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Optimization and antibacterial response of *N*-halamine coatings based on polydopamine

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Abstract: Due to the ability of microorganisms first to adhere to material surface and then to lead to 13 the formation of a biofilm, it is essential to develop surfaces having antimicrobial properties. It is 14 well known that N-halamine coatings allow to prevent or minimize such phenomena. In the present 15 work, various polydopamine (PDA) coatings containing chloramine functions are studied. Actually, 16 three PDA-based films were formed by simple immersion of a gold substrate in a dopamine solution 17 either at pH 8 in the presence or not of polyethyleneimine (PEI) or at pH 5 in the presence of 18 periodate as oxidant. These films were characterized by polarization modulation reflection 19 absorption infrared spectroscopy and X-ray photoelectron spectroscopy analyses and by scanning 20 electron microscopy observations. The chlorination of these PDA films was performed by their 21 immersion in a sodium hypochlorite aqueous solution in order to immobilize Cl(+I) into the 22 (co)polymers (PDA or PDA-PEI). Finally, antibacterial assays towards a Gram-negative bacteria 23 Escherichia coli (E. coli) and a Gram-positive bacteria Staphylococcus epidermidis (S. epidermidis) were 24 conducted to compare the bactericidal properties of these three N-halamine coatings. Whatever the 25 bacteria tested the PDA coating having the best antibacterial properties is the one obtained using 26 periodate. 27

Keywords: polydopamine coating; N-halamine; antibacterial surfaces; XPS; PM-RAIRS; microbiological tests

1. Introduction

The fight against the adhesion and proliferation of bacteria on surfaces is a constant 32 concern and a major medical and socio-economic issue for our society. In the medical and 33 food industry, bacterial contamination of surfaces is responsible for many nosocomial and 34 food infections through, among other things, prostheses, in operating theaters, in water 35 pipe circuits or even in food industry installations [1-5]. In addition, this biocontamination 36 of the surfaces may also lead to a deterioration of the structural and functional properties 37 of the materials affecting the hulls of ships as well as civil engineering works or even 38 cultural heritage [1, 6]. Treatments to fight against biocontamination of materials generate 39 a significant economic impact and are sometimes ineffective, the biofilm lifestyle indeed 40 conferring great resistance to microorganisms [7]. Thus, the prevention of bacterial 41 adhesion appears essential and results in the development of antibacterial coatings as 42 evidenced by the numerous research projects aimed at the development of such systems. 43 One can cite surface functionalization with various antimicrobial molecules, such as 44 enzymes, peptides, organic compounds (aldehyde, quaternary ammonium) or even oxide 45

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protective layers [8-12]. Three main classes of antibacterial coatings can be designed in 46 such a way: either to limit bacterial adhesion, which is called antiadhesive coatings and/or 47 to inhibit the development of bacteria, these are said to be bacteriostatic films, or even to 48 kill them as biocidal coatings [13]. These can act by contact and/or by release of 49 antibacterial substances. Quaternary ammonium salts, silver ions or antibiotics are most 50 often found in literature as biocidal substances incorporated or deposited on matrices, 51 often polymeric, or chemically grafted on surfaces [14-18]. However, the increased 52 resistance of microorganisms to these substances, the surrounding toxicity of these 53 products and the complexity of grafting or deposition are obstacles to large-scale 54 industrial use. Bio-based compounds such as enzymes or antimicrobial peptides have 55 been considered as alternative routes but their high cost of production and purification, 56 as well as their relative instability in the face of variations in pH or temperature, restrict 57 their use [19-23]. 58

As alternatives to these compounds, a class of antibacterial compounds, the N-59 halamines, has attracted great interest in recent years [24-26]. These compounds contain 60 nitrogen-halogen covalent bonds which are formed by halogenation of imide, amide or 61 amine groups. The antimicrobial properties of N-halamine compounds are due to the 62 halogen which is at the oxidation state (+ I) and which therefore has oxidizing properties. 63 It can react with suitable biological receptors such as thiol groups of amino acids within 64 bacteria. This reaction can interfere with the metabolism of cells such as respiration, 65 especially protein related processes, resulting in the death of the bacteria [24]. The 66 disinfectant effectiveness of N-halamines is similar to that of bleach (sodium 67 hypochlorite), but these compounds are more stable, less corrosive and relatively easy to 68 generate. Indeed, after immobilization of the precursors (containing NH groups: primary 69 or secondary amine, amide or imide functions) of N-halamine on a surface, they are 70 converted into haloamine functions by a halogenation process (substitution reaction of H 71 by Cl(+ I) or Br(+ I)), thanks to a sodium hypochlorite or a sodium hypobromite solution 72 to generate chloramine or bromamine functions, respectively [24, 25, 27]. 73

Various methods for bonding N-halamines to substrate surfaces have been described, 74however, these methods often require either complex surface pretreatment or lengthy 75 coating formation procedures and often lack universality, strategies employed being 76 specific to the physical and chemical properties of the surfaces of materials. In this context, 77 polydopamine (PDA), an aqueous insoluble biopolymer produced by the 'auto-oxidation' 78 of a catecholamine neurotransmitter, dopamine (DA), has become a highly studied 79 polymer in materials science as a functional bio-system inspired used in a very wide range 80 of applications. PDA provides exceptional adhesion properties producing a universal 81 coating and offering the possibility of a large repertoire of post-functionalization, which 82 has paved the way for many applications, both in the biomedical sciences and in the 83 process, energy conversion or water treatment devices [28, 29]. 84

In a previous study, we have demonstrated the possibility to elaborate a new 85 antibacterial coating formed be a thin chlorinated PDA film [26]. This film is obtained by 86 dopamine monomer polymerization in mild basic aqueous solutions followed by a 87 treatment in sodium perchlorate (NaOCl) solution. This treatment leads to the formation 88 of chloramine functions inside the polymer estimated between 10²¹ and 10²² at·cm⁻³ [26]. 89 Microbiological tests towards Escherichia coli (E. coli) bacteria showed that chlorinated 90 PDA coatings reduced E. coli adhesion up to 45 % compared to uncoated surfaces, while 91 in the same time bacterial viability was reduced by 34% on chlorinated PDA coating 92 compared to initial PDA films. 93

However, the maximum thickness obtained for a deposition of PDA in alkaline 94 conditions in the presence of O₂ as oxidizing agent is less than 50 nm, which hinders any 95 optimization of the antibacterial effect of the coating by increasing the thickness of the 96 deposit in order to maximize the number of amine functions available. Moreover, many 97 materials or molecules sensitive to pH, such as cellulose, polyester, phenolic resins, 98 proteins or certain gels are not suitable for functionalization by dopamine in an alkaline 99 aqueous medium. At the end the fabricated PDA films are unstable in a strong alkaline 100 environment [30-32]. 101

To address those issues, various ways of optimizing the coating were considered 102 during this study. First of all, increase the thickness of the PDA film by modifying the 103 oxidant, replacing O_2 with sodium periodate which allows (i) deposition in an acidic 104 medium with faster kinetics and (ii) to obtain thicker films [32]. The second path explored 105 is based on the introduction of more amine functions through polyethyleneimine (PEI), 106 forming a PDA-PEI composite. Moreover, amino-rich PEI has been frequently introduced 107 as a cross-linking agent, which affords free-standing PDA/PEI composite films by Michael 108 addition or Schiff base reaction and improves the chemical stability in strong alkaline 109 environment [33]. This N-halamine based on co-deposition of PDA and PEI was studied 110 and it was showed that the antibacterial ability of the coatings increased with increasing 111 the PEI content. In addition, the chlorinated co-deposition coatings had significantly 112 increased antibacterial properties compared to the unchlorinated ones, the chlorinated co-113 deposition coatings inactivating >99.99% of Staphylococcus aureus (S. aureus) and >99.9% of 114 E. coli after contact of less than 10 min with PDA antibacterial materials [27, 34]. 115

Thus, in the present work, the optimization of the synthesis of PDA coating 116 containing chloramine functions was studied. For this purpose, first, the formation of 117 three PDA-based films on gold surface was performed by simple immersion of the 118substrate in a dopamine solution either at pH 8.5 in the presence or not of PEI or in 119 dopamine solution in the presence of sodium periodate as oxidizing agent at pH 5. Those 120 films were characterized by polarization modulation reflection absorptions infrared 121 spectroscopy (PM-RAIRS) and X-ray photoelectron spectroscopy (XPS) analyses and by 122 scanning electron microscopy (SEM) observations. The chlorination of the PDA films was 123 performed by immersion in a NaOCl aqueous solution in order to immobilize Cl(+I) 124 oxidative species into the polymers. Finally, antibacterial assays against E. coli and 125 Staphylococcus epidermidis (S. epidermidis) were conducted to compare the bactericidal 126 properties of those N-halamine coatings. 127

