A green and bio-inspired process to afford durable anti-biofilm properties to stainless steel

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A bio-inspired durable anti-biofilm coating was developed for industrial stainless steel (SS) surfaces. Two polymers inspired from the adhesive and cross-linking properties of mussels were designed and assembled from aqueous solutions onto SS surfaces to afford durable coatings. Trypsin, a commercially available broad spectrum serine protease, was grafted as the final active layer of the coating. Its proteolytic activity after long immersion periods was demonstrated against several substrata, viz. a synthetic molecule, \textit{N} -\textit{a}-benzoyl-DL-arginine-\textit{p}-nitroanilide hydrochloride (BAPNA), a protein, FTC-casein, and Gram-positive biofilm forming bacterium \textit{Staphylococcus epidermidis}.

\textbf{Keywords:} trypsin; DOPA; cross-linking; multilayer; anti-biofilm; stainless steel

Introduction

Microorganisms tend to attach, grow and form a biofilm on surfaces in contact with liquids. At this stage of material colonization, bacteria are embedded in a slimy matrix of extracellular polymeric substances (EPS) that protects them efficiently from physical and chemical biocidal agents, and so makes the biofilm very difficult to remove. Biofilms develop on many types of materials and affect a wide variety of industries ranging from offshore oil and gas and food processing, to marine equipment, cooling systems and medical devices. This inevitably comes with associated economic loss, maintenance costs and public health concerns (Mah and O’Toole 2001; Stoodley et al. 2002; Gilbert et al. 2003).

There are several methods for avoiding biofilm development. One is to use antimicrobial surface coatings that kill the colonizers as they come close in contact (Klibanov 2007). Marine equipment is protected against biofouling and biofilm formation using paints containing metal or organometallic compounds such as tributyl tin or copper complexes. However, the use of eco-toxic tin based coatings is prohibited in Europe since 2008 according to directives 76/769/EEC and 99/51/EC. In the medical field, silver ions or antibiotics can be inserted in catheters and other indwelling devices to reduce biofilm development. Nevertheless, several studies report on the toxicity of silver (eg AshaRani et al. 2009) and the increasing occurrence of antibiotic multi resistant strains is well attested. So, environmentally friendly antifouling approaches which are safe to humans are still needed. Other methods aim at delaying, reducing the initial attachment of fouling organisms, or at interfering with the formation of EPS (Murata et al. 2007; Banerjee et al. 2011). Examples of anti-adhesion/anti-biofilm coatings comprise polymers (polyethylene glycol) (Statz et al. 2006) or poly(sulfobetaine methacrylate) (Cheng et al. 2007) as well as enzymes (Olsen et al. 2007). More particularly, the immobilization of hydrolytic enzymes such as proteases and glycosidases at surfaces is reported as an environmentally friendly anti-biofilm approach. Indeed, these biomolecules degrade the proteins/polysaccharides that bioorganisms use to anchor and colonize substrata (Caro et al. 2010). There are few publications reporting the immobilization of enzymes on materials to confer antibacterial biofilm properties to surfaces (Minier et al. 2005; Caro et al. 2009, 2010; Yuan et al. 2011).

In this study, the selected antifouling agent was trypsin, a commercially available broad spectrum serine protease. This endopeptidase cleaves peptides on the C-terminal side of lysine and arginine amino acid residues (Rawlings and Barrett 1994) and so may prevent biofilm formation by hydrolyzing the proteins responsible for bacteria adhesion.

Stainless steel (SS) is a material widely used in the food and building industry but also in medicine and
various other fields because of its corrosion and chemical resistances and its aesthetical and mechanical properties (Helsen and Breme 1998). Unfortunately, it is often prone to biofilm formation when ageing. Some publications report on the development of biofilm resistant SS using antimicrobial peptides (Vreuls et al. 2010a; Hequet et al. 2011), enzymes (Caro et al. 2010) or a combination of antimicrobial and antiadhesion ingredients (Caro et al. 2009; Vreuls et al. 2010b; Yuan et al. 2011).

