PREPARATION AND CHARACTERIZATION OF CHITOSAN-AGAR FILMS

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Abstract

Hydrogel films composed of chitosan and agar or chitosan, agar and honey/bee pollen were prepared; they could be used as active wound dressings. During this study, various types of gel films were prepared using chitosan with different molecular weights and ratios of the two biopolymers. In addition, compositions with different amounts of honey or bee pollen were obtained. The selected samples were characterized by determining typical properties that are important for wound dressings. The best two-component films had a chitosan to agar weight ratio of 2:1. From among the examined tri-component films, the 2:1:0.5 weight ratio of chitosan, agar and 50 wt% honey solution was the best composition.

Keywords: *chitosan, agar, honey, bee pollen, wound dressings*

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1. Introduction

The human skin is the outer covering and most significant organ of body; it has an essential role in preventing pathogen entrance to the body and is a barrier between the human body and the external environment [1]. It acts as an outermost layer to protect the human body and is essential to human health [1]. A wound is described as damage and any disorder in the healthy structure and function of the skin [2]. Due to healing time, wounds can be divided into acute and chronic. Acute wounds usually heal completely within 8–12 weeks, with a small scar [3, 4]. Chronic wounds, such as diabetic foot, leg ulcers or burn wounds, continue to pose a challenge to medicine. They require long-term patient care, are easily infected are a source of pain and generate significant costs [3, 5]. In recent years, there has been an opinion that the wounds themselves are very diverse and require an individual approach. The complexity of wound healing as well as the variety of tissue damage means that there is no perfect dressing that could be used in all situations. The dressing should be selected individually for the type of wound and the phase in which it is located [3, 6]. An ideal wound dressing should treat wounds at reasonable costs with minimal inconveniences for the patient (among other considerations) [2]. Therefore, new types of various dressings are being developed. It seems that biopolymers are a good direction for the preparation of this kind of medical material.

Biopolymers such as polysaccharides are getting more attention nowadays than synthetic polymers because they exhibit biodegradability, biocompatibility, low immunogenicity and antibacterial activity [7]. Due to these features, they can be of great importance in medical, tissue engineering and active dressing materials. They are the answer to the modern approach to wound healing. In addition to providing a physical barrier, they also fulfil the basic properties required from wound dressings, i.e. to provide a humid environment, gas exchange, be painless in application, non-toxic, favourably affect the site of injury and be active (e.g. have antimicrobial effects) [5, 6].

Polysaccharides are a large group of biopolymers. They are obtained from renewable raw materials: microorganisms (e.g. dextran, xanthan gum), higher plants (e.g. cellulose, starch, guar gum), algae (e.g. alginates, agarose) and animals (e.g. chitin, hyaluronic acid). They are made of monosaccharides connected by an O-glycosidic bond [8]. Many structures have been developed based on polysaccharides, including for use in medicine: drug carriers, hydrogels, fibres, micro- and nanoparticles and porous scaffolds used in tissue engineering [8]. In this study, two polysaccharides (chitosan and agar) were utilized to prepare materials that could serve as wound dressings.

Chitosan is obtained from chitin by chemical or enzymatic deacetylation. Chitosan is an amino polysaccharide and can be (depending on the degree of removal of acetyl groups) a homopolymer of β-(1-4)-2-amino-2-deoxy-D-glucopyranose or a copolymer of β-(1-4)-2-amino-2-deoxy-D-glucopyranose and β-(1-4)-2-acetamido-2-deoxy-D-glucopyranose. Chitin with a degree of deacetylation above 50% is deemed as chitosan [9]. This polysaccharide has become an interesting material in pharmaceutical applications, especially as a film-former due to its biodegradability, biocompatibility and low toxicity [10].

Agar is one of the hydrocolloids (along with alginate and carrageenan) obtained from seaweed. Agar is a mixture of two polysaccharides: agarose (approximately 70%) and agaropectin. Agarose is responsible for the gelling properties of agar, while agaropectin is used for thickening [11]. Agar gelation is possible thanks to hydrogen bonds. Agar forms physical gels, which means that their structure is formed only by the accumulation of hydrogen bonds. In addition, these gels can be re-dissolved and gel again and again – the process is reversible [12]. Depending on the preparation method, the agar may be in the

form of scales, powder, ribbons or spongy lumps [13]. The main area of agar application is the food industry (80%); the remaining 20% is the raw material for the biotechnology and pharmaceutical industries. Agar is a component of such food products as coatings, ice cream, meringues, candies, marshmallows, etc. Agar is also a stabilizer for canned products and sauces [11]. In medicine, agar is used in dentistry as a material for the production of impression masses (for occlusal models). Agar gels are commonly used in laboratories as bacterial culture media. In pharmacy, agar can act as a filler for tablets and capsules, and also affect their swelling and disintegration. Agar is, for example, a component of laxatives [11]. It is also used for dressing materials. For example, Rivadeneira et al. [14] checked the effect of the agar to soy protein ratio on the obtained dressing material. Agar improved the mechanical properties of the obtained material, reduced solubility and increased absorption. The combination of agar and type I collagen (cross-linked with glutaraldehyde, among others) was also studied [15].

