. (2009a). Briefly, the amount and activity of immobilized subtilisin A was dependent on the concentration of the immobilization solution and

of the copolymer film. Highly swelling PEMA copolymer layers enabled the immobilization of up to five times more enzyme than hydrophobic POMA copolymer films.

Since cellulase consists of a complex mixture of more than three protein components (for details see Beldman et al. 1985), the quantification of the immobilized amount was not possible by a.a. analysis using HPLC. Therefore, the amount of immobilized cellulase was estimated by measuring the thickness of the immobilized protein layer by ellipsometry (Fig. 2). The results show a thinner enzyme layer on POMA copolymer films as compared with PEMA. The compact, non-swelling properties of the POMA copolymer films (Pompe et al. 2005) possibly triggers conformational changes and spreading of the proteins onto the surface as a result of hydrophobic interactions between the surface and the hydrophobic moieties of the proteins. Immobilization onto PEMA copolymer films enabled the generation of enzyme layers up to 10 times thicker than onto POMA layers, with minor dependence on the concentration of the immobilization solution (within the range tested). The hydrophilic, highly swelling, 3-D-like PEMA copolymer film possibly enables the penetration of the enzyme into the swollen layer resulting in higher loading. In addition, cellulose-containing PEMA copolymer films prepared with enzyme concentrations below 2 mg/ml were slightly inhomogeneous (results not shown). The accelerated transport of the proteins to the surface resultant from more concentrated immobilization solutions possibly favors cellulase immobilization onto hydrophilic polymer film.

INSERT FIGURE 2

The activity of the cellulase layers was determined by following the degradation of CMC at 50 °C using DNS to detect the amount of released reducing sugars (Ghose 1987). The initial activity, expressed as the concentration of released glucose equivalent per unit time, was determined through the slope of the linear region of the reaction progress curve. The initial activity of the cellulase layers in dependence of the copolymer film and of the concentration of the enzyme immobilization solution is presented in Fig. 3.

INSERT FIGURE 3

The results show that for immobilization of cellulase below 10 mg/ml, the activity of the immobilized enzyme layer for the different MA copolymer films was similar. Since thicker enzyme layers were measured onto PEMA copolymer films as compared with POMA copolymer films in this concentration range, the results suggest a more efficient immobilization onto the hydrophobic coating (i.e. a higher amount of active enzyme per total immobilized amount). The low immobilization efficiency onto PEMA copolymer films may be attributed to limited substrate diffusion and/or to restrictions in structural transitions of the enzyme within the multilayer-like packed enzyme, as compared with POMA copolymer films. For immobilization solutions higher than 10 mg/ml, the immobilization of cellulase onto PEMA copolymer films enables generating cellulase films with approx.

double the activity than onto POMA copolymer films. The higher concentration of the immobilization solution results in an accelerated transport of the enzyme to the polymer surface, possibly resulting in a conservation of enzyme conformation and activity.

Bacteria attachment

The effect of the activity of immobilized cellulase and of immobilized subtilisin A on the initial attachment of two bacteria species associated with hospital-acquired infections was investigated by comparing active enzyme containing PEMA copolymer coatings of highest activity (see Table 1 for coating properties) with respective heat-inactivated controls, and with bare PEMA copolymer films.

INSERT TABLE 1

The effects of immobilized cellulase and subtilisin A on the attachment of *Staphylococcus epidermidis* are presented on Fig. 4. While cell attachment was significantly reduced onto active cellulase layers, with 67% less cells attaching to the active surface as compared with respective denatured control, no significant differences were observed between active and inactive subtilisin A layers. The results suggest that proteins are playing a minor role on the initial attachment of *S. epidermidis*.

