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Design of an antibacterial gelatin based on a covalent protein-protein coupling

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ABSTRACT: An antibacterial peptide (AMP), i.e., nisin, was covalently bound to gelatin through a protein–protein coupling. Various reaction conditions were tested to study and optimize parameters of grafting e.g., orientation and density of AMP, which could impact the final antibacterial activity of the modified biopolymer. Modification was investigated by Fourier transform infrared (FT-IR) spectroscopy and zeta potential. The antibacterial activity of the nisin-enriched gelatin was evaluated against two *staphylococci* bacterial strains, i.e., *Staphylococus epidermidis* and *Staphylococcus aureus*. A higher activity was found for gelatin modified at pH = 7.4 revealing an influence of the nisin orientation on the protein antibacterial property. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 41825.

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INTRODUCTION

Gelatin is a natural protein obtained by partial hydrolysis of collagen from bones and skins of animals and fish.[1] It is biodegradable, biocompatible and abundantly available with low cost.[2,3] Thanks to these characteristics, gelatin is used in numerous fields such in food and pharmaceutical industries as emulsifier, foaming agent or edible packaging or in medicine as wound dressing.[1,4]

For most of these applications, bacterial infections, in particular biofilms infections are very problematic. Thus, bacterial contaminations can slow down wound healing, leading to a long-term hospitalization of patients.[5] They can also reduce the shelf life of food and cause illness in food industry.[6] To limit these bacterial surface contaminations, antimicrobial agents, e.g., silver, chitosan, or nitrogen oxides are usually incorporated or mixed with gelatin to elaborate antimicrobial packaging or wound dressing for example.[7–9] Due to the non-covalent immobilization of the antimicrobial agents, these works led to the release of the compounds and to a possible loss of activity in time or an unsatisfactory activity. Furthermore, in some applications such wound dressing, a release in human body of some potential toxic products should be avoided. Thus, recently, Jiang *et al.* have covalently immobilized on gelatin backbone quaternary ammonium salt.[10] The modified gelatin exhibited antibacterial activity with minimum inhibitory concentration (MIC) of 80 μg/mL against *S. aureus* and 160 μg/mL against *Escherichia coli*. However, quaternary ammoniums which have a potential toxicity are not recommended in some applications and the emergence of multi-resisting bacteria toward this type of compounds begins to limit their used.

To overcome all these drawbacks, AMPs are very promising and present several advantages as antimicrobials: a broad-spectrum activity, a rapid onset of killing and a low level of induced resistance (compared with classical antimicrobial agents such antibiotics or quaternary ammonium). However, their susceptibility to proteolysis, their pH sensitivity and the cost of synthesis constitute some disadvantages. Numerous methods based on physical or chemical immobilization of AMPs, were explored to tether AMPs on solid surfaces in order to elaborate antibacterial coatings.[11–16] Recently, we elaborated an antimicrobial hyaluronic acid (HA) by covalently attach the nisin, which is widely used in food industry.[17–19] Nisin has also been incorporated or mixed with gelatin to add antimicrobial properties to the protein for active packaging.[20,21] However, such non-covalent approach could lead to a decrease (or a loss) of activity in time, as mentioned above, or to a low activity due to interaction between nisin and gelatin which induces a decrease of the AMP diffusion.

The aim of the present work was to obtain an antimicrobial gelatin, usable in solution or as gel, for wound dressing for

example, and exhibiting a long-term activity without AMP release by using an original protein–protein coupling. Mobility, density and orientation of the covalent immobilized AMPs being important for their antibacterial activity,[22] we investigated different experimental conditions, to optimize the peptide grafting and so the antibacterial activity of the new biopolymer. The physicochemical properties of the modified protein were investigated by Fourier transform infrared spectroscopy and zeta potential and its antibacterial activity tested against *Staphylococcus epidermidis* and *Staphylococcus aureus*, two bacterial species involved in biofilm infections.[23]

EXPERIMENTAL

Chemical Materials

Gelatin was provided by the Maia Woundcare Society (Cergy Pontoise, France). The protein was extracted from beef bones by a basic process (pI = 4.7) and presented a bloom of 250 and a molecular weight (M_w) of 1,00,000 g/mol. N-hydroxysuccinimide (NHS), 1-(3-dimethylaminopropyl)-N′-ethyl-carbodiimide hydrochloride (EDC), phosphate buffered saline (PBS) tablet, 4-morpholineethanesulfonic acid (MES), potassium hydroxide (KOH), hydrochloric acid (HCl) were purchased from Sigma Aldrich (Saint Quentin Fallavier, France). Nisin Z (3331 g/mol, purity 90%), was purchased from Anhui Mimetals Development (Hefei Anhui, China). Milli-Q water (resistivity higher than 18.2 MΩ.cm) was obtained from a Milli-Q Integral 10 system.

