Postprint of: Kaczmarek B., Wekwejt M., Nadolna K., Owczarek A., Mazur O., Pałubicka A., The mechanical properties and bactericidal degradation effectiveness of tannic acid-based thin films for wound care, Journal of the Mechanical Behavior of Biomedical Materials, Vol. 110 (2020), 103916, DOI: 10.1016/j.jmbbm.2020.103916

The mechanical properties and bactericidal degradation effectiveness of tannic acid-based thin films for wound care

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Abstract

The surface area is the most important aspect when considering the interactions between a material and the surrounding environment. Chitosan (CTS) and tannic acid (TA) were previously successfully tested by us to obtain thin films to serve as wound dressings or food packaging materials. However, surface properties as well as the antimicrobial activity of the material were not considered. They are important if the material is likely to find application in biomedical or food packaging application. Thereby, this study is a further investigation of chitosan/tannic acid films surface properties. The results showed that higher content of tannic acid increases the surface free energy and roughness, which is beneficial when considering the application of the materials as wound dressings. However, higher content of chitosan provides better antibacterial properties. Hence, the most optimal complex of chitosan and tannic acid for proposed application is the ratio 80/20.

Key words: tannic acid, chitosan, surface properties, bacteria, biofilm

Introduction

Materials for application as wound dressings should possess many special properties. Surfaces of biomaterials are exposed to adhesion of bacteria which are able to adhere to a flat

surface [1]. Most of the natural bacteria can form biofilm without causing any harmful reaction. However, also pathogenic microorganisms can adhere, which increases the risk of illnesses and inflammations developing [2]. For bacteria, there are numerous advantages of biofilm formation as protection from antibiotics, disinfectants and the dynamic environment [1]. The structure of biofilm is affected by different factors such as pH, oxygen, the presence of nutrients and surface roughness etc. Materials for medical purposes should be studied for interaction with bacteria [3]. An important factor is the surface morphology. A rough surface improves the prevention against pathogenic microorganisms and at the same time does not inhibit human cell adhesion [4-7]. Materials should act as a mechanical barrier against pathogens and should not change the mechanical properties after being soaked with body fluids. Tannic acid is a polyphenolic compound which has been studied as an active substance with antimicrobial properties [8]. Tannic acid was studied as an additive to graphene to obtain three-dimensional structures with antibacterial properties against Gran-negative and Gram-positive bacteria [9]. In another study, hyaluronic acid nanoparticles were loaded tannic acid as a microbial agent [10]. Moreover, tannic acid was precipitated as a coating on the AZ31 magnesium alloy. Such coating exhibits good antibacterial properties, due to the tannic acid's ability to interact with the bacterial cell wall and chelate metal ions to reduce the activity of metalloenzymes [11]. Chitosan and tannic acid have been reported as active compounds with antimicrobial properties. The aim of the experimental study was to detect a potential synergy of antibacterial properties of chitosan and tannic acid against biofilm formation. Thin films were characterized by the measurement of surface free energy, the contact angle, and mechanical properties after immersion in simulated body fluids (SBF). The roughness of the films was determined by atomic force microscopy. The concentration of tannic acid released from material after immersion in SBF, SGF (simulated gastric fluid), SIF (simulated intestinal fluid) was determined by spectrophotometric method. Also, microbiological studies were carried out as an inhibition of bacteria growth test and bacteria adhesion observation. The obtained results allow for the selection of the optimal composition of the chitosan and tannic acid films, which will ensure adequate properties and bactericidal effectiveness.

Materials and methods

Samples preparation

Chitosan and tannic acid were purchased from Sigma-Aldrich company (Germany). Chitosan (CTS; DD=78%, M_v=1.8 × 10^6, shrimp derived; CAS Number: 9012-76-4) and tannic acid (TA; M_v=1701.2 g/mol; CAS Number: 1401-55-4) were dissolved in 0.1M acetic acid,

separately, at a concentration of 2%. Complexes of chitosan and tannic acid were prepared in the weight ratios of 80/20 and 50/50, based on the previous research [12]. Obtained mixtures were placed on a plastic holder for solvent evaporation. The obtained films had thickness of 0.035 ± 0.003 mm which was measured with a thickness gauge (Sylvac, Switzerland).

