FISH COLLAGEN AND CHITOSAN MIXTURES AS A PROMISING BIOMATERIAL FOR POTENTIAL USE IN MEDICINE AND COSMETIC INDUSTRY

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Abstract

For the last three decades, an increasing interest in new materials based on blends of two or more polymers has been observed. Fish skin collagen and chitosan are constantly highly popular among scientists. This study aimed to obtain thin films from mixtures of low and medium molecular weight chitosan with fish collagen in three different ratios and examine their features for potential use in medicine and cosmetic industry. Polymer blends in ratios 25:75, 50:50, and 75:25 were made to obtain thin films. The infrared spectroscopy, mechanical properties study, contact angle measurements, topographic imaging, and swelling test were used to characterize the features of the films. A statistical appraisal of the results was conducted with the Q-Dixon's test. The infrared spectroscopy analysis showed that in the IR spectra of the examined biomaterials, there are shifts in the bands positions proving intermolecular interactions between collagen and chitosan in the blends. The mechanical properties in the mixtures were different from those of a single biopolymer film. Hydrophilicity and polarity of the blends decrease with the increasing collagen content, which may suggest that the adhesion to the skin will be enhanced. The surface topography of the obtained films varies depending on the ratio of biopolymers in the mixtures. The swelling tests indicated that chitosan absorbs more water than collagen. The properties of the films made of collagen-chitosan mixtures vary depending on the molecular weight of chitosan and the content of each biopolymer in the blend.

Keywords: *collagen, chitosan, biomaterials, blends, medicine, cosmetics*

Introduction

Chitosan is a cationic copolymer obtained by the chitin alkaline deacetylation process. It is constructed of 2-amino-2-deoxy-D-glucopyranose units, which are mostly devoid of acetyl groups and connected with a β-1,4-glycosidic bond [1,2]. Chitosan is present in some fungi species' cell walls but its content is much lower than in the case of chitin [3]. In the medical and cosmetic industries such properties of chitosan as biodegradability, biocompatibility, non-toxicity, mucoadhesiveness, anti-tumor, and antibacterial activity are the most desired. The possibility of processing chitosan in many different forms, such as thin films, membranes, nanoparticles, hydrogels, and scaffolds, is also of significance.

Collagen is the most abundant protein in the human body, where it plays an important role in providing the strength and right structure maintenance of the tissues and creating a scaffold for internal organs [4-6]. Currently, 29 types of genetically different collagen are known [7]. In the human body, type I, II, and III are primarily found [4]. Due to the risk of typical hoggery and cattle zoonotic diseases transmission, as well as religious aspects, the interest in the alternative for mammalian collagen increased [8,9]. The attention focused on fish waste which makes up about 50-70% of the seafood production [9,10]. In favor of using fish for collagen production speaks the 75% content of this protein in a fish body. Skin, head, scales, bones, fins, air bladders, and other entrails can be used in the extraction process of this biopolymer [11]. Due to the lower hydroxyproline content, fish collagen is marked by a significantly lower denaturation temperature in comparison to mammalian collagen. In this regard, collagen extracted from silver carp (Hypophthalmichthys molitrix) stands out. It shows quite a high denaturation temperature in contrast with other fish species [10,12].

Compared to their pure components, mixtures of chitosan and collagen gain unique mechanical and structural properties [13]. The materials obtained from the mentioned blends are bioresorbable, elastic, amenable to further modifications, and have higher resistance to enzymes than pure collagen [14]. The fact that after combining both biopolymers there is still a possibility to obtain different forms such as thin films, membranes, hydrogels, and sponges is also an eminent advantage. All of these features make collagen-chitosan mixtures, as well as the polymers that create them, widely applicable in the medicine and cosmetic industries [7].

Chitosan and collagen blends can mimic the extracellular matrix to a large extent, thus contributing to the growth and proliferation of cells. These properties are used in dermal matrixes production, which are applied for treating full-depth wounds [15]. The influence of the mixtures on matrix mineralization as well as on proliferation and differentiation of osteoblasts finds its use in artificial bones and bone tissue implants production [7]. Chitosan-collagen crossed-linked membranes can be used successfully for artificial cornea fabrication and other tissue engineering purposes [16]. Crosslinking of scaffolds made of mentioned biopolymers makes it possible to use them in cartilage regeneration and reconstruction [17]. The medicine also benefits from chitosan-collagen scaffolds made by the 3D-printing method, which are able to partially recreate the right environment for axons regeneration. This feature is used in the spinal cord injury treatment [18]. By crosslinking chitosan-collagen microspheres obtained in the emulsification process, it is possible to use them in the increasingly popular cytotherapy. These microspheres form a matrix for the 3D macrophage proliferation. The studies indicated that such microspheres increased the proliferation, lifetime, and functionality of the macrophages [19].

