THE PROPERTIES OF FISH COLLAGEN FILMS AFTER CROSS-LINKING WITH TANNIC ACID

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Abstract

Over the last three decades, an increasing interest in the preparation of new materials for wound healing has been observed. Collagen is a widely used biomaterial, and especially fish skin collagen is more and more popular among scientists. This study aimed to obtain thin films from native fish skin collagen and collagen cross-linked with tannic acid. Infrared spectroscopy, mechanical test, topographic imaging, and swelling test were used to characterize the features of the mentioned films. Statistical evaluation of the results was conducted with the Q-Dixon test. Infrared spectroscopy analysis showed that in the IR spectra of examined biomaterials, there are slight shifts in band positions after tannic acid cross-linking. The mechanical properties of the cross-linked material were different from those of the native collagen film. The Young's modulus was higher for cross-linked collagen, whereas the elongation at break was lower than for pure collagen. The swelling of the collagen films increased after cross-linking with tannic acid. Swelling tests indicated that collagen cross-linked with tannic acid absorbs more water than before cross-linking. The properties of collagen films were significantly improved after tannic acid cross-linking. All alterations can be a result of collagen cross-linking by tannic acid, probably by forming hydrogen bonds between collagen and tannic acid.

Keywords: *collagen, biomaterials, medicine, wound dressing, cross-linking, tannic acid*

Introduction

Collagen is the most abundant protein in the human body and plays an important role in providing strength and maintenance the right structure of tissues, as well as creating a scaffold for internal organs [1-3]. Currently, 29 genetically different collagen types are known [4], however, the most commonly used in medical applications is type I collagen. In recent years, an increase in the application of fish skin collagen increased [5,6]. It is the result of the transition of several diseases from mammals and also due to some religious aspects existing in some regions of the world. The attention is mainly focused on fish waste, which makes up about 50-70% of seafood production [7-8]. Collagen can be extracted not only from the fish skin, but also from heads, scales, bones, fins, air bladders, and other entrails [8]. Fish collagen has a significantly lower denaturation temperature in comparison to mammalian collagen due to the lower hydroxyproline content [9,10], but collagen extracted from silver carp (*Hypophthalmichthys molitrix*) shows a quite high denaturation temperature in contrast to other fish species [10]. To improve the stability and mechanical properties of collagen, several cross-linking methods can be used [11-19].

Glutaraldehyde is one of the chemical compounds often used to improve the mechanical properties of collagen-based biomaterials [12,13]. Next, genipin is also widely applied for collagen cross-linking [14,15]. This compound is much less toxic than glutaraldehyde and other commonly used cross-linking agents.

The chemical cross-linking of collagen can be performed using 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) [16,17]. This method of cross-linking of collagen leads to the formation of a covalent bond between the carboxylic acid groups from aspartic and glutamic acid. Another cross-linking agent used for collagen modification is dialdehyde starch (DAS).

DAS was used as a cross-linking agent for collagen [18] and gelatin [19] to improve the properties of the materials. DAS aldehyde groups interact with a free amino group of collagen during the cross-linking reaction. As a result, collagen can be intra- and intermolecularly linked via DAS bridges.

Chemical cross-linking is crucial for collagen properties because collagen-based materials are widely used in biomedical and cosmetic industries [20]. It may influence collagen properties for wound healing applications [21-23]. Collagen hydrogels can also be loaded with other active substances making up the base of various kinds of cosmetic products, e.g. beauty masks.

In this work, collagen from the skin of Silver carp was cross-linked with tannic acid and the properties of collagen materials were studied. Based on the results of our previous research on the cross-linking of biopolymers, we assumed that it can improve the collagen properties important for medical applications [24-26]. Despite the level of development of collagen-based wound dressings attained so far, there is still a pressing need for further improvements.

Materials and Methods

Materials and film preparation

Collagen (Col) from skins of Silver carp fish was purchased from SanColl Sp. z o.o., Poland. Acetic acid was purchased from Avantor Performance Materials, Poland. The solution of 0.4 M acetic acid solution was prepared by diluting concentrated acetic acid with distilled water. To obtain a 1% solution, collagen was dissolved in the previously prepared solvent. For cross-linking of collagen, 1% and 2% of tannic acid were added and the solution was mixed for 2 hours. Thin films were obtained by pouring 25 g of each solution onto plastic plates with dimensions 100 x 100 x 20 mm.