In the present work, a green and durable bio-inspired approach was developed to impart anti-biofilm properties to SS surfaces through trypsin grafting on a multilayered cross-linked thin film coating. A Layer-by-Layer (LbL) assembly process was selected because of its versatility towards the shape and the nature of the substrata and its green and simple mode of processing (Decher 1997). However, films made from electrostatic adsorption of water-soluble polymers are expected to be less stable when used in aqueous environments. In the present study, multilayer films were strongly anchored to SS surfaces due to a bio-inspired ‘glue’ layer composed of a water-soluble polycation containing 3,4-dihydroxy-L-phenylalanine amino acid (DOPA) groups, P(mDOPA)-co-P(DMAEMA$^+$) (2, Figure S1; 15 mol% mDOPA) [Supplementary material is available via a multimedia link on the online article webpage]. Its chemical composition has been inspired from the adhesive proteins present in mussels foot (Waite and Tanzer 1981; Yu et al. 1999) and described previously (Charlot et al. 2009). In addition to being responsible for mussel adhesion, DOPA can be involved in protein cross-linking when reacted with amino groups (Lee et al. 2006). This interesting feature led to the design of a tailored homopolymer bearing highly reactive quinone groups (Pox(mDOPA), (3, Figure S1) [Supplementary material is available via a multimedia link on the online article webpage] that was implemented for cross-linking multilayer films formed by alternating the deposition of a commercially available polymer bearing amino groups, poly(allylamine) (PAH, 1, Figure S1) (Faure et al. 2011).

These two bio-inspired polymers, P(mDOPA)-co-P(DMAEMA$^+$) and Pox(mDOPA), were the main building blocks for the design of cross-linked films containing strongly anchored trypsin that was required to impart long-term anti-biofilm properties to SS surfaces. The proteolytic activity of the grafted enzyme was confirmed towards various substrata ($\text{N}$-$\text{z}$-benzoyl-DL-arginine-$\text{p}$-nitroanilide hydrochloride (BAPNA) (4, Figure S1) [Supplementary material is available via a multimedia link on the online article webpage] and fluorescein-tagged casein (FTC-casein)) as well as against Gram-positive bacteria, eg Staphylococcus epidermidis.

**Experimental section**

**Nutrient media**

Bacto$^\text{Tm}$ Tryptone, Bacto$^\text{Tm}$ Yeast Extract and Bacto$^\text{Tm}$ casamino acids were obtained from Becton Dickinson (USA). Agar-agar was purchased from Merck (USA). Luria-Bertani medium (LB) was prepared with 10 g of Bacto$^\text{Tm}$ Tryptone, 5 g of Bacto$^\text{Tm}$ Yeast Extract, and 10 g of NaCl$^{-1}$ of deionized (DI) water. LB agar medium was obtained by adding 15 g of agar-agar$^{-1}$ of LB. M63 medium containing 100 mM $\text{K}_2\text{HPO}_4$, 15 mM ($\text{NH}_4$)$_2\text{SO}_4$, 1.6 mM, MgSO$_4$, 4 $\mu$M FeSO$_4$, pH 7.2 was supplemented with 0.5% casamino acids and 0.2% glucose.

**Polymers**

Synthesis of P(mDOPA)-co-P(DMAEMA$^+$) was described in a previous publication (Charlot et al. 2009). Briefly, $[2-(\text{methacryloxy})\text{ethyl}]$ trimethylammonium chloride (DMAEMA$^+$) was copolymerized with $\text{N}$-methacryloyl 3,4-dihydroxy-L-phenylalanine methyl ester (mDOPA) in water with V-50 as an initiator at 55°C for 24 h. Pox(mDOPA) was prepared according to a previously reported procedure (Faure et al. 2011). Briefly, P(mDOPA) was first synthesized by polymerizing $\text{N}$-methacryloyl 3,4-dihydroxy-L-phenylalanine methyl ester firstly protected with a borate function in water with V-501 as an initiator at 70°C for 24 h. P(mDOPA) was then oxidized into Pox(mDOPA) in water at 2 g l$^{-1}$ at a pH > 10 regulated by NaOH for 12 h.

