CHARACTERISTIC OF FISH COLLAGEN FILMS CROSS-LINKED WITH GLUTARALDEHYDE

Karolina Kulka-Kamińska[1]*, Patrycja Brudzyńska[1], Alina Sionkowska[1], Katarzyna Lewandowska[1], Łukasz Piwowarski[2]

[1] Department of Chemistry of Biomaterials and Cosmetics, Nicolaus Copernicus University in Toruń, ul. Gagarina 7, 87-100 Toruń, Poland
[2] SanColl Sp. z o.o.,
ul. Juliusza Słowackiego 24,
35-060 Rzeszów, Poland
*e-mail: kkulka@doktorant.umk.pl

Abstract

Collagen is a valuable biopolymer in many fields, especially in biomedical sciences. Thanks to its biodegradability and high biocompatibility, it is a desirable material for applications that require contact with the human body. There are many sources of collagen, of which marine-origin collagen has become an important one in recent times. Pure collagen has poor stability and is sensitive to the effects of heat and other external factors. The cross-linking process can improve the properties of collagen materials. Many different methods of cross-linking can be distinguished, including chemical ones. In this study, we were concerned to obtain collagen films modified with glutaraldehyde (GTA). The influence of this additive on the chemical, mechanical, swelling, and hydrophilic properties of the biopolymeric matrix was evaluated. Two different concentrations of collagen were used, as well as three different concentrations of GTA. Results of the analysis showed that the properties of the obtained films were affected by the addition of even a small amount of cross-linker. Spectroscopic measurements indicated minor changes that reflect interactions between GTA and the collagen matrix. Mechanical tests showed changes for modified samples in values of tensile strength, breaking force, and elongation at break. The hydrophilicity decreased slightly for films with GTA. The durability of the modified samples in the swelling test increased. Differences between 1% and 2% collagen films with additives were also observed. The GTA-obtained fish collagen films can be promising materials for biomedical applications.

Keywords: *collagen, glutaraldehyde, cross-linking, polymer films, marine sources*

Introduction

Collagen is a relevant biopolymer that occurs in living organisms. This fibrous protein constitutes 30% of vertebrates' bodies and supports the structural integrity and functions of tissues [1], and helps maintain mechanical and structural functions in organisms [2]. This polypeptide builds human skin, tendons, cartilage, bone tissue, and blood vessels [3,4].

There are 29 types of collagen that are genetically different. They differ in the sequence of the primary amino acids in their polypeptide chains [4]. Types I, II, and III are the main types of collagen in humans [5]. Type I mainly builds the skin and represents 80-95% of skin collagen; it is a crucial component of the extracellular matrix (ECM). Type II is predominantly found in cartilage, while type III is often found along with type I and usually makes up approximately 15% of skin collagen [6,7]. The helical structure, also known as the triple helix domain (COL), is the most characteristic feature of all types of collagen [8]. Collagens are highly organized supramolecular structures composed of three polypeptide chains with the characteristic amino acid sequence Gly-X-Y (Gly – glycine, X and Y usually represent proline, lysine, hydroxyproline, and hydroxylysine) which create tropocollagen. Tropocollagen molecules aggregate in fibrils that are subsequently cross-linked (maturation process) and finally create collagen fibrils [7,8]. Collagen biosynthesis involves a few steps: triple helix formation and post-translational modifications - enzymatic glycosylation, lysine hydroxylation, proteolytic cleavage of procollagen and formation of intra- and intermolecular cross-links [8]. Collagen triple helix is unique among other biopolymers due to the hydrogen bonds distribution that occurs only between polypeptide chains; there are no direct intramolecular hydrogen bonds [8].

Collagen as a biopolymer is a biocompatible, bioactive, and biodegradable material useful in biomedical applications. It is the best known protein-based material for skin scaffolds and wound dressings [9]. It can also be used in drug delivery systems, injectable dispersions, scaffolds for bone regeneration, cardiology, ophthalmology, and urology treatments [10]. The collagen used in these applications is of animal origin. Bovine collagen from skin and bone is of major industrial importance [10]. Another important source is the pig skin and bones. This collagen is very similar to human collagen, so it is much safer than the bovine in terms of allergenic potential [11], however, it is important to look for alternative sources due to the risk of zoonotic diseases [10]. Both bovine and porcine collagen carry the risks of contamination and, in addition, in the case of porcine collagen, religious issues remain a problem. In contrast to the collagen used in the industry, collagen obtained from rat tails is of great importance among the collagens used by scientists [9]. The search for new sources of collagen other than mammalian collagen is becoming more and more common [5]. A popular and convenient solution is marine-origin collagen, in particular from fish, derived from waste, which is safer and constitutes a part of the zero-waste trend. There are many advantages of marine sources, for example, high collagen content, environmental friendliness, low biological contamination and toxins content, minor regulatory problems, and low immunogenicity [10,12]. In addition to fish, other sources of collagen include jellyfish, sponges, starfish, octopus, and prawn [10,13]. For this purpose, almost all parts of the fish are used, including skin, bones, fins, and scales [9,10].