In addition to the polymers themselves, active dressings often include other additives. In the present study, honey and bee pollen served as such additions. Honey is a natural product made from flower nectar, primarily by honey bees, but also by some hymenopterans (e.g. wasps). The composition of honey, as well as its smell and colour, depend on its type (plants from which the nectar came), weather conditions, as well as the insects that produced it. The main component of honey (accounting for about 85%) are monosaccharides (glucose, fructose) and disaccharides. In addition, honey is formed by: water, organic acids, proteins, minerals and vitamins. The content of individual substances changes during storage – oxidative and fermentative processes take place in honey. Honey is acidic; the pH ranges from 3.2 to 4.5 [16, 17]. The antibacterial effect of honey is based on various mechanisms, one of which is the osmotic effect: high sugar levels can cause dehydration of bacterial cells and inhibit their growth. This effect may persist until the sugar concentration decreases to a critical level by absorbing more water [18]. Hydrogen peroxide also affects the antibacterial properties of honey. This compound is formed as a result of glucose oxidase activity. The enzyme is added by bees to the nectar they collect. This enzyme breaks down hydrogen peroxide [17, 18]. Honey is also anti-inflammatory [18]. All these properties make honey an almost perfect component of dressing materials.

Bee pollen, which is collected from the anthers of flowers, is used by bees to produce bee feathers (the so-called bee bread). Pollen, when mixed with a small amount of saliva or nectar, is formed into balls, attached to the hind legs and transported to the hive. The composition of pollen, like honey, depends on its origin, the breed of bees and the conditions that accompany the harvest. About 30% of pollen is proteins (including albumin, globulins and enzymes), and over 40% is sugars (mainly fructose and glucose). The bee pollen, in its composition, contains more than 100 enzymes (e.g. lysozyme, invertase, lipase and phosphatase). Bee pollen also contains small amounts of hormones, including growth hormone and gonadotropins. In addition, bee pollen contains lipids (including α-linolenic and linoleic acids), vitamins (which also have a significant effect on skin processes), micro- and macroelements and phenolic compounds, including flavonoids (about 0.7%, e.g. kaempferol, quercetin) [19, 20]. Bee pollen has been proven to inhibit microbial growth. For example, the use of ethanol extract from pollen inhibits the growth of microorganisms originating from the skin: *Candida albicans* and several bacteria (including *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*) [20]. Bee pollen extracts of various plants have exhibited different antimicrobial activity. Gram-positive bacteria are more sensitive to pollen than gram-negative bacteria [21, 22]. All these properties make honey and bee pollen an almost perfect component of dressing materials.

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2. Materials and Methods

2.1. Materials

Chitosan with low (20 kDa) (CHL) and medium (200 kDa) (CHM) molecular weight with a degree of deacetylation of 75–85% were obtained from Sigma-Aldrich. Sodium chloride (NaCl), glacial acetic acid and ethanol were purchased from Avantor Performance Materials Poland Ltd. Agar (A) was from TAR-GROCH-FIL, multifloral honey (H) was from a family apiary and bee pollen (BP) was purchased from an apiary from Bolesławiec (apiary No. 96, Jerzy Cyga). *S. aureus* PCM 458, *E. coli* PCM 2560, *Bacillus cereus* PCM 2025 and *P. aeruginosa* PCM 499 were used for antimicrobial tests.

2.2. Preparation of Chitosan-Agar Films

2.2.1. Preparation of Chitosan Solutions

Two types of chitosan were dissolved in 2 wt% aqueous solution of acetic acid, which contained 0.4 wt% NaCl. The solution was additionally filtered to remove impurities. Then, the obtained solutions were used as a component for gel preparation. The content of chitosan in the solution was 2 wt% [23, 24].

2.2.2. Preparation of Agar Solution

The 2 wt% agar solution was made directly before preparation of the dressings. Agar was dissolved by heating the solution in a microwave oven (bringing it to boiling several times until the so-called fluff disappears).

2.2.3. Method of Obtaining Two-Component Films

A 2 wt% solution of the appropriate chitosan was weighed into a 50 mL flask. The flask was placed in a water bath on a magnetic stirrer at 70°C. After reaching the set temperature, a 2 wt% agar solution at 90°C was added dropwise to the flask. The mixture was left on the stirrer for another 15 min, and after this time poured onto Petri dishes. After solidification, the consistency of the obtained gels was evaluated. The chitosan and ager were mixed with different weight ratios. The composition of the gels is given in the Table 1.

Table 1. Compositions of the prepared two-component films.

Sample No.		**01**	**1**	**2**	**3**	**7**	**8**	**9**	**02**	**4**	**5**	**6**	**10**	**11**	**12**
2 wt% CH	R	CHL							CHM						
		1	1	1	1	**2**	3	4	1	1	1	1	**2**	3	4
2 wt% AG		1	2	3	4	**1**	1	1	1	2	3	4	**1**	1	1

Abbreviations: **CH** –chitosan; **AG** – agar; **CHL** – chitosan with a low molecular weight; **CHM** – chitosan with a medium molecular weight; **R** – weight ratio of components [wt:wt].

2.3. Preparation of tri-component films

2.3.1. Preparation of Solutions of Honey and Bee Pollen

The 25 and 50 wt% bee pollen solutions and 50, 75 and 100 wt% honey solutions were obtained for the preparation of given films (see Table 2).

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Table 2. Compositions of the prepared tri-component films.