Dopamine hydrochloride, polyethylenimine (PEI) ($M_w = 25,000$ g mol$^{-1}$), polyallylamine hydrochloride (PAH) ($M_w = 15,000$ g mol$^{-1}$) and poly(acrylic acid) (PAA) ($M_w = 15,000$ g mol$^{-1}$) were purchased from Sigma Aldrich and used as received.

**Preparation of SS surfaces**

SS 304 2B surfaces (2 cm × 2 cm) supplied by CRM Group AC&CS (Belgium) were cleaned with acetone and ethanol (scrubbing with an optical tissue) and dried under argon.

**Cross-linked multilayer film assembly and enzyme grafting**

Covalent LbL film building was carried out according to a previously reported procedure (Faure et al. 2011). Briefly, SS coupons were dipped into the P(mDOPA)-co-P(DMAEMA$^+$) solution (2 g l$^{-1}$ in DI water) for 2 min at room temperature and then rinsed by dipping successively in two different DI water baths for 1 min. The following cycle was then...
repeated to obtain five bilayers: (1) immersion in Pox(mDOPA) at 2 g l$^{-1}$ DI water, at pH > 10 for 2 min, (2) two 1 min-rinses in DI water, (3) immersion in PAH at 2 g l$^{-1}$ DI water, at pH > 10 for 2 min, (4) two 1 min-rinses in DI water. The last layer was Pox(mDOPA).

Trypsin (Sigma-Aldrich, T1426) was then grafted by incubating 100 $\mu$l of enzyme solution Na$_2$CO$_3$ 100 mM at pH 10.2 (adapted from Caro et al. (2010)) on the Pox(mDOPA)-modified SS coupons at room temperature. Trypsin-grafted substrata were then rinsed 6x1 min at room temperature over 20 h at 6°C in 2 ml-aliquots of Na$_2$CO$_3$ 100 mM pH 7.2, and finally kept at 6°C before assessment of enzyme activity.

The same procedure was followed when PEI (8 g l$^{-1}$) was used as adherence layer instead of P(mDOPA)-co-P(DMAEMA$^+$).

**Electrostatic multilayer film assembly and enzyme insertion**

SS coupons were dipped into the P(mDOPA)-co-P(DMAEMA$^+$) solution (2 g l$^{-1}$ in DI water) for 2 min at room temperature and then rinsed by dipping successively in two different DI water baths for 1 min. The following cycle was then repeated as necessary to obtain five bilayers: (1) immersion in PAA at 2 g l$^{-1}$ DI water, pH 6.5 for 2 min, (2) two 1 min-rinses in DI water, (3) immersion in PAH at 2 g l$^{-1}$ DI water, pH 8.5 for 2 min, (4) two 1 min-rinses in DI water. The last layer was PAA, PAA and PAH being negatively and positively charged at the pHs used, electrostatic multilayers were easily built with the last layer being negatively charged.

Trypsin was then adsorbed by incubating 100 $\mu$l of enzyme solution Na$_2$CO$_3$ 100 mM at pH 7.2 on the PAA-modified SS coupons at room temperature. Trypsin-adsorbed substrata were then rinsed 6 x 1 min at room temperature over 20 h at 6°C in 2 ml-aliquots of Na$_2$CO$_3$ 100 mM pH 7.2, and finally kept at 6°C before assessment of enzymatic activity.

**Polydopamine coating and enzyme grafting**

Polydopamine coatings (films based on the self-polymerization of dopamine) were prepared following the procedure reported by Lee et al. (2007) with two immersion times: 17 h (duration reported in Lee et al. 2009) and 44 min (in order to fit the duration of the cross-linked multilayer presented above) and two 1 min-rinses in DI water.

