Influence of Nisin grafting on the antibacterial efficacy of AMP self-assembled monolayers (SAMs)

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Abstract:

The use of antimicrobial peptides (AMPs) covalently grafted on surfaces has been recognized in recent years as a promising strategy to fight against biofilms formation. However, after grafting, the understanding of AMPs-bacteria interactions is still debated in the literature.
In this study, Nisin, a cyclic AMP, was grafted onto gold surfaces via an indirect grafting on acidic thiol self-assembled monolayers using succinimide linkers. The physical and chemical properties of these SAMs were then finely characterized by XPS and FT-IR to confirm the covalent grafting of Nisin.
The anti-adhesion and bactericidal effects were then studied for *Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC 25923 and *Listeria ivanovii* Li4(pVS2) by a posteriori analysis of the culture supernatants (i.e. indirect technique) and *ex situ* by optical microscopy following crystal violet staining (i.e. direct technique). Statistical analysis reveals that Nisin coating has bactericidal and antiadhesive properties towards Gram positive bacteria, while no significant results were obtained for Gram negative bacteria.

Keywords: Nisin coating, AntiMicrobial Peptide, bactericidal effect, antiadhesive properties

1. Introduction

The contamination of surfaces, installations and equipment by the development of bacterial micro-organisms in the food and health fields (nosocomial infections, discharges, food poisoning), but also cosmetics, pharmaceuticals and industrial sectors (corrosion of ship hulls, corrosion of water pipes) constitutes a real public health problem [1], [2], [3].

Described by Donlan and Costerson in 2002 [4], these bacteria communities can organize themselves into groups within self-produced matrices that adhere to each other, and anchor themselves to numerous supports, forming a biofilm. The surfaces on which biofilms form are diverse in nature: natural or organic (rock, skin, internal organs, leaves or roots), industrial (ship hulls, pipes) and medical (protheses, implants, catheters) [5], [6]. Biofilms are present ubiquitous and are reservoirs for pathogens that may cause many infections: because of their complex structures and strong attachment to surfaces, their control can be complicated by their resistance to conventional treatments such as germicides and antibiotics [7], [8], [9].

Several strategies have been proposed to create sterile surfaces to combat bacterial contamination and biofilm growth, by adding aniadhesive, biocidal or antibacterial compounds via chemical grafting, impregnation or physical trapping [10], [11]. These include quaternary ammonium derivatives [12] or phenolic derivatives [13], antibiotics [14], or heavy metals such as silver or tin, in the form of coatings, dressings or nanoparticles [15], [16], [17]. Although a promising strategy, the use of such compounds is compromised by their potential toxicity to humans and to the environment. Moreover, in the case of biocidal compounds and antibiotics, bacteria become resistant after some time of use, and even led to bad reaction in patient bodies [3], [18]. This shows the urgent need to find an alternative for antibiotics, through the development of antibacterial molecules and compounds with high efficiency and low toxicity, and that do not induce bacterial resistance.

An alternative to antibiotics is the use of AntiMicrobial Peptides (AMPs), that are naturally produced by plants, mammals and microorganisms, enabling them to defend themselves against bacterial infections [19], [20], [21]. To date, the Antibacterial Peptide Database (APD, [22]) lists more than 4000 AMPs, which have different properties. These peptides are small, positively-charged molecules of 12 to 100 amino acids, naturally present in many organisms, and are produced in areas of infection and inflammation as the infected host's first immune barrier [23], [24]. Although they have similar physical properties, AMPs differ in their secondary structures, which give them different biological properties: antibacterial, antifungal, antiparasitic and antiviral. Recent studies have led to the isolation and characterization of thousands of AMPs in order to determine their biological activities and modes of action [24], [25]. Despite their promise, natural AMPs have medium range production costs and a narrower range of applications than synthetic AMPs. However, one natural peptide is an exception: Nisin, a bacteriocin produced naturally by the fermentation of *Lactococcus lactis bacteria* [26].

Nisin is one of the so-called "lantibiotics", a family of peptides made up of non-usual amino acids. It is 34 amino acids residues long and has several configurations, the best known and the most active being Nisin A and Nisin Z [26], [27], [28], [29]. Nisin Z has been discovered in 1991 and is one of the natural variants of Nisin A that differs in residue in position 27 that is Asparagine instead of Histidine [30]. While Nisin has been shown to be effective against Gram-positive bacteria such as *Salmonella, Pseudomnas, Staphylococcus* and *Listeria* [31], [32], [33], its effect on Gram-negative bacteria, such as *E. coli*, remains highly debated [34]. The insensitivity of Gram-negative bacteria to Nisin may be explained by the impermeability of the outer membrane of this type of bacteria, which is normally anionic due to the acid groups present on its surface, the latter annihilating the peptide's

effect [35]. In addition, some authors have studied several parameters that clearly affect the efficiency towards Gram negative membranes such as the structure characteristics related with bactericidal actions, including peptides constituents, molecular length, molecular charges and secondary structure. In particular, the absence of amphipathic design in the structure is responsible for low bactericidal activity [36]. This last point is clearly crucial in the mode of action of the peptide, namely pore forming and inhibition of the cell walls synthesis [37].

Early studies focused on unpurified Nisin, which had no antibacterial effect against Gram-negative bacteria. The Minimum Inhibitory Concentration (MIC) values of Nisin against *S. aureus* (Gram-positive) and *E. coli* (Gram-negative) were investigated by Kuwano, who again showed that unpurified Nisin has no bactericidal effect on *E. coli* (MIC > 75µM) [34]. However, he also studied the MIC values for purified Nisin on the same two bacteria. The work showed that the MIC for *E. coli* (600 nM) was 8 times higher than the MIC for *S. aureus* (75 nM). The antibacterial activity of Nisin against Gram-negative bacteria is still controversial, and a recent study showed some antibacterial activity of Nisin against 300 strains of Gram-negative bacteria, with a high sensitivity for *Helicobacter, Xanthomonas, C. freundii* but not for *E. coli* [38], [39].

The wide range of Gram-positive bacteria that can be treated with Nisin (alone or in combination with other molecules) makes it the most widely used antimicrobial peptide in the food industry especially in food packaging, to limit bacterial growth or even eliminate contamination [40], [41]. McAuliffe has widely studied the mechanism of action of Nisin, showing that the peptide targets lipids II on the surface of the bacteria, before adsorbing and starting to destabilize their cytoplasmic membrane structure, at very low concentration [42]. It has also been reported that Nisin is effective against the adhesion of some Gram-positive bacteria such as *Salmonella*, *Pseudomonas* or *Listeria ivanovii* on polymer coated stainless steel [43], [44], and against *Listeria monocytogenes* in many conditions [31].