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The fish that are often used for collagen extraction are as follows: cod *Gadus morhua*, white carp *Hypophthalmichtys molitrix*, yellowfish tuna *Thunnus albacares*, swordfish *Xiphias gladius*, tilapia *Oreochromis niloticus*, carp *Cyprinus carpio*, grouper *Epinephelus marginatus*, monkfish *Lophius piscatorius* [10,14-16]. Fish collagen contains less hydroxyproline in the polypeptide chain than mammalian collagen, which influences the denaturation temperature, which is much lower than in mammalian collagen. However, compared to collagen from other species of fish, collagen from silver carp is more resistant to high temperatures [17]. Slightly different characteristics, including denaturation temperatures, may occur in different species of marine organisms [16].

In terms of improving the physicochemical properties of collagen, some treatments can be performed, for example, the cross-linking process. Pure collagen is a structure that requires stabilization due to its poor resistance to high temperatures and other external factors [3]. Chemical and physical methods of cross-linking can be distinguished. Stabilization of the collagen structure can be achieved by the formation of new bonds, in the case of chemical cross-linking, functional groups are involved in this process [3]. Cross-linking is useful in the production of collagen scaffolds with properties similar to those of human tissues and organs [18]. The most commonly used chemical cross-linking agents are glutaraldehyde (GTA), dialdehyde starch (DAS), genipin, and EDC-NHS [3,18,19].

Glutaraldehyde (GTA) as a cross-linker provides enzymatic and thermal stability, as well as mechanical strength of the collagen biomaterial [18]. This chemical compound added to collagen generates a stiff network and causes the creation of covalent bonds between polypeptide chains [3]. Unfortunately, as a result of the toxicity of this substance, it cannot be used in all types of applications. It is important to use the appropriate concentration of GTA to maintain the balance between the desirable physicochemical properties of collagen-based material and low toxicity [18].

Researchers used different concentrations of glutaraldehyde as a cross-linking additive and different methods of treatment. Peng et al. prepared collagen sponges stabilized by GTA using the vapour cross-linking method [20]. They prepared more than 20 ml of glutaraldehyde solutions at different concentrations (5%, 10%, 15%, 20%, 25%) and placed in the lower part of the desiccators. The samples were exposed to GTA for up to 48 h. The results showed that complete cross-linking can be obtained with GTA concentrations of 20% and 25%, at lower concentrations cross-linking is not observed for this method [20]. In other research with dermal sheep collagen (DSC), scientists used 0.5% (w/w) of GTA solution to rinse the samples for 30 min. The results showed that this cross-linking compound can be very effective in the stabilizing of collagen-based materials [21]. The cross-linking mechanism of GTA is still under investigation. However, lysine or hydroxylysine is involved in the formation of cross-links with GTA [3].

In this study, we investigated the effect of the addition of glutaraldehyde (GTA) on the properties of fish collagen films. The films were obtained by the solvent casting method. To characterize the biopolymer films, a series of tests were carried out. Infrared spectroscopic analysis, mechanical test, swelling and degradation analysis, as well as contact angle measurements, were performed. The influence of different concentrations of GTA as an additive as well as two concentrations of collagen on the properties of the film was evaluated.

Materials and Methods

Preparation of biopolymer solution and films

1% and 2% (w/w) collagen solutions were prepared by dissolving freeze-dried collagen sheets from skins of silver carp fish (obtained by SanColl Sp. z o.o., Poland) in 0.1M acetic acid (Stanlab; Lublin, Poland). The obtained solutions were shaken and then stirred on a magnetic stirrer. The complete dissolution process took several days.

Collagen films were obtained following the solution casting method. Pure collagen films were prepared by pouring 30 g of solution onto a polystyrene plate (10 cm x 10 cm). GTA solution was prepared (12 mg/mL). To obtain the films, 1 ml, 2 ml and 5 ml of glutaric dialdehyde (GTA; Acros Organic; Geel, Belgium) were added to the weighted initial collagen solutions, respectively. The modified solutions were stirred on a magnetic stirrer for 5 min and then poured onto polystyrene plates and allowed to dry.

FTIR spectroscopy

Infrared spectra were recorded using Nicolet iS10 equipment with a diamond ATR prism crystal accessory (Thermo Fisher Scientific, Waltham, MA, USA). Measurements were conducted with a resolution of 4 cm^{-1}, in the wavenumber range of 400-4000 cm^{-1}, and using 64 scans.

Mechanical analysis

The mechanical properties of the samples were examined using a mechanical testing machine (Z.05, Zwick and Roell, Ulm, Germany). The following parameters were determined: Young's modulus (GPa), tensile strength (MPa), breaking force (N), and elongation at break (%). The speed starting position was 50 mm/min, the speed of the initial force was 5 mm/min, and the initial force was 0.1 MPa. The TestXpert II 2017 program was used to collect the data, the results were presented as average values and standard deviation. The average thickness of the collagen films was approximately 0.02 and 0.03 mm for 1% and 2% collagen, respectively.