Trypsin was then grafted by incubating 100 $\mu$l of enzyme solution at 10 mg ml$^{-1}$ Na$_2$CO$_3$ 100 mM, pH 10.2 on polydopamine-modified SS coupons for 4 h at room temperature. Trypsin-grafted substrata were then rinsed 6 x 1 min at room temperature over 20 h at 6°C in 2 ml-aliquots of Na$_2$CO$_3$ 100 mM pH 7.2, and finally kept at 6°C before assessment of enzyme activity.

**Multilayer characterizations**

Film growth was followed in real time using a quartz crystal microbalance coupled with the dissipation technique (QCM-D). A Q-Sense E4 was used in this study. The SS-coated AT-cut resonator (fundamental frequency: 5 MHz) was used as received. AT-cut is the technical term in the QCM-D technique for a crystal singularly rotated on its Y-axis cut in which the top and bottom half of the crystal move in opposite directions (thickness shear vibration). First, distilled water was introduced into the cell and the flow was maintained until a stable baseline was obtained. LbL deposition was then initiated by switching the liquid exposed to the crystal from distilled water to the glue solution (P(mDOPA)-co-P(DMAEMA$^+$); 2 g l$^{-1}$ with 0.15 M NaCl) at a flow rate of 250 $\mu$l min$^{-1}$ and a temperature of 25.09 ± 0.02°C. After 10 min, the substrate was rinsed by distilled water to remove the excess of unbounded glue. Then, the alternative deposition of Pox(mDOPA) (2 g l$^{-1}$) and PAH (2 g l$^{-1}$) solutions was carried out (∼10 min for each step) with rinsing steps with distilled water between each layer. Ellipsometry measurements were done using a SOPRA GES 5 working in the UV-Visible range from 300 up to 900 nm. The angle incidence was 75°.

**Measurement of proteolytic activity**

**Synthetic substrate:** N-$\alpha$-benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPNA, 4, Figure S1) [Supplementary material is available via a multimedia link on the online article webpage]

The proteolytic activity of the immobilized trypsin was monitored by measuring the increase in absorbance at 405 nm proportional to the increase in p-nitroaniline concentration (Figure 3). N-$\alpha$-benzoyl-DL-arginine-p-nitroanilide hydrochloride solution (BAPNA, 1 mM) (Sigma Aldrich ref B3133) was prepared by agitation over 12 h at room temperature in Tris buffer 50 mM, CaCl$_2$ 10 mM, at pH 8.2. One hundred and sixty $\mu$l of this solution were incubated on the modified SS coupons for 40 min at 37°C. Then 120 $\mu$l were then collected from the samples, transferred to a 96-well plate and absorbance measured at 405 nm using a spectrophotometer (Perkin Elmer).
agar. The plates were incubated at 37°C for 24 to 72 h. After incubation of an aqueous trypsin solution containing 25 mg l⁻¹ of 2,3,5-triphenyltetrazolium chloride dye (Fluka, ref. 93140). After incubation for 24 to 72 h at 37°C, strongly adherent living bacteria remaining on the samples appeared as purple CFU and were counted. All colony counts results are averages of 3 replicates.

**Casein substrate**

The Pierce® Fluorescent Protease Assay Kit (ref 23266) was used to assess the proteolytic activity of immobilized trypsin. FTC-casein solution was prepared by dissolving 10 μl of stock solution in 10 ml of TBS buffer (composition: 25 mM Tris, 0.15 M NaCl, pH 7.2). One hundred and sixty μl of this solution were incubated on the modified SS coupons at room temperature in the dark. After 1, 4, 15 and 24 h, 120 μl were collected from the samples, transferred to a 96-well plate and fluorescence intensity quantified using a plate reader (Enspire™ 2300 Perkin Elmer) fitted with a standard fluorescein excitation/emission filters (485/538 nm). As controls, buffer TBS and FTC-casein solution had fluorescence intensities around 25 and 1200, respectively.

**Assessment of biofilm formation and counts of viable bacteria**

A preculture of biofilm forming *Staphylococcus epidermidis* ATCC35984 was grown overnight at 37°C in 3 ml of LB medium and used the next morning to seed a fresh culture in 50 ml of LB. The bacterial concentration of the test inoculum was adjusted to ~10⁷ cells ml⁻¹ in M63 medium supplemented with glucose and casamino acids.

