Grafting of Lysozyme and/or Poly(ethylene glycol) to Prevent Biofilm Growth on Stainless Steel Surfaces

Anne Caro,[†] Vincent Humblot,[†] Christophe Méthivier,[†] Michel Minier,[‡] Michèle Salmain,[‡] and Claire-Marie Pradier*[,†]

Laboratoire de Réactivité de Surface, Université Pierre et Marie Curie - Paris VI - UMR CNRS 7609 - Tour 54, case 178, 4 place Jussieu, 75252 Paris Cedex 05, France, and Laboratoire de Chimie et Biochimie des Complexes Moléculaires, UMR CNRS 7576, Ecole Nationale Supérieure de Chimie de Paris, 11 rue Pierre et Marie Curie, 75231 Paris Cedex 05, France

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In the aim of protecting stainless steel surfaces against protein and/or bacterial adhesion, thin films including the glycosidase hen egg white lysozyme (HEWL) and/or the synthetic polymer poly(ethylene glycol) (PEG) were covalently coated onto flat substrates by wet chemical processes. Chemical grafting of both species was carried out by covalent binding to surfaces pretreated by the polyamine poly(ethylene imine) (PEI). Surfaces were characterized at each step of functionalization by means of reflection−absorption infrared spectroscopy by modulation of polarization (PM-RAIRS) and X-ray photoelectron spectroscopy (XPS) to determine the atomic and molecular composition of the interfaces, respectively. Then, the ability of the so-modified surfaces to prevent protein adsorption and bacterial adhesion together with their biocide properties were demonstrated by three local tests employing bovine serum albumin (BSA), and the bacteria *Listeria ivanovii* and *Micrococcus luteus*. A new test was implemented to assess the local enzymatic properties of HEWL. Cografting of PEG and HEWL resulted in a surface with both antiadhesion and antibacterial properties.

1. Introduction

Colonization of materials by living organisms occurs whenever they are in contact with biological environments. In the marine environment, this phenomenon is known as biofouling. It affects for instance ship hulls and induces considerable damages and subsequent additional maintenance costs. Antifouling paints that were loaded with tributyl tin until 2003, now include less toxic biocides such as copper-based compounds and metal-free compounds presenting activity against algae and other marine organisms.[1] Their antifouling activity is exerted by a slow leaching of the biocide initially included in soluble or insoluble matrices into the seawater surrounding the vessel.

Adhesion of microorganisms (mostly bacteria) is also encountered in the food environment (food processing equipments and packaging). Food that has entered in contact with contaminated surfaces may in turn become contaminated and allow transmission of infectious diseases.[2,3] In a closely related area, some of the nosocomial infections are known to be caused by the use of biomedical devices on which biofilms of Staphylococci bacteria have grown.[4] Surface cleaning procedures that are classically set in place to get rid of adherent bacteria may not be fully efficient against mature biofilms.[5] This is why extensive researches are currently carried out so as to prevent adhesion of microorganisms, among which one consists in altering the surface chemistry, for example by grafting species that would confer permanent antiadhesion or even biocidal properties to the surfaces.[6−9]

Poly(ethylene glycol) (PEG) coatings have been proposed as efficient tools to design surfaces resisting bioadhesion.[10] It is known to be particularly effective at preventing protein adsorption, providing their length is sufficiently long and the grafting density high.[11−15] Its unique coordination with surrounding water molecules in aqueous medium[16] gives rise to steric repulsion when proteins come close to the surface and thus contributes to the inertness of the PEG-terminated surfaces.[13,17] A range of techniques can be employed for the immobilization of PEG onto surfaces: covalent grafting of PEG with suitable terminal functions by solution-phase chemistry,[18−21] synthesis of PEG chains by polymerization of EG monomers, plasma polymerization of PEG precursors,[22−24] or even spontaneous assembly of PEG-grafted copolymers; in other words, the grafting of PEG side chains onto a polymer backbone, resulting in the formation of a comblike structure.[25] However, some authors have recently pointed out that PEG coatings were less efficient with respect to bacterial adhesion.[20,21]

Coming back to the biofouling issue, enzymes, in particular hydrolytic enzymes such as glycosidases and proteases, have been envisaged as alternative additives in antifouling paints.[26−28] The rationale is that these novel antifoulants are environmentally friendly and act more specifically that the traditional biocides mentioned above. Interestingly, hydrolases are also extensively used for cleaning of equipments used in the food processing industry.[29] Immobilization of glycosidase lysozyme at the surface of polymeric packaging films led to materials displaying bacterial growth inhibiting properties.[30,31]

Thus PEG and enzyme films are expected to provide surfaces with antiadhesive properties (by keeping away fouling species such as proteins and carbohydrates) and local biocide properties, respectively. Cografting of these polymers might confer to the surfaces both above-mentioned and complementary properties, thus ensuring an optimal protection of surfaces against protein and/or bacterial adhesion. Surprisingly, there have been very few reports dealing stricto sensu with the immobilization of

* To whom correspondence should be addressed. E-mail: Claire-Marie.Pradier@ccr.jussieu.fr. Tel: +33 (0)1 44 27 55 33. Fax: +33 (0)1 44 27 60 33.

[†] Université Pierre et Marie Curie - Paris VI - UMR CNRS 7609.

[‡] UMR CNRS 7576, Ecole Nationale Supérieure de Chimie de Paris.

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enzymes on surfaces in the purpose of inhibiting bacterial adhesion eventually leading to the formation of biofilm[32] and no report of attempts to cograft an enzyme and PEG together.

The objective of the present work is to chemically graft PEG, lysozyme, and both PEG and lysozyme to the surface of AISI 316 L stainless steel substrates and evaluate the antiadhesion and bactericidal properties resulting from these surface treatments using specially designed local tests. Lysozyme was chosen because it catalyzes the hydrolysis of 1,4-β-glycosidic linkages between *N*-acetymuramic acid and *N*-acetylglucosamine, which are components of the cell wall peptidoglycan of bacteria. As such, this enzyme is an efficient antibacterial (bacteriolytic) agent toward Gram(+) bacteria.