FTIR spectroscopy

The interactions between the polymer and the additive were evaluated by Fourier transform infrared spectroscopy using Nicolet iS10 equipment with an ATR accessory and a diamond crystal (Thermo Fisher Scientific, Waltham, MA, USA). For all spectra, 64 scans were recorded in absorption mode, with a resolution of 4 cm^{-1}. OMNIC 9 software was used to edit the spectra.

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Mechanical properties

Mechanical tests were carried out using a mechanical testing machine (Z.05, Zwick and Roell, Ulm, Germany). Young's modulus, tensile strength, and elongation at break were evaluated. Samples were cut in the shape of paddles (width 4 mm in the center). Testing program parameters were as follows: 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. Data were collected using the TestXpert II 2017 program, and results were presented as average values with standard deviation.

Scanning electron microscopy (SEM-EDX)

Surface imaging of the tested polymer samples was carried out using a scanning electron microscope manufactured by LEO Electron Microscopy Ltd. (Model 1430 VP). In addition, an EDX Quantax 200 X-ray spectrometer with a Bruker AXS XFlash 4010 detector was used for spot analysis of the chemical composition of the samples to confirm the presence of zinc.

Atomic force microscopy (AFM)

The microstructure of the polymer samples was analyzed using atomic force microscope images obtained by MultiMode Scanning probe microscope NanoScope IIIa (Digital Instruments Veeco Metrology Group, Santa Barbara, CA).

Swelling measurements

The swelling ratio was measured by immersing the composite fragments in phosphate buffered saline (PBS) solution, pH = 7.4, and citric buffer at pH 5.5. After 1, 2, 4, 8, 12, 24, and 48 hours of immersion, the materials were gently dried by putting them between two sheets of paper and then weighed [27]. The swelling ratios were calculated using the following equation:

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

m_t - weight of the material after immersion in PBS [mg],
m_0 - weight of the material before immersion [mg].
Samples of each type were measured in triplicate.

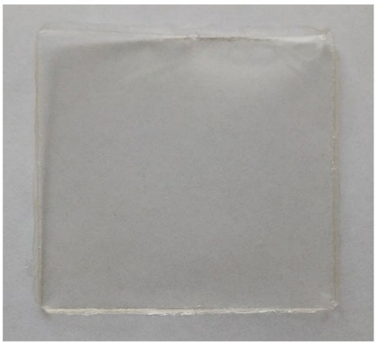

FIG. 1. An example of a film made from fish collagen.

Results and Discussions

Collagen films

After solvent evaporation, we obtained collagen film that could be removed easily from plastic plates. The example of obtained collagen film is presented in FIG. 1. The fish collagen film is translucent and mat. Collagen films cross-linked with tannic acid looked very similar.

FTIR spectroscopy analysis

For pure collagen films and collagen cross-linked with tannic acid, infrared spectroscopy analysis was done. IR spectra of the examined biomaterials are presented in FIG. 2.

FIG. 2 shows the FTIR spectra of collagen films before and after cross-linking with tannic acid. In general, the positions of the main bands in the IR spectra are the same. The analysis showed that the amide I peak observed in fish collagen at 1634 cm^{-1} after cross-linking with tannic acid can be found at 1631 cm^{-1}.

The amide II peak appears at 1544 cm^{-1} in collagen spectra, whereas after cross-linking it was found at 1542 cm^{-1}. The amide III peak characteristic for collagen appears in the spectra of collagen and cross-linked collagen at about 1450 cm^{-1}. In the range between 2000 cm^{-1} to 4000 cm^{-1}, all studied samples showed absorbance due to the presence of amide A and hydroxyl groups.