INSERT FIGURE 4

The effects of the activity of immobilized cellulase and of immobilized subtilisin A on the initial attachment of the pathogen *P. aeruginosa* is presented on Fig. 5. The results show that although immobilized subtilisin A reduced the attachment of *P. aeruginosa* by 44% when compared with its denatured control, immobilized cellulase had no significant effect on the attachment of *P. aeruginosa* when comparing active and denatured samples. In a first analysis, the results do not agree with the antifouling effect of free and immobilized cellulase towards *P. aeruginosa* as reported previously (Anderson et al. 2003; Loiselle and Anderson 2003). The different effects observed might be explained by the different experimental conditions used, as for example surface enzymatic activity, the different bacteria strains and biofilm growth conditions, as well as the different methodologies used for biofilm quantification.

INSERT FIGURE 5

The results suggest that different biomolecules are involved in the initial steps of attachment of the different bacteria species tested. Proteins may be playing a larger role in the initial attachment of *P. aeruginosa* to surfaces as compared with *S. epidermidis*, for which polysaccharides seem to play the major role.

The mechanisms by which enzymes may exert an antifouling effect include the degradation of molecules anchoring the cells to the surfaces (e.g. adhesins), and the degradation of the extracellular matrix thereby

weakening the biofilm structure and leading to dispersion or to the facilitated penetration of other antimicrobial compounds into the biofilm (Cordeiro and Werner, in press 2011). Previous studies showed that free subtilisin was more effective at inhibiting adhesion than at enabling detachment of the marine bacteria *Pseudoalteromonas sp.* D41 (Leroy et al. 2008b). In contrast, the enzyme inhibitory effects were comparable to the detachment effects for a multispecies biofilm (Hangler et al. 2009) suggesting that, in this case, the enzyme effectively degrades both protein-based adhesives and proteins contained in the matrix (Hangler et al. 2009). Recent work also showed that differences in chemical composition of the EPS are reflected on the vulnerability of biofilms to enzymatic treatment (Lequette et al. 2010; Chaignon et al. 2007; Augustin et al. 2004). For example, while staphylococcal biofilms producing substantial amounts of the polysaccharide adhesin poly-*N*-acetylglucosamine (PNAG) could be degraded by Dispersin B, biofilms not producing PNAG could not be dispersed by Dispersin B but by proteases (Chaignon et al. 2007).

Our results suggest that proteinaceous adhesives play a larger role in the attachment of *P. aeruginosa* than in the attachment of *S. epidermidis*. Further studies on the effects of immobilized cellulase and subtilisin A on the adhesion strength of the attached bacteria (i.e. testing the enzyme dispersal properties), and on possible synergetic effects of the activity of the immobilized enzyme and other antimicrobial agents (e.g. antibiotics) would be particularly important when considering the design of novel antimicrobial bioactive surfaces. Altogether the results demonstrate that due to the complexity and variability of microbial attachment mechanisms and of EPS composition, the production of enzyme-based antimicrobial coatings targeting the degradation of adhesive components should involve the co-immobilization of different enzymes to achieve high and broad-spectrum antifouling efficacy.

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Tables

Table 1 Description and properties of the enzyme containing PEMA copolymer layers tested for the effect of the activity of the immobilized enzymes on bacteria attachment.

Enzyme	Immobilization solution (mg/ml)	Immobilized amount ($\mu g/cm^2$)[a]	Thickness (nm)[b]	Activity
Subtilisin A	30[c]	2.18 ± 0.2[c]	20.2 ± 3.2[c]	236.3 ± 25 [pNa] / min $\times 10^3$ [c]
Cellulase	20	N.A.[d]	26.6 ± 0.8	4.1 ± 0.7 mg glucose eq. / l min[e]

[a] Determined by HPLC

[b] Determined by ellipsometry

[c] Data from Tasso et al. (2009a)

[d] See text for details

[e] Determined by following the release of reducing sugars from CMC at 50 °C (detection using DNS)