Numerous strategies for grafting these efficient molecules onto surfaces have been developed. Among them, we have (i) polymer brushes used by Glinel [45] or Hadjesfandiari [46], (ii) multilayer polyelectrolyte films functionalized by the insertion of defensin [11] and (iii) chitosan film onto which is covalently grafted the Dhvar5 peptide onto titanium and gold surfaces [47]. Although these methods have interesting properties, they do not always prevent the formation and growth of biofilms on the surface [48]. This fact leads to develop strategy that first prevents bacterial adhesion on the surface and kill bacteria with one or more biocidal agents. Humblot and al in 2009, used one of the widely-used strategy to graft Magnanin I, creating self-assembled monolayers (SAMs) on which the biological active compounds can be immobilized [49]. Indeed, molecules used to create SAMs are usually long and form a dense layer on the surface, onto which antibacterial molecules can be grafted, thus preventing bacterial adhesion and providing a biocidal effect to the surface [50].

Some studies have shown antiadhesive and antibacterial effect of Nisin against algae and *Listeria monocytogenes* [44], [51]. In the present paper, we studied antibacterial and antiadhesive properties of Nisin Z against *Escherichia coli* (Ec), *Staphylococcus aureus* (Sa) and *Listeria ivanovii* (Li). The strategy used for the grafting of Nisin on gold surfaces is the fabrication of a mixed thiols (25:75, MUA:C_6OH) SAMs which involves grafting acid thiols via a succinimide linker. Covalent binding of Nisin was performed by conversion of carboxylic terminal groups of MUA into esters, thanks to the reaction between N-hydroxysuccinimide (NHS) and carbodiimide (EDC), followed by reaction between esters and one of 4 NH_2 of Lysin residue or N-ter group (Figure 1).

Physico-chemical characterization has been performed by FT-IR and XPS spectroscopies to confirm the grafting of Nisin. Then anti-adhesion and antibacterial effects were evaluated for the three

bacteria: *Echerichia coli* ATCC 25922, *Listeria ivanovii* Li4(pVS2) and *Staphylococcus aureus* ATCC 25923 by optical microscopy and culture of supernatants. A statistical analysis of the results is performed to determine if Nisin has antiadhesive or antibacterial effects once grafted on the surface.
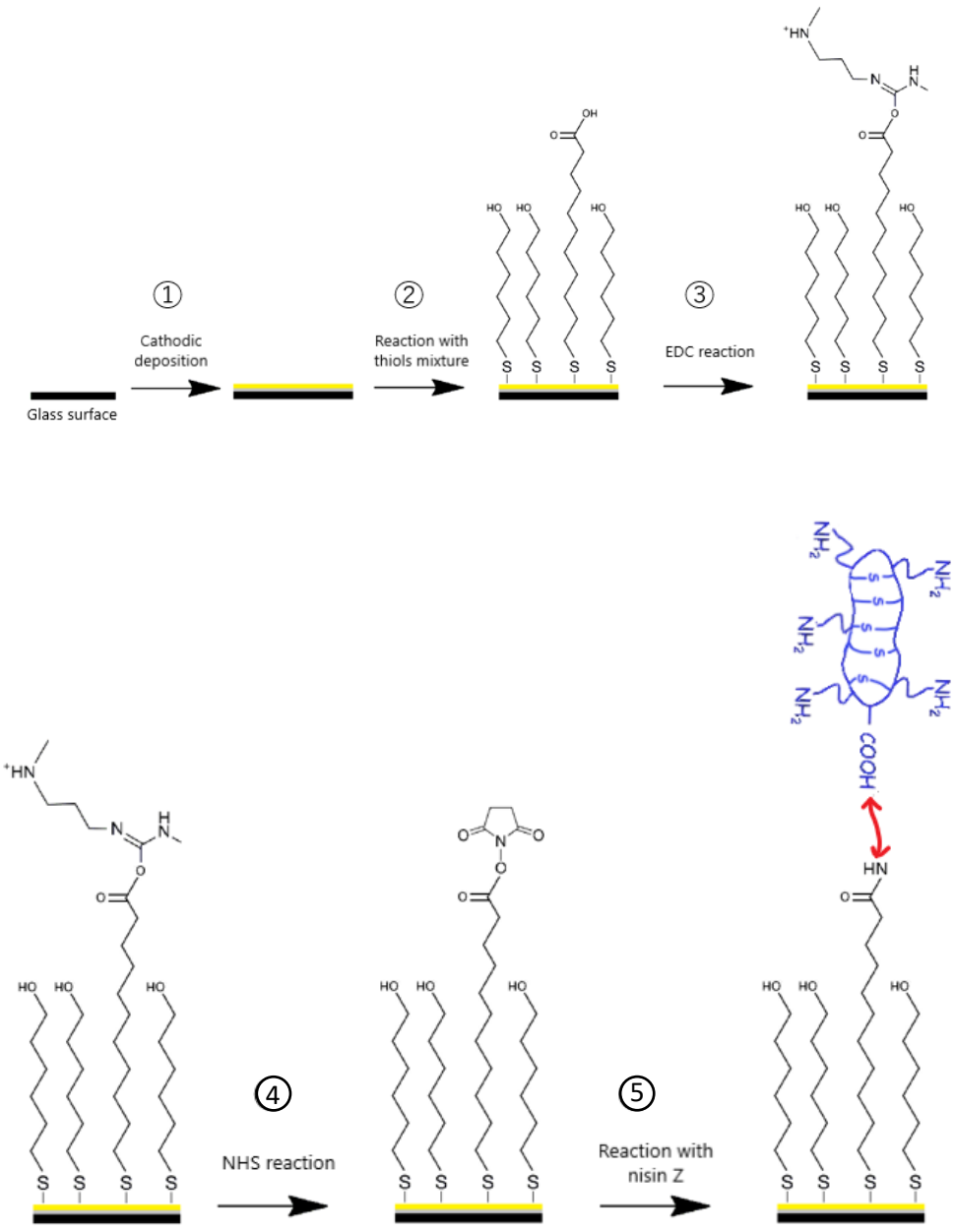

Figure 1. Nisin grafting strategies on COOH thiols self-assemblies.