SS coupons coated with trypsin (grafted or inserted) films were placed in Petri dishes containing damp blotting paper. The test inoculum (200 μl) was pipetted onto each substratum. The Petri dishes containing the inoculated coupons were closed and incubated at 37°C for 24 h.

The SS substrata were rinsed twice with 10 ml of sterile DI water to remove non-adherent bacteria, and then placed face downward in glass jars containing 20 ml of 500-fold-diluted LB and 4-mm glass beads. The jars were shaken horizontally for 10 min, and then their contents were sonicated in a water bath (50–60 KHz) for 2 min. Living bacteria were counted by plating 10-fold dilutions on LB agar containing 25 mg l⁻¹ of 2,3,5-triphenyltetrazolium chloride dye (Fluka, ref. 93140). After incubation for 15 min at 22°C ± 2°C in the dark and removal of the excess inoculums, biofilms were observed with an epifluorescence microscope (Olympus BX60/URFL-T, optical filters: excitation 486 nm and emission 520 nm, oil immersion, 1000× magnification). Cells that fluoresced green had intact membranes whereas cells that fluoresced red had compromised membranes. Adherent cells were motionless whereas non-adhering cells were in motion.

**Results**

**Multilayer building and enzyme immobilization**

Durable amine/quinone cross-linked multilayered films were built on SS surfaces following the strategy reported by Faure et al. (2011). Briefly, after coating the substratum with a layer of the bio-inspired glue P(mDOPA)-CO-P(DMAEMA⁺) (2, Figure S1) [Supplementary material is available via a multimedia link on the online article webpage], alternative deposition of Pox(mDOPA) (3, Figure S1) and poly(allylamine hydrochloride) (PAH; 1, Figure S1) was realized by dipping SS in aqueous solutions of these polymers at room temperature until a 5 cross-linked bilayer film was formed. The film building was demonstrated by a quartz crystal microbalance coupled with Dissipation (QCM-D). This technique follows the frequency change of a vibrating SS sensor vs time when a polymer solution is flowing over it; a decrease in frequency results in polymer deposition (Marx 2003). Figure 1 shows that P(mDOPA)-CO-P(DMAEMA⁺), Pox(mDOPA) and PAH were successfully deposited on the crystal in these conditions. Rinsing of the substratum with water demonstrated that most of the polymer chains remained on the substratum. Only a small fraction was removed that should correspond to excess of unbounded polymer. Ellipsometry measurements revealed that the 5-bilayer film, P(mDOPA)-CO-P(DMAEMA⁺)/[(Pox(mDOPA)/PAH)₅], had a thickness of ~9–10 nm. Pox(mDOPA) was the last layer, in order to render the surface reactive towards the biomolecule grafting as described below.

The incubation of an aqueous trypsin solution (100 μl, pH = 10.2) onto the modified substratum at room temperature allowed strong anchorage of the enzyme to the coating. Indeed, amino groups of the biomolecule are highly reactive towards the quinone functions present in Pox(mDOPA), therefore leading to its covalent grafting through Schiff base formation and/or Michael addition reactions according to
previous studies (Lee et al. 2006; Faure et al. 2011). After immobilization, each sample was thoroughly rinsed with buffer, with a final immersion period of 20 h at 6°C to remove the excess of unbound trypsin.

**Enzymatic activity of surface-immobilized trypsin**

*Proteolytic activity against N-\(\alpha\)-benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPNA)*

Trypsin specifically cleaves the peptide bond at the C-terminal side of basic amino acids (lysine or arginine). The catalytic activity of the immobilized protease was assessed by measuring spectrophotometrically its ability to convert a specific substrate (\(N-\alpha\)-benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPNA)) into \(p\)-nitroanilide that absorbs at 405 nm (Figure 3). The trypsin concentration and incubation time were optimized using this rapid screening technique and the results are summarized in Table 1.