Sample No.		**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**
I	C [%]	Ratio of components [wt/wt]														
CH	2	CHM													CHL	
		2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
AG	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
BP	25			0.5			1									
	50	0.5								1						
H	50		0.5								1	1	0.5	0.25	1	0.5
	75				0.5			1								
	100					0.5			1							

For sample no. 21, a few drops of glutaraldehyde solution were added after moving it onto the Petri dish.
Abbreviations: **I** – type of component; **C** – concentration of given component in the water solution; **CH**- chitosan; **AG** –agar; **BP** – bee pollen; **H** – honey; **CHM** – chitosan with medium molecular mass; **CHL** – chitosan with low molecular mass.

2.3.2. Method to Obtain Tri-Component Films

A 2 wt% solution of appropriate chitosan was weighed into a 50 mL flask. The solution of honey or bee pollen was then added and mixed accurately. Next, the flask was placed in a water bath on a magnetic stirrer. The temperature was set to 70°C. After reaching the set temperature, a 2 wt% agar solution at 90°C was added dropwise into the flask. The solution was left on the stirrer for another 15 min. The mixtures were then poured onto the Petri dishes. After solidification at 35°C, the consistency of the obtained gels was evaluated.

2.4. Analysis of films

2.4.1 Water Content

The water regain (W_{H2O}) of the selected samples of membranes was determined after equilibrating a sample of film with deionized water with different pH (4, 7 and 9) at 35°C. The membrane samples were removed from the water and weighed immediately after blotting free surface water. They were then dried at 50°C. The water content was deduced from the difference in weight between the wet and the dry membrane in unit gram H_2O/g of a dry film (Eq. 1):

$$W_{H2O} = \frac{m_w - m_d}{m_d}, \quad (1)$$

where m_w is the weight of the swollen film [g] and m_d is the weight of dry film [g] [25].

2.4.2. Moisture Sorption Study

A gravimetric method via the difference in weight before and after impregnation was applied during the swelling test. First, the samples were dried and weighed (m_d). They were then placed inside the weighting vessels with 20 mL of 0.9 wt% NaCl solution (pH 6) and stored at 35°C. The weight gain of films was recorded (m_t) at predetermined time intervals over 90 min, and the percentage of moisture sorption *(%SR*) was calculated (Eq. 2):

$$\%SR = \frac{m_t - m_d}{m_d} \times 100\%, \quad (2)$$

where m_d is the weight of dry sample [g] and m_t is the weight of sample after given time [g] [10].

2.4.3. Vapour Permeability

Circles with a 2.5 cm diameter were cut from the obtained films. The thickness of the films was measured with a micrometer screw. To the polytetrafluoroethylene vessels with a hole in the lid, 20 mL of 0.9 wt% NaCl solution was pipetted. The cut film disc was then placed between the seals and mounted into the vessel; it was carefully closed and the whole apparatus was quickly weighed. Next, the vessels with samples of films were placed in an incubator previously set at 35°C and weighted after a given time. This weighing was carried out over a period of 4–5 days. The rate of vapour permeability (R) was determined from the slope of the curve (Eq. 3) [26]:

$$f(t) = m_i - m_t\,, \quad (3)$$

where: m_i – initial mass [g], m_t — mass after given time t [g]. The constant vapour permeability was determined from Eq. 4:

$$K = \frac{R}{A}\,, \quad (4)$$

where: K is the vapour permeability constant [g cm^{-2} min^{-1}], R is the vapour permeation rate [g min^{-1}] and A is the active surface area of the film [cm^2].

2.4.4. Antimicrobial Properties of Films

2.4.4.1. Culture Conditions

Two millilitres of overnight cultures of *S. aureus*, *E. coli*, *B. cereus* and *P. aeruginosa* (grown aerobically in Mueller broth at 28 or 37ºC) was centrifuged at 500 *g* for 5 min and the supernatant was discarded. The pellet was re-suspended in 100 mL fresh Mueller-Hinton broth supplemented with 1% glucose to yield an inoculum of approximately 1.4 10^3 colony-forming units (CFU mL^{-1}).

2.4.4.2. Studies on Bactericidal Activity

The tested films were cut into squares (10 10 mm), and each sample was placed in 5 mL of test bacterium suspension (the tests were carried out in triplicate). All samples were incubated for 24 h at 28 or 37°C (depending on the temperature requirements of the tested microbe). The cultures of the studied microorganisms were incubated under the same experimental conditions and were used as controls. After incubation, the number of colony forming units per millilitre (CFU mL^{-1}) was determined. To evaluate bactericidal effect the studied films, the percentage reduction (%) of bacteria cultures was calculated using Eq. 5:

$$R_w = \frac{N_0 - N}{N_0} \times 100\%\,, \quad (5)$$

where R_w is the reduction in viability and N_0 and N are the CFUs in the initial (1.4 10^3 CFU mL^{-1}) and remaining suspension (24 h under experimental conditions). Bactericidal activity was also converted into a reduction of $\log_{10}$ units [27].

2.4.4.3. Statistical Analysis

All experiments were carried out in triplicate, and average values are reported. Mean separation and significance were analysed with STATISTICA data analysis software (version 10.0) and Microsoft Excel. The quantitative variables were characterized by the arithmetic mean of the standard deviation or median or max/min (range) and 95% confidence interval. Statistical significance of differences between two groups was processed with the Student's *t*-test. In all the calculations, a *p* value of 0.05 was used as the cut-off for statistical significance.

