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Addition of antimicrobial properties to hyaluronic acid by grafting of antimicrobial peptide ☆

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ABSTRACT

Nisin (an antimicrobial peptide) has been attached to hyaluronic acid (HA) to obtain an antimicrobial biopolymer under solution or gel form.

Various amounts of peptide have been grafted onto HA through a controlled reaction, to obtain a covalently grafting by formation of amide bonds. The modification has been confirmed and/or quantified by zeta-potential, ^{1}H NMR and Bradford assay. The antimicrobial activity of the modified polysaccharide was tested against *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* bacteria. In solution, modified HA exhibited a great antimicrobial property on the three tested bacterial species. Experiments then performed with hydrogels, allowed to confirm the feasibility to use nisin enriched HA as antimicrobial coating. Such a polymer is of great interest to avoid bacterial contamination in various applications as wound dressings, contacts lenses, cleaning solutions for contact lenses, cosmetics formulations, etc.

1. Introduction

Bacterial contamination of materials is of crucial importance in diverse fields such medical, food or cosmetic industries. Once adhered on a surface, bacteria form colonies and subsequently biofilms that serve as reservoirs for the development of pathogenic infections. Bacterial biofilm infections are particularly problematic because sessile bacteria can withstand host immune responses and are drastically more resistant to antibiotics, biocides and hydrodynamic shear forces than their planktonic counterparts [1].

Consequently, there is a considerable interest in the development of innovative techniques to remove or to kill micro-organisms present in aqueous solutions for sanitizing biomedical, pharmaceutical, industrial, and cosmetic formulations [2].

Considering the high resistance of sessile micro-organisms to inhibitors, the eradication of biofilms needs high concentration of disinfectants or antibiotics, causing severe environmental damages and/or facilitating the emergence of multi-drug resistance. In this context, prevention of biofilm formation is clearly preferable to any treatment strategy [3].

An efficient approach to prevent biofilm formation consists in immobilizing a bactericidal molecule on the support. Thus, various synthetic approaches based on the coating, grafting or release of bactericidal substances such as metal derivatives, poly(ammonium salts) and antibiotics have been extensively explored to produce antimicrobial materials. However, depending on the application, they are most often not completely satisfactory due to their limited efficiency, their toxicity or their role in the emergence of multi-resisting pathogens.

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To overcome these disadvantages, antimicrobial peptides (AMPs) are promising candidates. They are secreted by numerous living organisms (micro-organisms, vegetables, insects, fishes, and amphibians or mammalians) to protect themselves against invading micro-organisms. AMPs exert their activity by permeabilizing the bacteria cell membranes through the formation of pores or structural defects. Compared with conventional agents, AMPs offer the advantages to act at very low concentrations of about a few μg/mL and to have broad spectrum antibacterial activities. Thus, some AMPs, e.g., gramicidin [4], magainine [5,6] or nisin [7,8], have been immobilized on surface, others, e.g., temporin L [9] and nisin [10], have been encapsulated. It has been demonstrated that these peptides kept their antimicrobial activity after immobilization.

Nisin Z is a small (34 amino acids) cationic and hydrophobic peptide [11]. It exhibits an activity against Gram-positive bacteria which are often involved in biofilm infections [12]. It is the unique AMP which has been approved by the US Food and Drug Administration (FDA) for use in human food and veterinary products [13].

Today, the challenge is to propose antimicrobial materials which prevent biofilm formation by elaborating, for example, solutions or gels with antimicrobial properties. Biopolymers have known a real boom for some years, due to their biological origins. Indeed, these natural polymers have already many applications particularly in the medical field.

Hyaluronic acid (HA) is a linear polysaccharide consisting of alternating units of a repeating disaccharide, β-1,4-D-glucuronic acid-β-1,3-N-acetyl-D-glucosamine. HA is found in human body in the extracellular matrix of connective tissues [14]. Due to its viscoelastic, biocompatibility, biodegradability properties, its capacity to retain a high amount of water [14] and to promote healing [15], HA is used in a wide range of medical applications such as dermal filling [16], viscosupplementation in deteriorated joints [14], ophthalmic surgical aids [17] or drug delivery [18].