Surface free energy

The surface free energy was measured by the sessile drop method, whereby the contact angle is measured by observing the liquid drop from the side. Surface free energy (ɣs), its polar (ɣsP) and dispersive (ɣsD) components can be calculated by the contact angle measurement, in which non-covalent forces between the liquid and film surface are formed by Owens-Wendt method. The contact angles of two liquids: water and diiodomethane were measured at a constant temperature using an optical tensiometer (Attention Theta Life, Biolin Scientific, USA) equipped with a system of drop shape analysis (DSA 10 Control Unit, Krüss, Germany).

The morphology of surface

Topographic images were obtained using a multimode scanning probe microscope with a NanoScope IIIa controller (Digital Instruments, Santa Barbara, CA) operating in the tapping mode at room temperature. Surface images were acquired at fixed resolution (512 × 512 data points) using scan width 1 μm with a scan rate of 1.97 Hz. Silicon tips with spring constant 2–10 N/m were used.

Mechanical properties

The analysis was carried out to observe changes in the material properties related to the contact with body fluids. In order to perform mechanical testing films, the samples were immersed in SBF solution for 1, 3, 5, 24, 48, and 72 h. The maximum tensile strength tests (n=5) were conducted in the universal testing machine Z.05 (Zwick/Roell, Germany) with the initial force 0.1 MPa and the velocity of 5 mm/min.

Tannic acid release

Tannic acid release was carried out in three different types of conditions - simulated body fluid (SBF; pH=7.4), simulated gastric fluid (SGF; pH=1.2) and simulated intestinal fluid (SIF, pH=6.8) which contained corresponding digestive enzymes. Selected solutions were prepared as traditional media and reference the appropriate conditions for film testing [13,14]. The total content of polyphenols was determined by the Folin-Ciocalteu method. To perform this

analysis, 0.5 mL of Folin-Ciocalteu reagent was mixed with 1 mL of Na_2CO_3, 1 mL of sample and distilled water to complete 10 mL. The mixture was stored in 40°C for 30 min. Then the absorbance of the samples was measured at 725 nm, in triplicate, by means of a UV–Vis spectrophotometer (UV-1800, Shimadzu, Switzerland). Gallic acid was used as a standard and the results were expressed as mg/ml.

Inhibition of bacterial growth

Inhibition of bacterial growth was evaluated by measuring the turbidity of cultured bacterial broth with the tested materials according to McFarland standards [15]. Before testing, the films (n=5) were soaked in 70% EtOH for 1 h and then washed in a sterile phosphate buffer solution. The *Staphylococcus aureus* strain (ATCC 29213) with the initial concentration $1.5x10^8$ CFU/ml was cultivated in 2 ml Trypticase Soy Borth (Merck, Poland) at 37°C. The optical density was measured using The DensiCHEK Plus (BioMerieux, USA) and the readings were made after: 0.5, 2, 4 and 6h. The maximum measuring range of this device is 4 McFarland index /MSi/. The following conversion of optical density to the number of bacteria was used (Tab. 1) and it is consistent with the assumptions of the McFarland method.

Table 1. The conversion of optical density to the number of bacteria /Adapted [9]/

McFarland index /MSi/	Approximate Bacterial Suspension / ml
0.5	$1.5 x10^8$
1.0	$3.0 x10^8$
2.0	$6.0 x10^8$
3.0	$9.0 x10^8$
4.0	$12.0 x10^8$

Adhesion of bacteria to the surface

Evaluation of bacterial adhesion to the film surfaces was performed by immersing the specimens in a bacterial solution, drying, covering them by gold, and then assessing with the use of scanning electron microscope (LEO Electron Microscopy Ltd, England).

The *Staphylococcus aureus* strain (ATCC 29213) with the initial concentration of $1x10^8$ CFU/ml was added to 30 ml of the Tryptic Soy Bulion (Merck, Poland) and incubated with specimens at 37°C for 14 days. Before the tests, the films (n=3) were soaked in 70% EtOH for 1 h and then washed in a sterile phosphate buffer solution.

Statistical analysis

Statistical analysis of the data was performed using commercial software (SigmaPlot 14.0, Systat Software, San Jose, CA, USA). The Shapiro–Wilk test was used to assess the normal distribution of the data. All of the results were presented as a mean ± standard deviation (SD) and were statistically analyzed using one-way analysis of variance (one-way ANOVA). Multiple comparisons between means were performed using the Bonferroni t-test with the statistical significance set at $p < 0.05$.