3. Results and Discussion

3.1. Films Composed of Chitosan and Agar

The two-component films were obtained in the first stage of the study. Several ratios of agar or chitosan were used for their preparation; in addition, two kinds of chitosan with different molecular weight were used. Films containing higher amounts of chitosan (3 or 4 parts to 1 part of agar) did not solidify. The other ratios of chitosan to agar (1:1, 1:2, 1:3, 1:4 and 2:1) yielded coherent gels. Fig. 1 show photos of dried gels that had a coherent structure.

Before checking the physicochemical properties of the prepared films, they were organoleptically evaluated for consistency: colour, transparency, flexibility, stiffness, fragility and extensibility. The best notes were awarded to films No. 7 (with CHL) and No. 10 (with CHM), with a weight ratio of chitosan to agar of 2:1. These films were characterized by good flexibility and low rigidity and fragility. They were also transparent. Their only drawback was they that they were not very stretchy. During analysis, films with a higher proportion of agar were of a lower quality, mainly due to their stiffness. In addition, the films prepared with higher amounts of chitosan (chitosan to agar weight ratio 3:1 or 4:1) had better flexibility and reduced stiffness than samples with a chitosan to agar ratio 2:1, respectively, but less transparency, so this gels were also rejected at this step of the study. After this organoleptic study, two films (No.7 and 10) were further examined and characterized with regard to properties that are important for wound dressings. This composition was also used for the preparation of tri-component gels.

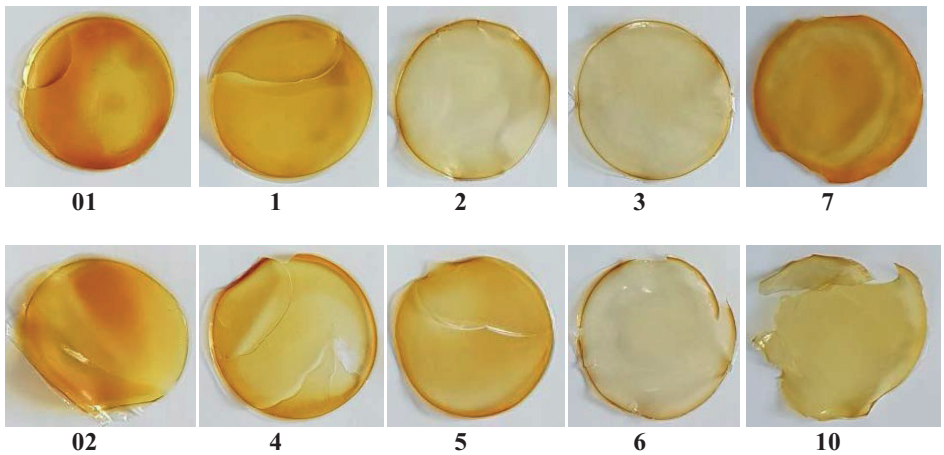

Figure 1. Films with a ratio of chitosan to agar 1:1 (No. 01-CHL and 02-CHM), 1:2 (No. 1-CHL and 4-CHM), 1:3 (No. 2-CHL and 5-CHM), 1:4 (No. 3-CHL and 6-CHM), 2:1 (No. 7-CHL and 10-CHM).

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3.2. Tri-Component Films

The tri-component films, with agar, chitosan and honey or bee pollen, were obtained by heating the solutions of chitosan and honey or chitosan and pollen be up to 70°C and then adding dissolved agar. Gels with different proportions of ingredients were obtained in this way (the weight ratio of chitosan and agar was 2:1 in all gels). The best of the films are shown in Fig. 2. The first series of tests was performed with medium-molecular-weight chitosan because these compositions worked better than those prepared with low-molecular-weight chitosan.

Films obtained with pollen bee were inflexible; hence, only one sample (No. 19), which contains 1 part by weight of 50% pollen, was chosen for further analysis. However, even this selected sample was not proper for wound dressings it was inflexible, non-transparent, fragile and stiff. Films with a high amount of honey (17 and 18) were also rejected because they were sticky and left a sticky mark on the skin. Their stickiness made it difficult to receive and store. Samples No. 14 and 15 had the appropriate structure with relatively high honey content (75 and 100% honey solution, respectively). Despite their good properties, these films were also rejected because they were prepared with more concentrated honey solutions, and on the stage of preparation of mixtures it was difficult to prepare homogeneous mixtures of all components. Samples No. 20–23, with the smallest amounts of honey, were also consistent and flexible. These gels were obtained from 50 wt% solution of honey, so in this case it was easier to mix all ingredients altogether. In the case of film No. 21, glutaraldehyde was added to obtain a compact structure. Hence, it was rejected because of the toxicity of this crosslinking compound [28].