2. Experimental Procedures

2.1. Materials. PEI (branched poly(ethylenimine), average M_w ~25 000), glutaraldehyde (GA) 25% in water (w/w), lysozyme from chicken egg white crystallized three times (HEWL) (50 000 units/mg protein), and BSA (albumin from bovine serum) were obtained from Sigma-Aldrich. PEG (ButyrALD - poly (ethylene glycol) - ButyrALD, MW 3400 Da) was obtained from Nektar Therapeutics. The Gram-positive bacterial strain *Micrococcus luteus* CIP A270 (same strain as *M. lysodeikticus* ATCC 4698) was obtained from the Collection of Institut Pasteur. The Gram(+) bacterial strain, *Listeria ivanovii Li4pVS2*was obtained from the Laboratoire de Chimie et Microbiologie de l'Eau (Poitiers - France). It was chosen for the cell adhesion tests because it belongs to the same genus as the foodborne pathogen *Listeria monocytogenes*, which is known to adhere and form biofilms on stainless steel surface, which is a major cause of food products contamination.[33] Moreover, *L. ivanovii* is easy to handle and nonpathogenic to humans.[34] AISI 316 L stainless steel coupons (10 × 10 × 2 mm), polished on one side to 0.5 μm, were purchased from Goodfellow.

2.2. Pretreatment of Coupons. Coupons were polished down to 1/4 μm (abrasive/polishing disk and diamond suspension), leading to an average roughness equal to 2−4 nm as checked by atomic force microscopy. They were rinsed with ethanol, then successively ultrasonically washed 15 min in cyclohexane, three times 10 min in water, and 20 min in acetone. Coupons were then etched by sulfochromic acid (6 g of potassium bichromate per 100 mL of sulfuric acid) at 60 °C during 10 min to form a reactive, Cr-enriched, hydroxylated oxide top layer.[35] They were then extensively rinsed with water and dried with a flow of N_2. The resulting coupons will be named SS-SC.

2.3. Grafting and Cografting of HEWL and PEG. The grafting process for HEWL or for PEG is schematized in Figure 1. Stainless steel surfaces were first functionalized with amino groups, resulting from the physisorption of PEI.[21] In brief, a layer of PEI was deposited on the hydroxylated surface by immersion overnight in a 3% (w/v) solution of PEI in water, followed by extensive washing in water. The resulting coupons will be named SS-SC-PEI. Grafting and cografting of enzyme and PEG were then carried on these functionalized substrates as follows.

Grafting of HEWL was performed in 2 steps as follows. SS-SC-PEI samples were allowed to react with glutaraldehyde (1 volume of 25% stock solution in 4 volumes of absolute ethanol) and $NaCNBH_3$ at 3% (w/v) to yield SS-SC-PEI-GA samples. Then, they were treated overnight by a mixture of HEWL (1 mg/mL) and $NaCNBH_3$ (3 mg/mL) in 0.1 M phosphate buffer

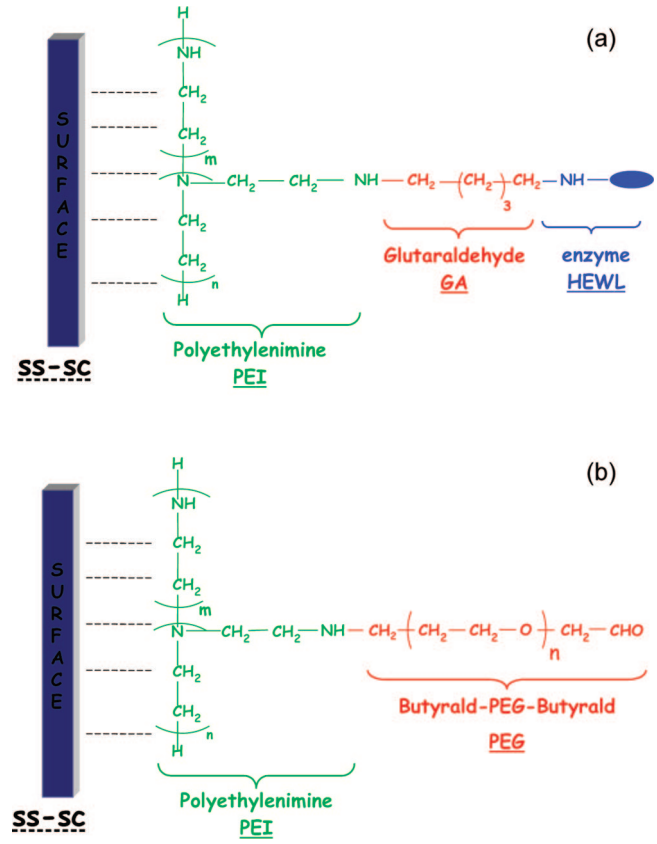

Figure 1. Schematic representation of the stainless steel (SS) surfaces functionalized by PEI-GA-HEWL (a) and by PEI-PEG (b).

(pH 8.0) at 4 °C and washed with phosphate buffer solution and pure water. These coupons will be named SS-SC-PEI-GA-HEWL.

Grafting of PEG was carried out according to ref 19 with slight modifications. PEG chains with terminal aldehyde groups were covalently bound to the amino-terminated side chains of PEI physisorbed to the stainless steel surface by reductive amination reaction. SS-SC-PEI substrates were immersed overnight in PBS solution of PEG dialdehyde (1 mg/mL) and $NaCNBH_3$ (3 mg/mL) at 60 °C overnight. The resulting SS-SC-PEI-PEG surfaces were thoroughly rinsed with sterile water.

For the cografting of PEG and enzyme, SS-SC-PEI-PEG surfaces were treated with GA and HEWL as above. PEG was grafted first because its immobilization required a heating step at 60 °C that may have denatured the enzyme if applied after enzyme grafting. These coupons will be named SS-SC-PEI-PEG-HEWL. Note that such an experiment may lead to the grafting of HEWL on the C═O ending group of either the PEG polymers or the GA grafted on the still available sites of PEI.

2.4. Characterization Techniques. The various stages of grafting were characterized by reflection−absorption infrared spectroscopy with modulation of polarization (PM-RAIRS) (characterization of the chemical functions present at the surface) and X-ray photoelectron spectroscopy (XPS) (elementary chemical surface characterization).