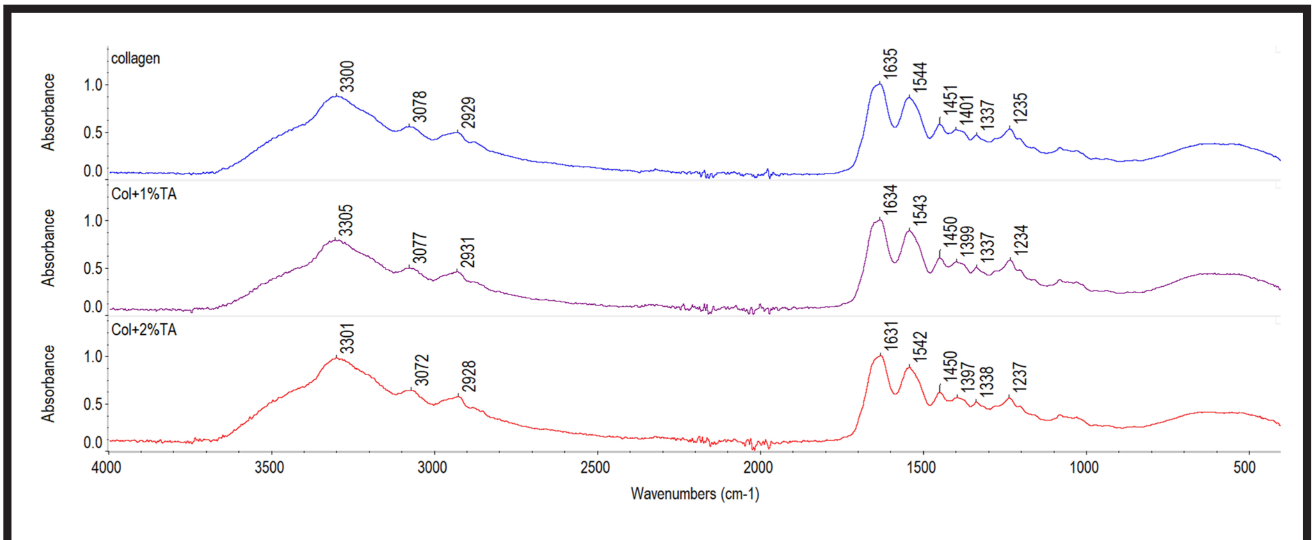

FIG. 2. FTIR spectra of films made of non-cross-lnked collagen films and collagen films cross-linked with tannic acid.

The analysis indicated that in the IR spectra of cross-linked collagen the amide A was shifted to lower wavenumbers. The amide B peaks are in the same position before and after cross-linking of collagen.

The small changes observed in the peak positions may show that the addition of tannic acid influences the interactions between collagen macromolecules, which are mainly via hydrogen bonds. Hydrogen bonds can be formed between collagen molecules and also between collagen and tannic acid molecules.

Mechanical properties

For each film obtained in this research, mechanical properties were measured. The results are shown in FIGs. 3-5.

The results of the mechanical properties measurements indicated that the film made of collagen from fish skin showed a lower Young's modulus than those cross-linked with tannic acid (FIG. 4). The film with a 2% content of tannic acid showed the highest Young's modulus in this research. However, with increasing amounts of tannic acid, the elasticity of the films decreased (FIG. 5). It can be assumed that after cross-linking of fish collagen with tannic acid, the flexibility of the films is smaller. For medical applications such as wound dressing, mechanical properties are crucial because they decide about the possibility of application without destroying. The values of standard deviation may indicate that the films absorb the humidity that is present in the laboratory during the measurements, which could further change their properties. However, the values of mechanical properties are good enough for the applications mentioned above. Nevertheless, another cross-linking agent should also be considered in the future.

Topography imaging

The image of the surface topography and the surface structure of the studied collagen thin film is presented below (FIG. 6). The value of the surface roughness for collagen film was about 8 nm when we consider the Rq value. The structure of the surface observed by AFM for cross-linked collagen films was similar.

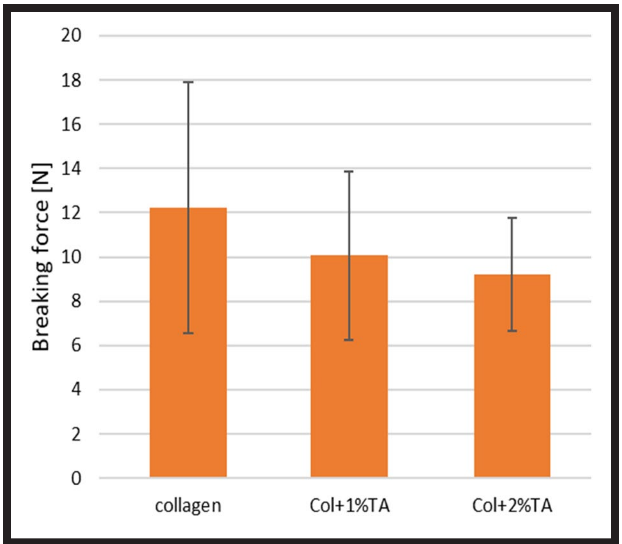

FIG. 3. The breaking force for non-cross-linked collagen films and collagen films cross-linked with tannic acid.

