International Journal of Biological Macromolecules 47 (2010) 570–577

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Chemical and thermal cross-linking of collagen and elastin hydrolysates

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ARTICLE INFO

Article history:
Received 7 July 2010
Accepted 6 August 2010
Available online 14 August 2010

Keywords:
Collagen
Cross-linking
Elastin hydrolysates

ABSTRACT

Chemical and thermal cross-linking of collagen soluble in acetic acid and elastin hydrolysates soluble in water have been studied. Solutions of collagen and elastin hydrolysates were treated using variable concentrations of 1-ethyl-3(3-dimethyl aminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS). Moreover, diepoxypropylether (DEPE) has been used as cross-linking agent. Films made of collagen and elastin hydrolysates were also treated with temperature at 60 °C and 100 °C to get additional cross-links. The effect of cross-linking has been studied using FTIR spectroscopy, thermal analysis, AFM and SEM microscopy. Mechanical and surface properties of materials have been studied after cross-linking.

It was found that thermal and mechanical properties of collagen and elastin materials have been altered after thermal treatment and after the reactions with EDC/NHS and/or DEPE. Surface properties of collagen materials after chemical cross-linking have been modified. Thermal and chemical cross-linking of collagen films lead to alteration of polarity of the surface.

1. Introduction

Both, elastin and collagen, are structural proteins and may undergo varied sequences of photochemical and chemical reactions [1,2]. Collagen is the main protein of connective tissue and the main component of the skin. As an extracellular matrix protein it is widely used as a biomaterial for tissue regeneration and implantation. Elastin is an extracellular matrix protein in mammals where it is the main component of skin, blood vessels, such as the aorta, and tissues of the lung. Elastin can provide an excellent basis for biomaterials, such as arterial prosthesis, dermal substitute and hydrogels [3]. It is a highly insoluble structural protein and usually elastin hydrolysates are more useful for biomedical applications. However, the material made of elastin hydrolysates only is very elastic with low mechanical strength. A good way for obtaining new materials can be a preparation of a blend containing elastin hydrolysates and collagen [4,5]. In mammals, collagen and elastin are mixed together in appropriate ways depending on the functions [6]. In this context, elastin could represent a valid alternative to synthetic biomaterials, for application in tissue replacement and/or tissue regeneration.

For cross-linking of protein materials one can use physical or chemical methods. As physical cross-linking agent gamma radiation can be used [7,8]. However, the energy of gamma radiation can destroy the native structure of the protein. Next to gamma radiation physical cross-linking agent is UV irradiation. It is less harmful for proteins than gamma radiation, but UV light can also destroy protein structure [1,9,10].

Chemical cross-linking can give highly cross-linked material in a very short time [11]. There are several chemical compounds capable to cross-link proteins.

Covalent cross-linking using 1-ethyl-3-(3-dimethylaminopropyl)-1-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) is a widely used method [12–14].

The aim of this work was to study the chemical cross-linking of collagen soluble in acetic acid and elastin hydrolysates soluble in water. Solutions of elastin hydrolysates and collagen were treated using variable concentrations of 1-ethyl-3(3-dimethyl aminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS). Moreover, diepoxypropylether (DEPE) has been used as cross-linking agent. Collagen and elastin hydrolysates have been cross-linked by the temperature to compare chemical cross-linking with physical cross-linking, namely thermal cross-linking. Cross-linked collagen and elastin materials are usually required for the preparation of scaffolds containing hydroxyapatite. Such materials can be applied as bone tissues substitutes.

2. Materials and methods

2.1. Collagen

Collagen was obtained in our laboratory from tail tendons of young albinos rats (Medical University, Poznan, Poland). After washing in distillate water, the tendons were dissolved in 0.1 M acetic acid for 3 days at 4 °C. Tendons were blended in a Waring blender in 0.5 M acetic acid, and then spun at 10,000 rpm in a Sorvall

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doi:10.1016/j.ijbiomac.2010.08.004

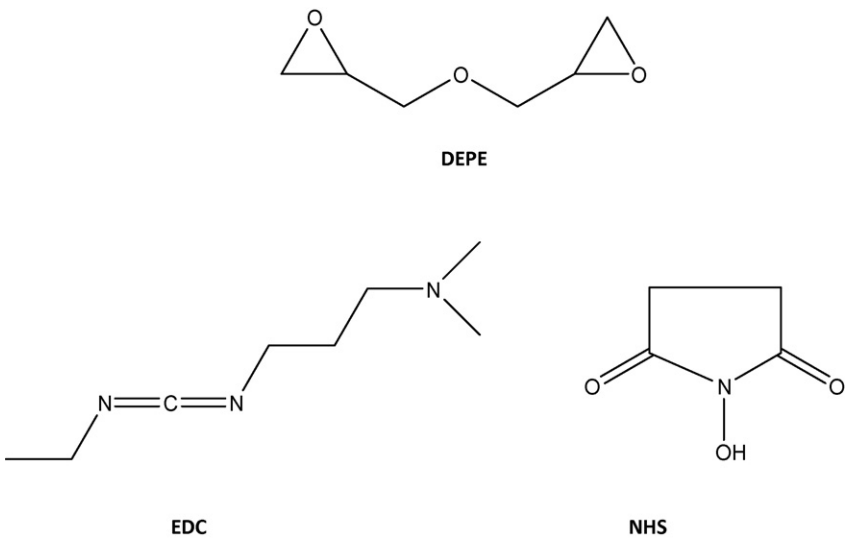

Fig. 1. Chemical structure of diepoxypropylether (DEPE) and 1-ethyl-3(3-dimethyl aminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS).

