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Sustainable and Bio-inspired Chemistry for Robust Antibacterial Activity of Stainless Steel

Emilie Faure,[a] Philippe Lecomte,[a] Sandrine Lenoir,[a] Christelle Vreuls,[b] Cécile Van De Weerdt,[b] Catherine Archambeau,[c] Joseph Martial,[b] Christine Jérôme,[a] Anne-Sophie Duwez[d] and Christophe Detrembleur*[a]

Abstract: We report on the original synthesis of a poly(methacrylamide) bearing (oxidized) 3,4-dihydroxyphenylalanine specially designed to (i) insure film growth by covalent coupling, (ii) covalently bind an antibacterial peptide and (iii) contribute to the film cross-linking that is essential for the durability of the properties.

Stainless steel is widely used in daily life because of its resistance to corrosion and chemicals, and its mechanical and aesthetic properties. It can be found in many areas such as medicine and household appliances, building and food industries.[1] One main limitation of stainless steel is its inability to stop bacteria proliferation. Therefore for hygienic reasons, there is a strong need in developing robust surface modifications to give antibacterial (AB) properties to stainless steel. The literature is extremely rich in describing strategies for coating substrates with inorganic (silver,[2-4] copper,[5]…) and organic (antibiotics,[6, 7] peptides,[8] (co)polymers,[9-12]…) biocides. However, most of the time, the AB activity is time limited by the diffusion of the active species out of the coating. Stainless steel producers and users are now expecting long-lasting coatings essential for the durability of the functionality. It is therefore necessary to strongly bind the biocides to the surface. Furthermore, considering environmental issues, current development of new products has to prevent toxic wastes. Green coating processes are thus urgently required.

As far as stainless steel (SS) is concerned, the direct covalent grafting of organic biocides onto the surface is not trivial because of the absence of appropriate functional groups allowing strong anchoring. Only few examples can be found in the literature such as the electrografting of acrylates[13, 14] post-modified to obtain AB properties,[15-17] the grafting of lysozymes or trypsin onto polyethylene imine adsorbed onto SS,[18] or the post-quaternisation of amino groups grafted onto SS pre-functionalized by cold plasma treatment[19, 20] or by silane coupling agents.[21] However, these strategies are multi-steps, sometimes complex processes and require the use of aggressive chemicals and/or toxic molecules that make difficult the implementation at the industrial scale.

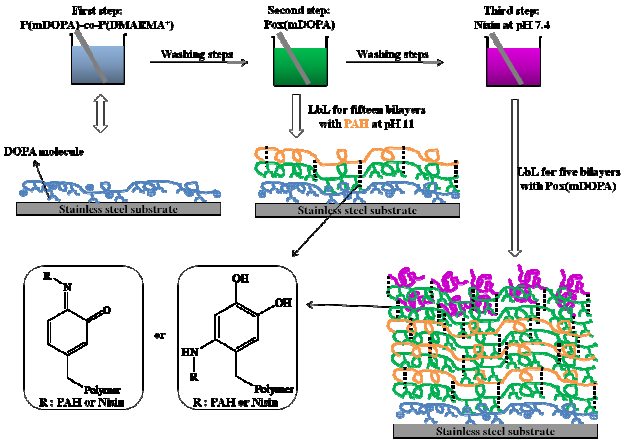
Here we report on a very simple and robust strategy to impart durable antibacterial properties to stainless steel by the Layer-by-Layer (LbL) technique[22] using a tailored multifunctional polymer. It relies on the original synthesis of a homopolymer of methacrylamide bearing (oxidized) 3,4-dihydroxyphenylalanine (mDOPA) specially designed to (i) insure LbL film growth by covalent coupling, (ii) covalently bind an AB peptide and (iii) contribute to the film cross-linking that is essential for the durability of the properties. Furthermore the whole process occurs in aqueous solution without specific surface treatments, providing a green approach. We therefore exploit the redox property of DOPA moieties, an important component responsible for the adhesion of mussels on various substrates,[23-26] present in a tailored polymer (P(mDOPA)) to get cross-linked multilayers and biocides grafting. This catechol derivative can be switched from a water insoluble (reduced state) to a water-soluble and highly reactive state towards primary amine (oxidized state). This reactivity makes this polymer unique and versatile for adjusting properties as demonstrated hereafter and for growing multilayers faster than works previously described elsewhere[26, 27].