2. Materials and Methods

Dopamine hydrochloride (98% purity), branched PEI (average Mw ~800 g·mol⁻¹ by 129 light scattering, average Mn ~600 motifs by gel permeation chromatography), sodium 130 periodate (99.8% purity), tris-(hydroxylmethyl)aminomethane hydrochloride (Tris-HCl), 131 sodium acetate (99% purity), and phosphate buffer saline (PBS) were obtained from 132 Sigma-Aldrich (St-Quentin Fallavier, France). A house-hold bleach solution at 2.6% of 133 active chlorine was diluted before being used for the chlorination of the PDA films. Ultra 134 pure water was obtained from a Milli-Q system (Millipore, resistivity of >18 M Ω ·cm⁻¹) 135 from EMD Millipore Corp. (Billerica, MA, USA). 136

Glass substrates (11 mm × 11 mm) coated with a 5 nm-thick layer of chromium and a 137 200 nm-thick layer of gold were purchased from Arrandee (Werther, Germany). Before 138 PDA coating, the gold-coated substrates were annealed in a butane flame to obtain a 139 crystal reconstruction of the first atomic layers, and a UV–ozone cleaning procedure for 140 15 min was then applied prior to ultrapure water and absolute ethanol rinsing for a period 141 of 10 min each [35].

PDA coatings

PDA-O₂

For the formation of the PDA coating, the substrates were immersed in dopamine 146 solution, prepared by dissolving 0.5 mg·mL⁻¹ of dopamine in 10 mM of Tris at pH 8.5. This 147 PDA film deposition protocol was inspired by the protocol proposed by Messersmith 148 group [36, 37]. Then, ultrasonic rinsing treatment in distilled water was carried out on the 149 substrates for 1 minute; finally, the samples were dried with dry nitrogen. The samples 150 are subsequently named PDA-O₂.

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For the deposition of PDA using sodium periodate (NaIO₄) as an oxidant at a 153 concentration of 5 mM, the substrates were immersed in dopamine solution, prepared by 154 dissolving 0.5 mg·mL⁻¹ of hydrochloric dopamine in 50 mM sodium acetate at pH 5.5. This 155 protocol was inspired by the study conducted by Ponzio et al. [32]. Subsequently, the 156 substrates were washed with ultrapure water for 5 minutes under sonication and then 157 dried with dry nitrogen. The samples are subsequently named PDA-IO4-. 158

PDA-PEI

The deposition of the PDA-PEI composite was carried out by immersing the substrates in a solution formed from a mixture of dopamine at 0.5 mg·mL⁻¹ and PEI at 0.25 162 mg·mL-1 in 10 mM of Tris at pH 8.5, under stirring. This PDA-PEI composite film 163 deposition protocol was inspired by the study conducted by Xu et al. [33]. This 164 functionalization is followed by ultrasonic treatment of the substrates in ultrapure water 165 and followed by drying with dry nitrogen. The samples are subsequently named PDA-PEI. 167

PDA film chlorination

For chlorination, PDA-XX films were soaked in 1 or 10 mM NaOCl solution at pH 10, at room temperature [26, 38, 39]. After chlorination, the substrates were washed with 171 deionized water thoroughly and nitrogen-dried. After chlorination, the coatings are 172 named PDA-XX-Cl.

Polarization Modulation Reflection Absorptions InfraRed Spectroscopy (PM-RAIRS)

PM-RAIRS measurements were carried out on a Nicolet Nexus 5700 Fourier-176 transform infrared (FT-IR) spectrometer (Madison, WI, USA) equipped with a wide-band 177 HgCdTe detector cooled with liquid nitrogen. Infrared spectra were obtained by addition 178 of 128 scans at 8 cm⁻¹ resolution. A ZnSe photoelastic modulator and a ZnSe grid polarizer 179 were placed prior to the sample to modulate the incident beam between p and s 180 polarizations (PM90, HINDS Instruments, Inc., Hillsboro, OR, USA), the modulation 181 frequency was 36 kHz. Interferograms (sum and difference) were processed via Fourier 182 transformation to obtain the resulting PM-RAIRS signal, which is the differential 183 reflectivity:

$$\Delta R/R^0 = (Rp - Rs)/(Rp + Rs) \tag{1}$$

with R^0 is the reflectivity of the initial IR beam and R the one after reflexion on the 186 substrate; Rs and Rp are the signals perpendicular and parallel to the incident plane, 187 respectively. 188

X-ray photoelectron spectroscopy (XPS)

XPS analyses were performed using an Omicron Argus spectrometer (Taunusstein, 191 Germany) equipped with a monochromated Al K α X-ray source (hv = 1486.6 eV) working 192 at an electron beam power of 300 W. Photoelectron emission was analyzed at a take-off 193 angle of 45°. The analyses were carried out under ultrahigh vacuum conditions ($\leq 10^{-10}$ 194 Torr) after introduction via a load lock into the main chamber. Spectra were obtained by setting up a 100 eV pass energy for the survey spectra, and a pass energy of 20 eV was 196 chosen for the high-resolution regions. Element peak intensities were corrected by 197 Scofield factors [40]. CasaXPS software (Casa Software Ltd., UK) was used to decompose 198 XPS spectra using a Gaussian/Lorentzian ratio of 70/30.

Water Contact Angle (WCA)

Static water contact angles (DSA100 apparatus, Krüss, Hamburg, Germany) were 202 measured under ambient conditions (at 20°C and 40% relative humidity) by analyzing the 203

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profile of sessile drops (1 µL droplet of milliQ water) deposited on a given surface. The 204 drop profile was recorded by a CCD camera, while the angles were measured by the 205 image analysis software. For each analyzed surface, at least for different location were 206 chosen for the deposition of the droplet; each test was performed in triplicate on three 207 different samples. The reported values for a given surface are thus the averages of these 208 36 measurements. 209

Chemical 5-thio-2-nitrobenzoic acid (TNB) titration

The presence of chloramine functions was confirmed by measuring the bleaching of 212 a 5-thio-2-nitrobenzoic acid (TNB) solution at 412 nm [41]. Fresh TNB solution was 213 produced before each experiment via addition of 2 equivalents of cysteine (Cys) to 1 214 equivalent of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) following the reaction: 215

$$DTNB + 2 Cys \rightarrow 2 TNB + cystine$$
(2)

For this aim, in equal volumes, 2×10⁻³ M cysteine and 10⁻³ M DTNB were mixed in 50 218 mM phosphate buffer solution (PBS) at pH 7.4, giving a highly colored yellow/orange 219 solution [42]. Then, this stock solution was diluted 10-fold with 50 mM PBS, pH 7.4. The 220 different substrates were immersed in this solution for 24 h. The yellow-colored TNB 221 reacts with haloamine functions to regenerate colorless DTNB. The UV/visible absorbance 222 measurements were carried out using a spectrometer with 1 cm path length cuvettes. 223 According to Beer–Lambert's law, the density of chloramine functions (da), in at cm-3, was 224 calculated according to the following equation: 225

$$d_{Cl} = \frac{A_{PDA} - A_{PDA-Cl}}{2 \varepsilon \, l \, V_{coating}} \, V_{TNB} \, N_A \tag{3}$$

where APDA and APDA-CI are the absorbance of the TNB solution containing the 228 substrate with the PDA coating and with the chlorinated PDA coating, respectively; ε is 229 the molar absorptivity coefficient of TNB, $\varepsilon = 14,100 \text{ M}^{-1} \cdot \text{cm}^{-1}$, l is the length of the 230 spectrometer cuvette and is equal to l = 1 cm, $V_{coating}$ is the volume of the PDA film in cm³, *V*_{TNB} is the volume of the TNB solution in L and *N*_A is Avogadro's constant.