Synthesis of Modified Gelatin

Fifty milligram of gelatin (corresponding to a final concentration of 1 mg/mL) was solubilized in 40 mL of PBS solution (pH = 7.4) or MES solution (pH = 4.5), at 35°C during 30 min. Different volumes (0.5, 1 or 5 mL) of a nisin solution, previously prepared at 17 mg/mL in 0.02*M* hydrochloric solution (HCl), were added to the protein solution in the aim to obtain various final peptide concentrations (i.e., 0.05, 0.1 and 0.5 eq for one carboxylic acid group of gelatin, respectively). Then, 500 µL of each solution of coupling agents, i.e., EDC and NHS, were successively added to the solution under magnetic stirring (i.e., an EDC/NHS molar ratio of 1/1 or 4/1 for 1 nisin eq). The pH of the reaction was adjusted to pH = 7.4 or 4.5 with KOH (0.1*M*) and the volume of the reaction was adjusted to 50 mL with PBS or MES solution, according to the pH. The reaction was maintained for 3 h at 35°C under magnetic stirring. The resulting mixture was sealed in a semi-permeable membrane bag (molecular weight cut-off of 25 kDa) and dialyzed for 3.5 days in a large amount of Milli-Q water. The resultant solution was lyophilized.

Synthesized products are noted G-$N_{0.05}$, G-$N_{0.1}$ and G-$N_{0.5}$, corresponding respectively to 0.05, 0.1, and 0.5 eq of nisin used in the reaction relative to one carboxylic acid of gelatin.

FT-IR Spectroscopy Measurements

The FT-IR spectroscopy analyses were performed using a Thermo Scientific Nicolet iS50 spectrometer equipped with a grazing angle attenuated total reflection (GA-ATR) accessory (VariGATR from Harrick Scientific, Pleasantville, NY). FT-IR spectra were recorded at a resolution of 4 cm^{-1} in the frequency range from 600 to 4000 cm^{-1}. The angle of incidence was fixed at 60°. The background was recorded on the GA-ATR unit without any substrate pressed against the crystal.

Zeta-Potential Measurements

Each sample (0.5 mg/mL) was dissolved in Milli-Q water at 35°C during 30 min. The pH was then adjusted at 5.5 with KOH (0.1*M*) and zeta-potential values were measured with a Zetasizer Nano-ZS (Malvern Instruments, Malvern) at 25°C. Each sample was measured three times and means values were reported. Each test was performed in triplicate.

Bacterial Strains and Cultural Conditions

S. epidermidis (ATCC 35984) and *S. aureus* (ATCC 29213) strains were used. All materials were previously autoclaved at 121°C for 15 min. Bacteria were maintained at −20°C in 30% glycerol. Bacteria were precultured under aerobic conditions in BHI (Brain Heart Infusion) broth (Difco, Becton Dickinson France, Le Pont de Claix, France), for 24 h at 37°C on a shaker. Cells were harvested by centrifugation (1500*g* for 15 min) and finally resuspended in sterilized PBS solution. The Optical Density (O.D) of the suspension was then measured at 595 nm. If necessary, additional PBS solution was added to the suspension to adjust the $O.D_{595}$ to 1 corresponding to about 1×10^9 colony forming units (CFU)/mL. Enumerations of bacteria were conducted on BHI agar plates, after incubation for 24 h at 37°C.

Agar Well Diffusion Assay

Wells of 1 cm^2 were aseptically punched in BHI agar plates previously inoculated with 350 µL of a bacterial inoculum containing approximately 1×10^9 CFU/mL, to obtain a final concentration of bacteria in the agar plates of 1×10^7 CFU/mL.[24,25] For the assay, wells were filled with 200 µL of G-N solutions at 2 mg/mL. Tests with unmodified gelatin at 2 mg/mL and nisin solutions (concentration of 0.17, 0.34 and 1.7 mg/mL) were used as controls. After cooling for 2 h at 5°C to allow pre-diffusion of nisin, plates were incubated at 37°C for 24 h. For each well, the inhibition diameter (mm) was then measured. Each test was performed in triplicate.