2. Results and Discussion

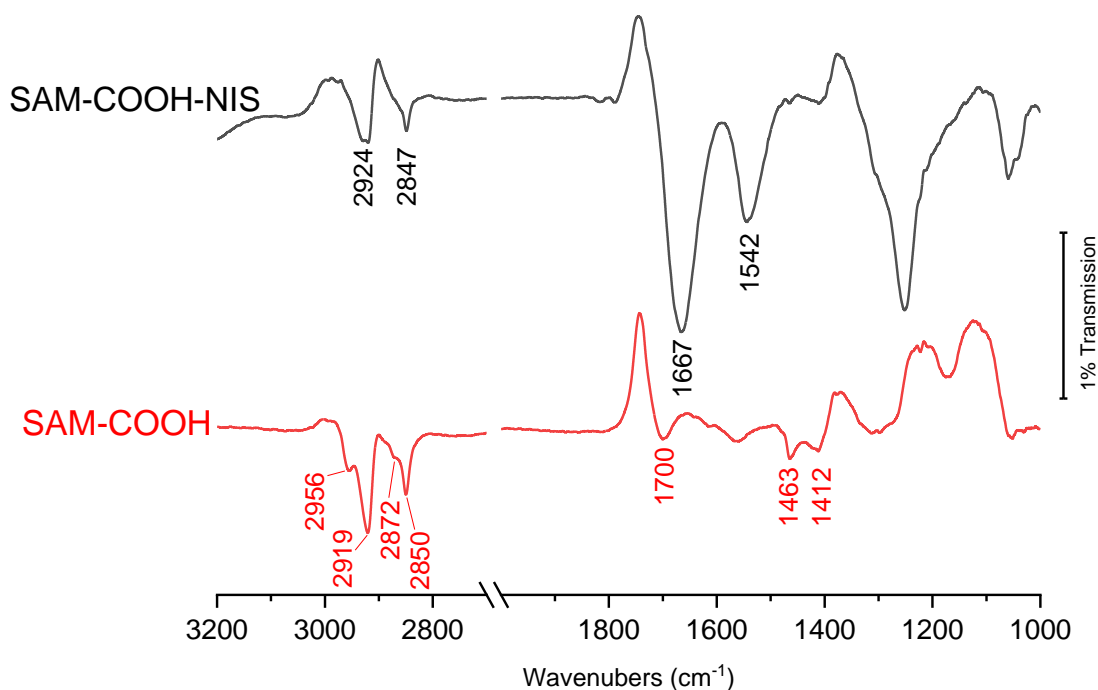### 2.1. Grafting characterizations

Figure 2. FT-IR ATR spectra of Nisin grafted on SAM-COOH thiol.

Figure 2 presents the FT-IR ATR data obtained for COOH thiol self-assembled monolayers (SAM-COOH) and after the grafting of Nisin (SAM-COOH-NIS) on Au surfaces. First the SAM-COOH spectrum exhibits features at ~1700 and in the 1412-1463 cm^{-1} region assigned to $v_{C=O}$ mainly [52], [53], [54], [55], and to several modes of vibrations of CH$_2$ moieties from the backbone of the molecule. Important to note that both spectra exhibit strong signal for sym and asym stretching of CH$_2$ at 2919-2924 and 2850-2847 cm^{-1}. In addition, SAM-COOH spectrum exhibits traces of sym and asym stretching of CH$_3$ due to purity of the thiols around 95%. Nisin was thus grafted on SAM-COOH via an amino group on activated SAM-COOH (Figure 1). On the spectrum at the top of Figure 2, one can see the appearance of Amide I and II bands at 1667 and 1542 cm^{-1}, respectively, showing the successful grafting of Nisin.

In order to confirm this point and to obtain quantitative data on these SAM, XPS analyses were performed on the 2 surfaces, and the data are presented on Figure 3. First, Figure 3(a) shows the S2p high resolution region, where the S2p$_{1/2}$ and S2p$_{3/2}$ doublets can be observed confirming the successful functionalization of both SAM-COOH. After Nisin grafting, these signals are enlarged due to the contribution of 5 disulfuric bridges in the Nisin molecules [30], confirming again the successful grafting of the peptide. Atomic percentages are presented on Table 1. The atomic percentage of S2p does not vary much as the S2p signal coming from the thiol anchoring moieties are attenuated by the Nisin molecule, together with the contribution from the 5 S atoms of the peptide.

When looking at Figure 3 (b), one can see even more clearly the successful grafting of the NIS molecule with the presence of intense broad peak in the 400 eV region, suggesting the presence of protonated and non-protonated amino groups. Again, the Nisin grafting is confirmed when looking at the atomic percentage of Table 1 for the N1s region, with a huge increase of around 800% of the N1s atomic percentage. Finally, the decrease of the Au4f signal also confirm the increase of the organic layer on top of the SAM-COOH, hence the grafting of Nisin.
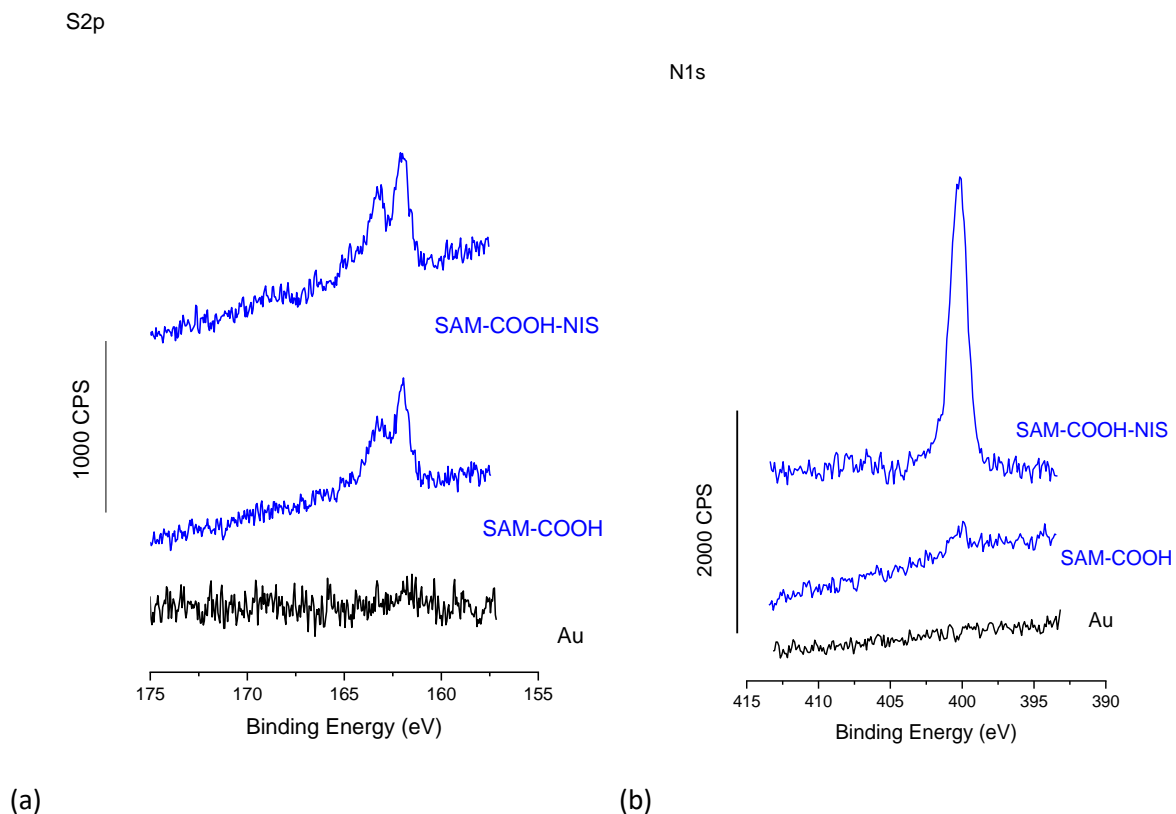