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For the cosmetic industry, the mechanical properties, the surface roughness and wettability of polymeric thin films are significant. It is possible to adjust these features by combining collagen and chitosan in different ratios [11]. The film forming properties of the mentioned biopolymers make both of them valuable ingredients for various kinds of cosmetics. They create a thin layer on the skin surface and limit transepidermal water loss, which contributes to better hydration of the epidermis [11]. The chitosan-collagen blend with an addition of hyaluronic acid can make a valuable component of haircare products [20]. Collagen in the form of peptides is used for stimulating the production of this protein in the skin, protecting skin lipids from degradation and preventing photoaging [21,22]. Chitosan-collagen hydrogels can be loaded with vitamins, antioxidants, or other active ingredients, making up the base for various kinds of cosmetic products and reducing the use of preservatives thanks to the antibacterial properties of chitosan [23]. Collagen peptide nanoparticles and chitosan nanoparticles can be used as emulsion stabilizers. The studies showed that emulsions with the addition of the mentioned stabilizer were marked by very high stability during storage [24]. Chitosan-collagen composites in the presence of proper additives are used for making 3D models of the human skin. It is an increasingly used alternative for testing new active substances on animals. Such components also enable the determination of the efficacy of a given ingredient and its potential to cause phototoxic reactions [25].

In this work, the blends of collagen from skin of Silver carp and chitosan were prepared, and their properties were studied. To the best of our knowledge such blends have not been studied yet.

Materials and Methods

Collagen (Col) from the skins of Silver carp (Hypophthalmichthys molitrix) was purchased from WellU Sp. z o.o., Gdynia, Poland. Low molecular weight chitosan (LCh) and medium molecular weight chitosan (MCh) were purchased from Sigma-Aldrich, Iceland. Sodium chloride was purchased from STANLAB, Poland. 99.5%-99.9% acetic acid and glycerine were purchased from CZDA – POCH, Poland. A 0.5M acetic acid solution was prepared by diluting a concentrated acetic acid with distilled water. To obtain the 2% solutions of collagen and both types of chitosan, each biopolymer was dissolved in the previously prepared solvent, which is 0.5M acetic acid. Mixtures of the previously prepared polymeric solutions were made by combining them in three different ratios: 25:75, 50:50, and 75:25. Thin films were obtained by pouring 25 g of each solution onto plastic plates measuring 100x100x20 mm. To investigate the films properties, the following equipment was used: Thermo Fisher SCIENTIFIC PIKE GladiATR NICOLET iS10, Waltham, MA, USA for obtaining IR spectra and OMNIC 9 software to edit the spectra; Zwick/Roell Z 0.5 testing machine, Ulm, Germany for the mechanical properties analysis; goniometer with a system of drop shape analysis (DSA 10, Krüss, Germany for the contact angle measurement; Multimode scanning probe microscope with a Nanoscope IIIa controller (Veeco Digital Instruments, Santa Barbara, CA, USA) working in air atmosphere, room temperature and tapping mode for the topographic imaging and NanoScope Analysis 1.40 software for editing the images. Using the contact angle measurements, the values of surface free energy were calculated by the Owens-Wendt method. A swelling test of the film samples in a PBS buffer solution with a pH of 7.4 was done. The statistical appraisal of the results was conducted with the Q-Dixon's test. All photographs were taken by one of the authors with a POCO X3 Pro smartphone camera.

Results and Discussions

Obtaining biopolymeric films

Photographs of the obtained polymeric films are presented in FIG. 1. The fish collagen film is translucent and mat (FIG. 1a). The chitosan films are clear and glossy (FIGs. 1b, 1f). The films made of chitosan-collagen blends are mat, the higher the collagen content is, the more opaque they are (FIGs. 1c-e, FIGs. 1g-i).

FTIR spectroscopy analysis

For each obtained film, Infrared spectroscopy analysis was done. The IR spectra of examined biomaterials are shown in FIG. 2.

FIG. 2a shows that the FTIR spectra of LCh and MCh are almost the same. The analysis revealed that the amide I peak observed in fish collagen at 1631 cm^{-1} is present in the chitosan spectra at 1640 cm^{-1} in LCh and 1636 cm^{-1} in MCh. The amide II peak appears at 1544 cm^{-1} in the collagen spectra and 1556 cm^{-1} in the chitosan spectra. The amide III peak characteristic for collagen disappears in the spectra of chitosan. In the range between 2000 cm^{-1} and 4000 cm^{-1}, chitosan shows a greater absorbance than collagen.

The analysis indicated that in the IR spectra of the blends (FIGs. 2b-g) the amide A and amide I peaks are shifted in comparison to the corresponding peaks in the pure polymers spectra. The amide B peaks are in the same position as in the chitosan spectra.

The amide II peak in the blend with the 75% MCh content (FIG. 2e) does not change its position in regard to the same peak in the pure collagen spectra. As for the same blend with LCh instead (FIG. 2b), shifts are present regarding the collagen, and LCh spectra. In the remaining blends with LCh (FIG. 2c, 2d) the amide II peak position is consistent with its position in the pure LCh spectra.

In the 75:25 (FIG. 2e) and 50:50 MCh-Col blends (FIG. 2f), there is no peak of the CH_2 group present in the fish collagen spectra. Only in the 25:75 MCh-Col blend (FIG. 2g), the CH_2 peak appears without a significant shift. As for the LCh-Col blends, the shifted CH_2 peak is present when the LCh content comes to 25% (FIG. 2d) and 50% (FIG. 2c). In all of the blends, the CH_2 peak does not change its position with regard to the pure LCh, and MCh blends spectra.

The amide III peak appears in the blends spectra at the higher wavelength number; the higher the chitosan content in the given mixture is. At the 75% collagen content (FIGs. 2d, 2g) the amide III peak has a position similar to that in the pure collagen spectra.