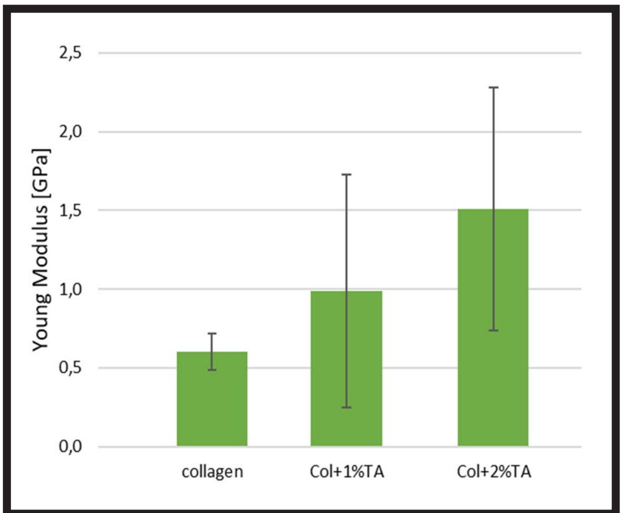

FIG. 4. Young modulus for non-cross-linked collagen films and collagen films cross-linked with tannic acid.

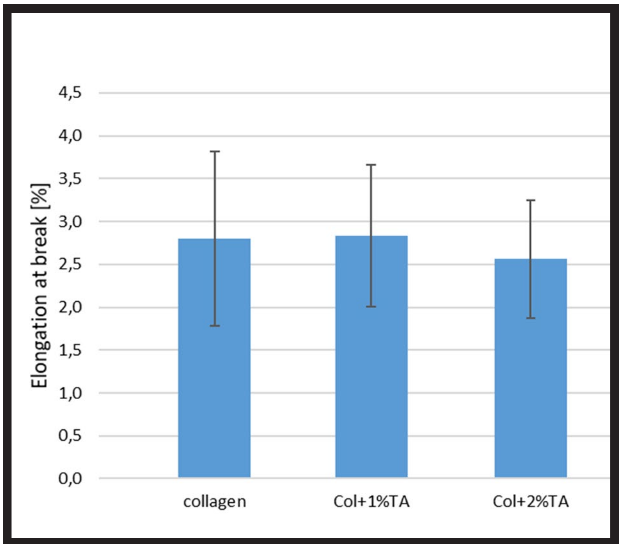

FIG. 5. The elongation at break for non-cross-linked collagen films and collagen films cross-linked with tannic acid.

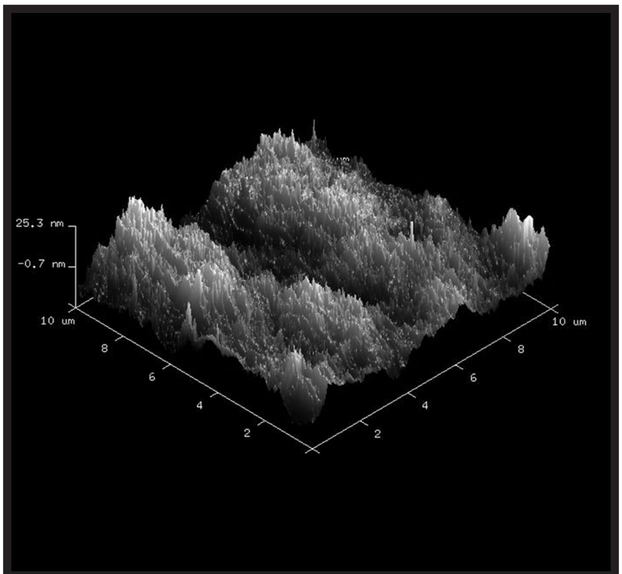

FIG. 6. AFM image of the fish collagen thin film.

Swelling test

Pictures of thin film samples in PBS solution after soaking for several periods are presented below (FIG. 7). The degree of swelling in PBS solution and citric buffer solution (pH 5.5) is shown in FIG. 8 and FIG. 9, respectively.

