Nisin Immobilization on Plasma Treated Polyvinyl Alcohol Films

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Nisin protein attachment on chemically crosslinked polyvinyl alcohol surface activated in atmospheric Dielectric Coplanar Surface Barrier Discharge was carried out in this work. Plasma activated PVA surfaces show improved level of nisin attachment in comparison with untreated films. In addition, the extent of the nisin surface immobilization and its release strongly depends on crosslinking degree of PVA substrate.

Keywords: nisin, polyvinyl alcohol, surface modification, antimicrobial activity, crosslinking

1 INTRODUCTION

Antimicrobial peptides such as bacteriocin, Nisin (approx. 3.4 kDa), have been studied as a preserving agent since fifties of the last century [1-3]. Nisin is an effective inhibitor of Gram positive bacterial strains and promising antimicrobial agent for polymer materials and coatings [3-6]. Novel strategies for the antimicrobial properties of the polymer materials include bacteriocin binding to create active surface. Controlling protein and peptide adhesion is an important issue in many fields especially in medicine [6-8].

Polyvinyl alcohol (PVA) is commonly used biocompatible water soluble polymer material which offers functional groups for possible protein binding and crosslinking for hydrogel preparation.

Surface immobilization of the nisin of various surfaces has been already reported [8, 9]. However, its immobilization of plasma activated xerogel system (i.e. solvent free crosslinked PVA) is unique.

Atmospheric plasma is used in this study because of sufficient uniformity and easy applicability for continuous low-cost surface plasma treatment [9-13]. In this study, we used the Dielectric Coplanar Surface Barrier Discharge (DCSBD).

This work deals with description of (i) the nisin surface immobilization on the crosslinked PVA films activated by DCSBD technique, (ii) nisin surface antibacterial activity and (iii) release kinetics in a physiological environment.

2 EXPERIMENTAL

PVA (PVA Mowiol 8-88, Sigma Aldrich) films were prepared by solvent cast technique (10 wt. %, 85 °C) from aqueous solution. Glutaric acid (GA, Sigma Aldrich) was used as crosslinking agent. The several crosslinking degrees were chosen: 0, 5, 10, 20 and 40% [14]. The surface of the foils was activated by Dielectric Coplanar Surface Barrier Discharge (DCSBD), RPS 40 systems (Roplass s.r.o., Czech Republic) [15, 16], in ambient air under the following conditions: power density 7 W/cm^2, frequency 25 kHz, with the treatment time of 10 s. The distance of the sample surface from the discharge plate was 0.3 mm. The samples were moved through plasma layer to obtain homogeneous treatment. Then, on the surface of treated PVA films, 40 µl of commercial nisin (Sigma-Aldrich) solution were applied (nisin was dissolved in phosphate saline solution, the pH value was set at 4.5 and the nisin concentration was 525 µg/ml). The samples were incubated for 20 minutes at room temperature. Then, the surface was dried, shortly rinsed off by demineralized sterilized water and dried again. The plasma treatment was applied again as a final processing.

The nisin release from the PVA surface into the physiological solution and nisin stability were studied by using Tricine SDS-PAGE (at 16 % and 4 % separating and stacking acrylamide gel containing 3 % bis-acrylamide, proteins and peptides were visualized by staining solution methanol/acetic acid/ water/

Coomassie Blue G250 and also by silver staining. After electrophoresis, the bands from the electrophoregrams were cut out and after spot detaining the peptide was extracted by 5% formic acid/acetonitrile (1:2) solution, the nisin concentrations were analysed by means of UV-VIS with Sunrise microplate ELISA reader (Tecan, Switzerland) at the wavelength of 595 nm). Water contact angle (WCA) of the treated/untreated films was measured by means of optical method, at room temperature; three 10 µl drops were leaved on the surface for 20 s. Measurements were taken from 3 different locations by using Surface Energy Evaluation system (See System, Advex instruments s.r.o., Czech Republic). Atomic force microscopy (AFM) was proceed on ICON, Bruker, USA. The antimicrobial assay was carried out by using the agar (Mueller-Hinton) diffusion testing against the sensitive bacterial strains: *Staphylococcus aureus* CCM 885 to analyse possible antimicrobial activity loss [8, 17]. The activity was expressed as diameter of zones of microbial inhibition (IZ) growth occurring around the sample. Reduction of antibacterial activity was calculated as IZ ratio of samples after given time from their preparation and freshly prepared samples.