Figure 3. (a) XPS S2p High Resolution (HR) region and (b) N1s HR region for Au, SAM-COOH and SAM-COOH-NIS.

From XPS quantitative data presented on Table 1, it is possible to estimate the equivalent thicknesses of the different layers created on the gold surfaces and the associated surfaces coverage using Equations 1 and 2, described in the Material and Methods section. Thus, for the SAM-COOH, the thiol surface coverage is calculated at 5.6 thiols/nm^2, from data of Table 1 and Equations (1) and (2). This surface coverage is relatively close to the optimal theoretical thiol coverage of 6-8 thiols/nm². When looking at the coverage obtained once Nisin has been grafted on acidic SAM, we obtain an equivalent coverage of 0.55 nis/nm^2. These values are quite common when looking at equivalent mass and size molecules grafted on surfaces, with for instance 1.3-1.9 temporine/nm² on titanium (13 AA for Temp vs 34 AA for Nisin) [56].

Table 1: Atomic percentages obtained by XPS for both SAM-COOH and SAM-COOH-NIS

	C1s	O1s	N1s	S2p	Au4f
SAM-COOH	59.5	11.4	0.9	2.6	25.6
SAM-COOH-NIS	61.7	13.4	8.1	2.4	14.4

2.2. Microbiological tests

6

Several tests were carried out to attest the behavior of our coatings towards several bacterial strains. Bacterial adhesion as well as killing experiments were performed and killing efficiency will be discussed as a function of the reference chosen for these calculations. They were carried out following an indirect route, based on the recovery of adhered bacteria and viable cell culture counting on agar plates. The first test was carried out in order to evaluate the adhesive properties while the second one will enable the calculation of the bactericidal activity of the grafted Nisin coating.

2.2.1. In vitro tests

First tests in solution were performed in order to evaluate the potential activities of Nisin in solution, and Table 2 presents the Minimal Inhibition Concentration (MIC) and Minimal Bactericidal Concentration (MBC) obtained for these 3 bacteria.

Table 2: MIC and MBC of Nisin towards *E. coli*, *S. aureus* and *L. ivanovii* bacteria.

Nisin	*L. ivanovii*	*S. aureus*	*E. coli*
MIC	156 µg/mL	312 µg/mL	> 20 mg/mL
MBC	312 µg/mL	312 µg/mL	> 20 mg/mL

First, one can observe that Nisin has no antibacterial activity or a potential very low one outside of the range of our experimental concentration for determining MIC (> 20mg/mL) towards Gram negative *E. coli* bacteria as expected from data from the literature [57] since the outer membrane (OM) of Gram-negative bacteria acts as a impermeability barrier for the cell and prevents Nisin from reaching the cytoplasmic membrane [42].

Charest [38] studied Nisin activity against 17 genera of Gram-negative bacteria and published range of MIC for each. The different strains were then classified in 4 groups: high sensitivity, moderate sensitivity, low sensitivity and no detected sensitivity. 51 strains of *E. coli* were tested and 50 of them showed a MIC mean value around 171.81 µg/mL, surprisingly low compared to what we obtain [38]. Other Nisin-sensitive genera include *Xanthomonas, Erwinia* and *Helicobacter* that are sensitive to Nisin for mean concentration between 5 and 130 µg/mL. Nisin bactericidal effect against gram-negative is still debated nowadays, especially the mechanism of action against *E. coli*. Then, Nisin cannot been considered as an antimicrobial peptide against *E. coli*. The tendency of Nisin to be not bactericidal towards Gram negative bacteria could be a problem as Nisin is one of the rare AMP to be FDA approved.

Around 84% of the natural AMPs of the antimicrobial peptide database (APD3) have been registered as being antibacterial, having Gram-positive activity, anti-Gram-negative effect or against both of them [22]. Unfortunately, these promising molecules are still in study in trials or *in vitro* investigation to show their effect against Gram-negative bacteria. Only one of the seven peptides approved by FDA has an effect against Gram-negative bacteria, and two are still in investigations [58], [59]. Oncocin and its derivates seem to be efficient against some Gram-negative bacteria such as *E. coli* and *M. luteus*. Depending on the sequence of the peptide used, derived from *Oncopeltus,* 13 peptides have been discovered [60]. For example, peptide 4 is efficient against *E. coli* and *M. luteus* with a MIC about 8 to 128 of µg/mL, peptides 10 and 11 are efficient against both cited and also against *P. aeruginosa*, *K pneumoniae*, *E. cloacae* strains, showing a large broad spectrum of MIC from 0.25 to 8 µg/mL [60].

However, when turning to Gram positive bacteria, Nisin shows a much better antibacterial activity in the range of 156 µg/mL towards *Listeria* and 312 µg/mL for *S. aureus*, that are close values to those that can be found in the literature [33].

Tyrocidin has been found to be efficient against both Gram positive (Sa) but not Gram negative (Ec) bacteria but it was also exhibiting a toxicity for blood human cells [61]. Another small peptide, indolicilin, composed of only 13 amino acids is efficient against both Gram-positive and Gram-negative bacteria [62]. In the work of Falla and al [63], MIC values are relatively low against notably the wild type *E. coli* (4 to 16 µg/mL), the defensin supersensitive *S. typhimurium* (8 to 64µg/mL) and *Staphyloccocus spp* (4 and 8 µg/mL). Two other peptides were also tested and MIC that are shown are also low against the previous cited strains, from 0.25 to 32 µg/mL for Gentamicin and from 0.25 to 64 µg/mL for Polymixin B. King and Phillips [64] have tested vancomycin and penicillin against a large range of Gram-positive strains of *Staphylococci*, including methicillin-susceptible and methicillin-resistant. Daptomycin was efficient against all tested strains, with a MIC between 0.03 to 1mg/mL, depending on the method used.