Figure captions

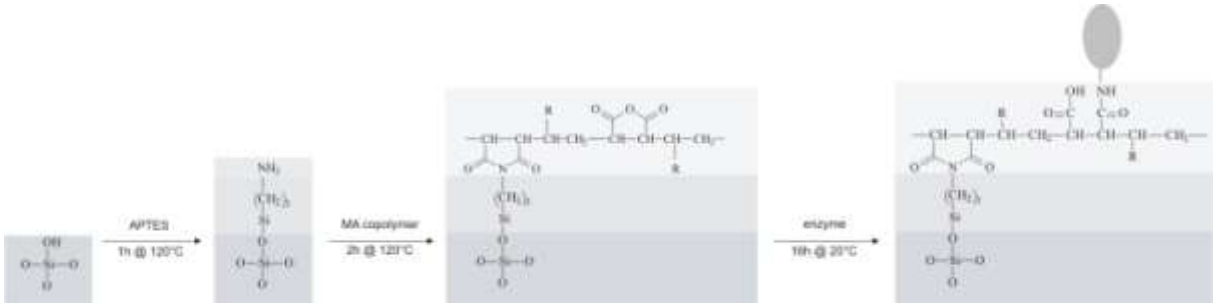
Fig. 1 Schematic representation of the strategy used to graft MA copolymer layers onto silica surfaces and for the covalent immobilization of biomolecules. R = H for PEMA and $(CH_2)_{15}CH_3$ for POMA. Dry thickness (ellipsometry): SiO_2 = 1-2 nm; APTES + MA copolymer = 5-11 nm; enzyme = up to 27 nm.

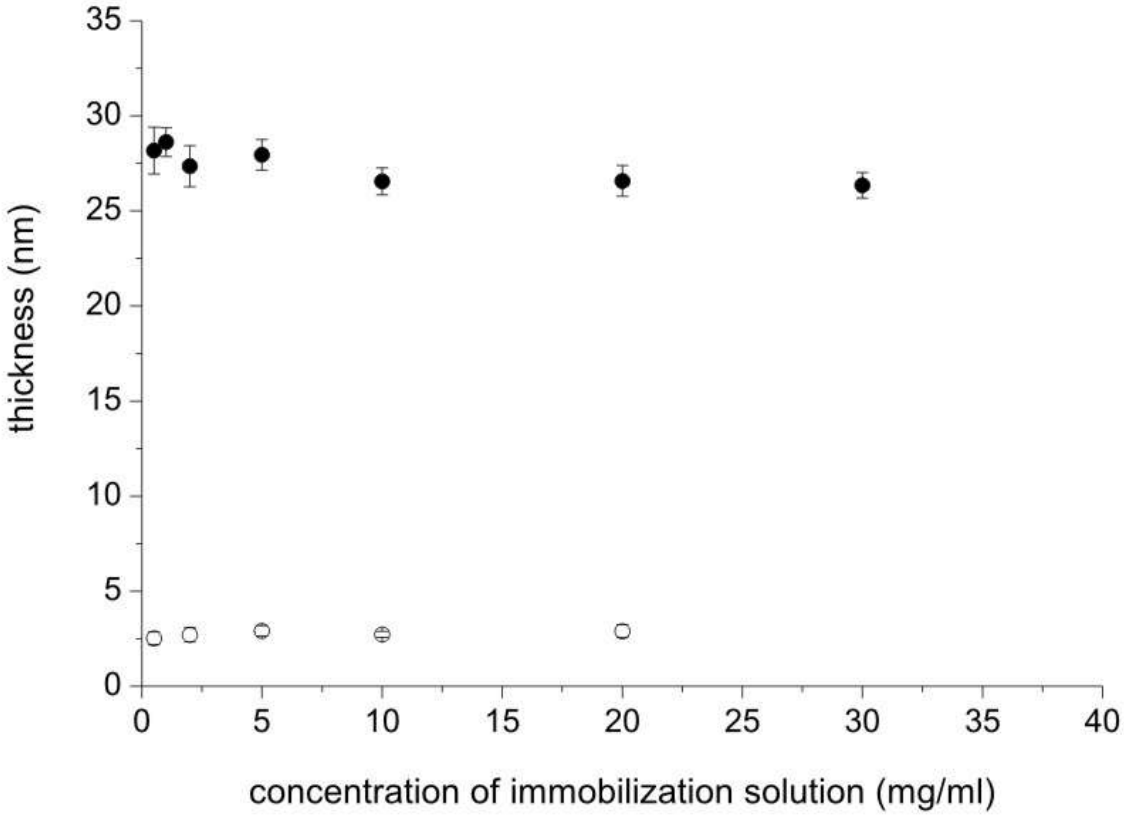
Fig. 2 Thickness of the immobilized cellulase layer onto PEMA (●) and POMA (○) copolymer films in dependence of the concentration of the immobilization solution determined by ellipsometry. Enzyme immobilization performed overnight at RT in 50 mM sodium citrate pH 4.0. Error bars = ± SD.