Swelling tests indicated that all samples absorb water and are stable even after 48 hours. However, after 8 hours of soaking in PBS solution citric buffer, all samples fell apart while being removed from the solution. Only films made of collagen cross-linked with tannic acid remained integral for up to 8 hours. The degree of swelling is greater for collagen cross-linked with tannic acid.

Materials made of collagen and collagen cross-linked with tannic acid were easily wettable by polar solvents such as PBS and/or citric buffer. All materials prepared in this research showed high swelling ability. This is because collagen contains a large number of functional groups capable of binding water [27-29]. The PBS solution with pH = 7.4 may correspond to the pH of blood, so the swelling in this pH can be crucial from a practical point of view. For healthy skin, the pH is about 5.5, so in this research, the swelling properties were also measured in a citric buffer in the above-mentioned pH to compare the swelling behavior in two different pH.

Although the biocompatibility test was not performed in this research, we have previously found, that cross-linking of biopolymer blends with tannic acid leads to biocompatible materials [26,30].

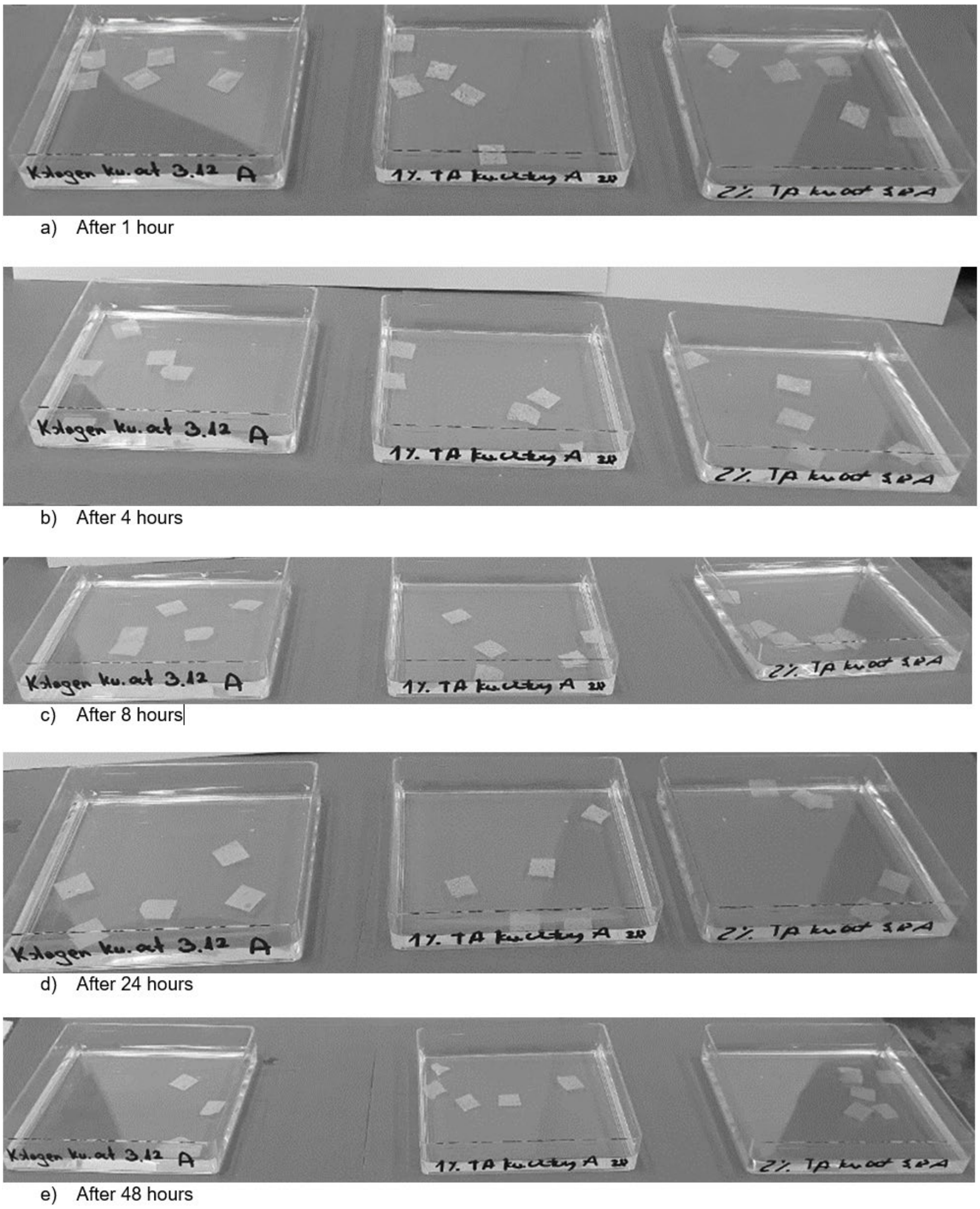

FIG. 7. Swelling of collagen films in PBS (from the left: non-cross-linked collagen, collagen + 1% of tannic acid, collagen + 2% of tannic acid).