Swelling and degradation test

Collagen films were cut into squares of weight about 0.0050 g. Each type of sample (five squares in one series) was placed in a container with 50 ml of phosphate buffer saline (PBS). The weight measurements of the samples were conducted after 1 h, 2 h, 4 h, 8 h, 24 h, 48 h, 72 h, 1 week, and 2 weeks. The swelling degrees were calculated using the equation:

$$\text{swelling} = \frac{(m_t - m_0)}{m_0} \cdot 100\% \qquad [\%]$$

m_t – weight of the material after immersion in PBS [g]
m_0 – the initial weight of the material [g]

Contact angle

Contact angle measurements of two liquids (glycerine (G) and diiodomethane (D) were performed using a goniometer equipped with drop shape analysis (DSA 10 produced by Krüss, Germany). All measurements were carried out at room temperature. The result of the contact angle for each sample is an average value of 10 measurements of individual droplets. The Owens-Wendt method was used to calculate surface free energy.

Results and Discussions

FTIR spectroscopy

In order to examine the chemical structure of the obtained films, IR spectra were made. The spectra of the pure collagen film and samples with additives are presented in FIG. 1 and 2. The infrared spectra show the characteristic bands of collagen. The bands at approximately 3300 cm^{-1} correspond to amide A [22]. Amide B appears at 3060-3080 cm^{-1} and bands at about 2930 cm^{-1} are related to the asymmetric stretching vibrations of $-CH_2$ [22,23]. The intensity of the amide III band that occurs at about 1230 cm^{-1} is related to the triple helical structure [24]. Wavenumbers for subsequent bands are shown in TABLE 1. It was observed that amide I and amide II in each sample are at the same positions. The addition of GTA does not influence the band position of amide I and II, as well as of amide III for 1% collagen. The amide III band in the 2% collagen film is shifted to lower wavenumbers. For amide B, the addition of GTA results in a shift to higher wavenumbers for both collagen concentrations (1% and 2%). A shift of a band attributed to $=CH_2$ stretching vibrations to lower wavenumbers is observed for 1% collagen films with the addition of the cross-linking agent, while for 2% films, there are no significant changes in this band. Small changes occur in amide A for 1% films with additives. The addition of GTA influences the intensity of bands (FIG. 1 and 2).