The C-O-C group peaks present in the chitosan spectra do not exist in the pure collagen spectra due to the absence of a glycosidic bond in this protein's structure. The C-O-C peaks appear in all the blends spectra without significant shifts, except the blends with a 25% LCh and MCh content, where the peak at 1071 cm^{-1} is shifted and the blend with a 75% LCh content, where the 1029 cm^{-1} is shifted.

The changes observed in the peaks positions are indicative of interactions between fish collagen and chitosan as well as of good miscibility of the mentioned polymers in each studied ratio. The effect of these interactions is the formation of new hydrogen bonds between the carboxylic, amine, and hydroxyl groups present in the studied biopolymers. Furthermore, in the blends, ionic interactions might appear between protonated amine groups of chitosan and anionic groups of the collagen.

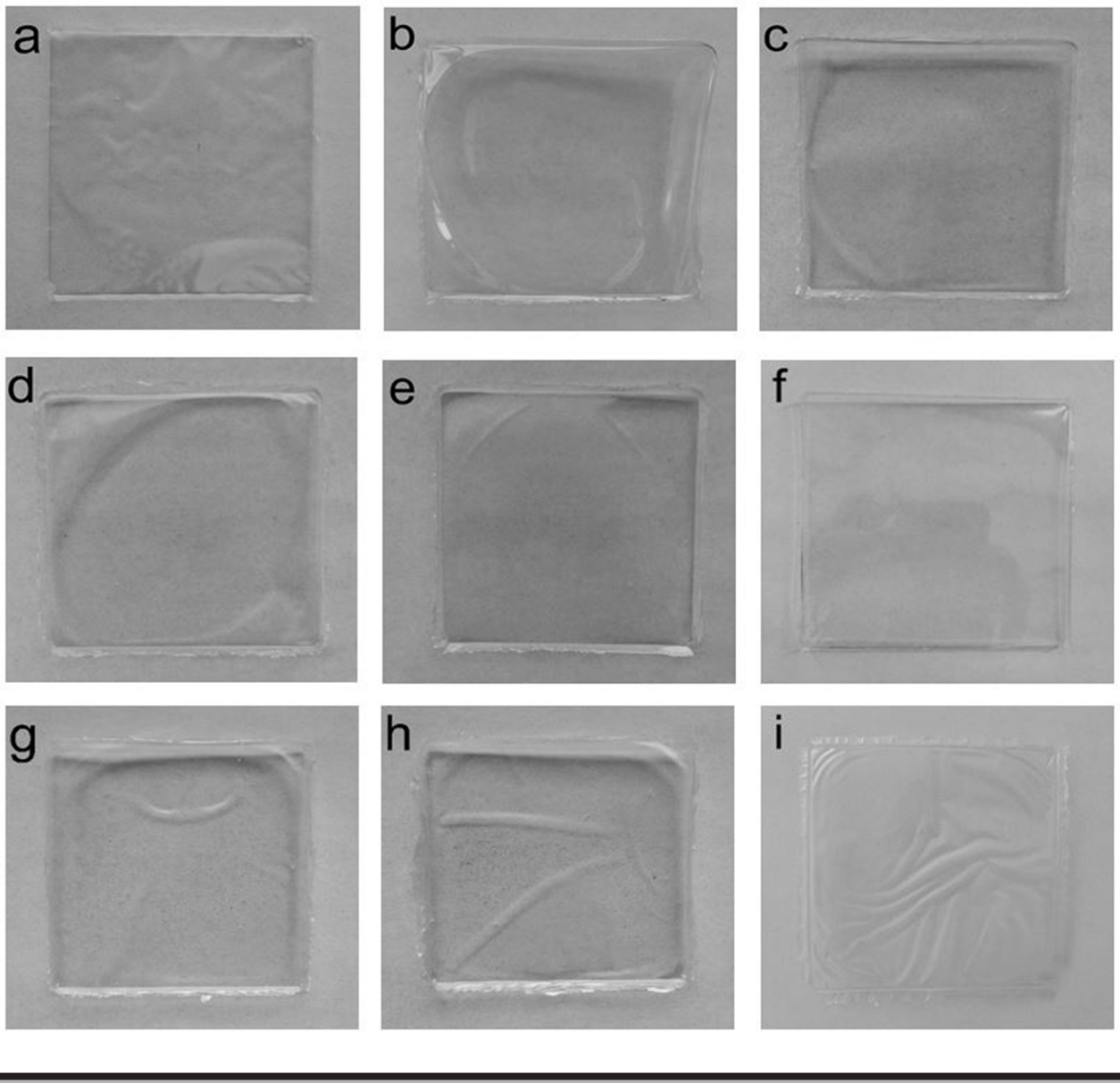
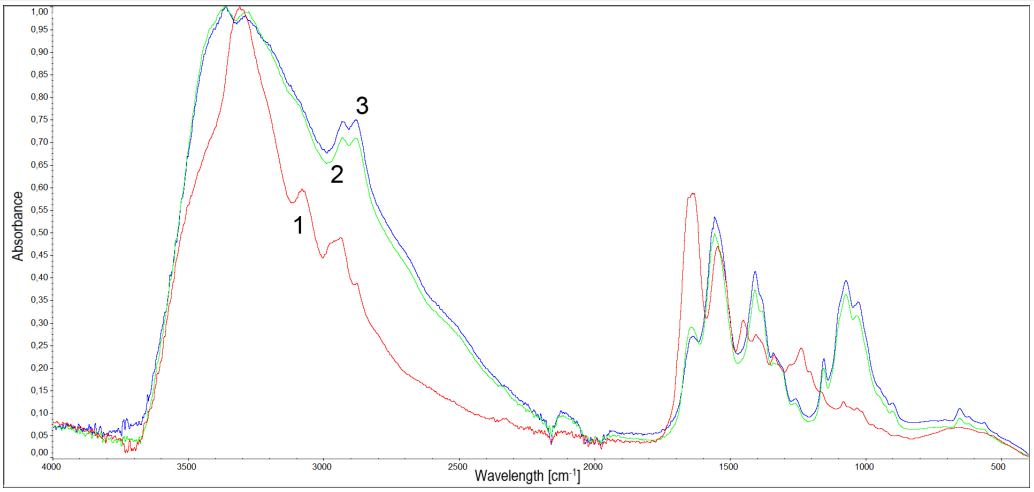