Using the experimental conditions described in Caro et al. (2010), the trypsin concentration was first fixed at 1 mg ml\(^{-1}\) and the incubation time at 1 h at room temperature. In these grafting conditions, the final absorbance at 405 nm was 0.19 ± 0.04 on four replicates. According to a calibration curve of the trypsin activity conducted in solution in a 96-well plate (volume/well = 120 \(\mu\)l) (Figure 2), this absorbance value corresponded to about 140 ng of trypsin per well, thus around 35 ng of catalytically active grafted trypsin per cm\(^2\) of substrate. However, no significant absorbance was observed when trypsin was inserted at pH 7.2 (the enzyme was positively charged) in a multilayer film built exclusively on electrostatic interactions between PAA and PAH instead of covalent ones (PAA was negatively charged and was the last layer of the film) (Table 1). Indeed, the absorbance was almost as low as that corresponding to the BAPNA solution in the absence of enzyme (0.06). The same behaviour was observed when poly(ethylenimine) (PEI) was used as the adherence layer instead of the...
bio-inspired glue (P(mDOPA)-co-P(DMAEMA\textsuperscript{+})). The film formation (PEI/(Pox(mDOPA)/PAH)\textsubscript{5}) and the grafting of trypsin on this film was evidenced by QCM-D (see ESI, Figure S2) [Supplementary material is available via a multimedia link on the online article webpage]. These comparisons indicated the importance of the strategy used for the coating activity. Indeed an optimal combination between an adherent bio-inspired layer and a green cross-linking pathway for the whole film building was necessary for maintaining an active film on the surface.

By increasing both the dipping period (from 1 to 4 h) and the concentration of trypsin (from 1 to 10 mg ml\textsuperscript{-1}) for the grafting, the content of immobilized enzyme on the cross-linked multilayer was increased from 35 ng cm\textsuperscript{-2} to 52 ng cm\textsuperscript{-2} (Table 1).

Finally, for comparison, these optimized experimental conditions were tested on the versatile polydopamine coating prepared by self-polymerization of dopamine over 12 to 24 h as reported elsewhere (Lee et al. 2007, 2009; Kang et al. 2009). These authors demonstrated successful trypsin immobilization on this robust polydopamine coating. When dopamine was self-polymerized in the presence of SS for 44 min instead of 12–18 h (such a time scale was not realistic for industrial applications on large surfaces), no significant absorbance was measured (~0.09) indicating that almost no enzyme was immobilized. Moreover, when self-polymerization was kept going for ~17 h, the proteolytic activity of the trypsin was not significantly increased (Table 1). It can result from the incubation time of trypsin (4 h) being too short to reach the embedded reactive sites compared to the work of Lee et al. (2009) who reacted trypsin for 1 night with the polydopamine coating.

### Proteolytic activity against fluorescein-tagged casein (FTC-casein)

The biofilm matrix is a complex mixture of polymeric biological components including proteins. In this study trypsin was aimed at impairing the development of mature biofilm by acting on the matrix proteins as the biofilm was formed. Casein was used as a substrate in order to confirm that the immobilized enzyme retained its catalytic activity not only against a small and easily diffusing substrate such as BAPNA, but also against macromolecular substrates such as those that the protease is targeted for in a real biofilm formation situation. The proteolytic activity of the immobilized trypsin was therefore assessed against fluorescein-labelled casein. Upon digestion of the native FTC-casein, the protease released small fluorescein-labelled fragments, decreasing the fluorescence quenching (Figure 3). Fluorescence resonance energy transfer (FRET) measurements would therefore allow determination of the enzyme activity. The results expressed as RFU as a function of the contact time between the immobilized enzyme and the fluorescein-labelled substrate are shown in Figure 4. The proteolytic activity of the grafted enzyme was observed after contact with the substrate for 15 and 24 h. Greater proteolytic activity was observed when the trypsin was grafted on top of cross-linked films as compared to trypsin adsorbed on the top of the electrostatic-based films.