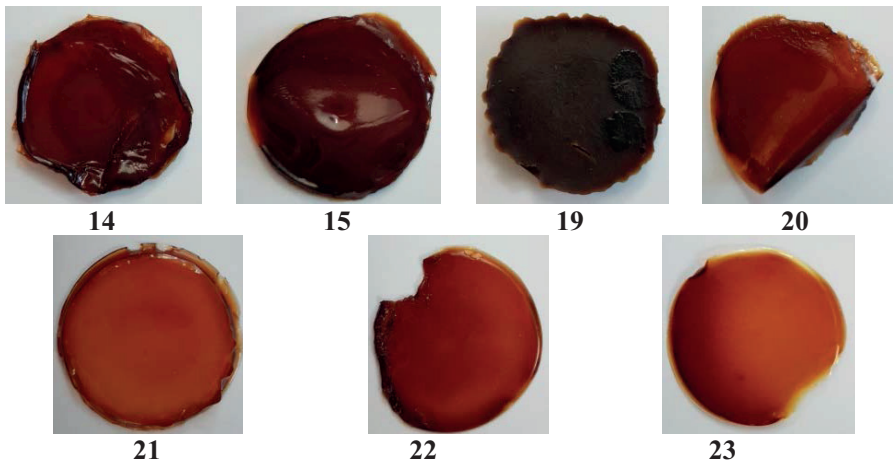

Figure 2. Pictures of selected tri-component films: No. 14, 15, 20, 21, 22 and 23 with honey, and No. 19 with bee pollen.

The organoleptic analysis revealed that samples No. 20 and 22 had the appropriate properties required for wound dressings. Samples No. 24 and 25, prepared with CHM, had the best wound dressing properties; analogous gels prepared with CHL were also obtained (see Table 2). However, samples No. 20 and 24 were stickier and left an unpleasant trace on the skin, so for further research it was decided to use films 22 and 25, prepared with less amount of honey, were subjected to further evaluation.

After preliminary analysis, further studies were carried out to characterize the physicochemical properties of the selected two-component (No. 7 and 10) and tri-component (No. 22 and 25) films. For these samples the water regain, swelling properties, vapour permeability and antimicrobial properties were studied.

3.3. Analysis of Selected Films

3.3.1. Water Content

Adsorption of water was studied depending on the pH, as well as on the composition of the tested film. The swelling ability of a wound dressing is an important aspect to evaluate its suitability for wound healing. It should adsorb, for example, the exude liquid from the wound. Thus, gels were swollen at 35°C to simulate the body temperature in the wounded area of the skin [29]. The swelling properties were evaluated by immersing the given sample in the proper water solutions for 24 h. The calculations of this parameter must consider the possible dissolution and elution of individual gel components. The values of the water regain parameter of selected films are shown in Fig. 3.

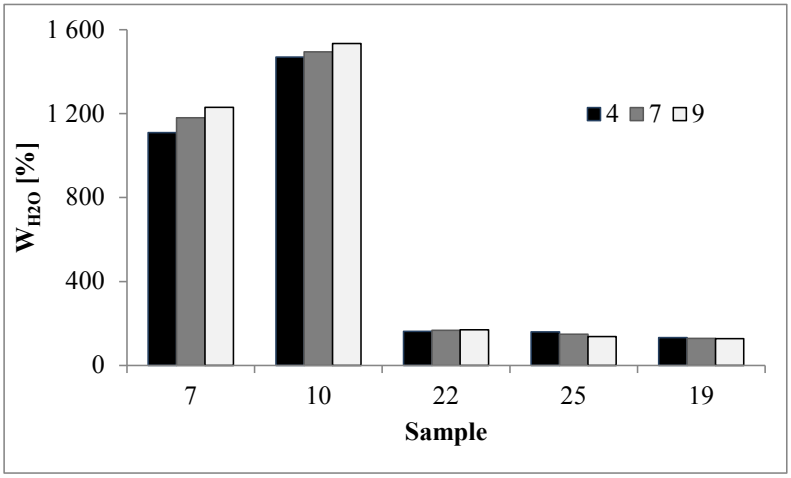

Figure 3. Water regain (W_{H2O}) values of selected films at 35°C and at pH 4, 7 and 9.

The two-component films absorbed water to a much greater extent, regardless of the pH, compared with their analogues prepared with honey. In addition, two-component gels prepared with CHL had a lower water adsorption capacity than their two-component analogue prepared with CHM (see Fig. 3, samples 7 and 10). Both films had the same amount of agar, so the type of chitosan influenced the water adsorption ability. This phenomenon had also been observed by Tangsadthakun et al. [30], who prepared films composed with collagen and chitosan, and Nunthanid et al. [10], who prepared chitosan films with different molecular weights and degrees of deacetylation. The swelling properties of chitosan/agar films were different in various media. The ability of water adsorption increased as pH increased. The highest values of water regain for the two-component films occurred at pH 9. For tri-component films, the values of water regain was much lower than for their two-component analogues. This difference was likely because the free spaces between agar and chitosan chains were taken by honey or bee pollen and thus limited possible water absorption. Notably, pH had no effect on the tri-component get swelling properties.

3.3.2. Moisture Sorption Study

The next parameter studied was the moisture sorption speed of films over time. These observations were made on selected films, which were immersed win 0.9 wt% NaCl solution (pH 6) at 35°C for 90 minutes. Those conditions reflect physiological liquid that could be exuded from a wound, e.g. the pH of a difficult-to-heal wound [31, 32] or its temperature [29]. The tri-component films (No. 22 and 25) increased their mass only about 20 wt% compared with their initial weight (Fig. 4). By contrast, two-component films (No. 7 and 10) increased their mass more than 120 wt%. The largest change in mass occurred in the first 10 min; subsequently, the mass of the two-component films gradually increased, while the mass of the tri-component films remained at a similar level. A smaller change in the mass of films with the addition of honey is probably due to due to the fact that potential free spaces between agar and chitosan networks were in this case filled with honey, similar to what was observed for water regain values (Fig. 3).