2.4.1. PM-RAIRS Measurements. Samples were placed in the external beam of a Fourier Transform infrared (FT-IR) spectrometer (Nicolet 5700), and the reflected light was focused onto a nitrogen-cooled mercury cadmium telluride (MCT) detector, at an optimal incident angle of 80−85°. A ZnSe grid polarizer and a ZnSe photoelastic modulator to modulate the incident beam between p and s polarizations (HINDS Instruments, PM90, modulation frequency = 36 kHz) were placed

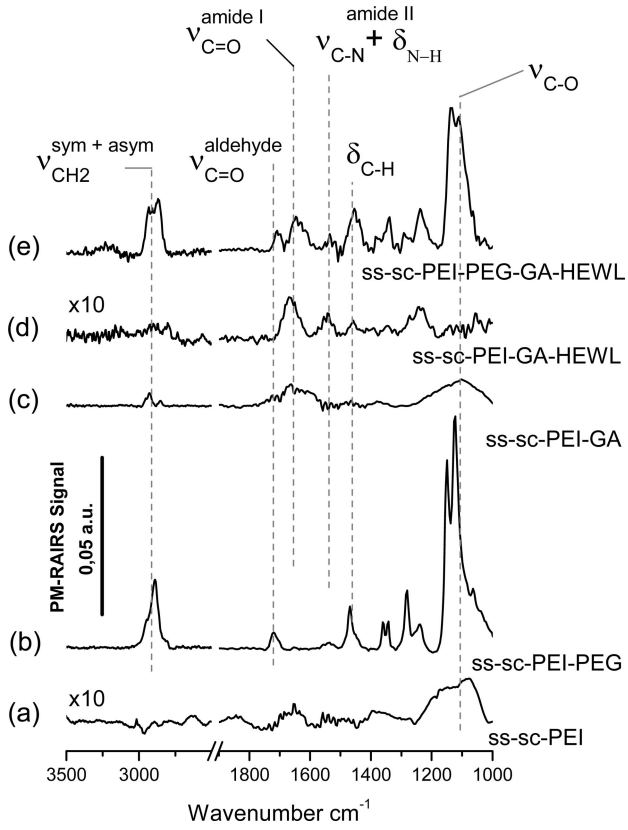

Figure 2. PM-RAIRS spectra of coated stainless steel surfaces (a) SS-SC-PEI, (b) SS-SC-PEI-PEG, (c) SS-SC-PEI-GA, (d) SS-SC-PEI-GA-HEWL, and (e) SS-SC-PEI-PEG + HEWL (cografting).

prior to the sample stage. The detector output was sent to a two-channel electronic device that generates the sum and difference interferograms. Those were processed and underwent Fourier transformation to produce the PM-RAIRS signal ($\Delta R/R^0$) = (Rp − Rs)/(Rp + Rs). All reported spectra, recorded at 8 cm^{-1} resolution, were obtained by coaddition of 128 scans; using a modulation of polarization enabled us to perform rapid analyses of the sample after immersion without purging the atmosphere or requiring a reference spectrum.

2.4.2. XPS Analyses. XPS analyses were performed on all samples using a PHOIBOS 100 X-ray photoelectron spectrometer from SPECS GmbH (Berlin, Germany) with a Mg Kα X-ray source ($h\nu = 1253.6$ eV) operating at 10^{-10} Torr or less. Spectra were carried out with a 20 eV pass energy for the survey scan and 10 eV pass energy for the C 1s, O 1s, and N 1s regions. High resolution XPS conditions have been fixed as the following: "Fixed Analyser Transmission" analyses mode, a 7 × 20 mm entrance slit, and an electron beam power of 150 W (12.5 kV and 12 mA), leading to a resolution of 0.1 eV for the spectrometer. Such a low e-beam energy was used to keep the adsorbed layers as intact as possible. A takeoff angle of 90° from the surface was employed for each sample and binding energies were calibrated against the Cr $2p_{3/2}$ binding energy of the oxidized chromium Cr_2O_3, which was set at 576.2 eV; this calibration yielded a CC-CH contribution at 285 eV in agreement with conventional XPS analyses; when the metal substrate is totally screened by the organic layer, the later contribution will be used. The spectra were fitted using the Casa XPS v.2.3.13 Software (Casa software Ltd. UK) and applying a Gaussian/Lorentzian ratio, G/L equal to 70/30. The constraint applied in the fitting procedure was the following: fwhm = 1.5 ± 0.2 eV. In addition, the error in the peak fitting process was estimated at 3% or less with respect to the integration of the raw data. The reproducibility of the XPS data was checked by the analysis of four identically prepared samples for all types of functionalization except for the SS-SC-PEI-PEG-GA-HEWL for which only two samples were submitted to XPS analysis.

2.5. Surface Activity Tests. Three tests were performed to assess the effect of surface functionalization against protein or bacterial cell adhesion, and bacterial cell growth respectively.

***2.5.1. Protein Adsorption Test, Test* 1.** To estimate the antiadhesive or repellent effect of PEG-coated surfaces toward proteins, the following test was performed using BSA. This protein was chosen for its known very strong adsorption capacity on many types of materials, in particular stainless steel.[36,37] SS-SC, SS-SC-PEI-PEG, and SS-SC-PEI-PEG-HEWL were immersed overnight in a solution of BSA (20 mg/L) in saline medium (NaCl, 23.75 g/L, $CaCl_2$, 1.15 g/L, and $MgCl_2$, 11.1 g/L). This particular medium was chosen because it is known to promote BSA adsorption on SS.[38,39] They were washed with sterile water and dried in a flow of N_2. Adsorption of proteins was measured by analyzing the surface composition by PM-RAIRS and XPS.

***2.5.2. Bacterial Adhesion Test, Test* 2.** A second test was applied to all the coated surfaces to evaluate their efficiency against bacterial adhesion. Cultures of *L. ivanovii* were grown in a BHI (Brain Heart Infusion containing a mixture of proteins and salts) culture medium at 37 °C in a closed incubator.[40] After 24 h, the cell concentration reached about 10^9 cells/ml ($OD_{600\ nm} = 1$). A drop of bacterial suspension (50 μL) was dispensed on SS-SC, SS-SC-PEI-PEG, SS-SC-PEI-GA-HEWL, and SS-SC-PEI-PEG-HEWL samples with a micropipette and left overnight under a wet atmosphere to avoid evaporation. Samples were then washed with sterile water and dried. Bacterial adhesion was controlled by analyzing the surfaces by PM-RAIRS.