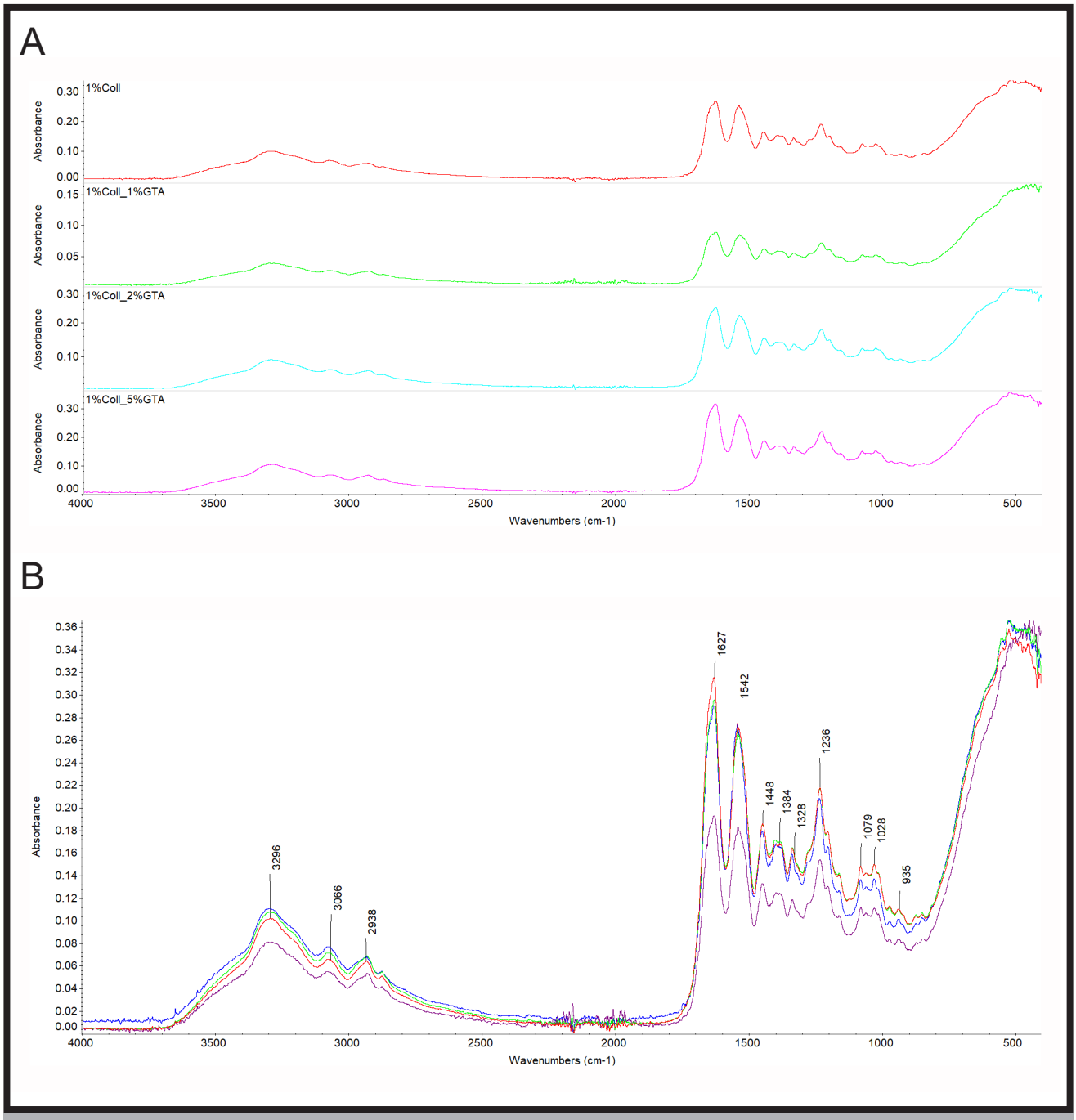

FIG. 1. The IR spectra of 1% collagen films cross-linked with GTA. Spectra are presented in stack (A) and overview (B). Wavenumber values are present for the main bands (B).

