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# BACTERIAL INHIBITION VIA CARBOXYMETHYL CELLULOSE-CONTAINING ELECTROSPUN MATS LOADED WITH NISIN Z PEPTIDE FOR POTENTIAL CHRONIC WOUND CARE

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## ABSTRACT

Nisin Z is an amphipathic peptide with a significant antibacterial activity against Gram-positive bacteria and low toxicity in humans. Here, we report the production of nanofibrous mats by co-electrospinning of the peptide with carboxymethyl cellulose (CMC) and poly(vinyl alcohol) (PVA) polymers for prospective wound dressing applications. The mats were studied against four of the most common bacteria found in chronic wounds. The evaluation of bacteria membrane permeability and the minimum inhibitory concentration (MIC) showed that chelating agent ethylenediaminetetraacetic acid (EDTA) can expand the activity of Nisin Z against Gram-negative bacteria. PVA (10 wt%)/CMC (3 wt%) electrospinning nanofibers were successfully produced at a 70/30 % (v/v) polymer ratio. The addition of Nisin Z to the polymer solutions did not alter the properties of the fibers.

## KEYWORDS

Antimicrobial peptides, polymers, electrospun nanofibers.

## INTRODUCTION

The resistance of bacteria to traditional antibiotics is raising a serious global public health problem with huge economic and societal consequences. Antimicrobial peptides (AMPs) have been the focus of great interest as alternatives to antibiotics. Nisin Z belongs to the lantibiotics class of AMPs and is produced by the non-pathogenic bacteria *Lactococcus lactisis*. This AMP has a significant antibacterial activity against Gram-positive bacteria [1]; however, its activity against Gram-negative bacteria is limited and can be improved with the addition of the non-antibiotic chelating agent ethylenediaminetetraacetic acid (EDTA) [2], or by surfactants such as Tween 80 and Triton X-100. Nisin Z has been studied for food preservation applications, but very little research has been done to explore its potential in biomedicine. Polymers are large molecules found everywhere and play a vital role in daily life, falling into two broad categories, natural and synthetic. Carboxymethyl cellulose (CMC) is a pH-sensitive, natural, chemically modified cellulose derivative, soluble in water and capable of forming a gel after water absorption [3]. However, its mechanical performance is limited. Blending CMC with a mechanically resilient polymer, such as poly(vinyl alcohol) (PVA), may raise its flexibility and give rise to a highly effective composite for biomedical applications, combining the advantages of both components [4,5]. PVA is a non-toxic, water-soluble, biodegradable and biocompatible synthetic polymer with good mechanical features [6]. CMC/PVA blends can be processed by electrospinning in the form of nanofibrous mats with large surface area, high porosity and an architecture resembling the native extracellular matrix (ECM). Electrospinning is a cost-effective, simple and straightforward technique that allows the production of

nanofibers with a tuned composition to achieve desired properties, making it suitable for wound dressing production, as compared to other methods [7]. This study envisaged the production of CMC/PVA/Nisin Z nanofibrous mats by co-electrospinning and their examination against four of the most common bacteria found in chronic wounds.

## MATERIALS AND METHODS

The bacteria membrane permeability was evaluated by procedure described by Felgueiras et al. [8]. The relative electric conductivities (REC) of bacteria suspensions by the addition of EDTA, Tween 80 and Triton X-100 were measured. The Gram-positive bacteria *Staphylococcus aureus* (ATCC 6538) and the Gram-negative bacteria *Escherichia coli* (ATCC 25922) were sub-cultured in Mueller Hinton Broth (MHB) overnight, and then collected by centrifugation at 4000 rpm for 10 min. These microbial cells were used as representative of each type of Gram bacteria. Their concentration was adjusted to $1\times10^5$ CFUs/mL and washed in a 5 % glucose solution until their electric conductivities were close to that of 5 % glucose (isotonic bacteria). EDTA (200 µM), Tween 80 (0.01 %) and Triton X-100 (0.02 %) were added to the 5 % glucose solution and electric conductivities of the mixtures were marked as L1. The surfactants were also added into the isotonic bacteria solution and incubated at 37 ºC and 120 rpm for 1 h, then the conductivities were measured and market as L2. The control was the bacteria in 5 % glucose treated in boiling water for 5 min, marked as L0. The bacterial cell membrane permeability was expressed as the ratio of REC, which was calculated as in Eq. [1]:

$$REC(\%) = \frac{(L2 - L1)}{L0} \times 100 \quad [1]$$

Minimum inhibitory concentration (MIC) of Nisin Z/EDTA was established via broth microdilution assay [9] against the Gram-positive bacteria *S. aureus* and *Staphylococcus epidermidis* (ATCC 35984), and the Gram-negative bacteria *E. coli* and *Pseudomonas aeruginosa* (ATCC 25853). Stock solutions of Nisin Z (1024 µg/mL) and EDTA (200 µM) were prepared in phosphate buffer saline solution (PBS, pH 7.4) by ultrasonication for 10 min. To establish the MIC, Nisin Z was serial diluted (1/2 v/v) in MHB and EDTA was added without undergoing dilution. Then, a bacteria suspension prepared at $2\times10^7$ CFUs/mL in MHB was added to the solutions. Free Nisin Z and EDTA were also tested. As control, agent-free bacteria suspensions (positive) and MHB (negative) were used. Absorbance measurements were performed at times 0 h and 24 h, after incubating at 37 ºC. The MIC was established as the concentration at which bacteria did not show any growth. The minimum bactericidal concentration (MBC) was determined by culturing the microorganisms at MIC and its vicinities. The plates were incubated at 37 ºC for 24 h at which point grown colonies were observed and counted.

PVA (Mw 78,000; 88 % hydrolyzed) and CMC (Mw 250,000; DS= 0.7) were prepared in distilled water ($dH_2O$). PVA at 10 % (w/v) and CMC at 3 % (w/v) were dissolved separately for 5 h at 40 °C by continuously stirring followed by 1 h of ultrasonication. PVA/CMC polymer ratios of 100/0, 90/10, 80/20 and 70/30 % (v/v) were prepared and blended with the most effective Nisin Z concentration (antimicrobial profile). The solutions were electrospun to generate a porous, nanofibrous mats, at specified processing conditions of positive voltage of 25 kV, feed rate of 0.8 mL/h, needle inner diameter of 18 gauge (1.02 mm) and an aluminum collecting sheet at working distance of 18 cm. Glass slides were used to collect the mats for microscopy observation.

The thermomechanical properties of the electrospun mats were characterized by differential scanning calorimetry (DSC), thermogravimetry (TGA), Fourier-transform infrared spectroscopy (FTIR) and dynamometer. Nisin Z content will be estimated by elemental analysis and by sulfo-succinimidyl-4-O-(4,4′-dimethoxytrityl)-butyrate (sulfo-SDTB).

## RESULTS AND DISCUSSION

Differences in REC of bacteria suspensions by the addition of agents can be used to determine their capacity to penetrate the membrane, as conductance has been suggested as an indicator not only of pore size but also of interaction of permeating ions with wall channels in various bacteria [10]. The effect of the agents on S. aureus and E. coli cell membrane permeability was evaluated through the percentage of REC (Table 1) after 1 h of incubation. Data reported an increase in the solution's conductivity upon incubation with the agents, which indicated the cell membranes became permeable, at different levels, after treatment. This was particularly noticeable on EDTA, a chelating agent that has been used to control microorganism infections by combining it with other bioactive agents [2].

**Table 1. Effect of surfactants in the cell membrane permeability of *S. aureus* and *E. coli* bacteria (n = 3, SD < ±0.5 %). The bacteria inoculum without any agent (C) was used as control.**

| | REC (%) | | | |
|---|---|---|---|---|
| **Bacteria** | **C** | **EDTA** | **Tween 80** | **Triton X-100** |
| ***S. aureus*** | -1.93 | 23.22 | 3.87 | 9.43 |
| ***E. coli*** | -1.99 | 21.66 | 1.70 | 0.96 |

The obtained MBC values for the Gram-positive bacteria *S. aureus* and *S. epidermidis*, and Gram-negative bacteria *E. coli* and *P. aeruginosa*, are shown in Table 2. No activity was observed in free EDTA (200 µM) against Gram-negative bacteria; however, against the Gram-positive bacteria *S. aureus* and *S. epidermidis* a reduction of 91.12 % and 99.33 % was evidenced, respectively. Regarding the activity against Gram-negative bacteria, the results confirmed that Nisin Z activity is limited, requiring EDTA to destabilize the outer bacterial membrane and facilitate peptide permeabilization [11].