The results presented further represents average values from at least 5 measurements. Standard deviation value was always up to 10 % of average value.

3 RESULTS AND DISCUSSION

Figure 1 shows effect of plasma treatment on nisin immobilization and thus on the surface topology of the PVA substrates. The changes in roughness indicate the nisin absorption.

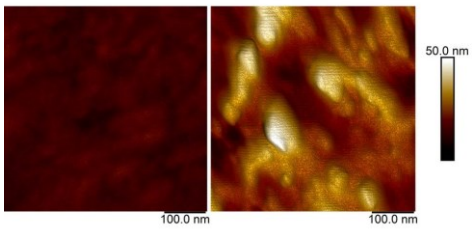

Fig. 1: AFM image taken 14 days after treatment; PVA/GA, crosslinking degree: 20%, RF power 7W/cm^2, treatment time 10s, without (left) and with nisin (right)

A hydrophilic character (wettability) of PVA films significantly increased after the plasma treatment, see Figure 2. The significant decrease of WCA was observed because characteristic polar functional groups were introduced onto the PVA surface, after plasma treatment [12]. According to the measured contact angle, the total surface free energies γ^{total} and its components: Lifschitz-van der Waals and electron acceptor/electron donor (γ^{LW}, γ^{AB}) were calculated by Owens-Wendt fit, using water/diiodomethan, see Table 1[1]. The γ^{AB} component of the surface free energy increases with the plasma treatment, what indicates polar groups (attachment via covalent bonds). Also the total surface energy increases, what enables nisin adhesion. With nisin immobilization on the surface, the γ^{LW} increase, thus, the van der Waals interactions predominate. The total surface free energy increased too. The different trend (WCA increase) with nisin adhesion demonstrates decreased wettability, suggesting the hydrophobic nisin groups attendance. It was found out that water contact angle drop of plasma treated PVA films was sufficient for PVA wettability sustainment (WCA decrease is higher than 50 % of the initial value). Moreover, the treated films reveal homogeneity of their surfaces in comparison to the untreated films that showed different wettability at different places. While non-crosslinked PVA films and films with low crosslinking degree up to 5 % show only slightly decreased wettability in comparison with untreated PVA films, samples with higher crosslinking degree (10 and 20 %) show significant increase in WCA (Figure 2). Maximal WCA of 84° was obtained for PVA with at 20 % of crosslinking. This can be attributed to protein surface adhesion when nisin was bond on PVA substrate. It also reveals optimal PVA crosslinking degree for the protein efficient surface immobilization.

Effect of crosslinking degree on amount of nisin adsorbed on PVA substrate can be clearly recognized in the release study presented Figure 3. Initial concentration of nisin is in agreement with WCA measurements (Figure 2). It is also evident that crosslinking degree of PVA influences extent

of nisin adsorption as well as its release profile. Pure PVA (without crosslinking) show no affinity towards nisin. Nisin was washed off from the PVA surface after 1-24 hours in salt solution. However, PVA films cross-linked with di-carboxylic acid (GA) and plasma treated show enhanced surface affinity to the bacteriocin-nisin. The highest nisin surface adsorption was found for the samples with 10 % crosslinking degree. More intensive initial introducing of crosslinking connections leads to apparent change of material properties and formations of inhomogeneity on the surface.

Table 1. The influence of plasma treatment and nisin adhesion on γ^{AB} and γ^{LW} components of the surface free energy of PVA films with different crosslinking degree

crosslinking	Untreated		Treated		Treated+nisin	
%GA/mJm^{-2}	γ^{LW}	γ^{AB}	γ^{LW}	γ^{AB}	γ^{LW}	γ^{AB}
0	*24.5*	*11.25*	*4.06*	*40.27*	*39.1*	*19.8*
5	*32.7*	*11.79*	*5.63*	*47.44*	*40.26*	*21.59*
10	*33.1*	*12.05*	*9.21*	*45.82*	*42.05*	*28.34*
20	*31.06*	*13.67*	*11.35*	*52.21*	*37.95*	*34.19*
40	*32.44*	*14.64*	*0.72*	*64.83*	*36.68*	*19.08*