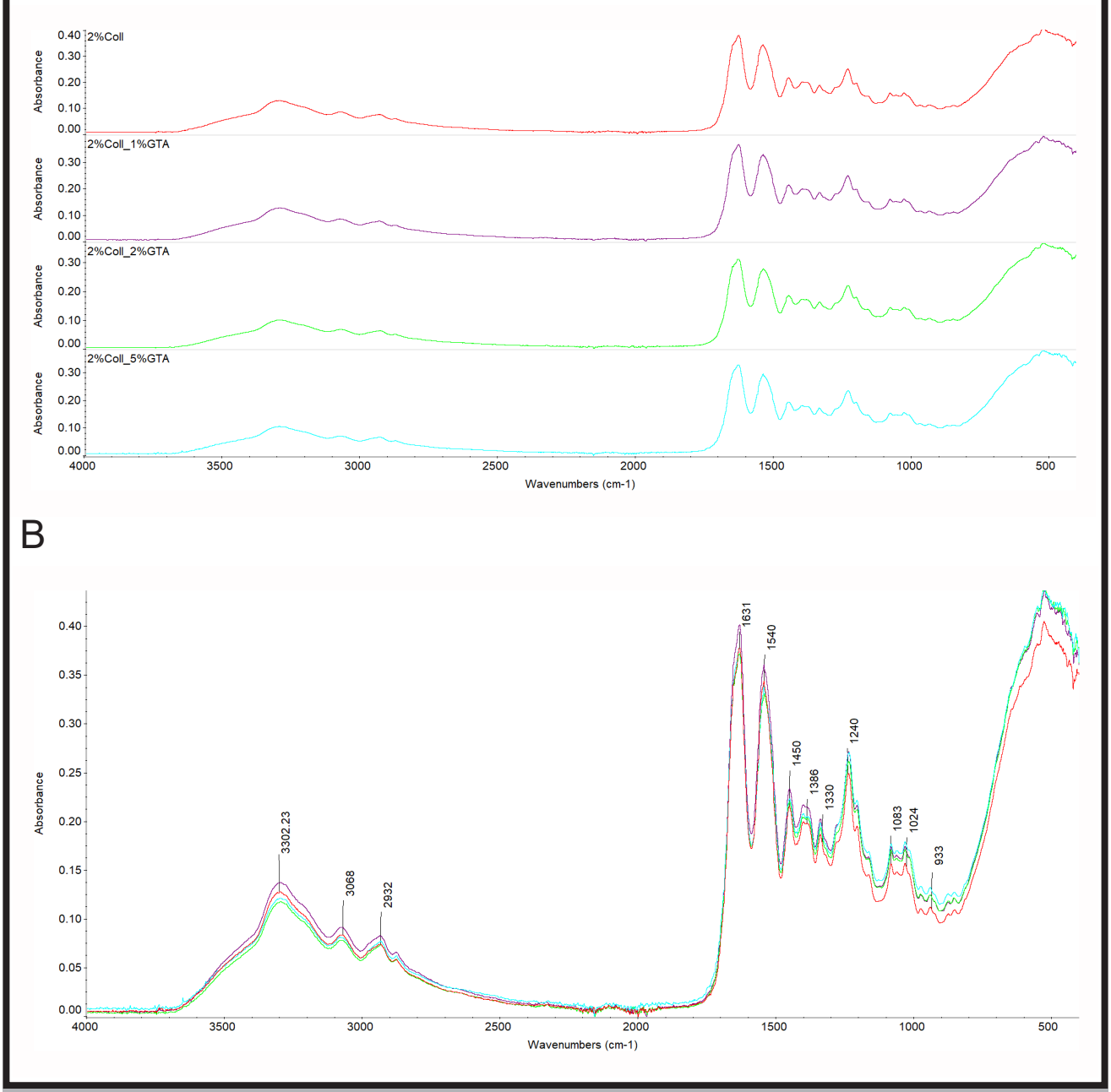

FIG. 2. The IR spectra of 2% collagen films cross-linked with GTA. Spectra are presented in stack (A) and overview (B). Wavenumber values are present for the main bands (B).

TABLE 1. Bands positions in IR spectra of collagen films with and without GTA.

Functional group vibrations		Amide A	Amide B	$=CH_2$	Amide I	Amide II	Amide III
Wavenumber [cm^{-1}]	1%Coll	3296	3066	2938	1627	1542	1236
	1%Coll_1%GTA	3286	3076	2931	1630	1540	1232
	1%Coll_2%GTA	3294	3078	2931	1629	1541	1233
	1%Coll_5%GTA	3292	3077	2932	1631	1541	1233
	2%Coll	3302	3068	2932	1631	1540	1240
	2%Coll_1%GTA	3294	3077	2935	1629	1541	1234
	2%Coll_2%GTA	3293	3077	2930	1630	1540	1233
	2%Coll_5%GTA	3298	3076	2930	1632	1541	1232

Mechanical properties

The results of Young's modulus, tensile strength, and elongation at break for thin collagen films without and with different concentrations of GTA are shown in FIG. 3. The concentration of collagen and addition of GTA affect the mechanical parameters of the samples.

Higher values of tensile strength, breaking force, and elongation at break were obtained for 2% collagen films in comparison to 1% films. Among all samples, the highest values of tensile strength, breaking force, and elongation at break were observed for samples with 1% of GTA, while the lowest for 5% GTA for 1% collagen. The latter was therefore the least flexible. One-way ANOVA was employed to examine differences in all mechanical properties between sample groups with different GTA concentrations.

Contact angle

The analysis indicated that all samples were hydrophilic. The addition of a cross-linking agent slightly decreased hydrophilicity. The values of the contact angle for glycerine were increased. The results are presented in TABLE 2 and FIG. 4.

Swelling and degradation

The swelling ratios of collagen films as a function of incubation time are presented in FIG. 5 and 6. The pure collagen films have fallen apart after 4 hours of incubation. The addition of the GTA increased the stability of samples up to 14 days, except for 2%Coll_1%GTA. The swelling degrees of the 2% collagen films are significantly higher than the 1% films. In both cases, the highest values were obtained for samples cross-linked with 1% GTA. The increased amount of cross-linker is responsible for the lower swelling ability.