FIG. 1. The photographs of the polymeric thin films:
a) Col;
b) LCh;
c) 75:25 LCh-Col blend;
d) 50:50 LCh-Col blend;
e) 25:75 LCh-Col blend;
f) MCh;
g) 75:25 MCh-Col blend;
h) 50:50 MCh-Col blend;
i) 25:75 MCh-Col blend.

a)


Absorbance
Wavelength [cm-1]
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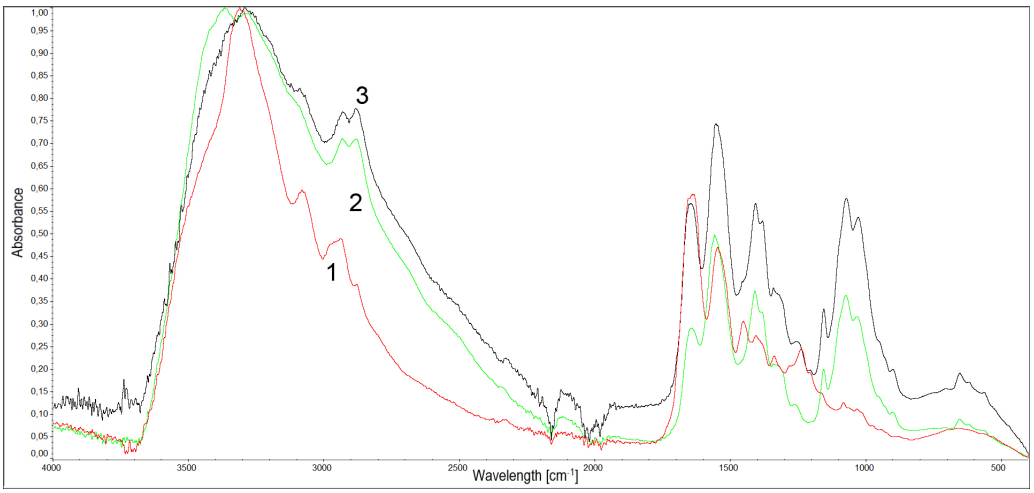
2
3

b)


Absorbance
Wavelength [cm-1]
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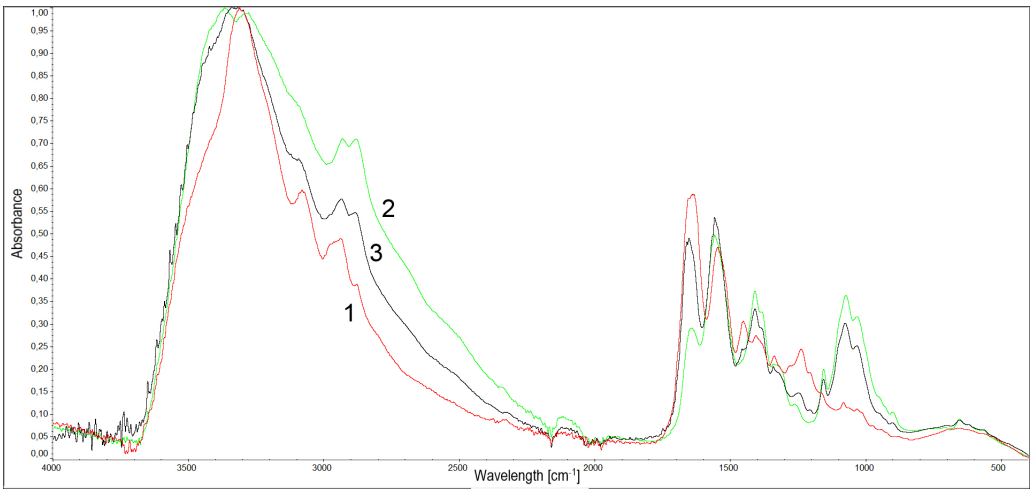
c)