Microbiological tests

Microbiological experiments were conducted with Escherichia coli ATCC 25922 236 described as a rod shape with an average height of 1 µm and *Staphylococcus epidermidis* 237 CIP 6821 described as a sphere ranging from 1 to 2 µm diameter. S. epidermidis strain was 238 chosen as non-pathogenic bacteria mimicking S. aureus bacteria. Three culture media were 239 used for these experiments: 2 nutritive media, Lysogeny-broth medium (LB) for E. coli, 240 Muller-Hilton medium (MH) (Sigma-Aldrich, St-Quentin Fallavier, France) for S. 241 epidermidis and a un-nutritive phosphate buffer saline (PBS) medium for killing 242 experiments. 243

Bacteria were stored at -80°C. Before the tests, they were incubated overnight on LB 245 agar plate for E. coli and MH agar plate for S. epidermidis, at 37°C. Then, a liquid preculture was prepared with one colony of E. coli or S. epidermidis in LB or MH media, 247 respectively, and incubated for 18 h at 37°C under stirring (90 rpm). Bacteria were then 248 harvested by centrifugation (3500 rpm, 4°C, 20 min). Bacteria were re-suspended in the 249 PBS medium and bacterial suspensions were adjusted to an absorbance at 620 nm of 0.01 250 (5x106 CFU·mL-1). 251

Bacteria Growth Capacity (Cultivability of Adhered Bacteria)

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Bacterial growth capacity after contact with functionalized surfaces was determined 254performing killing tests. Before bacterial inoculation, the surfaces were disinfected by 255 washing them five times with ethanol solution at 70%. The killing test was performed in 256 sandwich configuration. For this, 20 µL of the bacterial suspension in PBS media were 257 deposited on a first plate; then, a second plate was placed onto the first one, on the coating 258 side so that the bacterial suspension is sandwiched between the two substrates. After 3 h 259 of contact, at room temperature, the surfaces were mildly rinsed with sterile PBS in order 260 to remove non-adhered bacteria, then transferred into a tube containing 2 mL of sterile 261 PBS solution and sonicated (Bandel in Sonorex RK 31, Berlin, Germany; f= 35 kHz and P= 262 90 W) for 2 min to recover the adhered bacteria without damaging them. After the 263 sonication, SEM observations of the plates were performed to verify that most of the 264 adhered bacteria were detached during the sonication process. Traditional 265 dilution/counting was carried out in in triplicate on LB or MH agar plates. The plates were 266 incubated at 37°C overnight before enumeration. Results were expressed in the number 267 of attached and cultivable bacterial cells onto the different surfaces per mL (CFU·mL-1). 268 The percentage of killing (%killing) was calculated using equation (4): 269

$$\%$$
 killing = 100 (CFUref-CFUchlorinated surface)/CFUref (4)

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Each test was done in triplicate, and the number of CFU·mL⁻¹ is the average of the results obtained for each sample.

Epifluorescence optical microscopy observations

Samples were immersed in 4 mL of bacterial suspension and incubated for 3 h at 276 37°C. It is important to keep in mind that this incubation time corresponds to the limit of 277 the reversible phase in the biofilm formation process. Surfaces were then thoroughly and 278 carefully rinsed 8 times by replacing 1 mL of the bacterial suspension by 1 mL of fresh 279 PBS solution to remove non-attached bacteria. Adhered bacteria on the surfaces were 280 fluorescently stained by adding 1 µL of 5x10-3 M Syto9® (Invitrogen, US) solution and 3 281 µL of 5x10-3 M Propidium Iodide® (Invitrogen, US) solution and were incubated for 15 282 min at 37°C. 283

Then the surfaces were directly observed in the last rinsing solution by using the 284 fluorescence/reflection mode of an upright epifluorescence microscope (Axio Observer, 285 Zeiss) equipped with a long working distance water objective (W Plan-Apochromat 286 63X/1.0, working distance 2.0 mm, Zeiss®). On each surface, micrographs were taken on 287 10 random locations. Experiments were conducted with two surfaces of each type and 288 reproduced by 3 independent experiments. Micrographs were analyzed by ImageJ 289 V.1.44d software® (NIH). Each image was processed to select the color channel and adjust 290 thresholds by Otsu or Intermodes method depending on the intensity histogram of each 291 image. Then, the number of adhered bacteria (N) and their size on each micrograph were 292 calculated with help of the analyze particle plug-in. The %killing was calculated using 293 equation (5): 294

% killing = 100 (Ngreen + red bacteria - Nred bacteria)/Ngreen + red bacteria (5)

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The significance of two-by-two differences between averages cells number in the 298 diverse culture conditions was evaluated by bilateral Student's t tests (application 299 conditions: independent data and equal variances assessed by F-test) with significance 300 thresholds of 0.01 and 0.05. The alternative hypothesis ($\mu_1 \neq \mu_2$) was assumed to be true 301 when the main hypothesis ($\mu_1 = \mu_2$) was rejected, where μ_1 and μ_2 are the two mean values 302 to be tested for significant difference. 303

3. Results

3.1. PDA Coating Elaboration

In aerated solution, dopamine monomers are known to self-polymerize upon 310 oxidation within the right concentration and pH conditions. The polymerization is usually 311 accompanied with a change of the solution color from colorless to dark brown [26]. In this 312 study, we have studied several ways of obtaining PDA films by changing the oxidant 313 agent, atmospheric O_2 or sodium periodate, or by adding a small polymer, PEI, which is 314 known as a good reticulate agent [34, 43]. 315

The DA polymerization was followed upon increasing contact time between the gold 316 substrates and the DA solutions (Figure S1). Figure 1 presents the PM-RAIRS data 317 obtained for the three routes of PDA grafting, showing similar profile. According to 318 previous study on the formation of PDA films using atmospheric O₂ as oxidant [26], in 319 Figure 1(a) a broad IR massif is observed in the 1600 cm^{-1} region, with a second group of 320 features is visible at lower wavenumbers. These IR features indicate the presence of a PDA 321 film, with the main characteristic peaks observed at 1620 and 1515 cm⁻¹, assigned to the 322 stretching of the (C=C) group within the DA ring together with the stretching v(N-H)323 vibrations [44], respectively. In the second region, IR peaks are observed at 1450, 1350, and 324 1290 cm⁻¹, respectively assigned to the stretching vibrations (C-N-C) of the DA ring and 325 to free catechol moieties of free dopamine, v(C-O) and v(C=N). These PM-RAIRs features 326 are consistent with the formation of PDA coating on gold surface [44-47]. 327



Figure 1. PM-RAIRS spectra of gold surface coated with (a) PDA-O₂, (b) PDA-IO₄, (c) PDA-PEI.

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PM-RAIRS analyses of the spectrum obtained using periodate is presented in Figure 332 1(b); the spectrum obtained after 5 hours reveals the same characteristic groups of PDA 333 observed previously for the PDA-O₂ film at 24 hours. However, one can also note that a 334 new peak appears at 1720 cm⁻¹ assigned to the stretching vibration of a C=O bond of a 335 carboxylic group. This peak suggests a hyper oxidized state of the PDA due to the strong 336 oxidizing power of periodate, the material is probably richer in carboxylic group or 337 quinonoid structures and has undergone a partial loss of carbon [31]. This is in accordance 338 with the known chemistry of periodate, which, when used in excess, can cause *o*-quinone 339 oxidative cleavage [48]. The spectrum obtained for the PDA-PEI film after 24 hours, Figure 340 1(c), is also similar to the one obtained for PDA-O₂. In addition, IR features are also 341 observed in the 2800-2900 cm⁻¹ region, assigned to the stretching vibration of CH₂ groups 342 from the PEI backbone. Finally, it is important to note that the intensity of the N-H 343 vibration at 1560 cm⁻¹, compared to the C-C, C=N at 1660 cm⁻¹ is greater in the latter case, 344 suggesting the presence of more NH groups in the case of the PDA-PEI film [34]. 345

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PDA film growth can be quantitatively investigated using PM-RAIRS data, by 347 integrating the area under the IR features as a function of immersion time (Figure S1), as 348 presented in Figure 2. Growth profiles observed are quite different for all three 349 polymerization routes. For PDA-O₂ films, a rapid increased of the peak's intensity is 350 observed for the 12 first hours, followed by a slowest increase to end up to a plateau after 351 48 hours. This stationary phase can be attributed to the depletion of the monomer in the 352 supernatant solution, being able to crosslink on the PDA film, in favor of non-reactive 353 quinone molecules [49]. The ellipsometry data (Figure S2B) confirms the logarithmic 354 kinetic deposition profile obtained by PM-RAIRS analysis. On the contrary, PDA-IO4-355 films show an even faster increase of the IR intensity during the first 3 to 5 hours to quickly 356 stabilize into a plateau regime. The thickness measurements obtained by SEM (Figures 357 S2A and S3) also confirm the deposition kinetics observed by PM-RAIRS, indeed a faster 358 deposition kinetics than during a conventional deposition is observed, for this a 359 logarithmic kinetics is observed. Finally, the data obtained for PDA-PEI film seems to be 360 a mix of both previous ways, with a fast increase during 5 hours, smaller than for PDA-361 IO₄⁻ though, followed by a slowest increased up to a steady-state obtained after 10 hours 362 of immersion. It is important to note that the intensity of the areas under peaks are 363 expressed in arbitrary units; thus, a calibration was applied using the thickness obtained 364 on the plateau regime using ellipsometry analysis and SEM observations [26] (Figures S2 365 and S3), showing similar thicknesses for both PDA-O2 and PDA-PEI around 50-55 nm and 366 60-65 nm, respectively, while for PDA-IO4⁻ the film thickness reaches 110-115 nm [32]. 367 Thus, one can conclude on the strong efficiency of periodate as oxidant agent compared 368 to atmospheric O₂, leading to greater thickness of PDA films, together with faster growth 369 rate. Consequently, the use of periodate allows to accelerate the oxidation kinetics of 370 dopamine, after 5 hours of immersion a thickness plateau around 100 nm is reached, i.e. 371 a gain of 60 nm in thickness compared to the conventional deposit obtained after 24 h. 372 Besides, copolymerisation of PDA and PEI leads to similar thickness of the film than PDA-373 O_2 one, with a kinetic growth rate increased by a factor 2 in the case of PDA-PEI, with the 374 hypothesis of a greater concentration of NH groups. 375