Results and discussion

Surface free energy

The surface free energy, polar and dispersive components results are shown in Table 2. Such analysis is a technique used to measure the integration possibilities with the surrounding tissue environment. The surface free energy is energy resulting from the “dangling bonds” exposed at material's surface [16]. The addition of tannic acid increases the surface free energy and polar component of the surface. Tannic acid has many hydroxylic groups in the structure and the hydrophilicity of the surface is enhanced.

Table 2. The polar (ɣsP) and dispersive (ɣsD) component, and surface free energy (ɣs) measured for films based on chitosan and tannic acid in 80/20 and 50/50 ratio (n = 10; mean ± SD, [123] significantly different between the groups: CTS – 1, 80CTS/20TA – 2, 50CTS/50TA – $p < 0.05$)

Specimen	ɣsP [mJ/m^2]	ɣsD [mJ/m^2]	ɣs [mJ/m^2]
CTS	0.91 ± 0.02 [2,3]	28.27 ± 0.14 [2]	29.19 ± 0.16 [2,3]
80CTS/20TA	5.23 ± 0.18 [1,3]	29.79 ± 0.56 [1,3]	35.03 ± 0.74 [1,3]
50CTS/50TA	9.51 ± 0.26 [1,2]	28.10 ± 0.55 [2]	37.61 ± 0.80 [1,2]

The morphology of surface

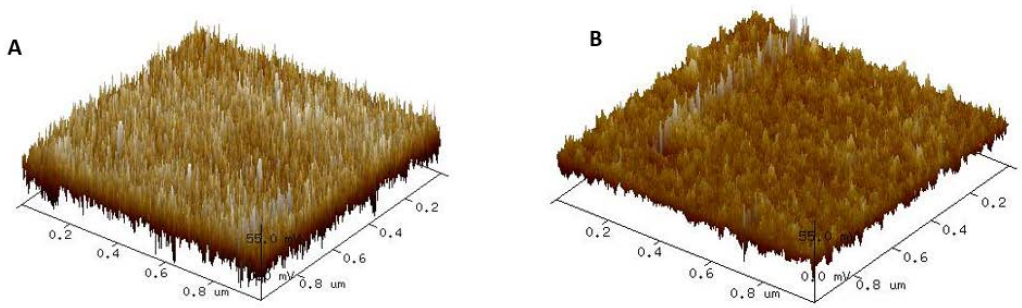

Figure 1. The AFM images of A) 50CTS/50TA B) 80CTS/20TA. The presented pictures are representative for 5 experiments.

The morphology of the films was evaluated with atomic force microscope (Fig. 1). The higher content of tannic acid results in the surface roughness improvement. Tannic acid interacts with chitosan by hydrogen bonds and the complex is formed. As a result the polymeric chain orientation is more chaotic and more functional groups are exposed on the film surface [17]. Such surface is better for human cell-biomaterial interaction and worse for the bacteria adhesion due to the presence of cell wall in bacteria.

Mechanical properties

The maximum tensile strength was different depending on the immersion time. For samples based on chitosan and tannic acid in 80/20 ratio the increase of maximum tensile strength was observed for the first 24h. After that it decreased. The reason is high ability of chitosan to swell in aqueous conditions (first 24h) but also its low stability in PBS (after 24h). For samples of chitosan and tannic acid in 50/50 ratio, first, a decrease of maximum tensile strength was noticed, and after 48h an increase was observed. This type of material has lower chitosan content and as a results it is not as susceptible to swelling as 80/20. Tannic acid is released from the immersed films and in the same time chitosan is solvated by water molecules, which enhances the mechanical parameters. The maximum σ_{max} value was noticed after 72h immersion in PBS. After another day of immersion samples lost their shape and the measurement was not possible to be made.