2.2.2. Surfaces tests

First experiments to be carried out were the influence of the coatings towards bacterial adhesion. In fact, it is known that, depending on the surface charges and some other parameters, such as surface free energy, the bacterial adhesion can be reduced which can be a first effect on the global efficiency of the coatings.

Figure 4 shows the results obtained after 3 hours of incubation of several bacteria (*Listeria ivanovii* (Li), *Staphylococcus aureus* (Sa) and *Escherichia coli* (Ec)) on three different surfaces : bare gold (absolute reference surface), SAM-COOH (functionalized reference surface) and finally SAM-COOH-NIS.
Looking first at Gram positive bacteria Li and Sa, the results are quite different when compared to the bare Au surface. Indeed, for Li a strong decrease of the adherence by one order of magnitude is observed for both functionalized surfaces. Contrary, for Sa the thiol layer increase by 50% the adherence while SAM-COOH-NIS reduce by a factor 3 the bacterial adhesion. These different behaviors could be explained by the intrinsic properties of the bacteria themselves, with *L. ivanovii* being a motile bacterium [65] while *S. aureus* does not have same mobility properties [66].
From another point of view, when looking specifically at the antibacterial coating on the adhesion, the Nisin coating doesn't influence much bacterial adhesion for *L. ivanovii* while it clearly has an influence for *S. aureus* with an adhesion decrease of almost a factor 5 when compared to the SAM-COOH surface. Nevertheless, the adhesion of Li is already drastically reduced compared to bare gold, thus the difference between SAM-COOH and SAM-COOH-NIS even if neglectable, could still show some interest compared to totally bare surface. Finally, in the case of Gram negative bacteria, i.e. *E. coli*, there is no significant differences for all three considered surfaces.
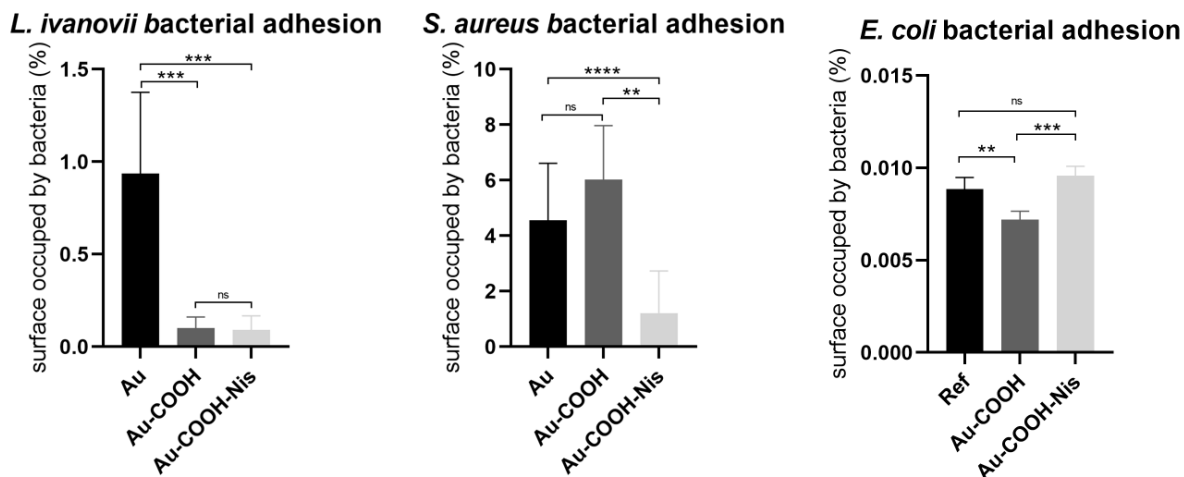
Figure 4. Bacterial adhesion on three surfaces: bare Au, SAM-COOH and SAM-COOH-NIS. Bacteria tested were *Listeria ivanovii* (Li), *Staphylococcus aureus* (Sa) and *Escherichia coli* (Ec). Adhesion incubation were performed at 30°C (Ec) and 37°C (Li and Sa) in PBS at an initial concentration of 10^6 CFU/mL. (ns: non significant, * : $p < 0.05$, **: $p < 0.01$, ***: $p < 0.005$, ****: $p < 0.001$).

Killing by contact experiments were carried out in order to evaluate the bactericidal efficiency of covalently grafted Nisin on surfaces towards the same 3 bacterial strains. Here, it is important to note that incubation was performed in PBS after washing the inoculum by centrifugation to be sure that no growing media was left, and that no increase in CFU due to bacteria proliferation could be possible. Figure 6 presents the data obtained expressed as CFU/mL of revivable adhered bacteria recovered from the different surfaces and counted after overnight incubation on agar gelose (*Cf.* Figure 5), where every single dot represents a bacterial CFU.
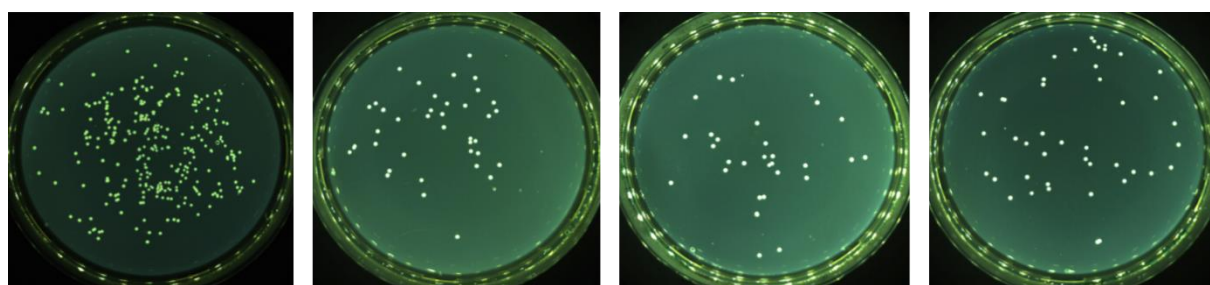

Figure 5. examples of CFU counting on Agar Plate for Sa, from left to right : inoculum, Au, SAM-COOH and SAM-COOH-NIS.

In addition, Table 3 presents the killing efficiency in % with respect to different references following the following formula (Eq. 3):

%Killing = 100 x ((bacteria CFU ref – bacteria CFU SAM-COOH-NIS)/ bacteria CFU ref)   (Eq. 3)

with Ref being: the initial bacterial inoculum, the bare Au surface or the SAM-COOH surface.