Contact angle measurements with water were used to assess the wettability of a 376 surface and to demonstrate a change in the chemistry of the latter following surface 377 modification (Figure S4). It is noted that for the PDA- O_2 coating, the contact angle 378 decreases over time to reach a plateau after 3 h of deposition, level around 55° which 379 approaches the value of the contact angle reported in the literature [32]. It can thus be seen 380 that this monitoring of wettability makes it possible to have, in a simple and rapid way, 381 an indication of the time at the end of which the coating becomes homogeneous at the 382 macroscopic level in terms of coverage of the substrate and thus completes the monitoring 383 by PM-RAIRS and ellipsometry. 384

Concerning PDA-IO4⁻, the evolution of the contact angle formed between the coating 385 and the water matches the profile obtained for the PDA-O₂ coating. In fact, a sharp 386 reduction in the angle is observed during the first 10 hours, then a plateau is observed 387 indicating the presence of an homogeneous coating on the gold surface. Note that this 388 change is similar to the profile found when monitoring the thickness of the deposit. Thus, 389 just as with the PDA-O₂ coating, the kinetic monitoring of the deposit by measuring the 390 contact angle turns out to be a simple method of monitoring the PDA deposit over time. 391 Finally, it is noted that PDA-O₂ and PDA-PEI composite have the same evolution profile 392 of wettability during the first 30 hours of deposition. Indeed, the contact angle with water 393 decreases over time to reach a plateau after 5 hours of deposition, around 50° which is 394 slightly lower than the value obtained from pure PDA. 395



Figure 2. PDA film growth. PM-RAIRS peak area (left axis) and film thickness (right axis) vs.397immersion time in DA solution. Data are from Figure S1, S2 and S3. Square: PDA-O2. Circle: PDA-398IO4⁻. Triangle: PDA-PEI.399

XPS analyses were performed on these three sets of the PDA-coated Au surface, the 400 survey spectra are presented in Figure 3. For all samples, the photopeaks C1s (285 eV), 401 O1s (530 eV), and N1s (400 eV) are observed, whereas the Au4f contribution (84 eV) is not 402 detected confirming film thickness greater than 15 nm [50]. In addition, for PDA-IO4, the 403 I3d signal, at 620-630 eV, is observed together with the counter-ion Na⁺ signal at 1051 eV. 404 For samples PDA-O₂ and PDA-IO₄, the chemical composition in carbon and nitrogen is 405 close to the theoretical composition of PDA (N/C = 0.125) taking into account the carbon 406 contamination, which slightly reduces the N/C ratio (Table 1). Finally, for the PDA-PEI 407 sample, the atomic percentage of N is much greater than for the two others films, due to 408 the presence of PEI as copolymer in the film, with the N/C ratio being accordingly twice 409 bigger than previously. 410



Figure 3. XPS survey spectra of gold surface coated with (a) PDA-O₂, (b) PDA-IO₄⁻ and (c) PDA-PEI.

Table 1. Atomic percentage and N/C ratio obtained from XPS data.

	С	Ν	Ο	Au	Ι	Na	N/C	
PDA-O ₂	75.5	7.3	17.2	ND	-	-	0.1	
PDA-IO4-	72.2	7.1	19.3	ND	0.9	0.5	0.1	
PDA-PEI	69.4	15.4	15.2	ND	-	-	0.2	
¹ ND=non detectable								

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The chlorination of the different PDA films was carried out using diluted NaOCl as 422 a function of contact time and concentration in order to optimize the chlorination process. 423 Figure 4 shows the evolution of the PM-RAIRS spectra upon increasing contact time 424 between the PDA films and the NaOCl solution. Two concentrations of the NaOCl 425 solutions were tested, 1 and 10 mM. After a certain time, drastic changes on the profile 426 spectra can be observed suggesting the degradation of the film as long contact time with 427 alkaline solution can degrade the polymer structure [38, 51, 52]. It is first observed that 428 after the chlorination, a change in the IR spectra of the three PDA films is observed. In 429 fact, the latter appear to show a strong decrease in the peak at 1515 cm⁻¹, a peak attributed 430 to the deformation vibration of the δ (N-H) amine bond, therefore suggesting the creation 431 of chloramine functions. There is also a shift in the peak characteristic of the aromatic cycle 432 from 1620 cm⁻¹ to 1645 cm⁻¹. This change can be a consequence of the grafting of chlorine 433 atoms on nitrogen atoms located near the aromatic ring modifying the chemical 434 environment of the latter. Finally, the appearance of a shoulder around 1720 cm⁻¹, the 435 intensity of which increases with the chlorination time (Figure 4) may be ascribed to the 436 appearance of carboxylic functions due to the oxidation of the PDA coating on contact 437 with hypochlorite ions. 438

For instance, on PDA-O₂ films, Figure 4(a,d), the intensities of the peaks of the 439 chlorinated spectra start to decrease for contact time of 15-20 minutes, with a complete 440 destruction of the film after 50 minutes of contact time with NaOCl solution. Thus, an 441 optimized contact time for the chlorination process for PDA-O₂ was chosen to be 20 442 minutes at 1 mM. The same processes were applied for the PDA-IO₄⁻ and PDA-PEI films, 443 Figure 4(b,e) and (c,f), ending up with optimized chlorination times of 20 minutes at 1 mM respectively. 445



Figure 4. Chlorination of PDA films as a function of immersion time in (a),(b) 1 mM, (c) 10 mM448NaOCl solution. (a),(b),(c): PM-RAIRS spectra. (d),(e),(f): area under IR peaks. (a),(d): PDA-O2;449(b),(e): PDA-IO4⁻; (c),(f): PDA-PEI.450

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Figure 5. XPS survey spectra (a-c) and high resolution Cl2p spectra (d-f) after chlorination of (a, d) 453 PDA-O₂, (b, e) PDA-IO₄⁻ and (c, f) PDA-PEI. 454

XPS experiments were carried out for the different PDA films chlorinated with 455 optimized conditions, the data are presented in Figure 5 and the quantitative data are 456 summarized in Table 2.

First, the survey spectra in Figure 5 (a-c) show additional contributions at 200 eV and 458 270 eV, assigned to Cl2p and Cl2s contributions, respectively, confirming the successful 459 chlorination of the PDA films. High resolution spectra of the Cl2p contribution are also 460 shown in Figure 5 (d-f), exhibit two distinct doublets centered for the Cl2p_{3/2} contribution 461 around 200 eV and 198 eV. The most intense one, at 200 eV, named Cl200, is usually 462 assigned to chlorine atoms in N-Cl bond, while the lower binding energy one is ascribed 463 to free chlorine atoms. Thus, one can first conclude that the chlorination process was 464 successful, and second that only a few amount of free Cl atoms remain at the surface even 465 after heavy rinsing procedure. It is interesting to note, that the amount of Cl200 differs from 466 one film to another, thus both PDA-O2 and PDA-PEI exhibit N atomic percentages about 467 4.0%, while PDA-IO₄ film shows only 2.7%. However, per se, such analyses are not 468 sufficient to conclude that one film or another contains the highest amount of haloamine 469 functions. In fact, one should consider the original amount of N1s atoms present in the 470 analyzed thickness of the films, and it is possible to calculate the percentage of chlorinated 471 NH groups by looking at the Cl200/N ratio. From Table 2, it can be concluded that the 472 efficiency of chlorination is the best for the PDA-O₂ film with more than 60% of the N 473 atoms that are chlorinated, while this value is only 30% for the 2 others PDA films. 474