***2.5.3. Bactericidal Activity Test, Test* 3.** A third and new test was specially conceived and setup in order to evaluate the local bactericidal ability of enzyme-coated surfaces; the enzymatic activity of grafted HEWL had to be tested locally, that is, by contacting a small volume of bacterial solution with the surface. To do so, cultures of *Micrococcus luteus* CIP A270 were grown in trypto-casein-soya (TCS) medium (Biorad) at 32 °C on a rotary shaker for 24−48 h. Serial dilutions of these cultures were prepared in sterile 0.1 M phosphate buffer pH 6.24 in order to determine the concentration of viable cells by the plate-counting method. After 24−48 h, the culture concentration reached about 10^9 to 2 × 10^{10} colony-forming units (CFU)/mL. A microdrop (50 μL) of bacterial suspension diluted in 0.1 M phosphate buffer pH 6.24 (chosen according to ref 41) was pipetted onto SS-SC, SS-SC-PEI-GA-HEWL, and SS-SC-PEI-PEG-HEWL samples. The initial cell concentration was chosen so that the 50 μL drop contained a countable number of bacteria, that is, between 1 and 600 CFU. Interaction between bacteria and surfaces was carried out overnight under a wet atmosphere, after which the drops were taken back and directly spread on TCS agar plates. Rinsing drops of sterile phosphate buffer (2 × 50 μL) were pipetted onto the samples, taken back, and transferred to the corresponding agar plates. The number of viable cells was counted after 3 days of incubation of the plates at ca. 30 °C. The tests were repeated three times in duplicate.

3. Results and Discussion

3.1. Functionalization of Stainless Steel with Poly(ethylene glycol) (PEG). In this section, grafting of PEG polymers to the SS-SC-PEI surface, as well as its consequence upon BSA and *Listeria* adhesion, will be characterized. A two-step

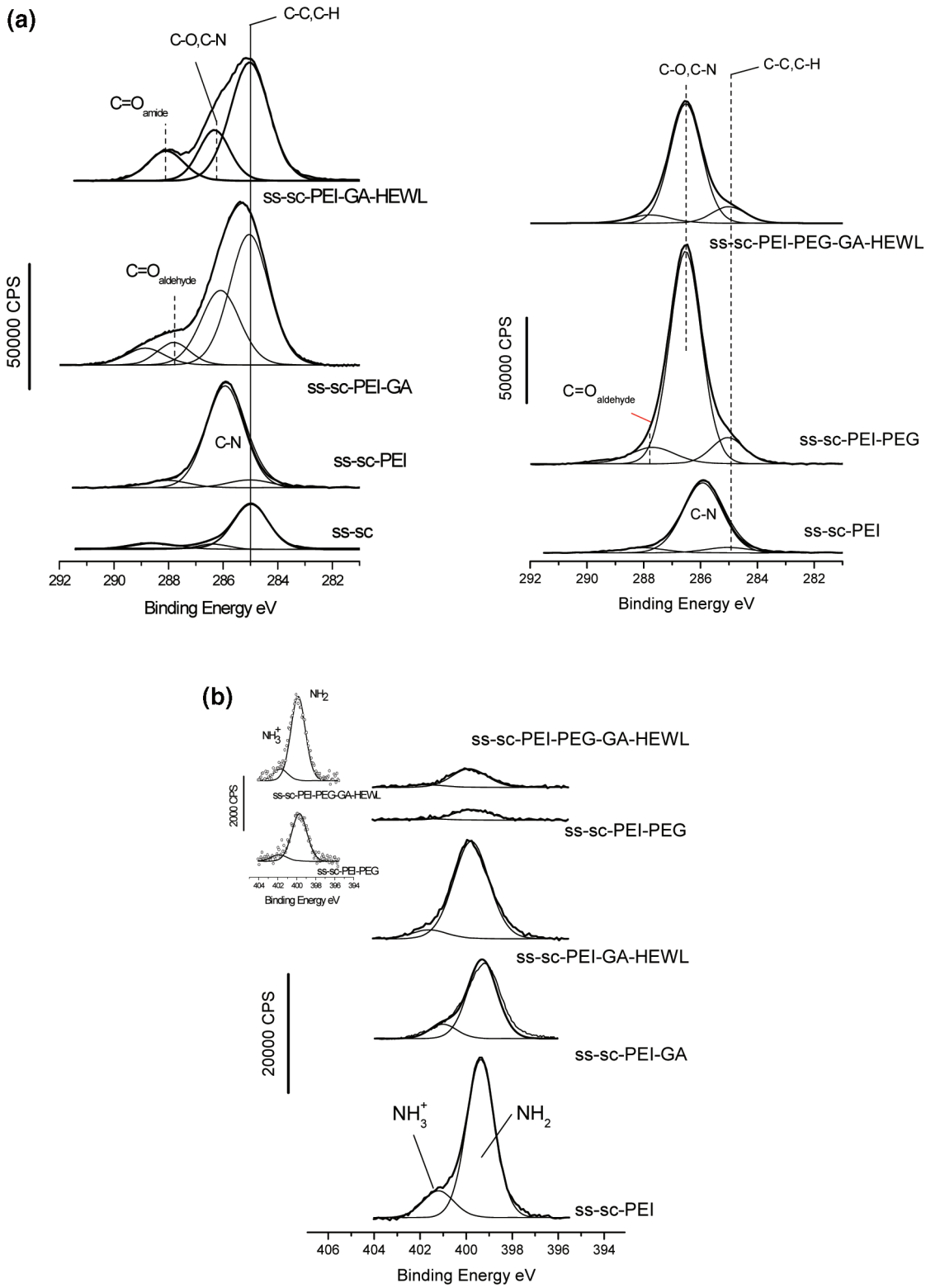

Figure 3. High-resolution XPS C 1s (Figure 3a) and N 1s (Figure 3b) spectra of coated stainless steel surfaces. Each spectrum is labeled by the type of coated layer.

procedure was chosen to construct the PEG coating. In the first step, stainless steel coupons were exposed to a solution of the polyamine PEI so as to generate reactive primary amines on the surface; as a matter of fact, a strong interaction is expected between the OH-enriched stainless steel surface and the positively charged amino groups of PEI.[42] Then, the PEG chains were covalently bound to this layer by reductive amination with a PEG dialdehyde in the presence of a reductant, $NaCNBH_3$, to stabilize the intermediate imine bonds (Figure 1a).