Fig. 3 Initial activity (expressed as the amount of released glucose equivalent per min) of immobilized cellulase onto PEMA (●) and POMA (○) copolymer films in dependence of the concentration of cellulase used for immobilization. Enzyme immobilization performed overnight at RT in 50 mM sodium citrate pH 4.0. Activity measured by detection of released sugars (using DNS) from 2% (wt/v) CMC in 50 mM sodium citrate buffer pH 4.8 at 50 °C. Error bars = ± SD.

Fig. 4 Surface adherent *Staphylococcus epidermidis* cells onto PEMA copolymer films containing no, active, or heat-denatured (a) cellulase and (b) subtilisin A after 24 h incubation at 37 °C. Error bars = ± SD.

Fig. 5 Surface adherent *Pseudomonas aeruginosa* cells onto PEMA copolymer films containing no, active or heat-denatured (a) cellulase, and (b) subtilisin A after 24 h incubation at 37 °C. Error bars = ± SD.

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20
25
30
35
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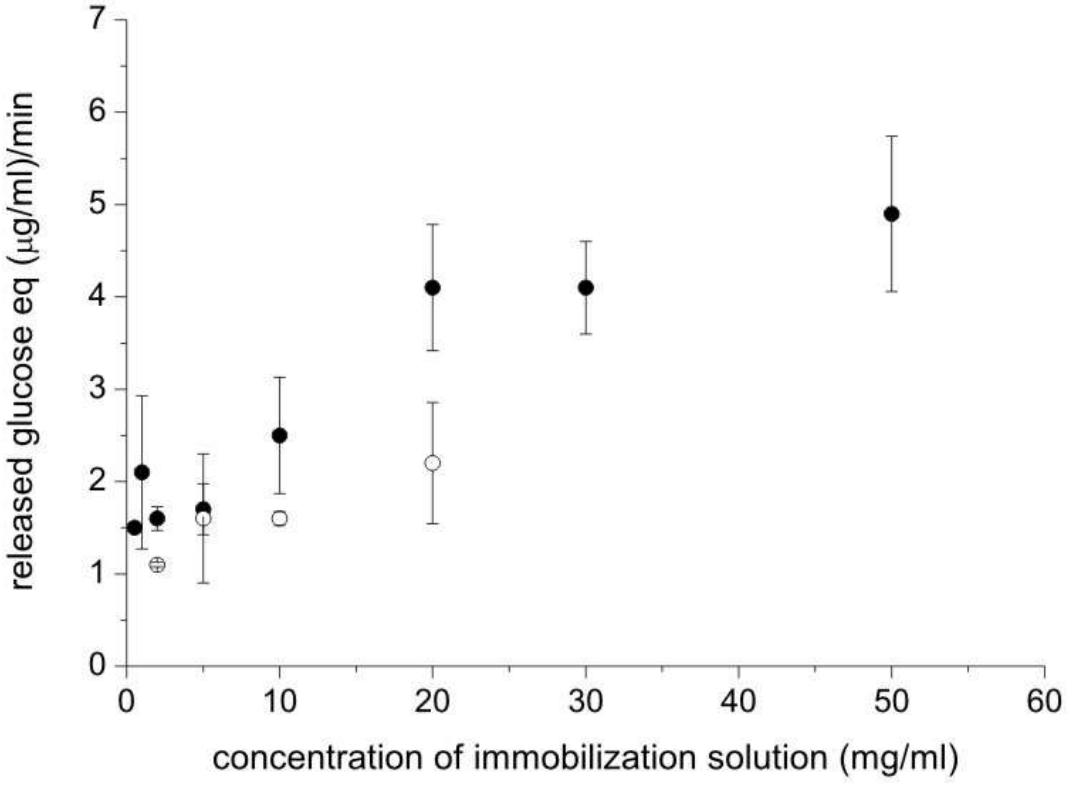
5
10
15
20
25
30
35
40
concentration of immobilization solution (mg/ml)