The developed strategy to build the multilayer (Scheme 1a) starts with a first immersion of the substrate in an aqueous solution of P(mDOPA)-co-P(DMAEMA$^+$) (2 g/L), a polycationic copolymer bearing 15 mol% DOPA moieties (Scheme 1b; chemical structure (3)) at room temperature.[4] This DOPA-functionalized polycation is designed to strongly anchor to the surface by DOPA/metal interactions.[28] The next layers are then built by the successive dipping of the surface into an aqueous solution of a poly(methacrylamide) bearing oxidized DOPA moieties on each monomer unit (Scheme 1b, formula (2))

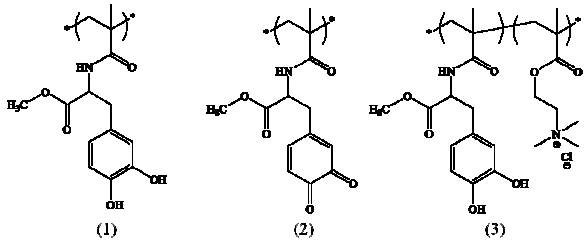
(Pox(mDOPA)), and then in a polymer bearing primary amines, polyallylamine (PAH). The appropriate combination of this polymer with PAH in LbL multilayers, by adequately controlling both the redox state of the P(mDOPA) polymer (Scheme 1b; chemical structures (1) and (2)) and the pH of the solutions, has been optimized to grow efficiently a durable LbL coating.

Oxidized DOPA moieties of Pox(mDOPA) are necessary for the covalent grafting of PAH through amine/quinone reaction and/or Schiff base formation at room temperature, and consequently for the interlayer cross-linking (Scheme 1a and S1, ESI†).[28-30] Preparation conditions are crucial for the success of the film build-up. First, P(mDOPA) is oxidized in aqueous media under basic conditions for 12 h to form the hydrosoluble Pox(mDOPA) (see ESI†) which is then deposited at a concentration of 2 g/L on the first P(mDOPA)-co-P(DMAEMA$^+$) layer by immersing the surface for 2 min. Importantly, the next solution of PAH (2 g/L) is deposited at pH=11 in order to obtain the polymer in the deprotonated state. The reaction between primary amines with the quinone groups of Pox(mDOPA) is therefore made possible (Scheme S1, ESI†).

(a)



(b)



Scheme 1 (a) LbL process for imparting long-lasting AB properties to stainless steel and (b) chemical structures of (1) P(mDOPA), (2) Pox(mDOPA) and (3) P(mDOPA)-co-P(DMAEMA$^+$).

In acidic media, amine groups are protonated and do not react with Pox(mDOPA), such that cross-linking and growth of the film cannot occur. All synthetic steps are performed in mild conditions and in aqueous media (see ESI), which make the building-block synthesis pathways relevant for the development of an environment-safe process.

Finally, in order to impart AB activity to these coatings, PAH is replaced by nisin in the 5 last layers (Scheme 1a). Nisin, a low molecular weight AB peptide (3500 g/mol), was selected because of (i) its high activity against a broad range of Gram-positive bacteria,[31] (ii) its wide use as food preservative, (iii) its lack of toxicity for humans[32] and (iv) its stability when involved in coatings.[33] For this purpose, nisin was dissolved in a phosphate buffer solution at pH = 7.4 to favor the peptide grafting through its N-terminal NH_2 group (pKa=6.8-8) and not

through the amino-groups of the three internal lysines (pKa=11) (Scheme S2, ESI†) that are mainly responsible for the AB activity of the peptide.[34] It is important to mention that the peptide grafting by the reaction of imidazole groups (pKa ~ 6) of nisin (Scheme S2, ESI†) with oxidized catechol[35, 36] cannot be excluded in these conditions.