3.1.1. Surface Characterization. Figure 2 shows the PM-RAIR spectra recorded after exposure of a SS-SC sample to PEI (spectrum a), and then to the PEG dialdehyde (spectrum b). PEI immobilization on the substrate sample was made clear by the presence of the C−N stretch band at ca. 1100 cm^{-1}. Grafting of PEG chains on the stainless steel surface was evidenced by the presence of several vibration bands characteristic of the polymer, that is, the C−H vibration bands at 2950−2850 and 1450 cm^{-1} assigned to ν_{C-H} and δ_{C-H} of CH_2

TABLE 1: Binding Energies and Intensities of the C 1s and N 1s XPS Contributions, O 1s and Cr2p Intensities, N1s/C1s Area and Atomic Ratios

		C1s				N1s							
		attribution	BE	%	fwhm	attribution	BE	%	fwhm	O1s	Cr2p	N1s/C1s (area)	N1s/C1s (atomic)
SS-SC	area		34076				21910			232068	195881	0.64	0.36
	at %		22.1				7.9			53.4	17.6		
		C−C,C−H	284.9	75.1	1.6	NH,NH_2	400.3	100	1.8				
		cont.	286.4	15.0	1.5								
		carb.	288.9	9.9	1.7								
SS-SC-PEI	area		82693				51862			92677	65996	0.63	0.35
	at %		53.1				18.6			20.7	6.9		
		C−C,C−H	284.9	5.0	1.3	NH,NH_2	399.4	84.4	1.5				
		C−N	285.9	87.7	1.6	NH_3^+	401.3	15.6	1.6				
		cont.	288.1	7.3	1.6								
SS-SC-PEI-GA	area		91725				34853			108637	22955	0.38	0.21
	at %		58.9				12.5			24.3	2.4		
		C−C,C−H	285.0	63.4	1.6	NH,NH_2	399.6	81.3	1.6				
		C−O,C−N	286.0	31.8	1.6	NH_3^+	401.1	18.7	1.7				
		$C{=}O_{aldehyde}$	287.9	9.1	1.6								
		carb	289.0	6.2	1.6								
SS-SC-PEI-GA-HEWL	area		95151				41545			103869	7651	0.44	0.24
	at %		61.1				14.9			23.2	0.8		
		C−C,C−H	285.0	63.4	1.6	NH,NH_2	399.7	86.4	1.7				
		C−O,C−N	286.3	21.7	1.4	NH_3^+	401.3	13.6	1.7				
		$C{=}O_{amide}$	288.1	14.9	1.5								
SS-SC-PEI-PEG-	area		125650				2916			170864		0.03	0.02
	at %		66.7				1.4			31.9			
		C−C,C−H	284.9	14.5	1.4	NH,NH_2	399.7	81.0	1.7				
		C−O,C−N	286.4	78.6	1.3	NH_3^+	401.4	19.0	1.7				
		$C{=}O_{aldehyde}$	287.7	6.9	1.6								
SS-SC-PEI-PEG-GA-HEWL	area		122513				7338			170026		0.06	0.03
	at %		65.8				2.2			32.0			
		C−C,C−H	285.0	11.9	1.4	NH,NH_2	399.8	88.2	1.7				
		C−O,C−N	286.5	80.9	1.3	NH_3^+	401.7	11.8	1.7				
		$C{=}O_{aldehyde}$	287.8	7.2	1.6								
		$C{=}O_{amide}$											

groups, respectively, as well as the aldehyde $\nu_{C=O}$ stretch at 1715 cm^{-1} and the ν_{C-O} at 1130 cm^{-1} characteristic of ether bonds. PEG grafting was stable after several rinses in pure water (3 cycles of 20 min in 4 successive bathes), that is, no change in the IR band intensities was observed.

The successive graftings of PEI and of PEG were confirmed by XPS analyses (Figure 3 a and b, and Table 1)). After PEI deposition, the C 1s peak is dominated by a contribution at 285.9 eV, characteristic of carbon in C−N bonds, while an intense and asymmetric N 1s peak revealed the presence of protonated and nonprotonated amino groups in a 15/85 ratio. After PEG binding, the C 1s high resolution spectrum revealed a main contribution at 286.4 ± 0.2 eV, assigned to carbon in C−O bonds present in the polymer. Two other contributions were identified at 284.9 and 287.7 ± 0.2 eV, corresponding to carbons in C−C/C−H, and C═O bonds, respectively. These contributions confirm the grafting of the aldehyde-terminated PEG. As expected, the N 1s peak was considerably attenuated. Table 1 summarizes the fractions of all C 1s and N 1s contributions. The N/C atomic ratio was equal to 0.02, that is, much below that measured on the SS-SC-PEI surface (N/C = 0.35), in good agreement with the fact that PEG does not contain N atoms and covers the PEI layer to a large or total extent. Note eventually that after PEG grafting, no metal contribution could be detected suggesting a total layer thickness above 9 nm taking the free mean path of Cr 2p electrons through an organic layer equal to 2.9 nm.[43] Given the obtained coverage of the surface, one may expect a brushlike organization of the PEG chains.[44]

***3.1.2. Efficiency against BSA Adsorption and L. ivanovii Adhesion, Tests* 1 *and* 2.** In order to estimate the effect of PEG immobilized to the surface, bare (SS-SC) and PEG-modified stainless steel surfaces were exposed overnight to a solution of BSA in saline water. The PM-RAIRS spectra recorded after rinsing and drying (Test **1**) are shown in Figure 4. As expected, a subsequent amount of BSA was adsorbed to the bare stainless steel coupon as evidenced by the presence of intense amide I and II bands at 1660 and 1550 cm^{-1} on spectrum a. Conversely, similar bands were almost undetectable on spectrum b, corresponding to the PEG-coated surface submitted to BSA. From the integrated area of the amide bands, one may deduce that PEG grafting reduced BSA adsorption by 97% with respect to the bare stainless steel sample. The intensity of the νC−O band at ca. 1130 cm^{-1} decreased upon exposure to BSA, indicating that a fraction of the PEG polymers desorbed during this step. Important is to note that, despite the presence of an aldehyde terminal group on the PEG layer, it did not seem to induce cross-linking of BSA as might be feared; this may be related to the pH of the BSA saline solution, which was equal to 5.7. This condition is not favorable to the formation of imines between BSA amino groups of aldehydes of the PEG layer as it is much too acidic.