released glucose eq (μg/ml)/min
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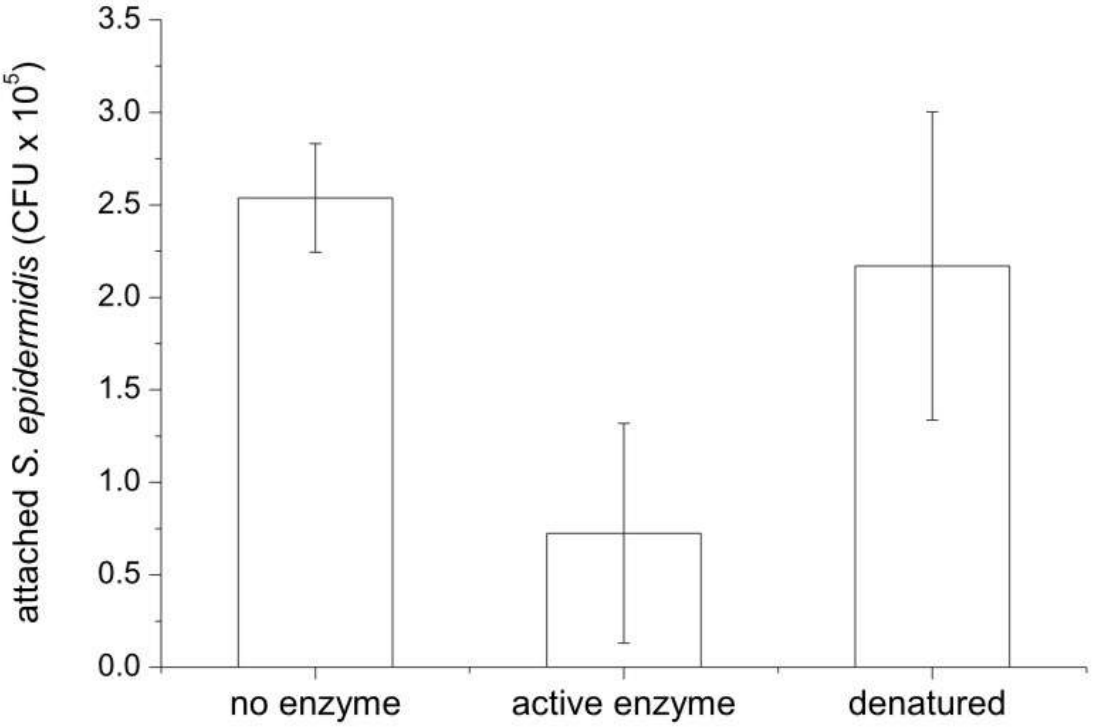
6
7
0
10
20
30
40
50
60
concentration of immobilization solution (mg/ml)