Absorbance
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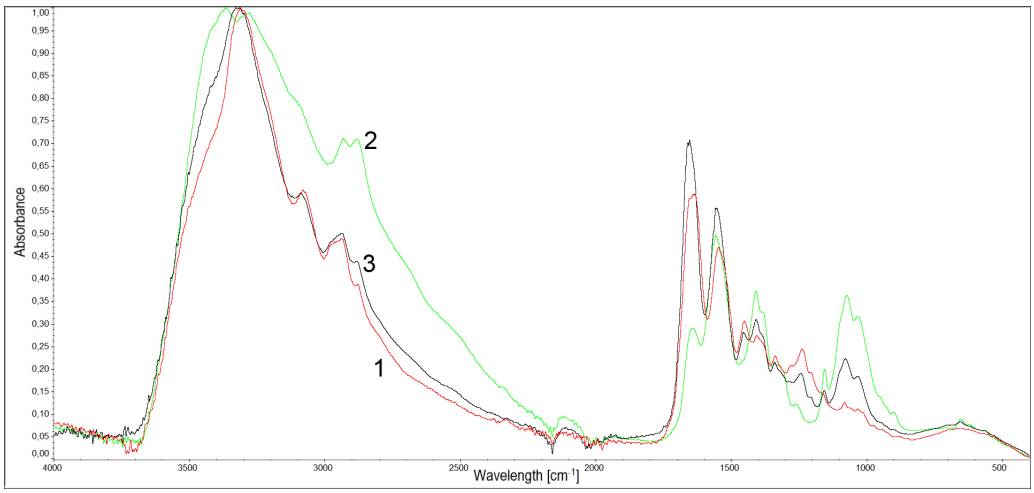
d)


Absorbance
Wavelength [cm-1]
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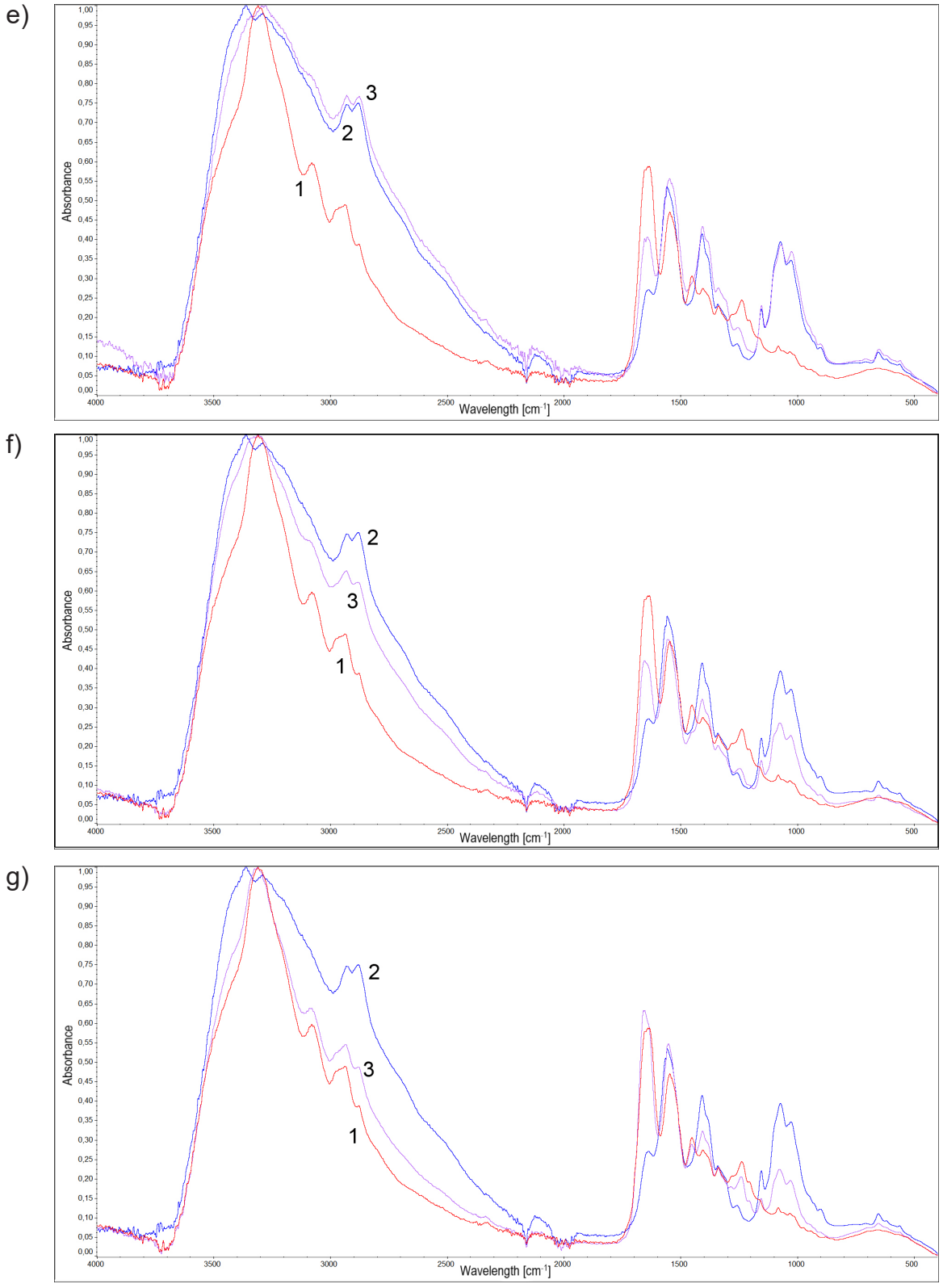

FIG. 2. The FTIR spectra of:
a) Col (1-red), LCh (2-green), MCh (3-blue);
b) Col (1-red), LCh (2-green), 75:25 LCh-Col blend (3-black);
c) Col (1-red), LCh (2-green), 50:50 LCh-Col blend (3-black);
d) Col (1-red), LCh (2-green), 25:75 LCh-Col blend (3-black);
e) Col (1-red), MCh (2-blue), 75:25 MCh-Col (3-violet);
f) Col (1-red), MCh (2-blue), 50:50 MCh-Col blend (3-violet);
g) Col (1-red), MCh (2-blue), 25:75 MCh-Col blend (3-violet).