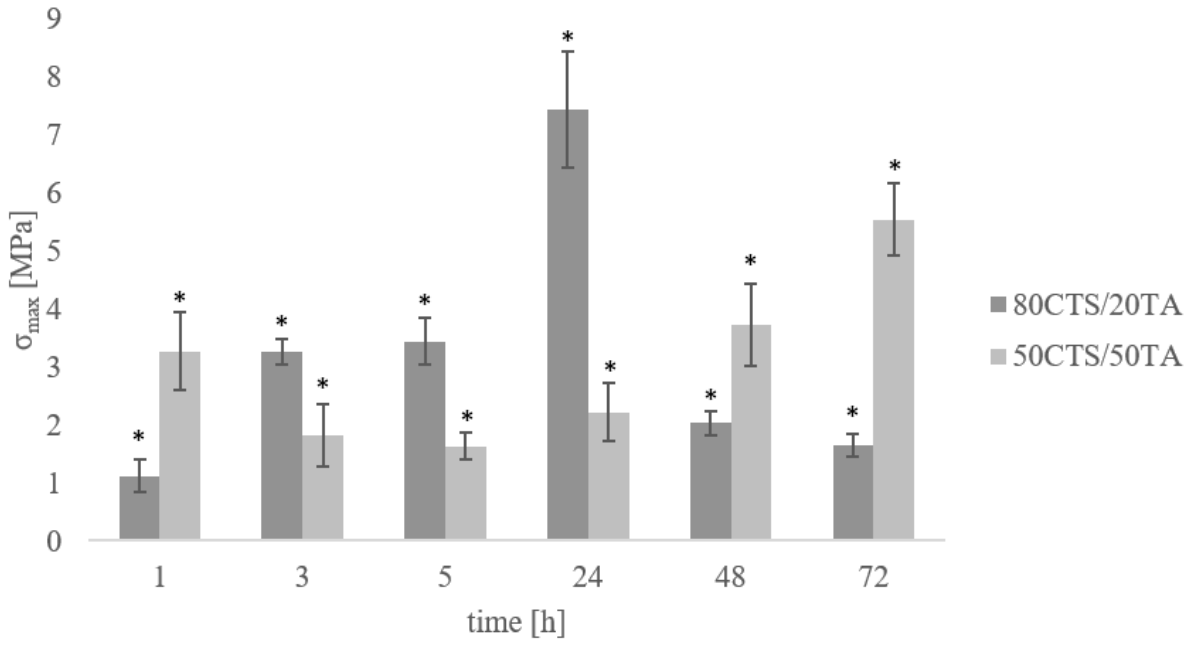

Figure 2. The maximum tensile strength (σ_{max}) measured for films after immersion in SBF for 1, 3, 5, 24, 48 and 72h (n = 5; mean ± SD, * significantly different between the groups – $p < 0.05$)

Tannic acid release

The concentration of tannic acid released was detected after films' immersion in three different media – simulated body fluid (SBF; pH=7.4), simulated gastric fluid (SGF; pH=1.2) and simulated intestinal fluid (SIF, pH=6.8). The results are presented in Figure 3. The released concentration was calculated per 1mg of film. Tannic acid was released firstly from the material surface and then after 4h, as a result of material swelling, more tannic acid was released. Higher concentration of tannic acid was noticed for materials based on chitosan and tannic acid in 50/50 ratio than for 80/20, which is similar to the film composition. Both types of films showed constant tannic acid release for 24-72h immersion time in SBF and SIF. In SGF, after 72h, maximum concentration of TA was noticed as a result of total material dissolution. In SBF and SIF conditions the films remained in a solid form. Thereby, the obtained materials are proposed to be applied in contact with body fluids or in intestinal parts (pH around 7). In stomach-like conditions, the proposed materials would totally dissolve, which may be beneficial for drug delivery purposes. The results showed that the released tannic acid concentration depends on the medium's pH as well as on time of contact.