As a first evidence of the multilayer film build-up, Quartz Crystal Microbalance coupled with Dissipation (QCM-D) was used to follow the film growth in real time on SS sensors by measuring the variation of the resonant frequency (Δf) *vs* time. A decrease in Δf indicates polymer deposition.[37] Fig. 1 shows that all partners are successfully deposited according to the selected deposition sequence and redox/pH conditions and remain on the substrate even after rinsing with water.

According to this procedure, 15 bilayers of Pox(mDOPA)/PAH, followed by 5 bilayers of Pox(mDOPA)/Nisin, were built on substrates provided by the steel industry (Arcelor-Mittal, SS 304 2B). Surface coverage and film thickness were probed by Field Emission Gun Scanning Electron Microscopy (FEG-SEM).

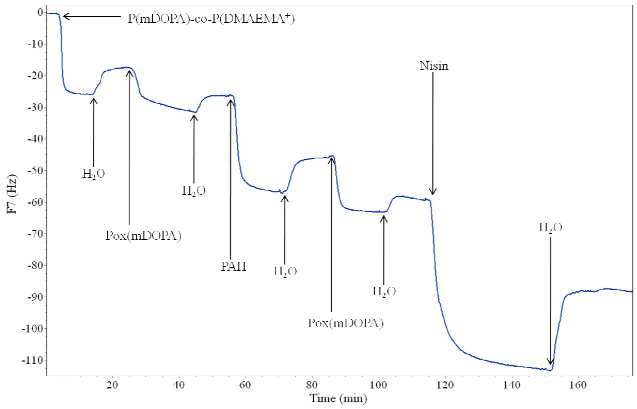


Fig. 1 Frequency change upon deposition of P(mDOPA)-co-P(DMAEMA$^+$), Pox(mDOPA), PAH and Nisin measured by QCM-D as a function of time at 25°C, where the overtone number is 7.

A cross-section of coated steel (Fig. S1, ESI) clearly evidences that the film is homogeneously covering the substrate, even in the substrate defects, with an average film thickness of about 50-60 nm. XPS analysis confirms the presence of nisin with its detected sulfur atoms (Fig. S2, ESI).

Antibacterial activity of the film has been assessed against *B. subtilis* using the standard JIS Z method specially designed for contact-killing coatings. Coated substrates were inoculated with about 10^6-10^8 cells/ml of bacteria for 24 hours and the number of survival bacteria was evaluated by counting them after spreading on agar plates. A strong AB activity was observed as demonstrated by a very high log decrease of survival bacteria compared to bare SS (Fig. 2, controls; according to this JIS Z 2801:2000(E) procedure, a 2 log decrease is considered as antibacterial). The permanent functionality of the coating was then evaluated by immersing the coated substrate in tap water during one night (the *immersion test*, Fig. 2) and by cleaning it with a wet sponge (30 back and forth movements, the *mechanical test*, Fig. 2). It was then tested again following the same procedure and AB activity was maintained at the same level, supporting the chemical grafting of nisin without denaturation. In order to test

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further the efficiency of the coating activity, the amount of bacteria was then considerably increased by one order of magnitude (from 10^8 to 10^9 bacteria/ml). The AB activity was still present but lowered, certainly due to the saturation of the surface by bacteria. Different control samples were prepared to assess the role of nisin grafting and of the developed strategy. First, films containing 20 bilayers of Pox(mDOPA) and PAH without nisin were tested and were shown to be inactive against *B. subtilis*, demonstrating the importance of nisin for the AB properties. Second, when P(mDOPA) is used in place of Pox(mDOPA), the film cannot be formed due to the lack of solubility of P(mDOPA) in water. Third, when polyacrylic acid (PAA) was used in place of Pox(mDOPA), AB activity is observed when nisin is present in the 5 last bilayers. However, this activity is almost completely lost when the immersion test is performed and partly lost with the mechanical test. It is important to note that this poor AB activity is observed even when using an amount of bacteria one order of magnitude lower than for the test carried out on the cross-linked system $(((Pox(mDOPA)/PAH)_{15}/Nisin)_5)$. This last experiment clearly demonstrates that cohesion between the layers by electrostatic interactions (PAA is negatively charged and nisin is positively charged) is not sufficient for preserving the durability of the functionality. Cross-linking of the film and covalent peptide grafting are thus necessary for the permanent activity.