### Effect of trypsin on the formation of a S. epidermidis biofilm

**Fluorescence microscopy**

The impact of trypsin on the formation of a S. epidermidis biofilm was qualitatively observed on SS coupons after labelling with fluorescent dye. Images taken with an epifluorescence microscope after biofilm formation for 24 h are summarized in Figure 5. Large clusters of glued and static bacteria in a matrix were observed both on bare SS (A, Figure 5) and on P(mDOPA)-co-P(DMAEMA\textsuperscript{+}))/(Pox(mDOPA)/PAH)\textsubscript{5} coated SS (B, Figure 5) demonstrating the formation of a thick biofilm on these two surfaces. By contrast, SS surfaces coated with P(mDOPA)-co-

### Table 1. Intensities of absorbance measured at 405 nm corresponding to trypsin proteolytic activity against BAPNA when involved in several multilayer films.

| Coating | Trypsin concentration (mg ml\textsuperscript{-1}) | Trypsin incubation time: 1 h | Trypsin incubation time: 4 h |
|---------|-----------------------------|------------------|------------------|
| P(mDOPA)-co-P(DMAEMA\textsuperscript{+})/(Pox(mDOPA)/PAH)\textsubscript{5} | 1 | 0.19 ± 0.04 (35 ± 7)\textsuperscript{a} | n.d.\textsuperscript{b} |
| | 10 | 0.24 ± 0.04 (44 ± 7)\textsuperscript{a} | 0.28 ± 0.07 (52 ± 13)\textsuperscript{b} |
| P(mDOPA)-co-P(DMAEMA\textsuperscript{+})/(PAA/PAH)\textsubscript{5} | 1 | 0 | n.d.\textsuperscript{b} |
| | 10 | 0.03 ± 0.01 (5 ± 2)\textsuperscript{a} | 0 |
| PEI/(Pox(mDOPA)/PAH)\textsubscript{5} | 1 | 0.03 ± 0.003 (5 ± 0.5)\textsuperscript{a} | n.d.\textsuperscript{b} |
| Polydopamine (44 min) | 10 | 0.28 | 0.03 ± 0.003 (9 ± 0.5)\textsuperscript{a} |
| Polydopamine (17 h) | 10 | n.d.\textsuperscript{b} | 0.05 ± 0.003 (9 ± 0.5)\textsuperscript{a} |

Note: a: amount of catalytically active grafted trypsin expressed in ng cm\textsuperscript{-2} of substratum. b: not determined. The BAPNA reference value of 0.06 was subtracted beforehand.
P(DMAEMA^+)/Pox(mDOPA)/PAH films grafted with trypsin (C, Figure 5) showed a large population of scattered bacteria, in constant motion when observed under the epifluorescence microscope, and few moving wadded clusters, an indication of less/no biofilm formation.

Quantitative measurement of biofilm

The anti-biofilm effect of the coatings with and without immobilized protease was evaluated against a biofilm forming Gram-positive strain, *S. epidermidis*. Table 2 shows that the number of viable adherent bacteria decreased by 92% (standard deviation (SD): 4%) for the cross-linked film grafted by trypsin (P(mDOPA)-co-P(DMAEMA^+)/Pox(mDOPA)/PAH)-Trypsin). For comparison, no anti-biofilm activity was observed for SS coated by the cross-linked film that did not contain trypsin, as was the case for uncoated SS. Importantly, a multilayer film formed by electrostatic interactions followed by deposition of trypsin on the last layer ((P(mDOPA)-co-P(DMAEMA^+)/PAA/PAH)_5-Trypsin), presented a low anti-biofilm activity of 58% that was not reproducible (SD: 39%). These comparisons clearly demonstrate that the incorporation of trypsin by electrostatic interactions was not enough to impart high anti-biofilm properties to the multilayer coating. Its covalent grafting was necessary to maintain this important activity.