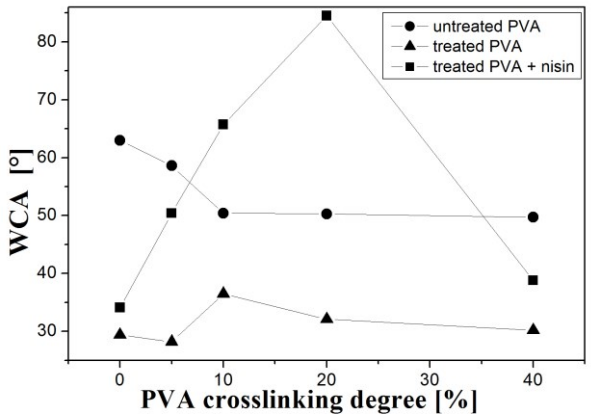

Fig. 2: The contact angle dependence as a function of PVA crosslinking degree; RF power 7W/cm^2, treatment time was 10 s

The microbial assay showed that nisin can be stable and active against Gram positive *Staphylococcus aureus* even after 90 days (Figure 4). Reduction of antibacterial activity of the cross-linked PVA films with attached nisin can be seen in Figure 4. Only 35 % reduction of antimicrobial activity was observed in the case of nisin containing PVA samples with 20 % crosslinking degree. It should be mentioned that synergy effect of the crosslinking agent, GA, cannot be omitted. Modest antibacterial activity of GA in the cross-linked PVA systems has been found. However, this factor does not represent significant contribution to antibacterial activity of the PVA/GA/nisin samples [18]. Therefore nisin can be supposed to the principal antibacterial agent in the studied systems.

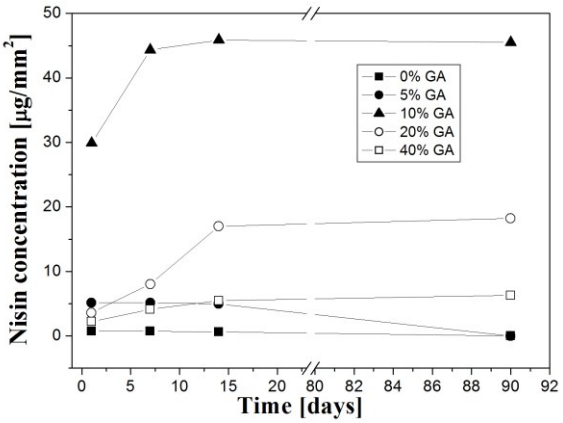

Fig. 3: Nisin release from the treated PVA films, after immersion in physiological solution

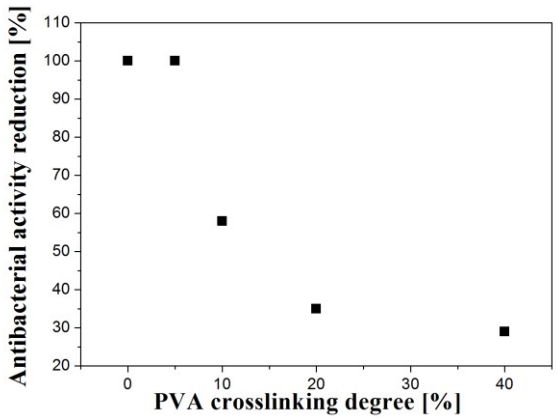

Fig. 4: Antibacterial activity change of PVA films with attached nisin after 90 days against Staphylococcus aureus (CCM 888)

4 CONCLUSIONS

Improved adhesion of nisin on biocompatible glutaric acid cross-linked polyvinyl alcohol surface due to plasma treatment by Dielectric Coplanar Surface Barrier Discharge activation was studied in this work. It was found that not only plasma treatment but also PVA crosslinking degree has the crucial effect on both extent of nisin surface attachment as well its antibacterial activity and release kinetics. Crosslinking degree in range 10 – 20 % was evaluated as optimal for preparation of relatively water-stable peptide coatings that can sustain also the antibacterial properties with potential applicability in medical field. The achievement of improved control by plasma treatment techniqueshave been attractive. The optimal PVA surface modification can be enhanced by combination of conventional techniques and solvent-free techniques, especially by using DCSBD plasma. The latter one can represent novel and promising method for biomaterials design.

Acknowledgements

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