Antimicrobial Activity Assay of G-N

G-N and gelatin were solubilized, without sterilization, at 2 mg/mL in 3 mL of sterilized PBS for 30 min at 35°C. Then, 15 µL of the bacterial inoculum (concentration, 1×10^9 CFU/mL) were introduced in the protein solutions to reach about 1×10^7 CFU/mL. After 24 h of incubation at 37°C on a shaker, a ten fold serial dilutions was realized and the appropriate dilutions were spread on petri dishes. After incubation for 24 h at 37°C, culturable bacteria were enumerated and expressed as CFU/mL. All the tests were performed in triplicate. The antibacterial activity of G-N was calculated using the following equation:

$$\text{Antibacterial activity} = [(\text{CFU/mL}_G - \text{CFU/mL}_{G\text{-}N})/(\text{CFU/mL}_G)] \times 100$$

RESULTS AND DISCUSSION

Synthesis

Gelatin modification was adapted from previous works on HA which used reaction between amino functions of nisin and

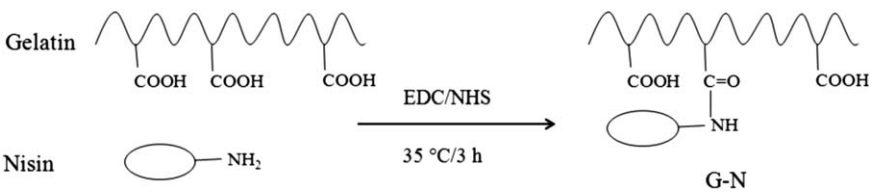

Figure 1. Scheme of gelatin modification.

carboxylic acid groups of the polysaccharide.[17] Compared with HA modification which was realized at room temperature, reaction was here heated at 35°C to allow gelatin solubilization, and limited to 3 h to avoid protein degradation.[26] Furthermore, to limit gelatin crosslinking, nisin was added before carbodiimides as shown in Figure 1.

We then optimized the nisin grafting in the aim to obtain the best gelatin antimicrobial activity. To this aim, three experimental parameters were investigated: the nisin concentration, the reaction buffer pH and the EDC/NHS molar ratio (Table I).

Three nisin concentrations, i.e., 0.05 eq, 0.1 eq, and 0.5 eq for one carboxylic acid group of gelatin, were used, for a synthesis performed at pH 7.4 or 4.5. The choice of pH = 7.4, was made to prevent undesirable modification of the ε-amine groups of lysine residues, i.e., residues 12, 22, and 34. Indeed, it has been shown that any modification of these residues is detrimental to the peptide antibacterial activity.[27] Furthermore, a reaction pH of 4.5 was also tested, because (i) carbodiimides chemistry is well known to exhibit an optimal pH range between 4.5 and 6 and (ii) nisin solubility is greater at weak acidic pH.[28–30] The last studied parameter was the EDC/NHS molar ratio. Indeed, even if the carbodiimides reaction is well documented, EDC/NHS amount seems to be often dependent of the molecules involved in the reaction and so might here altered gelatin crosslinking.[29,31–35]

Characterizations

Compared with that of gelatin, all G-N FT-IR spectra did not exhibit new band due to the structural similarity of gelatin and nisin, with two major bands around 1650 and 1550 cm^{-1}, corresponding to amide I and amide II bands (not shown). However, when the region between 1700 and 1450 cm^{-1} was magnified, some changes appeared, particularly on amide II band for G-$N_{0.05}$, G-$N_{0.1-1}$ and G-$N_{0.5-1}$ (Figure 2). Indeed, when gelatin exhibited only one wide band centered at 1550 cm^{-1}, G-N spectra had two symmetric bands at 1558 and 1540 cm^{-1}. This modification might reflect a slight crosslinking of gelatin, inducing a change of the protein secondary structure as suggested by the FT-IR analyses of gelatin modified by EDC/NHS but without nisin which exhibited two bands with similar intensity whatever the EDC/NHS conditions (spectrum not shown).[36–38] However, as observed in Figure 2, the ratio between the two bands depends on the nisin concentration. Indeed, an increase of nisin concentration led to an increase of the band at 1540 cm^{-1} (corresponding to the amide II band of nisin) compared with that at 1558 cm^{-1}.[39] These data prove that nisin had been coupled with gelatin. Identical spectra were obtained when pH or EDC/NHS molar ratio were changed (results not shown).