**Table 2. MBCs of Nisin Z and Nisin Z/EDTA against Gram-positive and Gram-negative bacteria (n = 3, SD < ±5.0 µg/mL).**

| | MBC (µg/mL) | |
|---|---|---|
| **Bacteria** | **Nisin Z** | **Nisin Z/EDTA** |
| ***S. aureus*** | 16 | 8 |
| ***S. epidermidis*** | 32 | 8 |
| ***E. coli*** | 1024 | 64 |
| ***P. aeruginosa*** | 1024 | 256 |

PVA and CMC polymers were selected based on their ease of process, biocompatibility, and biodegradability. It was only possible to produce nanofibers up to a maximum of 30 % CMC. After that point, the viscosity of the solution does not allow the formation of continuous fibers by electrospinning. Fiber production was verified by brightfield microscopy (Figure 1) using a microscope Leica DM750 with an integrated high-definition digital camera. Addition of Nisin Z to the polymer solution did not alter the properties of the fibers, maintaining their smooth and continuous morphology.

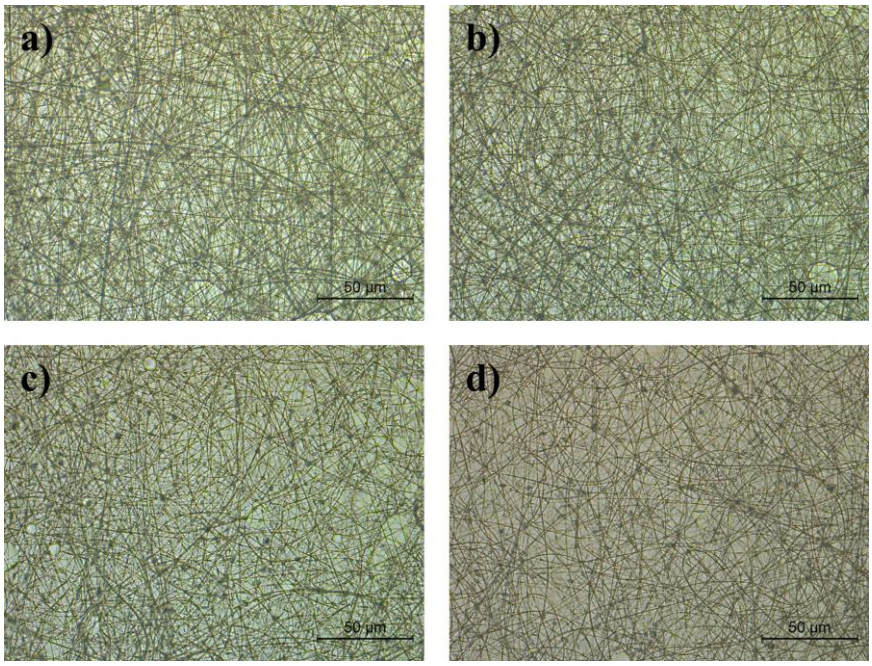


**Figure 1. Optic micrographs (brightfield) of a) 100/0, b) 90/10, c) 80/20 and d) 70/30 PVA/CMC nanofibrous electrospun mats. All images were collected with the objective magnification of 50× and ocular magnification of 10× (scale bar of 50 µm).**

## CONCLUSION

Data revealed that EDTA is more effective in allowing Gram-negative bacteria permeabilization by Nisin Z than the other tested agents. Its combination with Nisin Z is essential to improve its activity against Gram-negative bacteria. The PVA/CMC/Nisin Z co-electrospinning preliminary data are very promising, revealing the potential of this polymeric/antimicrobial peptide combination to promote the formation of complex nanofibrous mats for prospective wound dressing applications. Subsequent testing will be consisting in implementing a green crosslinking process on PVA so liberation of Nisin Z via CMC can be controlled and topically delivered.

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