9

Indeed, depending on the literature, the field of research or simply the research group the reference used for calculated the killing efficiency is very variable. Therefore, here for treating the raw data (i.e. before any correlation with bacterial adhesion) we have decided to calculate the % of killing with respect to 3 different references: the bacterial inoculum, the raw surface and the functionalized surface at the step just before Nisin grafting. Figure 6 presents the data obtained for revivable bacteria counted for each surface after inoculation of bacterial suspension.
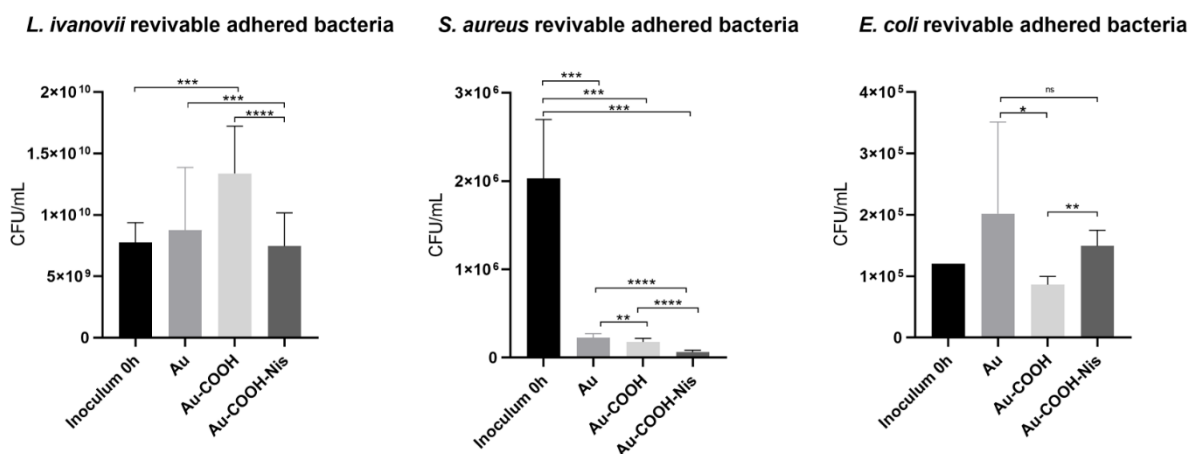

Figure 6. Revivable adhered bacteria recorded on three surfaces: bare Au, SAM-COOH and SAM-COOH-NIS. Bacteria tests were *Listeria ivanovii* (Li), *Staphylococcus aureus* (Sa) and *Escherichia coli* (Ec). Adhesion incubation were performed at 30°C (Ec) and 37°C (Li and Sa) in PBS; initial CFU/mL concentration are given as inoculum value for each separate experiment. (ns: non significant, * : $p < 0.05$, **: $p < 0.01$, ***: $p < 0.005$, ****: $p < 0.001$).

Table 3: Killing efficiency of Nisin towards 3 bacterial strain. % are calculated using Equation 3.

	% killing/inoculum	%killing/ref. surface	% killing/SAM-COOH
Listeria ivanovii	1.2	41.9	48.0
Staphylococcus aureus	96.8	72.2	64.4
Escherichia coli	<0	23.2	<0

First when looking at data from Table 3 and Figure 6, it is striking to see that depending on the reference used the result for the killing efficiency is very different, and with no logical scheme when turning from one bacterium to another bacterial strain.

When looking first at the functionalized surface at the step just before Nisin, i.e. % killing/SAM-COOH, it seems to be the best reference to attest the efficiency of the grafted Nisin peptide. As expected from the literature, Nisin seems to have no effect towards Gram negative *E. coli*, with a negative killing. On contrary for Gram positive strains, a moderate killing is obtained with around 48% and 64 % for respectively *L. ivanovii* and *S. aureus*. The difference is not significant and follows what was found in solution for the MIC/MBC experiments (Table 2), with similar values. In addition, previous studies have shown that other AMPS (namely Magainine I and Gramicidine) grafted on gold COOH SAMs exhibit killing efficiency similar for both *L. ivanovii* and *S. aureus* with respectively, 70 and 80 % for Magainine [49] and 70% and 65 % for Gramicidine [67].

When looking at the bare initial surface used as a reference, the killing efficiencies observed are within the same tendency, with a higher activity towards *S. aureus* than *L. ivanovii* with still a better activity for coqui than for bacillus. Surprisingly, when using the bare surface as a reference, Nisin

coating exhibits a very small activity towards *E. coli*, going thus against the *in vitro* MIC and MBC experiments.

Finally, using the inoculum as a reference for calculating the killing efficiency of grafted Nisin, the results are totally different with almost no activity towards *Listeria* (1.2 %) a much higher one for *S. aureus* (~97%) and still a negative activity for *E. coli*.

It is thus quite difficult to make final choice on how to present the killing efficiency data, as many debates are present in the literature and no one is using the same reference. However, an international normae was published stating that in the presence of an untreated control surface, if the killing efficiency between inoculum and untreated surface differs by more than 15%, the latest case should be preferred [68]. In our case, clearly the 15% difference is reached, therefore for the rest of our analysis, only the untreated surface will be used for calculating any killing efficiency.

Finally, another way of combining all the data obtained previously could be to make a correction of the adhered revivable bacteria by the adhesion factor calculated from Figure 4 (adhesion factor obtained by CFU normalization against Au surface. Corrected CFU are raw CFU x Adhesion factor). In fact, if a surface shows little adherence, the overall bactericidal efficacy will be increase with respect to the surface colonization by bacteria, thus Table 4 shows the corrected values for killing. The tendency is still the same with the Nisin coating being more efficient towards *S. aureus* than *L. ivanovii*. However due to low adhesion of Gram-positive bacteria (specially for *S. aureus*) on Nisin coating the killing values are increased to reach up to 66 % killing for Li and 92% for Sa.

Using a corrective factor linked to the bacterial adhesion enables one to obtain a general view of the potential efficacy of Nisin coating to reduce surface colonization and hence biofilm formation.

Table 4: killing efficiency of Nisin coated surfaces calculated using the non-coated surface as a reference and corrected or not by the adhesion factor.

	% killing/SAM-COOH	% killing/SAM-COOH corrected by adhesion
Listeria ivanovii	48.0	66.4
Staphylococcus aureus	64.4	92.6
Escherichia coli	<0	<0

3. Materials and Methods

3.1. Grafting strategies

Chemical and surface preparation

Nisin (Ile-Dhb-Ala-Ile-Dha-Leu-Ala-Aba-Pro-Gly-Ala-Lys-Aba-Gly-Ala-Leu-Met-Gly-Ala-Asn-Met-Lys-Aba-Ala-Aba-Ala-His-Ala-Ser-Ile-His-Val-Dha-Lys), 6-mercaptohexanol (C_6OH), 11-mercaptoundecanoïc acid (MUA), 1-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS), were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France). Reagents were used without any further purification and experiments were carried out at room temperature if not specified otherwise. The purity of Nisin is not given, no attempt to purify it was performed, despite the fact that it is more likely a mixture of Nisin A and Nisin Z.