attached S. epidermidis (CFU x 10^5)
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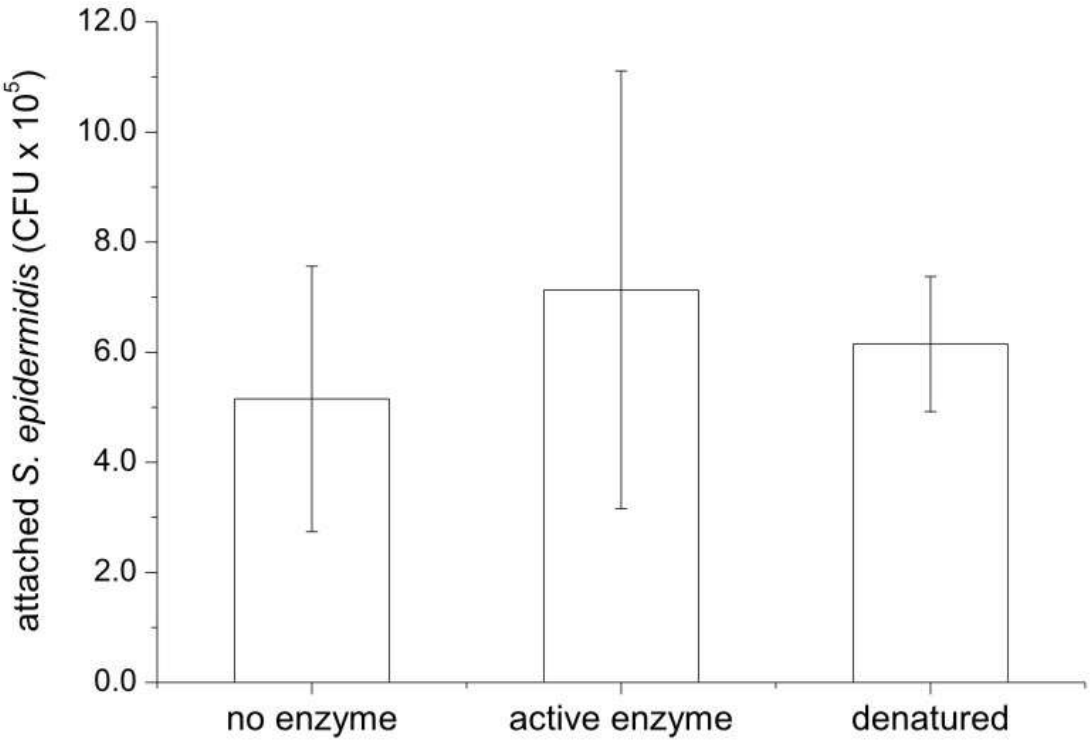
2.5
2.0
1.5
1.0
0.5
0.0
no enzyme
active enzyme
denatured


attached S. epidermidis (CFU x 10^5)
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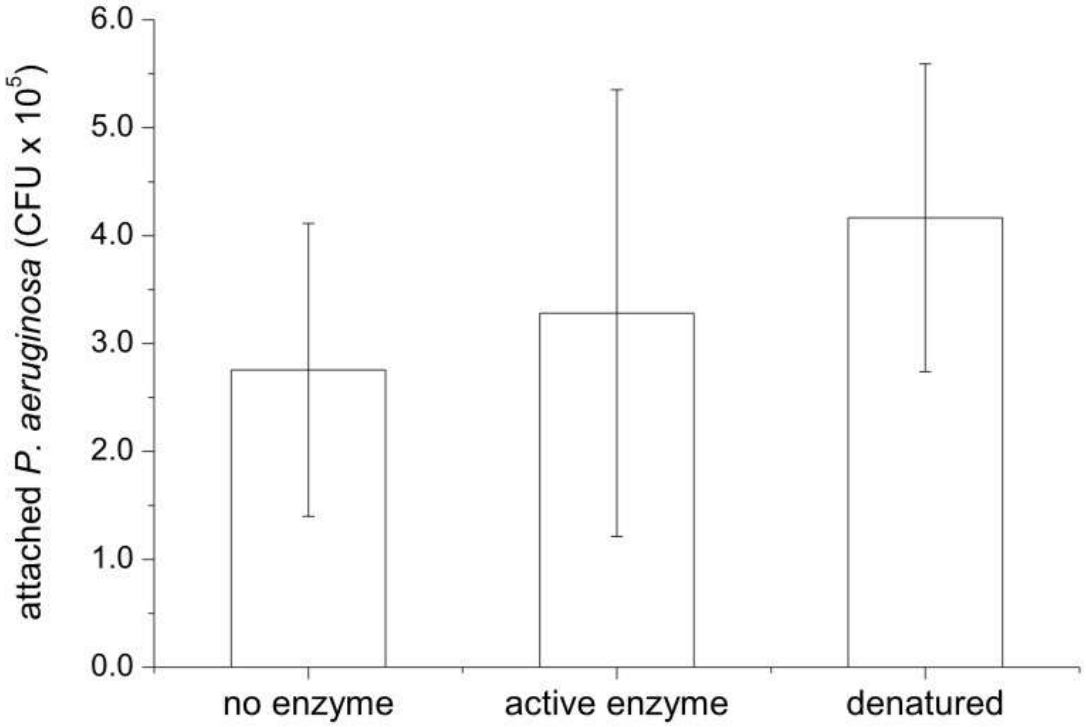
6.0
4.0
2.0
0.0
no enzyme
active enzyme
denatured