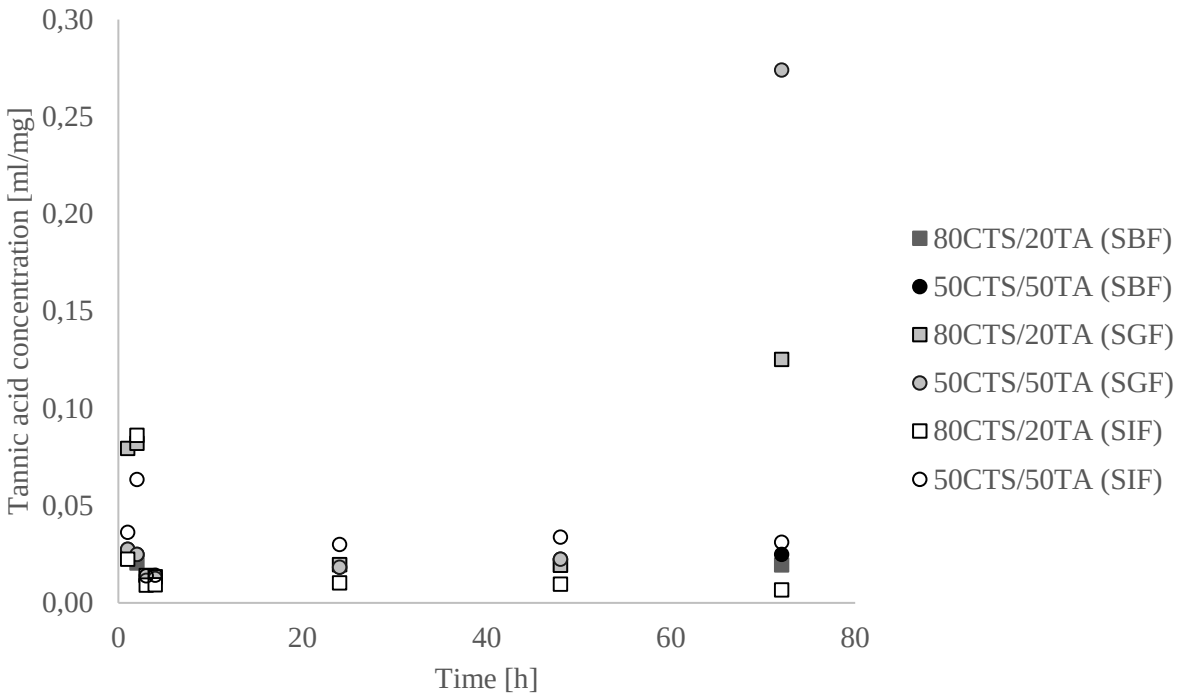

Figure 3. The tannic acid release in different media (SBF, SGF, SIF).

Inhibition of bacterial growth

The prepared films were immersed in the bacterial solution of *Staphylococcus aureus* and their effect on the multiplication of bacteria was evaluated. The obtained results are presented in Table 3.

Table 3. The influence of the studied films (n=5) on the multiplication of bacteria

Time:	*Staphylococcus aureus* stain (ATCC 29213)			
	K	The films composition		
		100CTS	80CTS/20TA	50CTS/50TA
0h	1.5			
2h	2.93	2.14#*	2.51#*	2.85#*
4h	>4	3.47#*	3.86#*	>4
6h	>4	>4	>4	>4
*Statistical analysis was performed between groups and control after 24h and the group, where the statistically significant difference occurred was marked. # max. SD ± 0.05				

In the case of bacteria incubated in the control (without film), their rapid multiplication to 4 MSi was observed within 4 h and this corresponds to 12x10^8 CFU/ml. Similar results were obtained for the film 50CTS/50TA. In contrast, for other films (100CTS and

80CTS/20TA), the growth of bacteria was slowed down. For scale approximation, it was estimated that the films inhibited the growth of bacteria by 14% and 25% for 2h incubation, which corresponds to about 1.2x10^8 CFU and 2.2x10^8 CFU. Hence, a significant inhibition of bacterial growth in the initial incubation phase with the material with a higher content of CTS is confirmed. According to literature, as well as to our results, chitosan shows bactericidal properties [18-20]. There are three proposed models of understanding the chitosan antimicrobial activity. First is the interaction between positively charged groups and negatively charged microbial cell membranes. The interaction is mediated by NH_3^+ groups and the negative residues, presumably by competing with Ca^{2+} for electronegative sites on the membrane surface. It promotes changes in the membrane properties and inhibits the bacterial growth. Other proposed mechanism is the binding of chitosan with microbial DNA and inhibition of mRNA and proteins synthesis. The third mechanism is the chelation of metals suppression of spore elements and binding the nutrients essential for microbial growth [21]. The exact mechanism is not known, however the antimicrobial activity of chitosan in the first hours after implantation has a key role in reducing infection. This is particularly important because the occurrence of an infection may lead to the rejection of the implant and a subsequent reoperation [22].