The entire modified gelatin showed an increased of zeta-potential values compared with unmodified gelatin as shown in Table II. This observation reflects a reduction in the net negative charge on modified gelatin which can be explained by the reaction between amino function of nisin and carboxylate groups of gelatin, leading to a decrease of anionic groups. However, nisin molecules containing one carboxylic acid and four amino functions, the increase of modified gelatin zeta-potential values might be due to residual free amino functions of grafted nisin. For G-$N_{0.05}$, G-$N_{0.1-1}$ and G-$N_{0.5-1}$, zeta-potential values were −23.9, −20.4, and −16.4 mV, respectively. The increase of the nisin amount during the synthesis led to an increase of zeta-potential values confirming FT-IR

Table I. The Different Gelatins Synthesized and the Reaction Conditions Used

Sample	Nisin concentration[a] (eq)	EDC/NHS concentration[b] (eq/eq ratio)	Reaction buffer pH (buffer type)
G-$N_{0.05}$	0.05	1	7.4 (PBS)
G-$N_{0.1-1}$	0.1	1	7.4 (PBS)
G-$N_{0.5-1}$	0.5	1	7.4 (PBS)
G-$N_{0.1-2}$	0.1	1	4.5 (MES)
G-$N_{0.1-3}$	0.1	4	7.4 (PBS)
G-$N_{0.1-4}$	0.1	4	4.5 (MES)
G-$N_{0.5-2}$	0.5	1	4.5 (MES)

[a] For one carboxylic acid group of gelatin.
[b] For 1 eq of nisin.

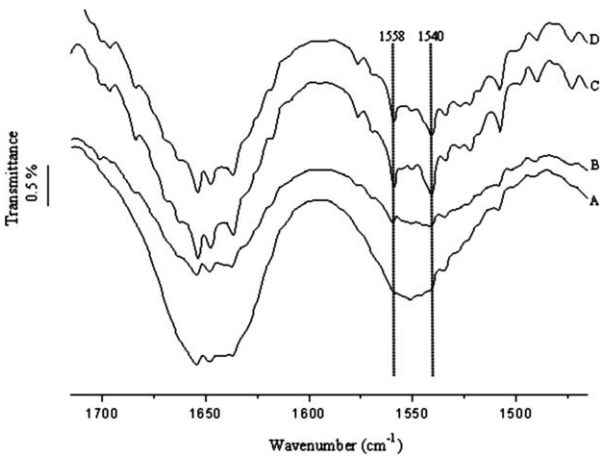

Figure 2. FT-IR spectra of the region between 1700 and 1450 cm^{-1} of gelatin (A), G-$N_{0.05}$ (B), G-$N_{0.1-1}$ (C) and G-$N_{0.5-1}$ (D).

Table II. Zeta-Potential Values of Unmodified and Modified Gelatins

Sample	Gelatin	G-$N_{0.05}$	G-$N_{0.1-1}$	G-$N_{0.5-1}$	G-$N_{0.1-2}$	G-$N_{0.1-3}$	G-$N_{0.1-4}$	G-$N_{0.5-2}$
Zeta-potential value (−mV)	26.5 ± 0.7	23.9 ± 0.6	20.4 ± 0.4	16.4 ± 0.6	19.4 ± 0.8	19.6 ± 0.7	17.5 ± 0.4	15.0 ± 0.7

results. For the same peptide concentration, an increase of the global charge was observed for gelatin modified at pH = 4.5 as compared with that at pH = 7.4. For example, zeta-potential value was −20.4 mV for G-$N_{0.1-1}$ (gelatin modified at pH = 7.4) and −19.4 mV for G-$N_{0.1-2}$ (gelatin modified at pH = 4.5), probably because a better activation reaction leads to a higher amount of grafted nisin. In the case of G-N synthesized with various EDC/NHS molar ratios, an increase of the global charge was found for compounds with a molar ratio of 4. This could be due to a higher number of grafted nisin, a consequence of a greater amount of activated carboxylic acid groups of gelatin when the EDC quantity increases. Furthermore, zeta-potential values of gelatin, modified by EDC/NHS and without nisin, showed no significant variation (around 0.2) compared with unmodified gelatin, whatever the EDC/NHS amount (results not shown), suggesting no N-acylurea formation, an irreversible and stable compound, probably due to the used of NHS.