attached P. aeruginosa (CFU x 10^5)
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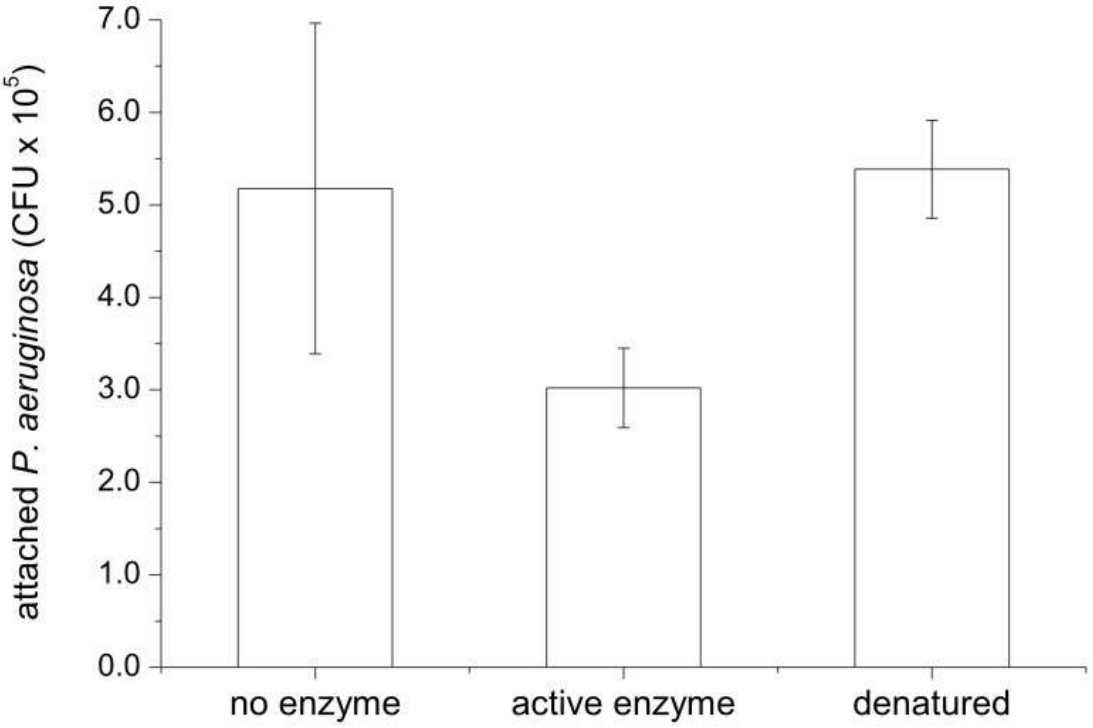
4.0
3.0
2.0
1.0
0.0
no enzyme
active enzyme
denatured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6.0
5.0
4.0
3.0
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0.0
no enzyme
active enzyme
denatured