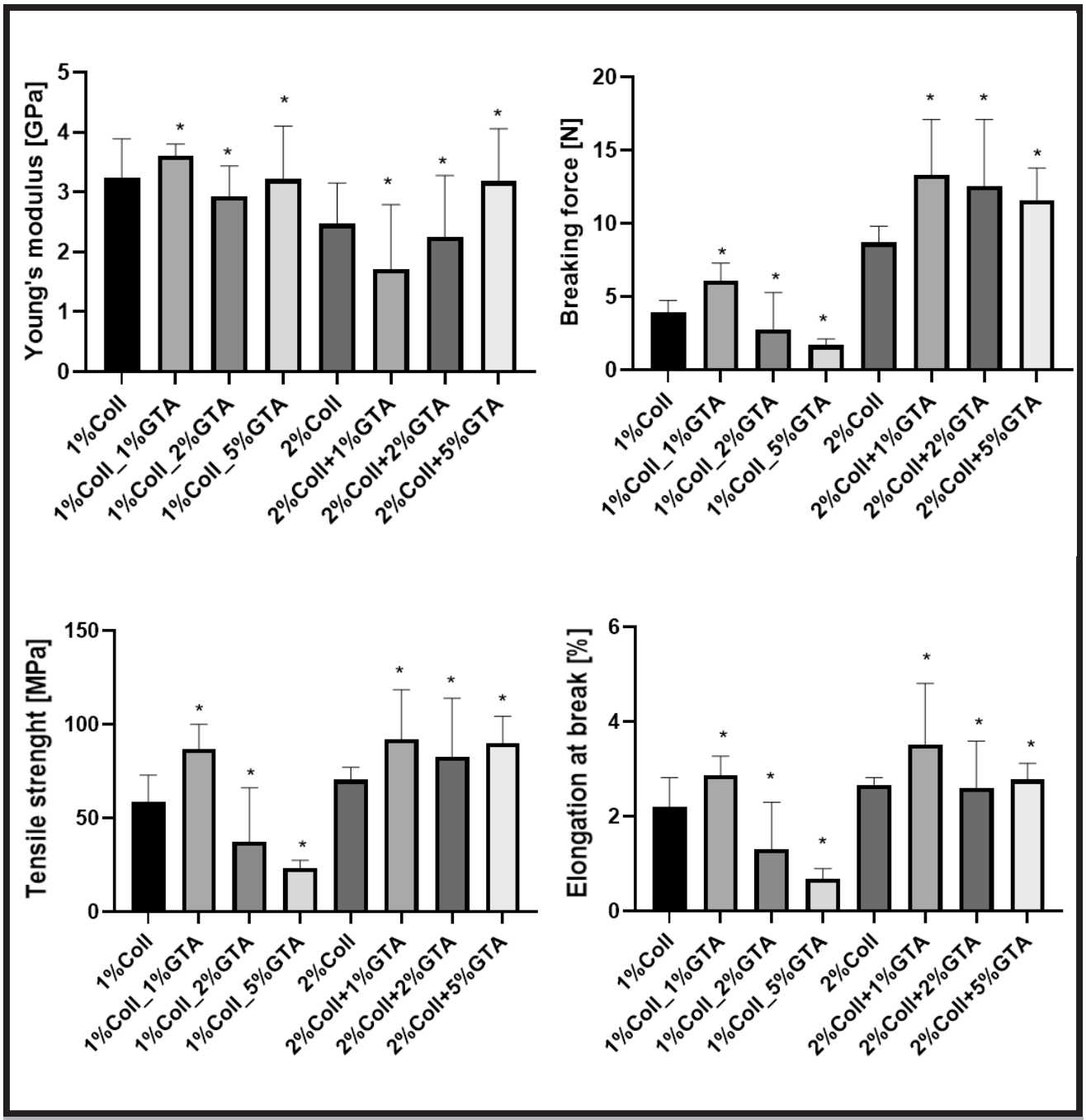

FIG. 3. Mechanical parameters of collagen films cross-linked with GTA; error bars represent standard deviation (SD) (significantly different from the respective control ($p < 0.05$) for all mechanical parameters).

TABLE 2. Contact angle results for collagen films not cross-linked and cross-linked with GTA.

Sample	Θ glycerine [°]	Θ diodomethane [°]	IFT (s) [mJ/m²]	IFT (s, D) [mJ/m²]	IFT (s, P) [mJ/m²]
1%Coll	58.7	36.8	44.01	33.33	10.68
1%Coll_1%GTA	59.8	43.5	41.5	29.89	11.61
1%Coll_2%GTA	63.9	46	39.19	29.37	9.82
1%Coll_5%GTA	62.7	46	39.61	29.1	10.52
2%Coll	61.3	38.5	42.62	33.04	9.58
2%Coll_1%GTA	63.1	44.1	40.12	30.30	9.82
2%Coll_2%GTA	64.5	43.8	39.78	30.81	8.97
2%Coll_5%GTA	63	42.5	40.71	31.21	9.51