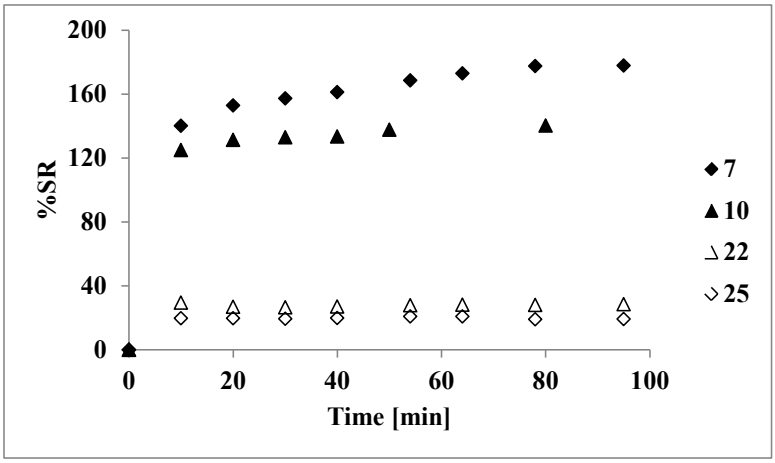

Figure 4. Moisture sorption percentage (%SR) of two-component (No. 7 and 10) and tri-component (No. 22 and 25) films incubated in 0.9 wt% NaCl solution (pH 6) at 35°C.

3.3.3. Vapour Permeability

It is important that active dressings maintain a moist environment, at the right temperature and pH, to ensure gas exchange. Therefore, the vapour permeability of select films was determined. The studies were conducted for a 0.9 wt% sodium chloride solution at 35°C for 4–5 days. Both two- and tri-component films were permeable to vapour. Gravimetric measurements allowed the observation of a change in the weight of the vessels with solutions, in which the only semi-permeable element was the film. During the measurements, the influence of the film composition and its thickness was examined. Two sample thicknesses – 10 and 70 μm – were examined for the two-component films (Fig. 5). The water vapour permeability was not affected by the molecular weight of chitosan, but it was affected by the film thickness: thicker films (70 μm) had less weight change than their thinner analogues. This phenomenon is probably because in thicker gels, the vapour molecules face a longer and more complicated path during evaporation.

For tri-component films, 70 and 200 μm thicknesses were considered (Fig. 6). Note that it was impossible to prepare 10-μm thick films because of the consistency of mixture with honey. As with two-component films, the chitosan molecular weight did not affect vapour permeability. Samples No. 25 (with chitosan CHL) and No. 22 (with chitosan

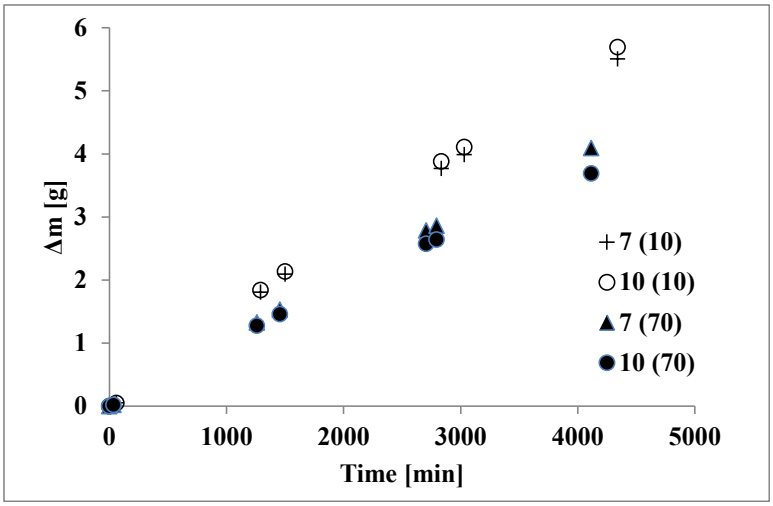

Figure 5. Change in mass ($\Delta m = m_i - m_t$, where m_i is the initial mass [g] and m_t is the mass after time t [g]) over time for chitosan-agar films (No. 7 and 10) at 35°C. The thickness of the films (in μm) is given in round brackets.