The surfaces were constituted of glass substrates (11 mm X 11 mm), modified by cathodic deposition with Plassys MP 700 at Mimento Renatech Technology Center clean room. Glass substrates were first activated by argon plasma then coated successively with a 5 nm thick layer of chromium and a 200 nm thick layer of gold. Gold surfaces were then cleaned in a bath of absolute ethanol for 5 min before adsorption of thiols.

Cleaned gold surfaces were immersed overnight at room temperature in a binary mixture at 1 mM (25/75) of MUA (0.25 mM) and C_6OH (0.75 mM) in absolute ethanol, under magnetic stirring, in order to insure an optimal homogeneity of the thiols layer. Surfaces were then rinsed in ethanol and MilliQ water baths for 5 min and dried under a flow of dry nitrogen.

The substrates were treated with a solution of NHS (200 mM) and EDC (50 mM) in ultrapure water for 60 min, rinsed in MilliQ water and dried under a flow of dry nitrogen.

Immobilization of Nisin (NIS, 50 mg/L in PBS) on gold surfaces was carried out by depositing a 150 µL drop of NIS/PBS solution on the Au-modified substrates at room temperature for 2 h. After the immobilization step, the surfaces were vigorously rinsed in PBS then MilliQ water with agitation, dried under a flow of dry nitrogen and stocked at 4°C.

For each step of functionalization, one serie of samples was characterized by Fourier-transform infrared spectroscopy (FT-IR) and by X-ray photoelectron spectroscopy (XPS).

3.2. Characterization techniques

3.2.1. ATR-FTIR
The gold samples were placed in the external beam of Diamond ATR FT-IR instrument (Perkin Elmer Spectrum Two) and the reflected light was focused on a DTGS (Deuterated TriGlycine Sulfate) wide band detector. Spectra were acquired at 8 cm^{-1} resolution with 1 minute of co-addition (around 60 spectra), thus ratioed towards a background collected in air. Baseline correction was applied with no further correction. Spectra were plotted as % transmission.

3.2.2. XPS analyses
XPS analyses were performed using an Omicron Argus X-ray photoelectron spectrometer, equipped with a monochromated AlKα radiation source (hv = 1486.6 eV), and a 150 W electron beam power. The emission of photoelectrons from the sample was analyzed at a takeoff angle of 45° for Omicron Argus X-ray under ultra-high vacuum conditions ($\leq 10^{-9}$ mbar). Spectra were carried out with 100 eV pass energy for the survey scan and 20 eV pass energy for the C1s, O1s, N1s, S2p and Au4f regions. Binding energies were calibrated against the Au4f binding energy at 84.0 eV and element peak

intensities were corrected by Scofield factors [69], 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.

Equivalent thickness (*d*) and surface density (*n*) were calculated using the following equations, respectively:

$$\frac{I_{S2p}}{I_{Au4f}} = \frac{\rho_{SAM+Nis}M_{Au}\sigma_{S2p}T_{S2p}\lambda_{S2p}^{SAM+Nis}(1-\exp(\frac{-d}{\lambda_{S2p}^{SAM+NIS}\sin(\theta)}))}{\rho_{Au}M_{COOH+NIS}\sigma_{Au4f}T_{Au4f}\lambda_{Au4f}^{Au4f}(\frac{-d}{\lambda_{Au4f}^{SAM+NIS}\sin(\theta)})} \qquad \text{(Eq. 1)}$$

where θ is the photoelectron collection angle. T_{Au4f} and T_{S2p} are the relative sensitivity factors of Au and S, respectively, provided by the spectrometer manufacturer. The Scofield photoionization cross-sections σ are equal to 14.4 for Au4f and 1.44 for S2p. λ_x^y is the inelastic mean free paths of electrons x in the matrix y. They were calculated using the Quases program (QUASES-IMFP-TPP2M Ver.3.0) based on the TPP2M formula. ρ_{SAM}, $\rho_{SAM+Nis}$ and ρ_{Au} are the densities of SAM, SAM+Nis and Au, respectively. M_{SAM}, $M_{SAM+Nis}$ and M_{Au} are the molecular weight of SAM or SAM+Nis and Au, respectively, and finally I_x is the raw intensity of element x.

$$n_{Nis}(nis.nm^{-2}) = \frac{d_{Nis}\rho_{Nis}N_A}{M_{Nis}} \quad \text{(Eq. 2)}$$

where d_{Nis} is the Nisin equivalent thickness, ρ_{Nis} is the density of Nisin, M_{Nis} is the molecular weight of Nisin an N_A is the Avogadro number.

3.3. Microbiological tests

3.3.1. Surface activity tests

Bacteria strains, media and culture conditions

The bacterial strains used in this work are *Listeria ivanovii* Li4pVS2*, Escherichia coli* ATCC25922 and S*taphylococcus aureus* ATCC25923. All strains were stocked at −80 °C in glycerol aliquot. The inoculum was prepared by first growing a solid culture on biological agar (15 mg/L) + LB (20 mg/L) for *E. coli*, biological agar (15 mg/L) + BHI (20 mg/L) for *L. ivanovii*, biological agar (15 mg/L) + MH (20 mg/L) for *S. aureus.* Petri dishes incubated overnight at 30 °C for *E. coli* and 37°C for *L. ivanovii* and *S. aureus*. Thus, liquid cultures were carried out by recovering 1 colony from the solid growth Petri dish and inoculated in 10 mL of associated media at 20, 15 and 20 mg/L for BHI, LB and MH and cultured overnight at 30 °C (*E. coli)* or 37°C (*L. ivanovii, S. aureus)* under 90 rpm agitation.

3.3.2. Contact Killing

Exponentially growing bacteria in media were harvested by centrifugation (5000g, 5 min, 25 °C), washed twice with PBS, and suspended in PBS to obtain a concentration of 10^9 CFU/mL. 3 solutions were prepared for each strain by dilution at 10^8 CFU/mL, 10^7 CFU/mL, 10^6 CFU/mL in PBS. 20 µL of the bacterial suspensions was spread manually onto 89 mm Petri dishes filled with agar + suitable media (LB for *E. coli*, MH for *S. aureus*, BHI for *L. ivanovii).* Thus, functionalized gold slides were deposited face-down on the freshly incubated Petri dishes by avoiding the creation of air bubbles. After overnight incubation at 30°C (*E. coli*) and 37°C (*S. aureus* and *L. ivanovii*), dishes pictures were recorded using an Interscience colony counter Scan 300.