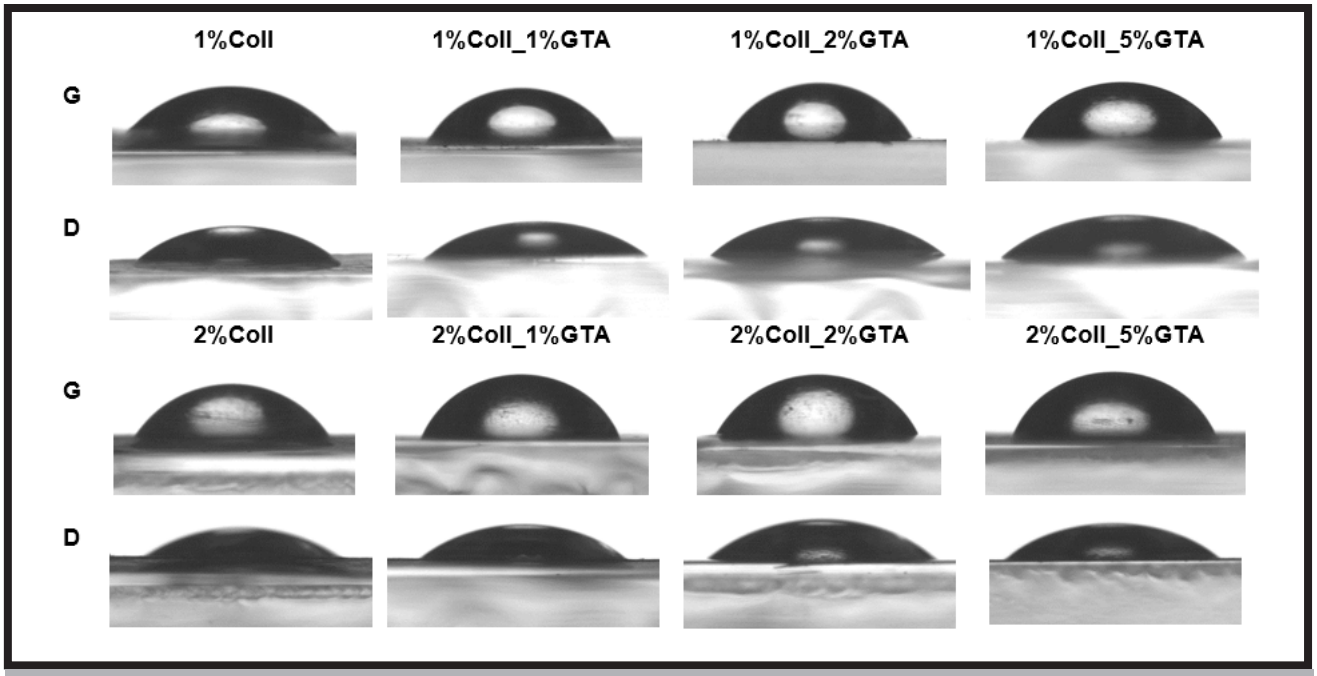

FIG. 4. Pictures of droplets during the contact angle measurement (G – glycerine, D – diodomethane).

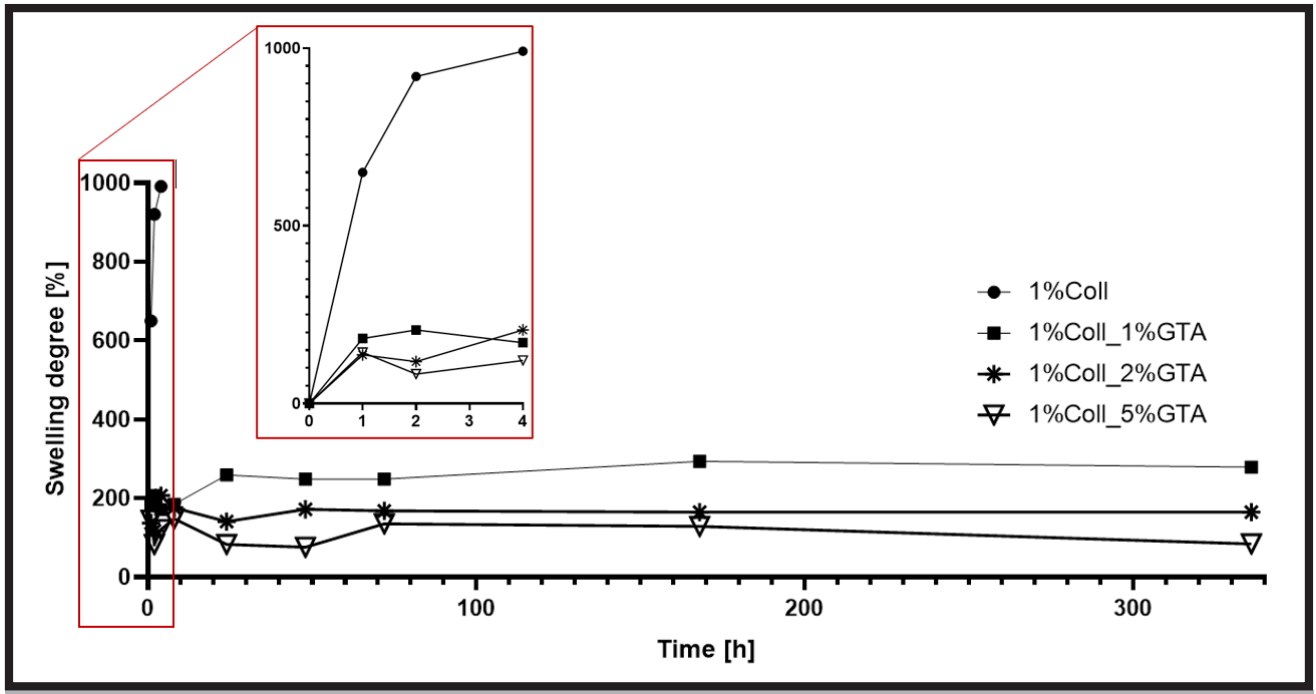

FIG. 5. Swelling degree of 1% collagen films non-cross-linked and cross-linked with GTA.