These results thus only give us information on the composition of the surface of the 475 coating one (10-15 nm of thickness probed). These differences between the PDA-O₂, PDA-476 IO₄⁻ and PDA-PEI coatings can be explained by the availability of N-H functions. Indeed, 477 let us recall that for the PDA-IO₄ coating, acid conditions and a strong oxidant were used 478to generate the PDA film, generating a different hyperoxidized structure compared to the 479 PDA-O₂ film, with a higher proportion of tertiary amine functions which does not allow 480 their chlorination. For the PDA-PEI coating, this drop in N-Cl can be due to the fact that 481N-H functions could be engaged in hydrogen bonds with the adjacent catechol functions 482 which do not allow the chlorination of these amine functions, Figure S5. 483

	С	Ν	0	Ι	Au	Na	N/C	Cltotal	Cl200	Cl200/N
PDA-O ₂ -Cl	69.85	6.45	18.9	-	-	-	0.1	4.8	4.0	0.6
PDA-IO4Cl	69.2	6.5	20.0	0.7	-	1.3	0.1	2.3	1.7	0.3
PDA-PEI-Cl	61.4	13.7	16.4	-	-	0.8	0.2	7.7	4.5	0.3

Table 2. Atomic percentage and ratio obtained from XPS data for PDA coatings after chlorination.484

Finally, it is possible to obtain the Cl/N ratio in the whole PDA film, not only from 486 the extreme surface as shown previously by XPS. In order to do that, a chemical dosage 487 using the oxidative properties of the chloramine film, to follow the oxidation of TNB into 488 the corresponding dimer DTNB, accompanied with a change of color of the solution, 489 Figure S6. Thus, by measuring the optical density at 412 nm, the amount of Cl(+I) 490 immobilized in the PDA film can be determined. The results are reported in Table 3, 491 together with the density of chlorine atoms for each film. The film having the highest 492 density of Cl(+I), hence N-Cl groups, is the PDA-PEI film. None the less, all three films of 493 PDA exhibit high amount of chlorine, ranging from 5x10²⁰ to almost 10²² at·cm⁻³. 494

This clear difference between XPS results and chemical assay with TNB comes first 495 of all from the fact that a quantification technique is used here which probes the depth of 496 the layer, unlike the XPS analysis which only gives information, as a reminder, on the 497 coating surface. In addition, the nature of the reagent used and the nature of the PDA-IO₄- 498 coating which are rougher and denser than the PDA-O₂ coatings Figure S3 can explain 499 this difference. Indeed, TNB being a bulky molecule would make its infiltration over the 500 entire thickness of the coatings difficult. 501

Table 3. TNB solution absorption (A) at 412 nm after 24 h of immersion of various samples, the number of chlorine atoms, density (Equation 3) of chloramine in PDA-O₂, PDA-IO₄⁻ and PDA-PEI coatings estimated by TNB dosage, and the Cl_{200}/N atomic ratio considering a N density of 5.3×10^{21} at cm⁻³.

	A at 412 nm	Cl (at.)	d⊂ı (at·cm⁻³)	Cl200/N
PDA-O ₂ -Cl	0.30	4.0 1016	2.5 1021	0.47
PDA-IO4Cl	0.50	5.8 1015	5.0 1020	0.10
PDA-PEI-Cl	0.20	9.25 1016	9.25 1021	0.29

3.3. Antibacterial properties of chlorinated PDA surfaces

Several microbiological tests have been performed in order to evaluate the different 509 antibacterial activities of the chlorinated PDA coatings. These tests were performed with 510 *E. coli* ATCC 25922 and *S. epidermidis* CIP 6821. They were carried out following two 511 routes: a direct one by optical microscopy using fluorescent stains to evaluate the bacterial 512 adherence and the cell mortality; a second indirect route was based on the recovery of 513 adhered bacteria and viable cell culture counting on agar plates. 514

The first test was carried out in order to evaluate the adhesive properties of PDA-XX 516 and PDA-XX-Cl coatings towards bacterial suspensions, the main results are presented in 517 Figure 6. The total adhered flora was evaluated by counting the green bacteria present on 518 the surface after recording several areas on the different surfaces; this green fluorescence 519 was obtained by marking bacteria with Syto[™] 9 fluorescent staining, that penetrates all 520 living and damaged bacteria. The numbering of adhered bacteria was done using ImageJ 521 software. Looking first at the results for E. coli, Figure 6(a), one can notice that all 522 chlorinated surfaces exhibit a decreased number of bacteria compared to the respective 523 non-chlorinated surfaces. Hence, for PDA-O2 and PDA-IO4, the net decrease of bacterial 524 adherence is 25% and 38%, respectively, with significant statistical differences (p < 0.01), 525

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see Figure S7. Concerning the PDA-PEI surface, a decrease of 21% is observed, Figure 6(a) 526 but with no significant difference (p > 0.01), Figure S7. Surprisingly, so such tendencies 527 were observed for S. epidermidis bacterial strain, Figure 6(b). All the adherence recorded 528 show no statistically significant differences, meaning that the chlorine substitution does 529 not affect the contact between the surface and the bacterial membrane. This can be 530 probably explained by the composition of the membrane of Gram positive bacteria 531 compared to the one of Gram negative bacteria, with the absence of charged 532 liposaccharide in the membrane in the latter case. In addition, only one relevant statistical 533 difference is observed in the case of PDA-PEI surfaces, which exhibit a much lower 534 adherence compared to its chlorinated counter-part, with 36% less adhered bacteria. This 535 could be explained again by the specific positive charges present on the outer membrane 536 of the Gram positive bacteria, resulting on charged repulsion between this one and the 537 positively charged amine groups of PEI under physiological conditions [53]. 538

Following the evaluation of total adherence properties by recoding green fluorescent 539 bacteria on the various surfaces, a second fluorescent staining was introduced in the 540 system and microscopy images were again recorded. This second fluorescent stain, 541



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Figure 6. Mean number of adhered bacteria observed by optical microscopy (green bars) and %544killing obtained from fluorescence optical microscopy (red bars) for (a) *E. coli* and (b) *S. epidermidis*545and for the three PDA coatings before and after chlorination.546

propidium iodide, penetrates only damaged bacterial membranes, and thus allows to evaluate the killing properties of surface using equation (5). 548

The results are presented in Figure 6 (red bars on top of green ones) for the six studied 549 surfaces and for both Gram negative and positive bacterial strains, presenting the 550 numbers of red bacteria recorded compared to the number of green bacteria recorded on 551 the same surface. For each surface, the percentage of killing is also indicated on the charts. 552 First of all, one can notice the very low killing efficiency obtained with this technique for 553 all surfaces, with less than 1% of killing on average for all PDA coatings towards S. 554 epidermidis, Figure 6(b), and a killing efficiency comprised between 2 and 11% towards E. 555 coli, Figure 6(a). It is also important to notice, that with this live/dead fluorescent 556 technique, one can evaluate the killing properties of surfaces without the bactericidal 557 molecules, for instance the fact that a PDA-PEI surface exhibits a 4% killing property 558 towards E. coli bacteria, to be compared with the 11% obtained once the PDA-PEI coatings 559 has been chlorinated, Figure 6(a). These results may be surprising when compared with 560 those reported in literature: for instance with PDA-PEI-Cl coating, Chien et al. have shown 561 a 99.99% of killing towards S. aureus and 99.9% towards E. coli, but these results were 562 obtained using an indirect method, ca. CFU numbering on agar plates [34]. 563

However, it is known also from literature, that the live/dead fluorescent techniques 564 are very efficient when the damaged caused to the bacteria by the antibacterial agents 565 affect directly the integrity of the membranes of the bacteria. In addition, the mode of 566 actions of haloamines moieties remains unclear, and seems to be more directed towards 567 growth inhibition by blocking of the cellular division than membrane disruption, that 568 could explain the poor number of red bacteria in our fluorescent optical microscopy 569 experiments. Indeed, the contact-active antibacterial mechanism inactivates the growth 570 bacteria by remaining bound to surfaces. The mechanism involves the direct transfer of 571 an oxidizing halogen from N-halamine to bacterial cells. Halogen has a strong tendency 572 to combine with another element, inhibiting the priming process of bacterial cells. In this 573 way, the antibacterial action occurs without the dissociation of the halogen from 574 haloamine bonds [54]. 575



Figure 7. Size distribution (on pixels) of adhered *E. coli* on PDA-X and PDA-X-Cl coatings, observed by optical microscopy with green epifluorescence staining (Syto[™] 9).