In some HA applications such as contact lenses [19–21] and wound dressings [22,23], infections are not still completely avoid. Even if some recent studies suggest a bacteriostatic effect of HA [24], antimicrobial activity of the polysaccharide is not clear and seems to depend on the concentration and molecular weight of HA and on bacterial species. Indeed, Ardizzoni et al. [24] have shown that solutions of HA (of high molecular mass) between 0.25 and 4 mg/mL had no effect on growth of Gram-negative bacteria (e.g., *Pseudomonas aeruginosa* and *Escherichia coli*) but a dose-dependent inhibition was found for *Staphylococcus epidermidis*. In the case of *Staphylococcus aureus,* a slight inhibition of the bacterial growth was observed for only high HA concentrations. In order to avoid these dependencies, HA must be used in combination with antimicrobial agents such as silver [25] or polyhexanide [26]. For example, Kemp et al. [25] showed that solutions of HA-silver nanoparticles exhibit an antimicrobial activity against *S .aureus* and *E. coli*, respectively, with a minimal inhibitory concentration of 0.025 and 0.1 μM compared to HA solutions which exhibit no activity for concentrations up to 1 μM for both bacteria.

The present study aims to elaborate an antimicrobial biopolymer combining properties of nisin and HA. Nisin was covalently grafted on HA using carbodiimide approach for carboxylic activation of HA. Various nisin concentrations were tested. All synthesized products were characterized by zeta potential, ^{1}H nuclear magnetic resonance (NMR) and Bradford assay. Antimicrobial activity of polysaccharidic solutions was tested against *S. epidermidis*, *S. aureus* and *P. aeruginosa,* bacterial species which are involved in biofilm infections. Antimicrobial activity of modified HA hydrogel were then investigated.

2. Materials and methods

2.1. Chemical materials

Hyaluronic acid sodium salt from *Streptococcus equi* (HA, 1,000,000 g/mol), N-hydroxysuccinimide (NHS), 1-(3-dimethylaminopropyl)-N′-ethyl-carbodiimide hydrochloride (EDC), Phosphate Buffered Saline tablet (PBS), 4-Morpholineethanesulfonic acid (MES), potassium hydroxide (KOH), hydrochloric acid (HCl), adipic acid dihydrazide (ADH), ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Aldrich. Nisin Z (3331 g/mol, purity 90%), was purchased from Anhui Mimetals Development (China). Milli-Q water (resistivity higher than 18.2 MΩ cm) was obtained from a Milli-Q Integral 10 system.

2.2. Synthesis of modified HA

One hundred milligram of hyaluronic acid (corresponding to a final concentration of 2 mg/mL) was solubilized in 40 mL of PBS solution (pH = 7.4) overnight. 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 for 1 eq of nisin). After 30 min, nisin, from a previously prepared solution at 1.75 mg/mL in 0.02 M hydrochloric solution (HCl), was added to the mixture to obtain various concentrations in the final solution (0.001 eq, 0.005 eq and 0.01 eq for one carboxylic acid group of HA corresponding respectively to 0.5, 2.5 and 5 mL). The pH of the reaction was adjusted with KOH (0.1 M) to pH = 7.4 and the volume of the reaction was adjusted to 50 mL with PBS solution. The solution was maintained for 24 h at room temperature under magnetic stirring. In order to remove small molecular weight products, NaCl (0.1 M) was added to the resulting mixture and sample was sealed in a semi-permeable membrane bag with a molecular weight cut-off of 50 kDa and dialyzed for 3.5 days in a large amount of Milli-Q water. The resultant solution was lyophilized.

Synthetized products are noted HA-$N_{0.001}$, HA-$N_{0.005}$ and HA-$N_{0.01,}$ corresponding respectively to 0.001, 0.005 and 0.01 eq of nisin used in the reaction relative to one carboxylic acid of HA.

For the elaboration of HA-N^*, used as negative control during agar well diffusion assays (see Sections 2.8 and 3.5), the same protocol was applied but without EDC/NHS addition.