The *L. ivanovii* adhesion test (test 2) was then applied to the bare and PEG-modified surfaces and the surfaces were analyzed by PM-RAIRS (Figure 5). A broadband centered at ca. 1650 cm^{-1}, together with a massif of bands between 1050 and 1150 cm^{-1}, were observed on the spectrum of the bare SS-SC coupon on which the suspension of bacteria was deposited. This result

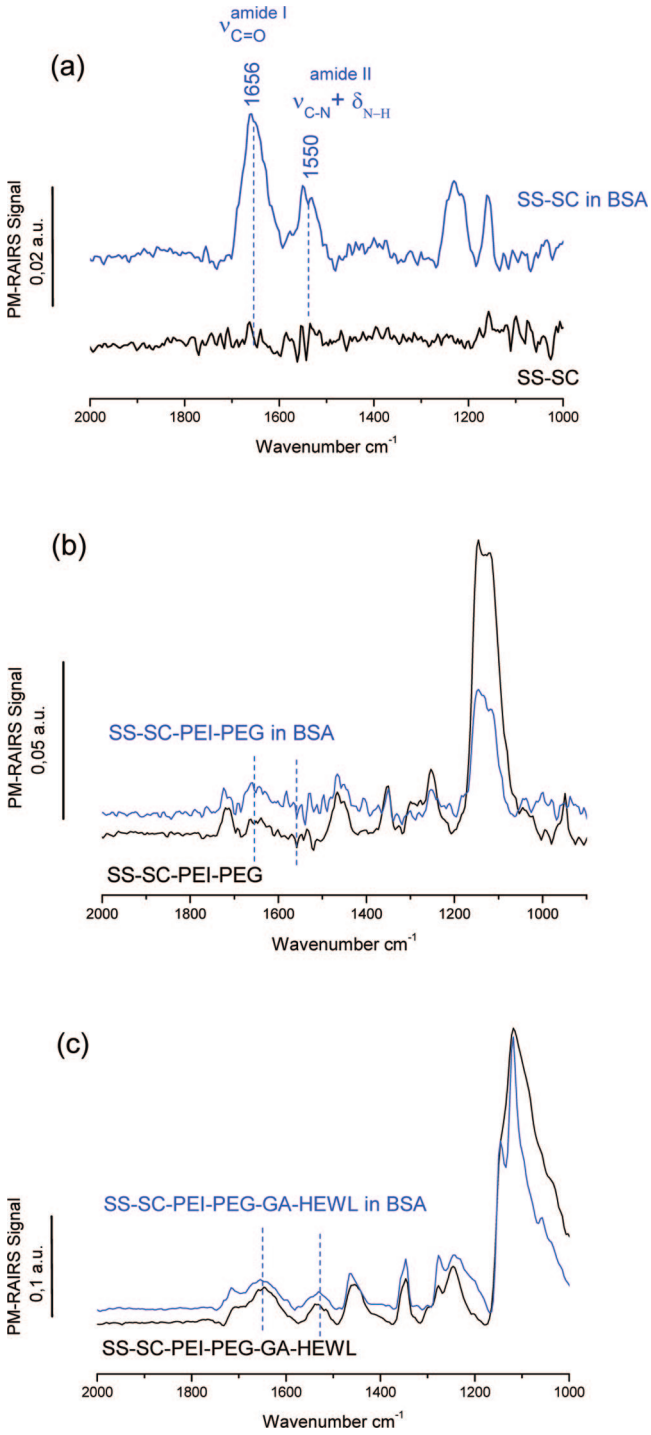

Figure 4. PM-RAIRS spectra of SS-SC (spectra a), SS-SC-PEI-PEG (spectra b), and SS-SC-PEI-PEG-GA-HEWL (spectra c) surfaces before and after immersion in BSA solution (Test **1**).

is in agreement with previously reported works on the IR analysis of bacteria physisorbed to ATR crystals;[45] it in particular proves that the adsorbed material is indeed adhered cells and not only the proteins from the BHI culture medium. Conversely, the IR spectrum of the SS-SC-PEI-PEG sample did not display such feature; a slight increase of the ν_{C-H} band was observed instead. One can clearly see the dramatic antiadhesive effect of the PEG coating toward *L. ivanovii* with only 4% of the amount of bacteria detected on the functionalized surface compared to the uncoated surface as calculated from the peptide bands areas. It thus appears that the PEG-modified surface inhibited protein/bacterial to a level that is almost undetectable by PM-RAIRS.

As a conclusion, we have clearly demonstrated that the PEG polymer was efficiently grafted to the stainless steel surface, and its repellent activity was evidenced both on proteins and a bacterial culture.

3.2. Functionalization of Stainless Steel with Hen Egg White Lysozyme (HEWL). Stainless steel samples were coated with HEWL so as to provide the surface with local bactericide properties. Starting from the same PEI layer, the sample was treated with a solution of the dialdehyde GA as a cross-linker then with a solution of HEWL in the presence of a reductant to covalently bind the enzyme by reductive amination via its lysine residues (Figure 1).

3.2.1. Surface Characterization. The spectra related to the binding of GA and then of the enzyme, HEWL) are displayed in Figure 2 (spectra c and d). The presence of the glutaraldehyde, GA, appears as a weak C═O stretch band at 1710 cm^{-1}; the weakness of this band may be explained by an orientation of the bond far from normal to the surface. Grafting of the enzyme (HEWL) was confirmed by the presence of absorption bands at about 1670 and 1530 cm^{-1}, corresponding respectively to the amide I and amide II bands, characteristic of proteins. In addition, it was important to note that the intensity of these bands was constant after several rinses in water (two hours), proving the stability of the enzyme coating to the SS-SC-PEI-GA surface.