In addition, we have noticed during our experiments that fluorescent intensity was 580 varying a lot when passing from the PDA coatings to the chlorinated ones, without any 581 experimental condition changes. Therefore, we have carried out statistical analyses on the 582 size of bacteria by average the numbers of pixels observed for each single bacterium. The 583 data are presented in Figure 7. It is quite clear that on non-chlorinated surfaces, the size 584 distribution of adhered bacteria is as often observed with a Poisson distribution profile 585 with a clear apex observed. However, when looking at the shape of this distribution for 586 chlorinated films, it is clear that the size distribution has changed, with no more Poisson 587 distribution profile, and a net decrease of the size of the bacteria. This size decrease was 588 also observed by scanning electron microscopy, Figure 8. The average size of E. coli is 589 around 1.5-1.9 µm for PDA-O₂ films, while the average size is closer to 0.8-1.2 µm when 590 the film has been chlorinated. Moreover, the shape of the bacteria is also affected, with no 591 longer a clear elongated bacillus shape but rather an oval one. 592

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Figure 8. SEM micrographs of E. coli on PDA-O2 (left) and PDA-O2-Cl (right) coatings.

The following experiments were carried out using an indirect method consisting on 596 recovering adhered bacteria from a given surface (either by mild sonication or by 597 vortexing) and to count the number of viable bacteria, after deposition/growth of 598 recovered bacteria on agar plates. The data obtained on agar plates are shown in Figure 599 S8 and Table S1, the results are summarized in Table 4. For each chlorinated surface, two 600 references were used to calculate the %killing, either the initial concentration of the 601 bacterial inoculum or by taking into account the respective non-chlorinated surface, using 602 equation (4). 603

Table 4. Killing percentage (%killing) of chlorinated coating obtained from CFU numbering on agar plates referenced towards the bacterial inoculum and towards the non-chlorinated coatings.

	PDA-Cl		PDA-IO4 ⁻ -Cl		PDA-PEI-Cl	
Reference	Inoculum	PDA	Inoculum	PDA-IO4-	Inoculum	PDA-PEI
E. coli ATCC 25922	99.0	34.0	97.7	91.4	97.4	86.2
S. epidermidis CIP 6821	70.0	59.4	83.0	76.6	66.6	58.7

When looking at results obtained for E. coli, and when comparing to the initial 607 bacterial inoculum, all three PDA-chlorinated surfaces enables an inactivation of bacterial 608 viability close to 2 log reduction, with the values ranging from 97.4% to 99.0%, as already 609 observed for PDA-O₂ in our previous study [26]. When comparing now with the non-610 chlorinated surface, these *%killing* are varying quite a lot as a function of the considered 611 surface. In fact, for the classical PDA-O₂-Cl, the *%killing* is only 34%, while both PDA-IO₄-612 -Cl and PDA-PEI-Cl exhibit %killing higher than 86%. In the case of PDA-IO4⁻Cl, this 613 difference can be explained by the fact that the film is thicker. However, at the point it 614 should be noted that I atoms could also participate to the antibacterial properties of this 615 PDA film. In the case of PDA-PEI, it can be explained by the fact that more NH groups are 616 available for chlorination (see XPS data of Tables 1 and 2) with overall more N-Cl 617 functions available. 618

The tendencies are more or less the same when looking at the data obtained for S. 619 *epidermidis,* with higher *%killing* of the chlorinated films when referenced to the inoculum 620 than referenced with the non-chlorinated coatings. However lower killing efficiencies 621 observed towards S. epidermidis are observed compared to those observed for E. coli. That 622 could be explained by the composition of the bacterial membrane of Gram positive 623 bacteria, as already observed by Targosz et al. [55], with an enriched peptidoglycan 624 concentration for Gram positive bacteria. Nonetheless, a coating seems to have better 625 efficiency than the others two: PDA-IO4-Cl, further experiments should be conducted to 626 specify the rule of the iodine atoms immobilized on/in the PDA coating 627

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4. Discussion

In this work, we studied the optimization of the antibacterial response of N-halamine 629 coatings based on PDA film. As reminder, a previous study demonstrated the elaboration 630 of a new antibacterial coating formed by a thin chlorinated PDA film containing 631 chloramine functions inside the polymer. The amount of these functions was estimated 632 between 10²¹ and 10²² at cm⁻³ [26] giving, for chlorinated PDA coatings, a E. coli adhesion 633 reduction up to 45% compared to uncoated surfaces and a bacterial viability reduction of 634 99% on chlorinated PDA coating compared to initial inoculum concentration and 34% 635 when compared to the non-chlorinated PDA film. 636

In order to optimize the amount of haloamine functions available on/in the material 637 to be protected, we have studied other PDA deposition pathways that make it possible to 638 increase the amount of amine functions. For this, two additional routes were considered. 639 The first one was to accelerate the kinetics of PDA film formation by using a more 640 powerful oxidant, ca. sodium periodate, and increasing the thickness of the deposit that 641 reachs 100 nm after 24 h. However, the PDA coating is rough and iodine atoms remain 642 trapped on/in the coating. The second route consisted of the use of a second polymer 643 richer in amine functions, PEI. PEI is known to act as a crosslinker within the structure of 644 PDA thus allowing more stable coatings to be obtained. The PM-RAIRS analyzes 645 confirmed the deposition of the composite and the XPS measurements showed an 646 enrichment of the PDA-PEI coating in amine functions. 647

The chlorination of the different PDA films was carried out using diluted NaOCl as 648 a function of contact time and concentration in order to optimize the chlorination process. 649 According to XPS analysis, probing the extreme film surface, we have concluded that the 650 efficiency of chlorination was the best for the PDA-O₂ films with more than 60% of the N 651 atoms that are chlorinated, while this value is only 30% for the two other films. These 652 differences between the PDA-O₂, PDA-IO₄⁻ and PDA-PEI coatings can be explained by the 653 availability of N-H function. Indeed, the PDA-IO4⁻ coating is composed by different 654 hyperoxidized structure compared to the PDA-O₂ film, with a higher proportion of 655 tertiary amine functions for which no chlorination is possible. For the PDA-PEI coating, 656 NH2 functions can be engaged in hydrogen bonds with the adjacent catechol functions 657 which do not allow the chlorination. The chlorination quantification of the whole films 658 was carried out by chemical assay with TNB and revealed that the PDA-PEI film showed 659 the higher density of N-Cl groups, which is not the one having the higher N/Cl ratio. The 660 three films of PDA exhibit high amount of chlorine, ranging from 5x10²⁰ to almost 10²² 661 atoms per cm³. We noticed difference between XPS results and chemical assay with TNB 662 coming first of all from the fact that a quantification technique is used here which probes 663 the depth of the fim, unlike the XPS analysis which only provides information on the film 664 surface. Moreover, due to the nature of the PDA-IO4⁻ coating which are rougher and 665 denser than the PDA-O₂ coating, the diffusion of the TNB molecules through the entire 666 thickness of the coatings could be difficult. 667

Next, the efficiency of the unchlorinated and chlorinated coatings in eliminating two 668 different bacterial strains: one Gram negative bacteria, Staphylococcus epidermidis CIP 6821, 669 and one Gram negative bacteria, Escherichia coli ATCC 25922, was studied. The first test 670 was carried out following a direct experiment by optical microscopy using fluorescent 671 stains to evaluate the bacterial adherence and the cell mortality. For E. coli, all chlorinated 672 surfaces exhibited a lower number of bacteria compared to the respective non-chlorinated 673 surfaces. For PDA-O2 and PDA-IO4, the net decrease of bacterial adherence was 25% and 674 38% respectively. Concerning the PDA-PEI surface, a decrease of 21% was observed but 675 with no significant difference (p> 0.01). Surprisingly, for *S. epidermidis* bacterial strain all 676 the adherences recorded showed no statistically significant difference, this finding could 677 be explained by the composition of the membrane of Gram positive with the presence of 678 charged liposaccharides and peptidoglycans [55]. Moreover, PDA-PEI surface exhibited a 679 much lower adherence with 36% less adhered bacteria compared to the chlorinated film. 680 This result could be due again to the positive charges present on the outer membrane of 681

S. epidermidis bacteria, resulting on charged repulsion between these ones and the positively charged amine groups of PEI. 683

Following the evaluation of adherence properties, the killing properties of surface 684 were evaluated. Bad killing efficiency was obtained with this technique for all the tested 685 PDA surfaces. We can notice that the live/dead fluorescent technique is very efficient 686 when the damages caused to the bacteria by the antibacterial agents affect directly the 687 integrity of the membrane of the bacteria. Thus, the mode of actions of haloamine moieties 688 seems to be more directed towards growth inhibition by blocking the cellular division 689 rather than membrane disruption, that could explain the poor number of red bacteria 690 observed during our fluorescent optical microscopy experiments. This was confirmed by 691 a statistical analysis of the size of bacteria grown on chlorinated film, looking at the shape 692 of this distribution it is clear that the bacteria size has changed with a net increase of the 693 number of small bacteria. In fact, the average size of E. coli that have adhered on 694 chlorinated PDA film were one third of the ones adhered on un-chlorinated PDA film. 695 Moreover, the shape of the bacteria was also affected, with an oval shape. 696