Discussion

Several strategies for trypsin grafting on various kinds of surfaces can be found in the literature (Kang et al. 2009; Lee et al. 2009; Caro et al. 2010; Sudheendran and Buchmeiser 2010). In particular, Caro et al. (2010) immobilized trypsin on SS surfaces; the enzyme was chemically linked to a thin polymer
layer that was physisorbed on the surface, bearing a high density of primary amine functions. The SS samples were pre-treated with organic solvents (ethanol, acetone and cyclohexane) and post-treated with sulfochromic acid to favor the adsorption of the adherence layer. The final enzymatic activity was assessed after three rinsing steps in PBS for 40 min.

In the present study, a proteolytic enzyme grafting strategy was proposed to impart high anti-biofilm properties on industrial SS surfaces. It relied on a set of environmentally friendly processes without the use of toxic and aggressive chemical reagents. The thin film coating comprised two newly developed water-soluble polymers containing DOPA, one of the natural constituents responsible for the well-known mussel versatile adhesion (Waite and Tanzer 1981; Yu et al. 1999). The anchoring of the coating onto SS was ensured by a polycation (P(mDOPA)-co-P(DMAEMA\(^{+}\)) that contained DOPA units required for adhesion onto the surface. Then, the alternative deposition of a homopolymer containing oxidized DOPA groups (Pox(mDOPA)) with a polymer bearing primary amine ones (PAH) provided the covalently cross-linked multilayer film, due to the quinone/amine reaction between the two partners. This reaction was implemented to graft trypsin on the last Pox(mDOPA) layer of the coating. The whole process was conducted in water at room temperature without the use of an additional chemical cross-linking agent.

Similar grafting strategies involving oxidized catechol functions were reported previously (Kang et al. 2009, Lee et al. 2009). However, the time required for surface modification can reach 24 h of substrate immersion in the Tris buffered dopamine solution and up to overnight incubation when enzyme grafting is considered. In the present work, about 45 min were necessary to build on SS surfaces a five-bilayer cross-linked coating based on the two newly developed DOPA containing polymers and four additional hours for enzyme grafting. As a basis for comparison, enzyme grafting realized on a polydopamine coating within this same time frame showed no catalytic activity against BAPNA. Using the grafting procedure, the durability of the enzyme grafting was demonstrated here after multiple rinsing steps followed by a long immersion period of 20 h.

In several publications (Kang et al. 2009; Lee et al. 2009; Sudheendran and Buchmeiser 2010), the proteolytic activity of the grafted enzyme was quantified on synthetic small size substrata such as BAPNA. These

| Multilayer film composition | % Reduction of adherent population |
|-----------------------------|-----------------------------------|
| Bare SS                     | 0                                 |
| P(mDOPA)-co-P(DMAEMA\(^{+}\))/ (Pox(mDOPA)/PAH)\(_3\) | 0* |
| P(mDOPA)-co-P(DMAEMA\(^{+}\))/ (PAA/PAH)\(_2\)-Trypsin | 58 ± 39 |
| P(mDOPA)-co-P(DMAEMA\(^{+}\))/ (Pox(mDOPA)/PAH)\(_3\)-Trypsin | 92 ± 4 |

Note: *final number of adherent bacteria was higher than on uncoated SS.

Figure 6. General strategies to characterize grafted trypsin activity on a SS surface.
molecules were able to diffuse easily and so reach the active site of the enzyme even if it is less accessible due to random immobilization. In the present paper, immobilized trypsin activity was first measured on BAPNA and confirmed on a high molecular weight protein, casein, displaying steric hindrance and limitation in the interaction with the trypsin active site (Figure 6). Finally, the anti-biofilm efficiency of immobilized trypsin was documented by both fluorescence microscopy and in vitro quantitative assessment against a biofilm of Gram-positive bacteria.

The DOPA based thin film coating may be further exploited for multiple enzyme grafting, combining proteases and/or glucanases, and allowing multispecies biofilm control/prevention to be addressed. Also, several experiments are under investigation in order to further decrease the film layer number in order to improve industrial scaling up of the coating process. This will be reported in future publications.

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