TABLE 1. The characterization of mechanical properties of collagen, chitosan and chitosan-collagen blends thin films.

Material	E_{mod} [GPa]	F_{max} [MPa]	Elongation at F_{max} [mm]	F_{Bruch} [N]	Elongation at break [%]
Collagen	2.29 ± 0.87	89.17 ± 19.05	1.65 ± 0.78	23.80 ± 5.34	6.59 ± 3.16
LCh	2.90 ± 0.47	76.45 ± 10.61	0.94 ± 0.13	26.20 ± 1.63	4.78 ± 1.69
75LCh/25Col	1.78 ± 0.33	69.40 ± 16.94	2.36 ± 0.87	33.98 ± 2.27	11.03 ± 1.93
50LCh/50Col	1.33 ± 1.03	86.56 ± 21.91	0.94 ± 0.41	31.79 ± 8.33	3.77 ± 1.64
25LCh/75Col	2.58 ± 0.67	99.31 ± 7.55	2.18 ± 0.33	30.99 ± 1.36	8.71 ± 1.33
MCh	2.63 ± 0.71	98.00 ± 6.10	1.19 ± 0.16	35.54 ± 4.69	13.29 ± 8.01
75MCh/25Col	1.47 ± 0.73	75.54 ± 5.03	1.18 ± 0.08	28.62 ± 2.19	6.00 ± 1.45
50MCh/50Col	0.99 ± 0.54	75.41 ± 9.94	1.45 ± 0.31	28.94 ± 0.64	6.58 ± 1.72
25MCh/75Col	1.79 ± 0.66	84.87 ± 6.85	1.47 ± 0.53	28.99 ± 3.34	5.88 ± 2.12

TABLE 2. The glycerine and diiodomethane contact angle and surface free energy values for the collagen, chitosan and chitosan-collagen blends.

Material	Contact angle Θ (glycerine) [°]	Contact angle Θ (diiodomethane) [°]	IFT (s) [mJ/m^2]	IFT (s,P) [mJ/m^2]	IFT (s,D) [mJ/m^2]
Collagen	60.5	56.6	37.43	15.13	22.27
LCh	72.4	58.1	31.95	8.11	23.84
75LCh/25Col	69.1	46.5	37.40	7.11	30.32
50LCh/50Col	71.6	54.5	33.57	7.63	25.94
25LCh/75Col	75.6	53.8	32.81	5.48	27.33
MCh	81.7	60.6	28.53	4.17	24.36
75MCh/25Col	75.3	59.1	30.67	6.81	23.86
50MCh/50Col	81.7	65.5	26.38	5.19	21.19
25MCh/75Col	85.3	65.1	25.83	3.59	22.24

Mechanical properties study

For each film obtained in this research, mechanical properties were measured. The results are presented in TABLE 1.

The results of the mechanical properties study indicated that the film made of pure LCh was the least deforming, ergo the stiffest. The 50:50 MCh-Col blend film was marked by the highest deformation and the lowest stiffness. The film with a 25% LCh content withstood the highest stress, while the film made of pure MCh withstood the highest breaking force. The 75% LCh film was the least resistant to the stress and the collagen film was the least resistant to the breaking force. In many cases, the values of standard deviation were high which made it difficult to decide which of the films was the most resistant to breaking. The elongation in millimetres is not proportional to the elongation percentage, ergo the conclusion about the elasticity of the studied materials is not unequivocal.

The observed variance might be caused by a slightly different sample mass and by a disparate mass of the polymers and the blends on the plates as well as by some differences in clamping the samples in the testing machine.

Contact angle measurements

Determining the contact angle informs about the quality of a material surface. The surface properties, such as roughness and wettability, are important in cosmetic and biomedical applications of biopolymer films. Wettability, i.e. the amount to which a liquid can spread on a surface, is determined by the intermolecular forces between the surface and the liquid.

TABLE 2 shows the results of the thin films surface wettability study. All of the examined biopolymer samples were marked by a glycerine contact angle below 90°, ergo it might be deduced that they are easily wettable and hydrophilic. The diiodomethane contact angle values were lower than in the case of glycerine and also lower than 90°. The reason for that might be the diiodomethane lower surface tension which lowers the contact angle. It is indicative of the more hydrophobic character of the samples, despite the earlier stated hydrophilicity. Furthermore, in the case of both liquids, the contact angles rose in the following order: collagen, LCh, and MCh. The addition of fish collagen to chitosan films caused the increase of a glycerine contact angle with the increasing content of this protein. In the case of diiodomethane, the tendency was similar, although at the lowest collagen content the contact angle value decreased.

The collagen film was marked by the lowest glycerine contact angle, ergo it was the most polar and hydrophilic. It was also confirmed by the highest value of the polar part and surface free energy. The high value of the polar part is indicative of the high polar, hydrogen, inductive, or acid-base interactions contribution between the biopolymers' molecules in the blends. In view of the mentioned properties, the collagen film might be marked by the highest adhesion to the skin which is hydrophobic. The 25:75 MCh-Col film is the least polar and hydrophilic one, which is also confirmed by the highest glycerine contact angle and the lowest value of the polar part and surface free energy. It is indicative of the mentioned film's lowest adhesion to the skin.