A second indirect route was based on the recovery of adhered bacteria and viable cell 697 culture counting on agar plates. When looking at results obtained for E. coli, and when 698 comparing to the initial bacterial inoculum, the three chlorinated PDA surfaces enable an 699 inactivation of bacterial viability close to 2 log reduction, with the values ranging from 700 97.4% to 99.0%, as already observed for PDA-O₂ in our previous study [26]. However 701 when comparing with the non-chlorinated surface, for the classical PDA-O₂-Cl film, the 702 %killing is only 34%, while PDA-IO4⁻-Cl and PDA-PEI-Cl films exhibit %killing higher than 703 86%. These differences can be explained, in the case of PDA-IO₄–Cl, by a thicker and more 704 dense film (Figures S2 and S3), rendering the haloamine functions more accessible for 705 antibacterial activity. In the case of PDA-PEI, it can be explained by the fact that more NH 706 groups are available for chlorination with overall more N-Cl functions available. The 707 tendencies were quite similar for S. epidermidis, with higher %killing of the chlorinated 708 films when referenced to the inoculum than referenced with the non-chlorinated coatings. 709 However lower killing efficiencies were observed towards S. epidermidis compared to 710 those for E. coli, and that could be again explained by the composition of the bacterial 711 membrane. 712

5. Conclusions

To conclude, we have shown in this study how to elaborate several PDA-based 715 antibacterial coatings composed of thin N-halamine films. Three routes have been 716 followed, a "classical" one with PDA formed with TRIS in the presence of atmospheric O2. 717 A second route was obtained by changing the oxidizing agent by replacing the 718 atmospheric O₂ by a stronger oxidant agent, NaIO₄, finally a copolymer PDA-PEI using 719 the conditions of the "classical" route. The three coatings exhibit similar haloamine 720 concentration in the order of 2.5x1021 and 9x1021 Cl at·cm-3 for PDA-O2 and PDA-PEI, 721 respectively. Noteworthy, for PDA-IO4⁻ coatings, the Cl(+I) density is four times lower. 722 Microbiological tests were carried out on two bacterial strains *E. coli* and *S. epidermidis*. 723 Direct tests, fluorescent staining observed by optical microscopy, have revealed a clear 724 decrease of bacterial adherence for *E. coli*, up to 40%, while no statistical decrease was 725 observed for S. epidermidis. In addition, these direct tests have shown that the morphology 726 of bacteria after contact with N-halamine coating has changed, with smaller size and 727 distorted shape. Indirect tests evidenced differences on the viability/growth of adhered 728 bacteria, with bactericidal properties higher in the case of Gram negative bacteria than for 729 Gram positive bacteria. The reduction of viable bacteria is higher when compared to the 730 initial inoculum rather than compared to the non-chlorinated surfaces. Finally, for both 731 bacterial strains, the best PDA coating appears to be the PDA-IO4⁻Cl one, especially when 732 considering that it contains four times less active haloamine functions for a 25% better 733 bactericidal efficiency. 734

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	Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1. PM-RAIRS spectra of gold surfaces coated with PDA-O ₂ , PDA-IO ₄ ⁻ and PDA-PEI films. Figure S2. PDA-O ₂ , PDA-IO ₄ ⁻ and PDA-PEI film thickness evaluated by SEM observations and ellipsometry measurements. Figure S3. SEM micrographs of PDA-O ₂ , PDA-IO ₄ ⁻ and PDA-PEI coatings on gold substrate. Figure S4. Evolution of the contact angle with water for PDA-O ₂ , PDA-IO ₄ ⁻ and PDA-PEI coatings. Figure S5. Representative structural components in PDA-IO ₄ ⁻ and PDA-PEI films. Figure S6. Numbering of chloramine functions by TNB. Figure S7. Statistical analyses of bacterial adherence. Figure S8 Optical photographs of agar plates presenting the killing tests results. Table S1 Average number of bacteria, in CFU.mL ⁻¹ , after killing tests against the different PDA coatings.	735 736 737 738 739 740 741 742 743			
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		761			
References		762 763 764			
1. de Carvalho, C. C. C. R. Marin	ne Biofilms: A Successful Microbial Strategy With Economic Implications. Front Mar Sci 2018, 5, 126.	765			
2. Hall-Stoodley, L.; Costerton,	J. W.; Stoodley, P. Bacterial biofilms: from the Natural environment to infectious diseases. Nat Rev	766			
Microbiol 2004 , 2, 95-108.					
3. Nickel, J. C.; Ruseska, I.; Wright, J. B.; Costerton, J. W. Tobramycin resistance of Pseudomonas aeruginosa cells growing as a biofilm					
on urinary catheter material. Antimicrob Agents Chemother 1985, 27, 619-624.					
4. Rabin, N.; Zheng, Y.; Opoku-Temeng, C.; Du, Y.; Bonsu, E.; Sintim, H. O. Biofilm formation mechanisms and targets for developing					
antibiofilm agents. <i>Future Med Chem</i> 2015 , 7, 493-512.					
5. Veerachamy, S.; Yarlagadda, T.; Manivasagam, G.; Yarlagadda, P. K. Bacterial adherence and biofilm formation on medical					
implants: a review. Proc Inst Mech Eng H 2014, 228, 1083-99.					
6. Donlan, R. M. Biofilms: microbial life on surfaces. Emerg Infect Dis 2002, 8, 881-90.					
7. Dufour, D.; Leung, V.; Lévesque, C. M. Bacterial biofilm: structure, function, and antimicrobial resistance. Endod Topics 2010, 22, 2-					
16.		776			
8. Alves, D.; Pereira, M. O. Mini	i-review: Antimicrobial peptides and enzymes as promising candidates to functionalize biomaterial	777			
surfaces. Biofouling 2014, 30, 483	-499.	778			
9. Chen, J.; Zhu, Y.; Xiong, M.; Hu, G.; Zhan, J.; Li, T.; Wang, L.; Wang, Y. Antimicrobial Titanium Surface via Click-Immobilization					
of Peptide and Its in Vitro/Vivo Activity. ACS Biomater Sci Eng 2019, 5, 1034-1044.					
10. Cox, H. J.; Li, J.; Saini, P.; Paterson, J. R.; Sharples, G. J.; Badyal, J. P. S. Bioinspired and eco-friendly high efficacy cinnamaldehyde					
antibacterial surfaces. J Mater Chem B 2021, 9, 2918-2930. 78					