To confirm the covalent grafting of nisin on gelatin and to confirm the purification method, agar well diffusion assays were performed using *S. epidermidis* as target bacterial species.

As shown in Figure 3, no inhibition zone was observed around the negative control well, which contained gelatin [Figure 3(A)], confirming the absence of an antimicrobial activity of the protein. In contrast, for positive controls, i.e., wells containing nisin solutions at the concentration of 0.17, 0.34 and 1.7 mg/mL, inhibition zones of 22, 23, and 26 mm diameter respectively were measured, demonstrating that the peptide easily diffused [Figure 3(B)]. No inhibition zone was observed for all G-N solutions as shown for G-$N_{0.1-1}$ solution [Figure 3(C)], confirming an efficient grafting of nisin on gelatin and no residual free peptide.

Antimicrobial Activity of G-N on *Staphylococci* Bacterial Strains

The antibacterial activity of soluble modified gelatins was evaluated in PBS for 24 h against *S. epidermidis*. Tests were performed

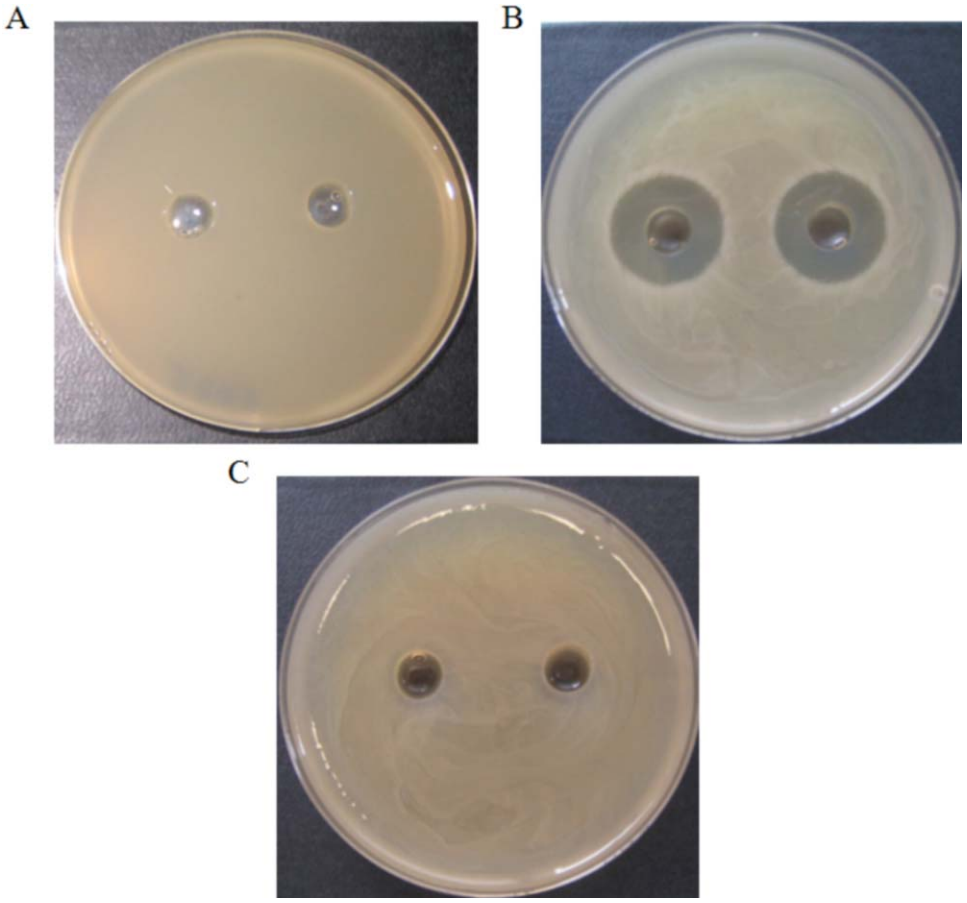

Figure 3. Agar well diffusion bioassays. Gelatin (at 2 mg/mL) (A), nisin (at 0.34 mg/mL) (B) and G-$N_{0.1-1}$ (at 2 mg/mL) (C) solutions. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table III. Antibacterial Activity of G-N Against *S. epidermidis* and *S. aureus*