Diiodomethane is a liquid with a preponderant dispersive part. The film with a 75% LCh content was marked by the lowest diiodomethane contact angle, which speaks to the fact that there is the highest dispersive interactions contribution between the biopolymers in this blend. It finds its confirmation in the highest value of the dispersive surface free energy. The least dispersive interactions appear in the 50:50 MCh-Col film, which is also confirmed by the lowest value of the dispersive part.

Topography imaging

The images of the surface topography structure of the studied thin films are presented below (FIG. 3). The values of the surface roughness are listed in TABLE 3.

The surface of the studied thin films is very diverse, whereby the films made of pure biopolymers were marked by a much lower roughness than the films made of the polymer blends (TABLE 3). The AFM analysis indicated that the collagen addition to the chitosan films modifies the roughness without a specific relationship. The films made of MCh-Col blends have a less rough surface than the films made of LCh-Col blends, which might be indicative of better miscibility, and thereby of a higher homogeneity of the first ones.

TABLE 3. The R_q and R_a parameters of collagen, chitosan and chitosan-collagen blend thin films.

Material	Rq [nm]	Ra [nm]
Collagen	7.78	6.10
LCh	5.92	2.60
75LCh/25Col	63.3	45.4
50LCh/50Col	70.8	56.7
25LCh/75Col	67.3	52.9
MCh	5.19	3.99
75MCh/25Col	34.2	23.5
50MCh/50Col	28.2	22.1
25MCh/25Col	58.4	45.7

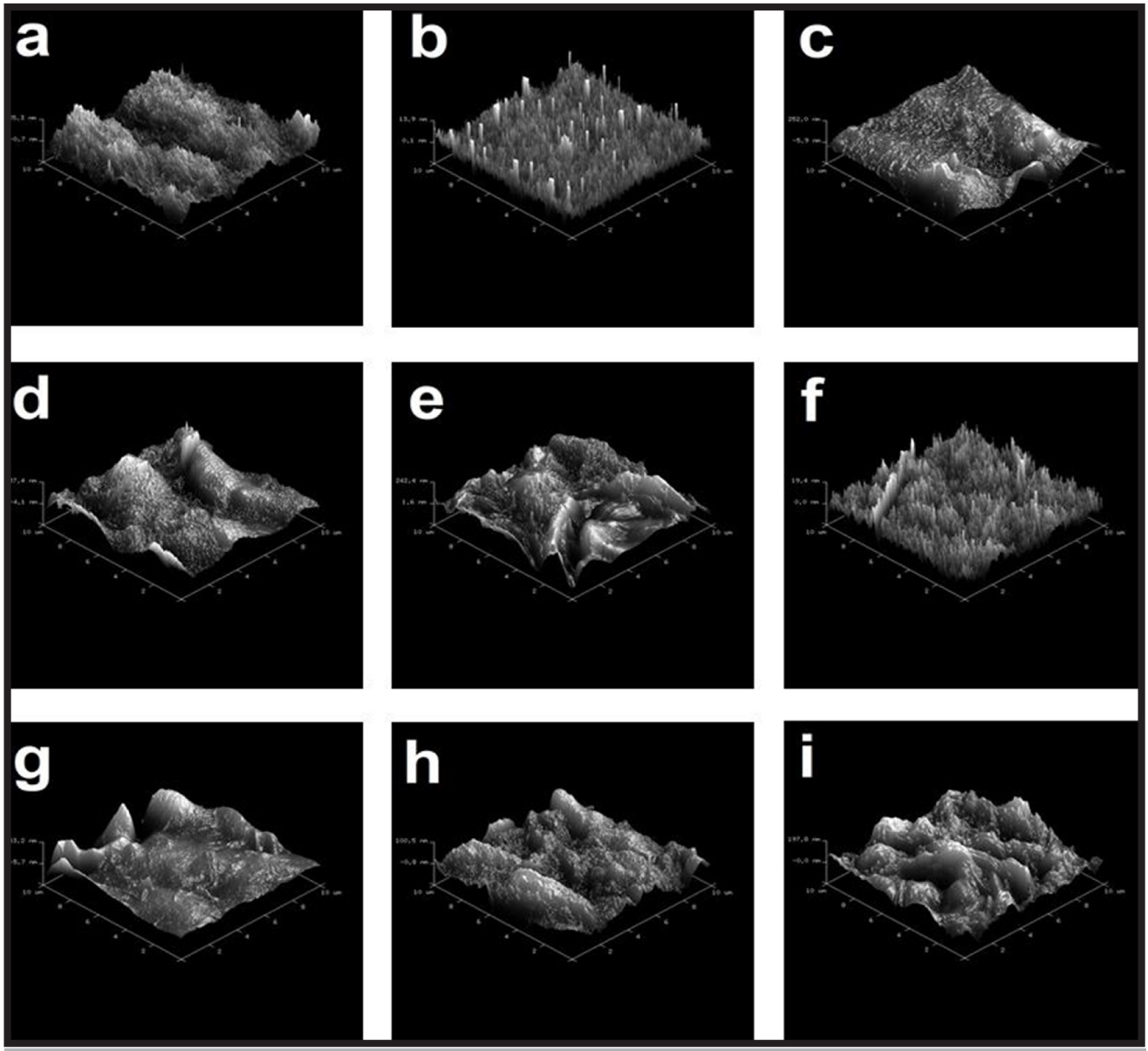

FIG. 3. The AFM images of: a) Col; b) LCh; c) 75:25 LCh-Col blend; d) 50:50 LCh-Col blend; e) 25:75 LCh-Col blend; f) MCh; g) 75:25 MCh-Col blend; h) 50:50 MCh-Col blend; i) 25:75 MCh-Col blend.