11. Nicolas, M.; Beito, B.; Oliveira, M.; Tudela Martins, M.; Gallas, B.; Salmain, M.; Boujday, S.; Humblot, V. Strategies for	783
Antimicrobial Peptides Immobilization on Surfaces to Prevent Biofilm Growth on Biomedical Devices. Antibiotics 2022, 11, 13.	784
12. Shahnawaz Khan, M.; Abdelhamid, H. N.; Wu, HF. Near infrared (NIR) laser mediated surface activation of graphene oxide	785
nanoflakes for efficient antibacterial, antifungal and wound healing treatment. Colloids Surf B: Biointerfaces 2015, 127, 281-291.	786
13. Siedenbiedel, F.; Tiller, J. Antimicrobial Polymers in Solution and on Surfaces: Overview and Functional Principles. Polymers 2012,	787
4, 46-71.	788
14. Fan, X.; Ren, X.; Huang, TS.; Sun, Y. Cytocompatible antibacterial fibrous membranes based on poly(3-hydroxybutyrate-co-4-	789
hydroxybutyrate) and quaternarized N-halamine polymer. RSC Advances 2016, 6, 42600-42610.	790
15. Kügler, R.; Bouloussa, O.; Rondelez, F. Evidence of a charge-density threshold for optimum efficiency of biocidal cationic surfaces.	791
Microbiology 2005 , 151, 1341-1348.	792
16. Olsen, I. Biofilm-specific antibiotic tolerance and resistance. Eur J Clin Microbiol Infect Dis 2015, 34, 877-886.	793
17. Olson, M. E.; Ceri, H.; Morck, D. W.; Buret, A. G.; Read, R. R. Biofilm bacteria: formation and comparative susceptibility to	794
antibiotics. Can J Vet Res 2002, 66, 86-92.	795
18. Timofeeva, L.; Klesncheva, N. Antimicrobial polymers: mechanism of action, factors of activity, and applications. <i>Appl Microbiol Biotechnol</i> 2011 , <i>89</i> , 475-492.	796 797
19. Baltzer, S. A.: Brown, M. H. Antimicrobial peptides: promising alternatives to conventional antibiotics. <i>J Mol Microbiol Biotechnol</i>	798
2011 , 20, 228-35.	799
20. Bang, S. H.; Sekhon, S. S.; Ahn, IY.; Kim, YH.; Min, J. Advances in antimicrobial agents based lysosomes. <i>Mol Cell Toxicol</i> 2014 .	800
10, 229-235.	801
21. Bechinger, B.; Gorr, S. U. Antimicrobial Peptides: Mechanisms of Action and Resistance. J Dent Res 2017, 96, 254-260.	802
22. Harris, F.; Dennison, S. R.; Phoenix, D. A. Anionic antimicrobial peptides from eukaryotic organisms. <i>Curr Protein Pept Sci</i> 2009,	803
10, 585-606.	804
23. Shima, S.; Matsuoka, H.; Iwamoto, T.; Sakai, H. Antimicrobial action of epsilon-poly-L-lysine. J Antibiot 1984 , 37, 1449-55.	805
24. Dong, A.; Wang, YJ.; Gao, Y.; Gao, T.; Gao, G. Chemical Insights into Antibacterial N-Halamines. <i>Chem Rev</i> 2017 , <i>117</i> , 4806-4862.	806
25. Hui, F.; Debiemme-Chouvy, C. Antimicrobial N-Halamine Polymers and Coatings: A Review of Their Synthesis, Characterization,	807
and Applications. <i>Biomacromolecules</i> 2013 , 14, 585-601.	808
26. Nazi, N.; Humblot, V.; Debiemme-Chouvy, C. A New Antibacterial N-Halamine Coating Based on Polydopamine. Langmuir 2020,	809
36, 11005-11014.	810
27. Chien, HW.; Chiu, TH. Stable N-halamine on polydopamine coating for high antimicrobial efficiency. Eur Polym J 2020, 130,	811
109654.	812
28. Liu, Y.; Ai, K.; Lu, L. Polydopamine and its derivative materials: synthesis and promising applications in energy, environmental,	813
and biomedical fields. <i>Chem Rev</i> 2014 , <i>114</i> , 5057-115.	814
29. Ye, Q.; Zhou, F.; Liu, W. Bioinspired catecholic chemistry for surface modification. Chem Soc Rev 2011, 40, 4244-58.	815
30. Ball, V. Physicochemical perspective on "polydopamine" and "poly(catecholamine)" films for their applications in biomaterial	816
coatings. Biointerphases 2014, 9, 030801.	817
31. Ponzio, F.; Ball, V. Polydopamine deposition at fluid interfaces. Polym Int 2016, 65, 1251-1257.	818
32. Ponzio, F.; Barthès, J.; Bour, J.; Michel, M.; Bertani, P.; Hemmerlé, J.; d'Ischia, M.; Ball, V. Oxidant Control of Polydopamine Surface	819
Chemistry in Acids: A Mechanism-Based Entry to Superhydrophilic-Superoleophobic Coatings. Chem Mater 2016, 28, 4697-4705.	820
33. Lv, Y.; Yang, SJ.; Du, Y.; Yang, HC.; Xu, ZK. Co-deposition Kinetics of Polydopamine/Polyethyleneimine Coatings: Effects of	821
Solution Composition and Substrate Surface. Langmuir 2018, 34, 13123-13131.	822
34. Chien, H. W.; Chiu, T. H.; Lee, Y. L. Rapid Biocidal Activity of N-Halamine-Functionalized Polydopamine and Polyethylene	823
Imine Coatings. Langmuir 2021, 37, 8037-8044.	824

35. Humblot, V.; Yala, JF.; Thebault, P.; Boukerma, K.; Hequet, A.; Berjeaud, JM.; Pradier, CM. The antibacterial activity of	825
Magainin I immobilized onto mixed thiols Self-Assembled Monolayers. Biomaterials 2009, 30, 3503-3512.	826
36. Lee, B. P.; Messersmith, P. B.; Israelachvili, J. N.; Waite, J. H. Mussel-Inspired Adhesives and Coatings. Annu Rev Mater Res 2011,	827
41, 99-132.	828
37. Lee, H.; Dellatore, S. M.; Miller, W. M.; Messersmith, P. B. Mussel-Inspired Surface Chemistry for Multifunctional Coatings. Science	829
2007 , <i>318</i> , 426-430.	830
38. Del Frari, D.; Bour, J.; Ball, V.; Toniazzo, V.; Ruch, D. Degradation of polydopamine coatings by sodium hypochlorite: A process	831
depending on the substrate and the film synthesis method. Polym Degrad Stab 2012, 97, 1844-1849.	832
39. Ma, K.; Jiang, Z.; Li, L.; Liu, Y.; Ren, X.; Huang, TS. N-halamine modified polyester fabrics: Preparation and biocidal functions.	833
Fibers Polym 2014 , 15, 2340-2344.	834
40. Scofield, J. H. Hartree-Slater Subshell Photoionization Cross-Sections at 1254 and 1487 eV. J Elec Spectrosc Relat Phenom 1976, 8, 120, 127	835
129-137.	836
41. Kiddles, P. W.; Blakeley, K. L.; Zerner, B. Eliman's reagent: 5,5 -dithiobis(2-nitrobenzoic acid) a reexamination. Anal Biochem 1979,	837
94,75-81	838
42. Deblemme-Chouvy, C.; Haskouri, S.; Foicher, G.; Cacher, H. An Original Route to Immobilize an Organic Biocide onto a Transparent Tin Dioxide Electrode. <i>Langmuir</i> 2007 , <i>23</i> , 3873-3879.	839 840
43. Segut, O.; Herlem, G.; Lakard, B.; Blondeau-Patissier, V.; Nardin, M.; Gree, S.; Rauch, JY. Electrochemically deposited	841
polyethyleneimine films and their characterization. Synth Met 2010, 160, 1359-1364.	842
44. Zangmeister, R. A.; Morris, T. A.; Tarlov, M. J. Characterization of Polydopamine Thin Films Deposited at Short Times by	843
Autoxidation of Dopamine. Langmuir 2013, 29, 8619-8628.	844
45. Chen, TP.; Liu, T.; Su, TL.; Liang, J. Self-Polymerization of Dopamine in Acidic Environments without Oxygen. Langmuir 2017,	845
33, 5863-5871.	846
46. Jiang, J.; Zhu, L.; Zhu, L.; Zhu, B.; Xu, Y. Surface characteristics of a self-polymerized dopamine coating deposited on hydrophobic	847
polymer films. <i>Langmuir</i> 2011 , 27, 14180-14187.	848
47. Zheng, W.; Fan, H.; Wang, L.; Jin, Z. Oxidative Self-Polymerization of Dopamine in an Acidic Environment. <i>Langmuir</i> 2015, 31,	849
11671-11677.	850
48. Weidman, S. W.; Kaiser, E. T. The Mechanism of the Periodate Oxidation of Aromatic Systems. III. A Kinetic Study of the Periodate	851
Oxidation of Catechol. J Am Chem Soc 1966, 88, 5820-5827.	852
49. Alfieri, M. L.; Panzella, L.; Oscurato, S. L.; Salvatore, M.; Avolio, R.; Errico, M. E.; Maddalena, P.; Napolitano, A.; D'Ischia, M. The	853
Chemistry of Polydopamine Film Formation: The Amine-Quinone Interplay. Biomimetics 2018, 3, 26.	854
50. Tanuma, S.; Powell, C. J.; Penn, D. R. Calculation of electron inelastic mean free paths (IMFPs) VII. Reliability of the TPP-2M IMFP	855
predictive equation. Surf Interface Anal 2003, 35, 268-275.	856
51. Xu, L.; Zuo, Y. Y. Reversible Phase Transitions in the Phospholipid Monolayer. Langmuir 2018, 34, 8694-8700.	857
52. Yang, W.; Liu, C.; Chen, Y. Stability of Polydopamine Coatings on Gold Substrates Inspected by Surface Plasmon Resonance	858
Imaging. Langmuir 2018, 34, 3565-3571.	859
53. Curtis, K. A.; Miller, D.; Millard, P.; Basu, S.; Horkay, F.; Chandran, P. L. Unusual Salt and pH Induced Changes in	860
Polyethylenimine Solutions. PLoS ONE 2016, 11, e0158147.	861
54. Luo, J.; Chen, Z.; Sun, Y. Controlling biofilm formation with an N-halamine-based polymeric additive. J Biomed Mater Res 2006,	862
77 <i>A</i> , 823-831.	863
55. Marcinkiewicz, J.; Biedroń, R.; Białecka, A.; Kasprowicz, A.; Mak, M.; Targosz, M. Susceptibility of Propionibacterium acnes and	864
Staphylococcus epidermidis to killing by MPO-halide system products. Implication for taurine bromamine as a new candidate for	865
topical therapy in treating acne vulgaris. Arch Immunol Ther Exp 2006, 54, 61-68.	866