Adhesion of bacteria to the surface

The prepared films were immersed in a bacterial solution and the adhesion of bacteria to their surface was evaluated. The obtained results are presented in Figures 4-6.

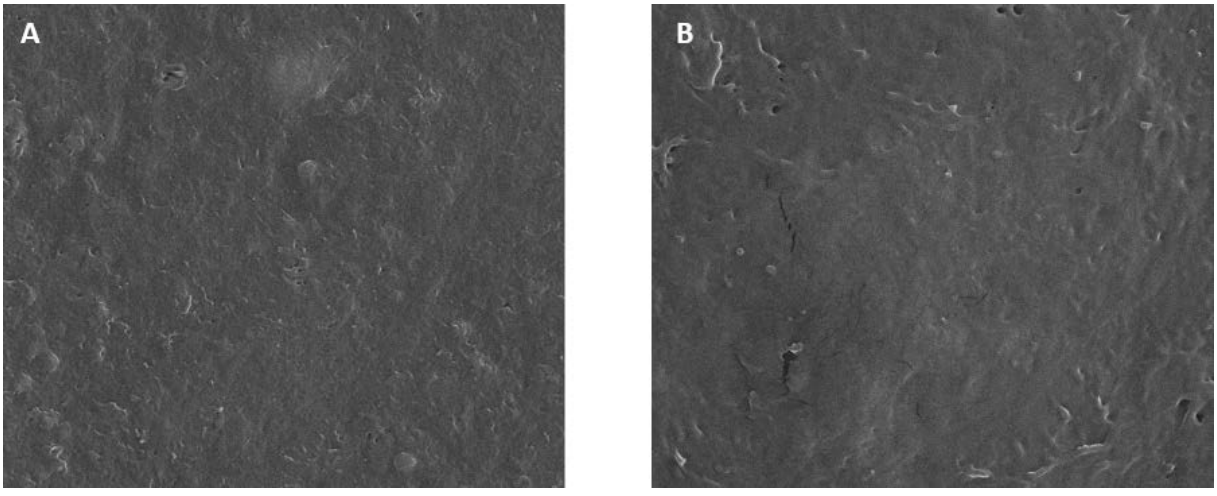

Figure 4. The SEM images of films based on chitosan with bacteria in magnification a) 10 000x b) 25 000x. The presented pictures are representative for 3 experiments.

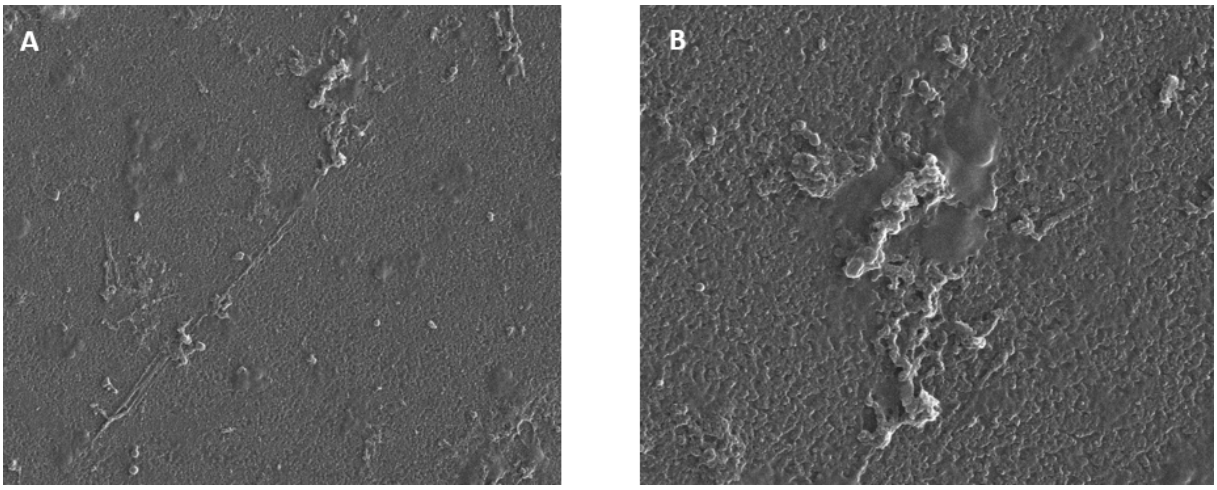

Figure 5. The SEM images of films based on chitosan an tannic acid mixed in 80/20 ratio with bacteria in magnification a) 10 000x b) 25 000x. The presented pictures are representative for 3 experiments.