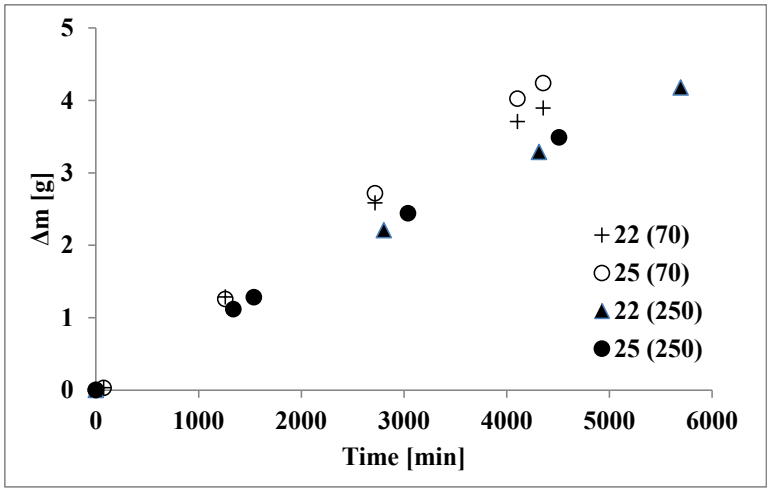

Figure 6. Change in mass ($\Delta m = m_i - m_t$, where m_i is the initial mass [g] and m_t is the mass after time t [g]) over time for samples composed with chitosan-agar-honey films (No. 22 and 25) at 35°C. The thickness of the films (in μm) is given in brackets.

CHM), with a similar thickness, exhibited a similar change in weight over time. The important parameter that influenced vapour pervaporation was the thickness of the prepared gels.

When comparing the two- and tri-component films, the weight change over time was similar for both samples. This proves that the addition of honey to the film composition does not affect the water vapour permeability.

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For a more complete analysis of the vapour permeation process, Table 3 presents the results of the permeability rate (*R*) and constant permeability (*K*) for all films for which water vapour permeability was studied. These results confirmed the previous conclusions based on the analysis of mass change over time. As already discussed, film thickness affected the measures. The composition of the analysed samples did not affect vapour permeability. *R* and *K* decreased as the thickness increased: the thinnest films (10 μm) had the highest values of these parameters.

Table 3. Analysis of the vapour permeability process for the studied films.

No.	Ratio of components				*L*	*A*	*R*	*K*
	2 wt% CH		2 wt% AG	50 wt% H	[μm]	[cm^2]	[$g \times min^{-1}$]	[$g \times cm^{-2} \times min^{-1}$]
	L	M						
7	2	-	1	-	10	5.72	1.3×10^{-3}	2.2×10^{-4}
10	-	2	1	-	10	5.72	1.3×10^{-3}	2.2×10^{-4}
7	2	-	1	-	70	5.72	1.0×10^{-3}	1.8×10^{-4}
10	-	2	1	-	70	5.72	9.0×10^{-4}	1.7×10^{-4}
22	-	2	1	0.5	70	5.72	9.0×10^{-4}	1.6×10^{-4}
25	2	-	1	0.5	70	5.72	1.0×10^{-3}	1.7×10^{-4}
22	-	2	1	0.5	250	5.72	7.0×10^{-4}	1.3×10^{-4}
25	2	-	1	0.5	250	5.72	8.0×10^{-4}	1.3×10^{-4}

Abbreviations: ***l*** – thickness of films [μm]; ***A*** – active surface area of films [cm^2]; ***R*** - permeability rate [$g \times min^{-1}$]; ***K*** – constant permeability [$g \times cm^{-2} \times min^{-1}$]; **CH** – chitosan; **L** – low molecular weight; **M** – medium molecular weight; **AG** – agar; **H** - honey.

3.3.4. Antimicrobial Properties of Films

The bactericidal activity of the selected films (No. 7, 10, 22 and 25) against well-known pathogens – *S. aureus, B. cereus, E. coli* and *P. aeruginosa* – was studied (Fig. 7). The studied films were not toxic to the gram-negative bacteria (*E. coli* and *P. aeruginosa*) and sporulating gram-positive *B. cereus* rods. The only bacterium that was sensitive to bactericidal effect of the films *S. aureus*. Film No. 10 had the highest bactericidal activity, with a 90 ± 5% cell mortality rate (in CFU, a reduction of 1 $\log_{10}$). Films No. 7 and No. 22 had the smallest killing effect of the studies gels: the mortality rate reached 75 ± 4% (a 0.61 $\log_{10}$ reduction; $p < 0.05$) and 47 ± 4% (a 0.33 $\log_{10}$ reduction, $p < 0.05$), respectively.

Based on the results obtained, it can be concluded that gram-positive bacteria (especially *S. aureus*) were more sensitive to the bactericidal effect of the tested films. These results are consistent with earlier data [33–36], which had demonstrated that chitosan shows higher antibacterial activity against gram-positive than gram-negative bacteria.

Recent data in literature have characterized chitosan as bacteriostatic rather than bactericidal [37], although the exact mechanism is still being discussed, and several other factors may contribute to the antibacterial action [38]. Three models of antibacterial activity have been proposed: (1) the interaction between positively charged chitosan molecule and negatively charged microbial cell membranes (in this model the interaction

is mediated by the electrostatic forces between the protonated NH^{+3} groups and the negative residues on the membrane surface) [39]; (2) the binding of chitosan with microbial DNA leads to the inhibition of messenger RNA (mRNA) and protein synthesis [40, 41]; and (3) metal chelation [42, 43].

The lack of bactericidal activity in the case of tri-component samples can be explained by, for example, the destruction of the antibacterial properties of honey by high temperature (during the preparation of films or during its '*processing*'). High temperature inhibits glucose oxidase activity (a by-product of the reaction carried out by this enzyme is hydrogen peroxide, which is responsible for the antibacterial properties of honey) [18]. The presence of honey reduces the chitosan weight in the film, so this reduces the amount of another potential antibacterial ingredient in this material.