As additional proofs of the cross-linking reaction through the amine/quinone reaction, aqueous solutions of Pox(mDOPA) and PAH (2g/l) at pH ~ 11 were mixed at room temperature. An insoluble product is instantaneously formed (Figure S3, ESI†) and its re-dissolution in any solvents (DMF, acetone, THF, …) is impossible, as expected for a cross-linked system. Moreover, solid state ^{13}C NMR analysis of the lyophilized product shows typical signals around 160 ppm assigned to the newly formed imine bonds (Figure S4, ESI†), coming from the Schiff base formation (Scheme S1 structure B, ESI†). The Michael addition is also expected to occur[28-30] (Scheme S1 structure A, ESI†) but cannot be evidenced on the basis of the NMR experiments.

In conclusion, we have developed a simple and robust green strategy for promoting long-lasting antibacterial properties to stainless steel using a water soluble polymethacrylamide bearing oxidized 3,4-dihydroxyphenylalanine groups. All the processing steps, including the synthesis of the polymers and the peptide grafting, were performed in aqueous solutions under mild conditions, making this process a sustainable alternative to current AB stainless steel coatings that slowly release biocides in the environment. Compared to other works reporting on mussel-inspired multifunctional coatings such as those made from self-polymerized dopamine,[27] our strategy is faster (~1h *vs* 24h[26]) and does not require the use of strong oxidizing agents. Moreover, our cross-linking process occurs at room temperature in water and does not require the use of any toxic reagent. This is in sharp contrast to conventional LBL PAA/PAH films that can only be cross-linked using some toxic activators (such as dicyclohexylcarbodiimide, DCC) and/or under thermal treatment that may denaturate the peptide. Our versatile approach might be applied to other biomolecules and surfaces, broadening considerably its general scope.

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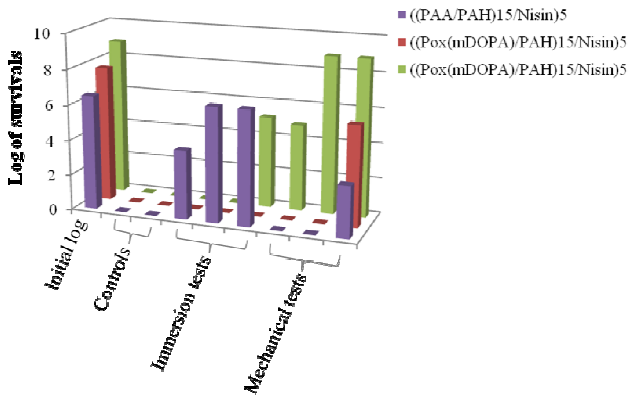

Fig. 2 Antibacterial assessments against *B. subtilis* using the JIS Z method, values are bacteria log survivals compared with bare stainless steel; all durability experiments were done in triplicate; purple colour corresponds to an initial bacteria log of 6.2, red colour to 7.7 and the green one to 8.9 (Control = AB coating on SS before mechanical and immersion tests).

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Notes and references

[a] *Center for Education and Research on Macromolecules (CERM)*
University of Liège, Sart-Tilman, B6, B-4000 Liège, Belgium
[b] *E-mail: christophe.detrembleur@ulg.ac.be*
Centre of Biomedical Integrative Genoproteomics
University of Liège, B34 Sart-Tilman, 4000 Liège, Belgium
[c] *ArcelorMittal Liège Research*
Bd de Colonster B57, 4000 Liège, Belgium
[d] *Nanochemistry and Molecular Systems (NANOCHEM)*
University of Liège, Sart-Tilman, B6, B-4000 Liège, Belgium
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