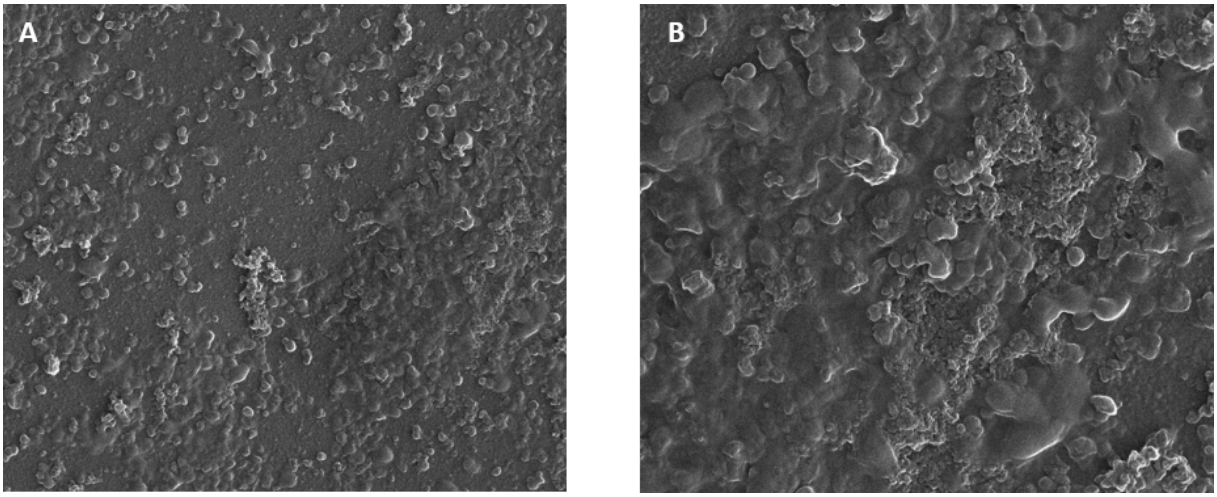

Figure 6. The SEM images of films based on chitosan an tannic acid mixed in 50/50 ratio with bacteria in magnification a) 10 000x b) 25 000x. The presented pictures are representative for 3 experiments.

A decrease of the amount of bacteria on the surface was observed along with the increase of CTS content in films (Figs. 4-6). In the case of 50CTS/50TA and 80CTS/20TA films, bacteria adhesion as well as biofilm formation was found on the surface (Figs. 3-4). However, on CTS film only single traces of bacteria were observed. (Fig. 2). Hence, a significant reduction in bacterial adhesion with increasing chitosan content in the films can be confirmed. The obtained results regarding the effect of chitosan on blocking the adhesion of bacteria are consistent with the literature [23-25]. Moreover, inhibition of biofilm formation was observed adequately as in position [26,27]. Therefore, it can be assumed that these films (chitosan and chitosan with tannic acid 80/20) can protect surface

from bacteria. The synergy of antibacterial properties of chitosan and tannic acid was not noticed in the presented study. In the case of films with a higher content of tannic acid, doping with antibiotics seems to be recommended due to the risk of pathogenic bacteria attachment on the surface. Furthermore, the bacteria growth on the film requires further studies, such as for example of bacterial biodegradation process.

Conclusions

In the experimental study, the surface properties of the films obtained from chitosan and its complex with tannic acid were compared. The most optimal solution seems to be a chitosan film with 20% addition of tannic acid. This choice is a compromise between properties of the material, effective biomaterial-surroundings interaction and bactericidal effectiveness that will protect against infection. The obtained results show that higher content of tannic acid increases the surface free energy and causes greater roughness. On the other hand, higher chitosan content resulted in the increase of mechanical properties and more effective inhibition of bacteria growth. The burst effect of tannic acid release was noticed, which suggests that the obtained films may be beneficial for the pharmaceutical application. Based on the results, we believe that these chitosan/tannic acid films could be potentially used as wound dressing materials.

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