The weak antibacterial activity of the studied films can be explained by the applied research method. Chitooligosaccharides, which are soluble in water, are considered to be bactericidal [44]. The obtained results suggest that no soluble bactericidal chitosan 'fragments' were released into the medium and therefore no biocidal activity was observed.

Several researchers have reported that acidic pH increases the bactericidal effect of chitosan, especially against gram-negative bacteria [39, 45]. Our experiments were carried out at pH 7.2–7.5, which may also cause a slight inhibition in the growth of the bacteria tested.

Another explanation of this phenomenon may be the architecture of microbial cellular envelopes. The cytoplasmic membrane of gram-positive bacteria is surrounded by a porous layer of peptidoglycan and lipoteichoic acid, and this type of cell wall structure allows various molecules to cross this barrier and get inside the cell. *S. aureus* has a relatively permeable cell envelope and perhaps this bacteria may be susceptible to the

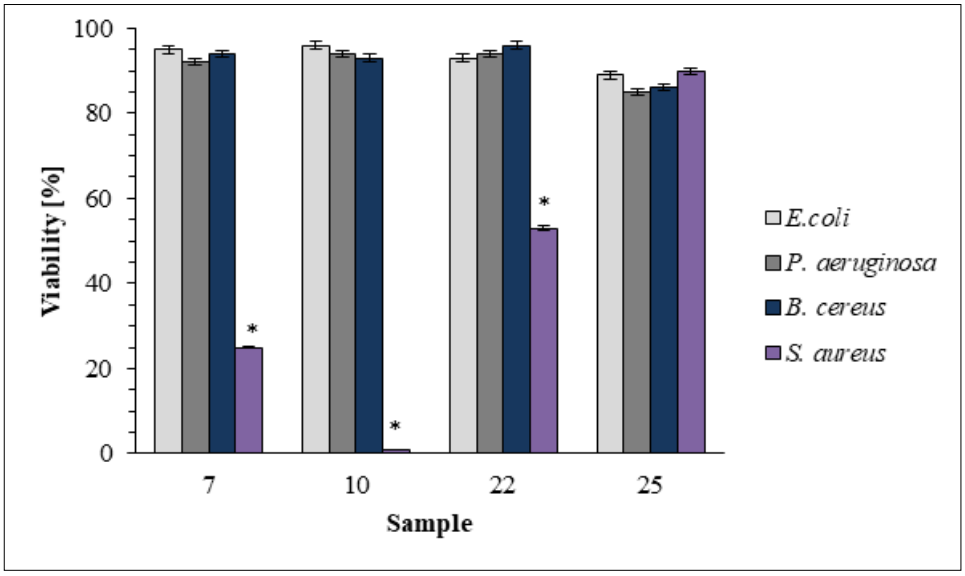

Figure 7. The effect of the selected films (samples designated as 7, 10, 22 and 25) on the viability of gram-positive and gram-negative bacteria. The bars represent the mean, with the error bars indicating the standard error of the mean. Statistical analysis was performed with Student's *t*-test; asterisks indicate a significant difference ($p< 0.05$). (***S. aureus*** **–** *Staphylococcus aureus, ,* ***E. coli*** *– Escherichia coli*, ***B. cereus*** *– Bacillus cereus,* ***P. aeruginosa*** *– Pseudomonas aeruginosa)*

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small amounts of 'soluble' chitosan that may enter the cells. The cell envelope of gram-negative bacteria consists of the inner membrane (a symmetrical phospholipid bilayer), the periplasm containing peptidoglycans and the outer membrane (lipopolysaccharides). This structure results in a complex electrostatic behaviour of bacteria, which is a key factor for controlling transport of chemical compounds [46]. The detailed explanation of our observations requires additional research.

4. Conclusions

The current research aimed to develop new dressing materials, especially active ones that can support the healing processes, possess antibacterial properties and will reduce the wound healing time and improve patient comfort. Hence, this paper examined several gels composed from agar and chitosan with and without honey or bee pollen. The method used to obtain films was very important, e.g. the order in which the components were added, the temperature of the individual components and of course the ratio of ingredients.

Organoleptic analysis identified the material that could be used as an active wound dressing. Almost all films obtained from the combination of chitosan and agar were coherent, flexible and transparent. The addition of bee pollen to the composition of the resulting films diminished their properties: the resulting films were fragile, stiff, inflexible and opaque. Chitosan-agar films with bee pollen did not meet the requirements for active dressings. While the addition of honey improved the flexibility and stretchiness of the resulting films, it reduced their transparency. Films with honey adhered better to the skin and were more plastic than two-component films. It seems that films with honey, due to these features, might be better active dressings than chitosan-agar films. The best two-component films had a chitosan to agar weight ratio of 2:1. They had good coherence; were flexible and transparent; and had high water absorption. The tri-component film with a 2:1:0.5 weight ratio of chitosan, agar and 50 wt% honey had the best composition. However, tri-component films absorbed less than two-component films. All selected films were vapour permeable. The vapour permeability was influenced by the thickness of the films, but not by their composition (molecular weight of chitosan or presence of honey). The selected films inhibited *S. aureus*, although the two-component gels were superior to the tri-component gels. The lower inhibition of samples with honey probably results from the preparation method. The tri-component dressing preparation needs to be improved so as not to deactivate the antibacterial properties of honey.

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