These results were confirmed by XPS analysis. Figure 3a,b shows the C 1s and N 1s XPS spectra before and after GA and enzyme binding. Clear changes in the C 1s and N 1s peak profiles were observed on these spectra. The C 1s spectrum of the PEI-GA surface exhibits a dominating CC,CH contribution at 285.0 eV as well as a new contribution at 287.9 eV, attributed to carbon in the C═O groups; the C−N contribution of PEI is still visible; a slight contribution at 289 eV is attributed to contamination by carbonates. Binding of GA also induces a slight attenuation of the nitrogen peak. After the enzyme grafting, the high resolution C 1s spectrum was fitted with three contributions at 285.0, 286.3, and 288.1 eV, corresponding to carbons in C−C/C−H, C−O/C−N and C═O bonds, respectively (see Table 1). The shoulder at 288.1 eV, assigned to carbon in C(═O)N groups, characteristic of peptide bonds, can be undoubtedly attributed to the enzyme. The N/C atomic ratio decreased, going from 0.35 for the PEI layer to 0.24 (see Table 1), when the enzyme was grafted to the PEI layer. From the attenuation of the metal contributions, the average thickness of the enzyme layer was found to be equal to 32 Å, suggesting the presence of one monolayer of HEWL; the size of hydrated lysozyme was indeed found to range from 30 to 55 Å, depending on the conditions of measurement.[46,47]

3.2.2. L. ivanovii Adhesion Test and Enzymatic Test against M. luteus (Tests 2 and 3). The *L. ivanovii* adhesion test was performed on the SS-SC-PEI-GA-HEWL sample. The PM-RAIRS analysis of the surface performed after rinsing and drying showed almost no change of the IR spectrum with respect to that recorded before (Figure 5). In particular, almost no increase of the broad amide band area was noticed, conversely to what had been observed for the control SS-SC coupon. Protection against adhesion of *Listeria ivanovii* might be due to a bactericidal effect as lysozyme is particularly effective for Gram(+) cells and is known to display antimicrobial activity against *L. monocytogenes*.[48]

Test **3** was then carried out on SS-SC-PEI-GA-HEWL samples in order to validate the local bactericidal properties of grafted enzymes. A microdrop of *M. luteus* culture suspension was deposited on the surfaces, and taken back after 18 h as

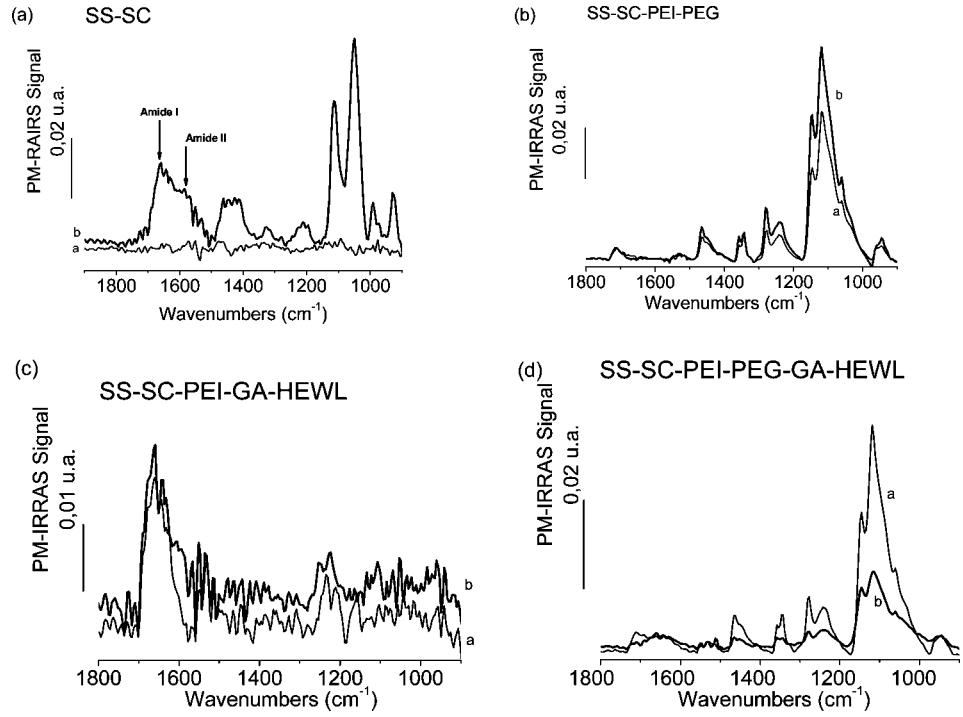

Figure 5. PM-RAIRS spectra of coated and uncoated stainless steel surfaces before (spectra a) and after contact with a *Listeria ivanovii* suspension (Test **2**) (spectra b).

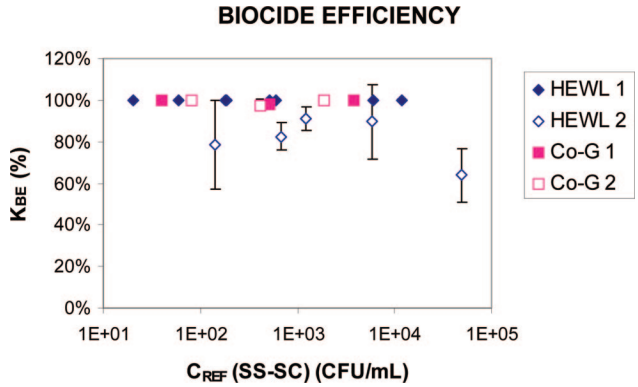

Figure 6. Activity of enzyme coatings toward *Micrococcus luteus*. Biocide efficiency factor K_{BE} ($K_{BE} = ((C_{REF} - C_{TEST}))/(C_{REF}) \times 100$) versus concentration of viable bacteria in microdrops left 16 h on surface; HEWL 1, first assay after HEWL grafting; HEWL 2, second microdrop deposition on a HEWL-coated surface; Co-G 1, first assay after cografting of PEG and HEWL; Co-G 2, second microdrop deposition on a PEG-HEWL-co-grafted surface (Test **3**). Each point of the figure is calculated from two assays on reference surfaces and two assays on treated surfaces performed with a same culture dilution.

described in Section 2.5.3. The biocide capacity of the SS-SC-PEI-GA-HEWL sample was quantified by calculating the biocide efficiency factor K_{BE} (eq. 1).

$$K_{BE} = \frac{(C_{REF} - C_{TEST})}{C_{REF}} \times 100 \qquad (1)$$

where C_{REF} and C_{TEST} are the concentration of bacteria remaining viable in the microdrop left overnight in contact with the reference (SS-SC) substrate and the HEWL-coated substrate (SS-SC-PEI-GA-HEWL), respectively. The K_{BE} factor was equal to 100% in the range of bacteria concentrations tested (between 20 and 1.2×10^4 CFU/ml), indicating that the maximum possible biocide efficiency was reached (Figure 6, HEWL 1).