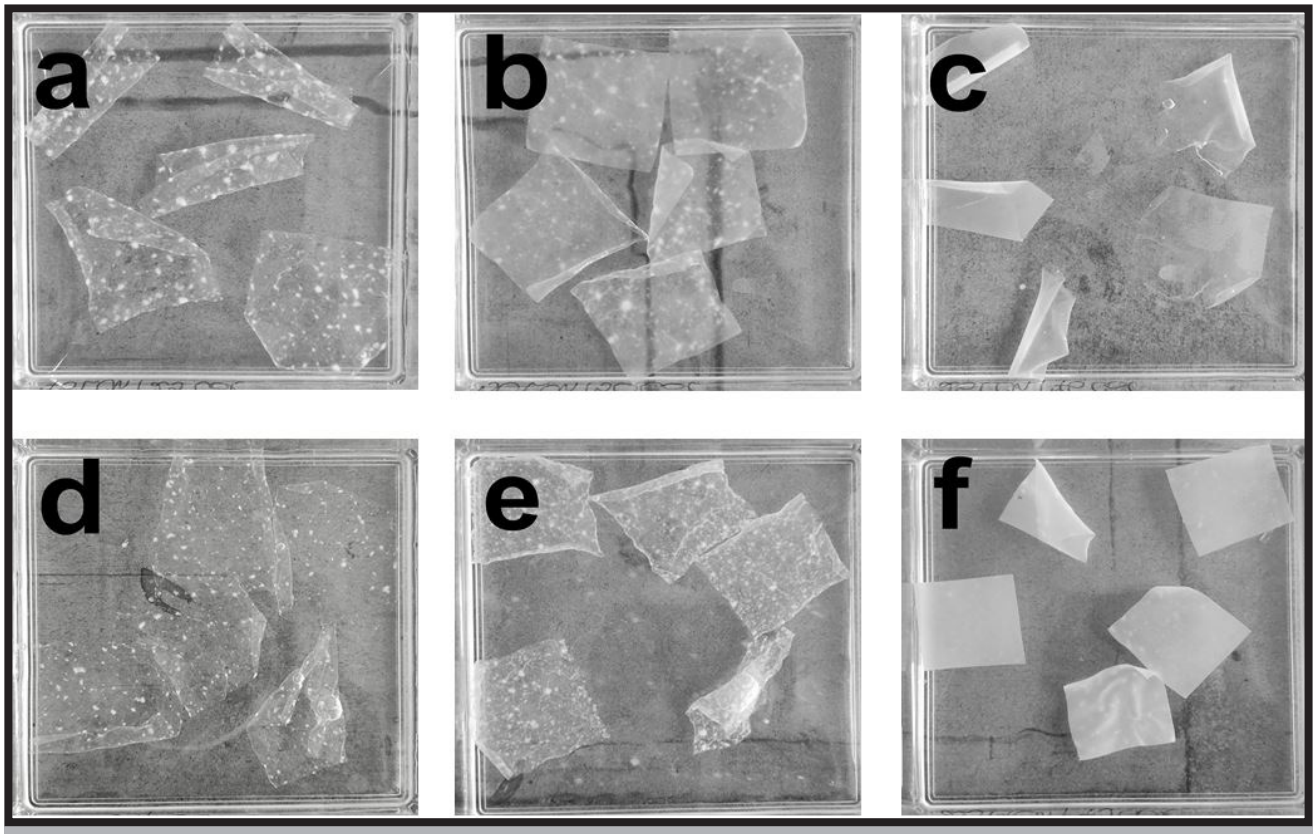

FIG. 4. The photographs of thin films samples after 1 h in PBS solution: a) 75:25 LCh-Col; b) 50:50 LCh-Col; c) 25:75 LCh-Col; d) 75:25 MCh-Col; e) 50:50 MCh-Col; f) 25:75 MCh-Col.

Swelling test

The pictures of thin films samples in PBS solution after soaking for 1 hour are presented below (FIG. 4).

The swelling tests indicated that chitosan absorbs more water than collagen, as the chitosan film samples increased their size while soaking much more than the fish collagen samples. After 1 h of soaking in PBS solution, almost all the samples fell apart while taking them out of the solution. Only a few ones made of collagen and blends with 75% collagen content remained integral for up to 4 h.

Conclusions

The interactions between collagen from skins of Silver carp and chitosan have been confirmed by IR spectra. The addition of fish collagen to chitosan films variously alters their mechanical properties. All of the studied thin films show good wettability and a hydrophilic character. The fish collagen film is marked by the highest hydrophilicity and polarity, while the 25:75 MCh-Col film is marked by the lowest hydrophilicity and polarity. The roughness of the films made of the blends is higher than the roughness of the films made of pure biopolymers and it changes irregularly, depending on the collagen content. The surface roughness of the thin films might be modified by altering the biopolymers ratio in the blend or the chitosan molecular weight.

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