	Antibacterial activity (%)	
Gelatins	*S. epidermidis*	*S. aureus*
G-$N_{0.05}$	88.40 ± 1.00	N.A
G-$N_{0.1-1}$	98.93 ± 0.13	90.20 ± 1.20
G-$N_{0.5-1}$	99.96 ± 0.02	N.D
G-$N_{0.1-2}$	95.32 ± 0.11	82.50 ± 2.40
G-$N_{0.1-3}$	99.66 ± 0.06	98.40 ± 0.70
G-$N_{0.1-4}$	99.08 ± 0.09	89.70 ± 1.70
G-$N_{0.5-2}$	99.72 ± 0.11	N.D

N.A = Not active.
N.D = Not determined.

with a protein concentration of 2 mg/mL. G-$N_{0.1}$ compounds were also tested against *S. aureus*. All results were gathered in Table III. A high biocidal activity was observed, i.e., a decrease of the bacterial population to 99.96%. However, a lower sensitivity of *S. aureus* toward modified gelatins was observed, confirming bibliographic data; thus no effect of G-$N_{0.05}$ was observed against *S. aureus*.[40] These results combined with previous diffusion experiments (see above) confirmed the success of the nisin grafting on gelatin. Furthermore, no antimicrobial effect was observed for all gelatins modified by only EDC/NHS compounds (i.e., without nisin). These results show that a slight crosslinking of gelatin (as shown by FT-IR analyses) does not alter bacterial behavior.

To optimize the gelatin antibacterial activity, we then investigated the impact of the nisin concentration, reaction buffer pH and EDC/NHS molar ratio on the biological activity.

Effect of the Nisin Concentration

As shown in Table III, the antibacterial activity of G-N against *S. epidermidis* increased with the nisin concentration since going from 88.4% for G-$N_{0.05}$ to 99.96% for G-$N_{0.5-1}$. These results are in agreement with the physicochemical characterizations which pointed out a positive effect of the nisin concentration on the grafted peptide amount. Moreover, the present data demonstrate that a low amount of nisin is sufficient to provide an antibacterial feature to gelatin.

Effect of Reaction Buffer pH

Though FT-IR analyses did not suggest variation of the peptide grafting according to the reaction buffer pH, antibacterial activity seemed pH dependent. Indeed, a higher antibacterial activity was observed for gelatins synthesized at pH = 7.4 as compared with those synthesized at pH = 4.5, whatever the nisin concentration and the bacterial species. Thus, an activity of 90.2% for G-$N_{0.1-1}$ and 82.5% for G-$N_{0.1-2}$ were observed against *S. aureus*. These observations disaccorded with zeta-potential analyses which suggested a higher amount of grafted nisin at pH = 4.5. This discrepancy might be explained by a better peptide orientation at pH = 7.4 thanks a grafting by the peptide N-terminal extremity.[27,41] Such observation suggests that the peptide orientation prevails over its density. This effect seems more important in the case of *S. aureus* compared with *S. epidermidis* with a higher difference of activity according to the experimental pH. This could be due to the lower sensitivity of *S. aureus* to nisin requiring a better orientation.

Effect of EDC/NHS Molar Ratio

EDC/NHS molar ratio had also a significant impact on the gelatin antibacterial activity. Thus, a molar ratio of 4/1 led to a higher activity whatever the bacterial specie. As suggested by zeta-potential measurements (see above), this could be due to a greater peptide density on the gelatin.

CONCLUSION

Gelatin has been modified by a covalent protein–protein coupling with an antimicrobial peptide through carbodiimides chemistry. Various grafting reaction conditions have been investigated to optimize biocidal activity of the protein. FT-IR analyses allowed to demonstrate the efficacy of the peptide grafting and zeta-potential measurements pointed out a higher global charge when gelatin was modified at pH = 4.5 and/or with an EDC/NHS molar ratio of 4/1. Antimicrobial experiments showed that nisin-enriched gelatin exhibited a high antibacterial activity against *Staphylococci* strains. The study suggested that the control of the grafted nisin orientation is predominant on peptide density for the biocidal activity.

Antimicrobial peptides actually constitute a natural alternative to chemical antibiotics and a potential for the biotechnology, permanently in search of sustainable strategies. The addition of an antimicrobial property to gelatin, *via* a "green" approach as here proposed, might enhance its use in numerous fields such medical (wound dressing for example), food industry (emulsifiers or foaming agents), and in cosmetic. For that, the antibacterial activity maintenance under gel form is required: experiments are yet in progress to control that.

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