To ensure that the bactericidal effect was really due to the immobilized enzyme and not to enzyme desorbed during test 3, the following control experiment was performed. A microdrop of sterile phosphate buffer was dropped onto SS-SC-PEI-GA-HEWL sample. After 17 h, the drop was recovered and transferred into phosphate buffer pH 6.24 inoculated to 1200 CFU viable *M. luteus*. After another 15 h, the number of cells was enumerated on agar plates. As a result, the number of cells was not different from the reference bacterial solution, indicating that no lysozyme was released from the surface and that the biocide activity was entirely due to the adsorbed enzyme.

For some samples, a second Test **3** was run one day after the first one. A slight decrease of the K_{BE} factor was noticed (Figure 6, HEWL 2). Nonetheless, the SS-SC-PEI-GA-HEWL sample remained active against bacteria.

Thus, coating of stainless steel surface with thin film containing HEWL conferred activity against two different Gram(+) bacterial strains. In particular, a high bactericidal effect was demonstrated on *M. luteus* strain.

3.3. Simultaneous Functionalization with PEG and HEWL. The final part of this work consisted in trying to combine the complementary properties of PEG and HEWL in order to optimize the antibiofilm surface treatment. To do so, those two molecules had to be cografted on the same stainless steel surface.

3.3.1. Surface Characterization. Following the protocol described in Section 3.2, PEG molecules were first grafted on the PEI-modified stainless steel surface, followed by reaction with GA and the enzyme. Since we showed that cross-linking of BSA did not take place on the PEG-modified layer, grafting of HEWL, another protein, is expected to take place by reaction with the GA molecules added after PEG immobilization. We cannot exclude however that cross-linking of lysozyme also took place with the aldehyde functions of the PEG layer. As a matter of fact, the PM-RAIRS analysis performed after the last step of functionalization (Figure 2, spectrum e) displayed vibration

bands characteristic of the two molecules, demonstrating that both PEG and HEWL were present on the surface. Note that, compared to the IR spectrum of the surface coated with HEWL alone, the amide bands integrated area was ca. 25% lower, indicating that the amount of HEWL, bound after PEG grafting, was only slightly reduced; this is a very promising result in the sense that the surface should exhibit both PEG and HEWL molecules in its topmost layer.

XPS spectra recorded for the coupon functionalized with both PEG and HEWL were very similar to those measured for the PEG-functionalized surface. A careful analysis of the N 1s region confirmed the presence of enzymes on the surface with a net increase of its intensity (see inset of Figure 3b and Table 1) when the enzyme was grafted after PEG immobilization. At this point, we have no obvious explanation for the apparent discrepancy between IR and XPS data, the former suggesting a significant amount of HEWL, while the latter shows little differences between the N 1s or C 1s peaks when only PEG or when HEWL and PEG were grafted; it might be due to the dehydration of the layer under vacuum required for XPS analysis that induces a collapsing of the PEG molecular brushes, some long chain molecules being possibly over HEWL molecules and thus screening their contribution. On the contrary, IR probes the whole layer thus being more representative of its true composition.

3.3.2. Grafting Efficiency. As previously, once the adsorption was characterized and the stability of the adlayers assessed, the efficiency toward protein/bacteria adhesion and the bactericidal ability of the surface coating were tested.

Test **1** confirmed that the adsorption of BSA was insignificant ca. only a few percent compared to protein adsorption on the bare surface (Figure 4c). A second coupon functionalized similarly was submitted to Test **2**, that is, adhesion of *L. ivanovii*, showing again the same repellent effect (Figure 5). The simultaneous presence of PEG and HEWL did not affect the repelling activity of the PEG.

Eventually, the local enzymatic test on *M. luteus*, Test **3**, was performed (Figure 6, Co-G 1). As for the previous test on the HEWL-modified surface, the biocide efficiency factor K_{BE} of the PEG-HEWL cografted surfaces was close to 100% for 3 orders of magnitude of bacterial concentrations on SS-SC surface ($40 \leq C_{REF} \leq 4000$ CFU/mL). What is more, K_{BE} remained at this 100% level for the second test run on the same surfaces (Figure 6, Co-G 2) suggesting that the presence of PEG protects the grafted HEWL from leakage or inactivation in the applied conditions.

These three tests attest the efficiency of the cografted PEG+HEWL surface, that is, its ability to significantly prevent the adsorption/adhesion of proteins/bacteria in contact with the functionalized surface by a combined antiadhesive and biocide activity.

4. Conclusion

Chemical grafting of two types of macromolecules to PEI-modified stainless steel surfaces was achieved. The thin films comprising the glycosidase Hen Egg White Lysozyme, and PEG were characterized by means of PM-RAIRS and XPS; the stability of these layers was assessed after successive rinses in sterile water. Surfaces coated with the enzyme displayed high antiadhesion activity toward *L. ivanovii* as compared to a bare stainless steel surface. Moreover, a marked local biocide activity was measured on *M. luteus* suspensions. Surfaces coated by PEG presented antiadhesive properties corroborated by adhesion tests with BSA and *L. ivanovii* with adhesion level reduced by 96% with respect to the bare surface. The efficiency of the "co-grafted" surfaces, that is, surfaces on which both PEG and HEWL were grafted, was assessed by three different tests. Reduction by 97% of BSA/*L. ivanovii* adhesion was measured, together with high and long-term antimicrobial activity against *M. luteus*. In conclusion, grafting of HEWL, PEG, or PEG+HEWL layers to stainless steel surface coated with PEI were proved to significantly decrease proteins/bacterial adhesion to these surfaces, and could be an interesting perspective as antifouling agents.

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