3.3.3. Minimal Inhibition Concentration (MIC)

MIC values toward *bacteria* were determined using the 2-fold dilution method. Experiments were performed in 96-well microplates in triplicate in culture media (LB for *E. coli*, MH for *S. aureus* and BHI for *L. ivanovii*), with an initial bacterial concentration of approximately 10^6 CFU/mL. The highest Nisin concentrations were prepared 80 mg/mL resulting in concentrations in the first well of 20 mg/mL, respectively. After overnight incubation at 30 °C and 37°C (respectively for Ec, and Sa, Li), MIC values were determined as the lowest concentration of the compound with no visible bacterial growth. Sterility control (culture broth only), growth control (culture broth with bacteria), and death control (culture broth with bacteria and ethanol: H_2O v/v 70/30) assessed the quality of each experiment.

3.3.4. Adhesion of bacteria on gold samples

Gold samples were washed successively in 70% ethanol and sterile water then dried in sterile environment. Surfaces were then immersed in bacteria suspension at 10^7 CFU/mL in PBS, in a 12-wells plate and incubated 3h at 30°C (*E. coli*) and 37°C (*S. aureus* and *L. ivanovii*) under 90 rpm agitation. Following incubation, samples were washed 3 times 5 min with sterile PBS and then dried under sterile environment.

3.3.5. Optical microscopy

Bacterial adhesion was measured by analyzing the plates that had undergone the contact tests. After incubation with the bacterial solutions, each sample was washed three times with milli-Q water, then immersed in a homemade 0.5% crystal violet solution in ethanol for 10 minutes. After staining, the surfaces were rinsed with milli-Q water until the staining disappeared from the well water and then the surfaces were dried. Each surface was observed under a Nikon ECLIPSE LV100ND optical microscope at magnifications of x50 and x100. 10 photos were taken for each magnification and each sample. The analysis was then carried out using ImageJ software.

3.3.6. Statistical analysis

Graphs and statistical analyses were obtained using GraphPad Prism 8.3.0 software (GraphPad Software Inc.). Mann-Whitney tests were performed to study the significance between conditions directly from raw data, all performed tests are nonparametric tests, unpaired, comparing ranks. The latter was considered statistical when the p-factor $p < 0.05$.

4. Perspectives and future work

Based on the results obtained with the coating of Nisin on gold surfaces via an acidic thiol self-assembled monolayer, few points of improvements could be explored.

It appears clearly that the purity of the antimicrobial peptide is of extreme importance with regards to the antibacterial activities of free peptides or immobilized peptides (physically or covalently) [34]. Therefore, some on-going work is performed in our research group to purify Nisin using High Performance Liquid Chromatography (HPLC) or Cation Exchange chromatography (IEC). In the same idea, optimization of nisin production (followed by high purification) can also help to increase to bacterial activity of home-made produced Nisin, using for instance a specific strain of L. lactis, less commonly used in industry [70].

Finally, orientation of the AMP with respect to the surface could also be crucial for bactericidal activities. Indeed, using the classical surface chemistry of acidic thiols for grafting of AMPs on surface is the purpose of this work. However, as Nisin possesses 5 possible amino anchoring groups, the orientation of the peptide will be random and heterogenous through the whole surface. On the other side, using amino thiol could solve this problem as Nisin possesses only one COOH group in its lateral

position, hence by using the reverse route (activation of the COOH of the Nisin and grafting directly on the amino SAM) will end up with the same orientation of the peptide on the overall surface. We have shown previously that this orientation can strongly influence the bactericidal activity of grafted AMPs [56], [71].

5. Conclusion

In this paper, we have successfully covalently grafted Nisin, an antimicrobial peptide of the bacteriocin family, on a mix acidic thiol Self-Assembled-Monolayer, as confirmed by FT-IR and XPS spectroscopies. In addition, XPS data have enabled average surface coverage of Nisin to be calculated to around 0.6 peptide/nm²; this value is within the average coverage usually observed considering the molecular weight and hence the steric hindrance of the AMP Nisin.
In vitro antimicrobial tests have shown that Nisin in solution has no activity towards Gram negative *E. coli* bacteria as expected from the literature, while it exhibits middle range activities towards Gram positive bacteria with MIC measured at 156 µg/mL and 312 µg/mL, for *L. ivanovii* and *S. aureus*, respectively. Antimicrobial activities were also tested on Nisin grafted gold surfaces, looking at 2 parameters: bacterial adhesion and bactericidal efficiency.

Looking first at adhesion, Nisin coatings have no effect on *L. ivanovii*, while the bacterial adhesion has decreased by 73% for *S. aureus*. In the case of *E. coli*, the AMP coating shows as small increase of bacterial adhesion.

Antibacterial activity was then measured by numbering the revivable adhered bacteria on the three kind of surfaces, the Au reference, the SAM-COOH and finally SAM-COOH-NIS. Percentages of killing were calculated by using different references according to several literature methods: the initial bacterial inoculum, the bare surface or the non-coated SAM-COOH. Surprisingly enough, the % killing calculated were very different depending on the reference used. For instance, for *S. aureus*, this value goes down from 96.8, 72.2 to 64.4 %, respectively. While for *L. ivanovii*, the tendency is reversed with % killing going up from 1.2, 41.9 to 48.0 %. The debate is not often discussed in the literature and all three references are used depending on the authors with sometime very different results from one study to another one. In our case, we have chosen to adopt the non-coated surface SAM-COOH as a reference, as recommended by ASTM normae. Using this reference, Nisin coating is more bactericidal towards *S. aureus* than *L. ivanovii*, and shows no efficiency towards *E. coli* Finally, a correction of the efficiency was carried out by introducing the adhesion factor in the calculation of the killing showing final efficiencies of the Nisin coatings of around 66 and 93%, respectively for *L. ivanovii* and *S. aureus*. Considering the mild activity measured *in vitro* (MIC determination) the overall surface efficiency of Nisin towards Gram positive bacteria can be considered as satisfactory. The results also suggest that Nisin coating can have a better activity towards non-motile Gram positive bacteria with a very low adhesion factor observed for *S. aureus* together with almost 1 log reduction of bacterial viability.

V.H..; project administration, V.H. funding acquisition, T.L and V. H. All authors have read and agreed to the published version of the manuscript."

Conflicts of Interest: Declare conflicts of interest or state "The authors declare no conflicts of interest."

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