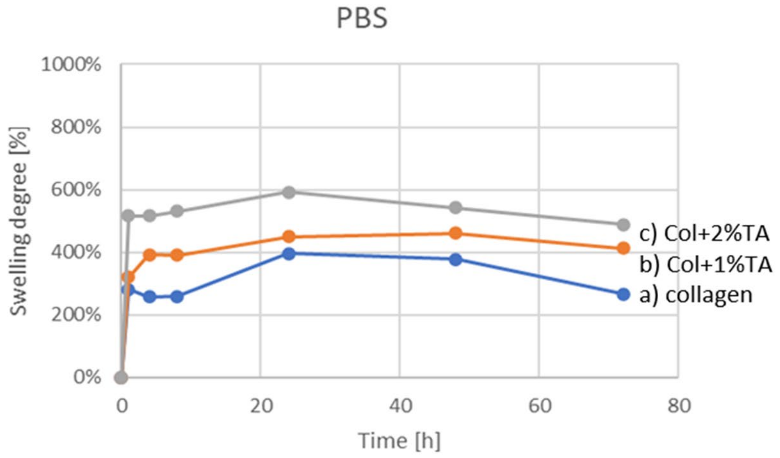

FIG. 8. Swelling of collagen films in PBS: a) non-cross-linked collagen; b) collagen + 1% of tannic acid; c) collagen + 2% of tannic acid.

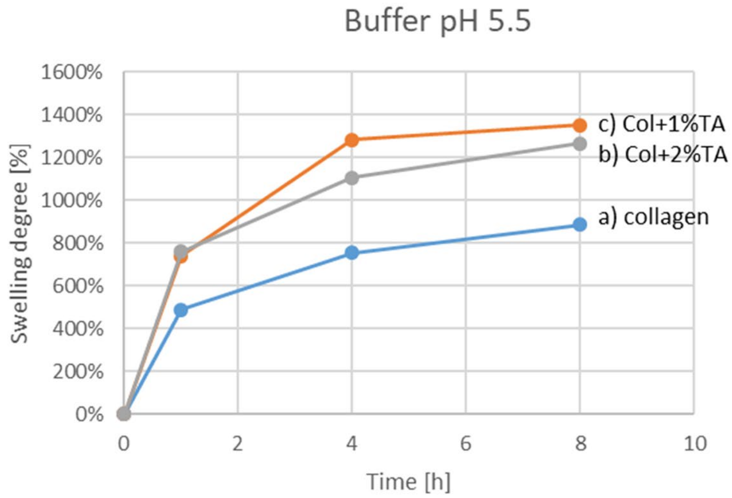

FIG. 9. Swelling of collagen films in citric buffer (pH 5.5): a) non-cross-linked collagen; b) collagen + 1% of tannic acid; c) collagen + 2% of tannic acid.

Conclusions

The addition of tannic acid to fish skin collagen leads to alterations in mechanical and swelling properties. These properties are crucial for application in wound dressing. Young's modulus was higher for cross-linked collagen, whereas the elongation at break was smaller than that for non-cross-linked collagen. The swelling of collagen films increased after cross-linking with tannic acid. The swelling tests indicated that collagen cross-linked with tannic acid absorbs more water than the non-cross-linked material, which may suggest also better absorption of exudate from the wound. To sum up, the properties of films made of fish skin collagen improved slightly after tannic acid cross-linking. The alterations can be a result of collagen cross-linking by tannic acid probably by forming hydrogen bonds. The slight shifts in the amide bands confirm the alterations in hydrogen bonding in collagen macromolecules. As there is still a need for the development of collagen-based wound dressings, our results may show a simple way for further improvements of these dressings and may encourage further research. However, other cross-linking agents should also be investigated in the future for further improvement of fish collagen materials.

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