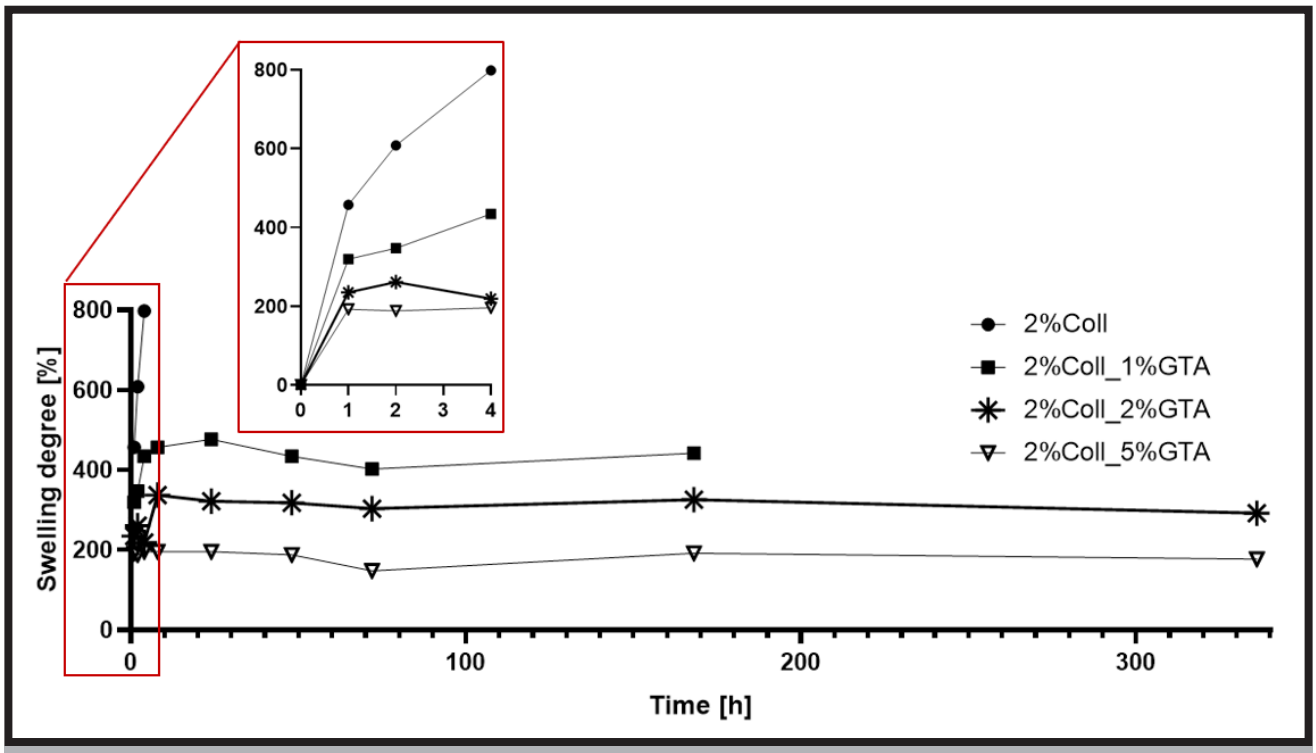

FIG. 6. Swelling degree of 2% collagen films non-cross-linked and cross-linked with GTA.

Conclusions

The results of our research show that even small amounts of glutaraldehyde (GTA) added to the fish collagen change the properties of the films obtained. The IR analysis confirmed small changes in the chemical structure of all samples with GTA, which may be the result of the creation of hydrogen bonds. The addition of GTA causes changes in the intensity of the bands. The addition of GTA to collagen films influences the mechanical properties, swelling ability, and contact angle. 2% collagen films are characterized by lower Young's modulus values and are more susceptible to elongation compared to 1% collagen samples. 1%Coll+5%GTA was the least flexible between all samples studied. The amount of cross-linking agent added in relation to the amount of collagen is crucial for the final properties of the film. Samples containing 2% collagen have a greater amount of polymer with which GTA can interact. The swelling degrees were increased for 2% collagen samples compared to 1% collagen films, which is connected with the amount of biopolymer that can absorb more liquid. Samples modified with GTA were more stable than unmodified collagen films. Both pure collagen samples (1% and 2%) disintegrated after 4 h in PBS. The remaining samples were stable for up to 2 weeks. Swelling behaviour is an extremely important value in the design of active dressings; this is due to the function of wound dressings to collect exudate. Native collagen films had a lower contact angle for glycerine than GTA-modified samples. However, all of the samples were hydrophilic. Fish collagen films modified with GTA may be promising materials for applications in the biomedical field after testing their potential toxicity. In the case of glutaraldehyde, it is important to maintain a balance between the amount of reagent used and the expected results. An advantage of the proposed method is its simplicity and quickness.

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ORCID iD

K. Kulka-Kamińska: https://orcid.org/0000-0002-0825-1774
P. Brudzyńska: https://orcid.org/0000-0002-6646-1534
A. Sionkowska: https://orcid.org/0000-0002-1551-2725
K. Lewandowska: https://orcid.org/0000-0002-8380-4009
Ł. Piwowarski: https://orcid.org/0009-0000-6889-9209

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