centrifuge and the soluble fraction decanted and lyophilised. The method used was the same as previously employed [15]. Collagen solution was freeze-dried to obtain 100% pure collagen (ALPHA 1–2 LDplus, CHRIST, −20 °C, 100 Pa, 48 h). Collagen solution with concentration of 0.5 mass % was prepared from lyophilized collagen in acetic acid.

2.2. Purification of elastin

Pig aortas were obtained from local butcher. Insoluble elastin was purified from aortas by Lansing's method [16]. Porcine aorta was cleaned from adhering tissues using a scalpel and cut into small pieces (about 0.5 cm wide rings). Remaining fat was removed by sequential extractions in ethanol (twice), mixture of ethanol/ether (50/50) (twice) and ether (also twice). The de-fatted tissue was placed in double amount of 0.1 M NaOH. Magnetic stirrer was placed into the flask and the mixture was heated to 95 °C for 50 min in a water bath. After cooling at room temperature samples were washed twice with cold 0.1 M NaOH in a Buchner funnel and then with demonized water. Dry material was minced in liquid nitrogen [16].

2.3. Hydrolysis of elastin

Elastin powder (1 g) was suspended in a mixture of 50 ml of tert-butanol and 50 ml of 1 M KOH and was stirred for 48 h at room temperature. Fifty milliliters of water was added and the resulting solution was neutralized with acetic acid. The solution of elastin hydrolysates was then dialyzed four times against demonized water.

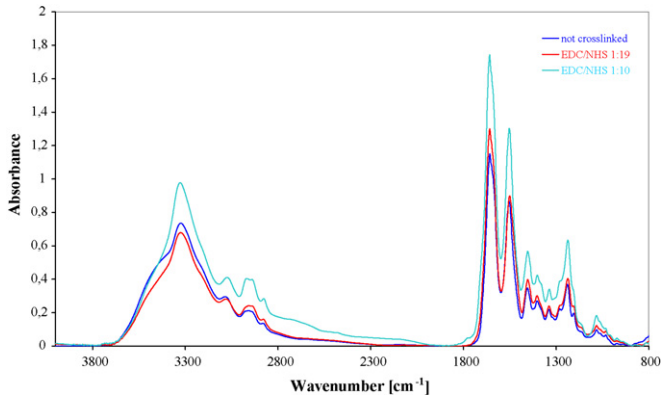

Fig. 2. FTIR spectra of collagen films after cross-linking with EDC/NHS (1:19 and 1:10).

2.4. Elastin/collagen mixtures

The mixtures of elastin/collagen were prepared by mixing of appropriate volumes of elastin hydrolysates and collagen in acetic acid solution (0.5 M) such that a series of solutions were produced in duplicate containing different percentage of elastin and collagen.

2.5. Cross-linking by EDC/NHS

Collagen solution (0.5%) was mixed with EDC/NHS (4:1). After solvent evaporation thin collagen film has been obtained.

2.6. Cross-linking by diepoxypropylether (DEPE)

Collagen solution was mixed with DEPE (20:1) and after solvent evaporation thin cross-linked films have been obtained.

Thermal cross-linking has been carried out in laboratory dryer with control temperature system at the 60 °C and 100 °C for 2 h and 24 h.

The changes in chemical structure of collagen and elastin were evaluated by attenuated total reflection infrared spectroscopy using a Genesis II FTIR spectrophotometer (Mattson, USA) equipped in ATR device (MIRacle^{TM} PIKE Technologies) with zinc selenide (ZnSe) crystal. All spectra were recorded in absorption mode at 4 cm^{-1} intervals and 64 scans.

The morphology of collagen films before and after cross-linking was studied using Scanning Electron Microscopy (SEM) (LEO Electron Microscopy Ltd, England). The measurements on all of the samples were repeated at different locations. The samples in the form of films were also observed by atomic force microscope (AFM). Topographic imaging were performed in air using a commercial AFM a MultiMode Scanning Probe Microscope Nanoscope IIIa (Digital Instruments Veeco Metrology Group, Santa Barbara, CA) operating in the tapping mode in air. Surface images, using scan widths ranging from 4 μm to 20 μm with a scan rate of 0.7 Hz, were acquired at fixed resolution (512 × 512 data points).

Contact angles of two liquids: diiodomethane (D) and glycerol (G) on collagen films were measured at constant temperature

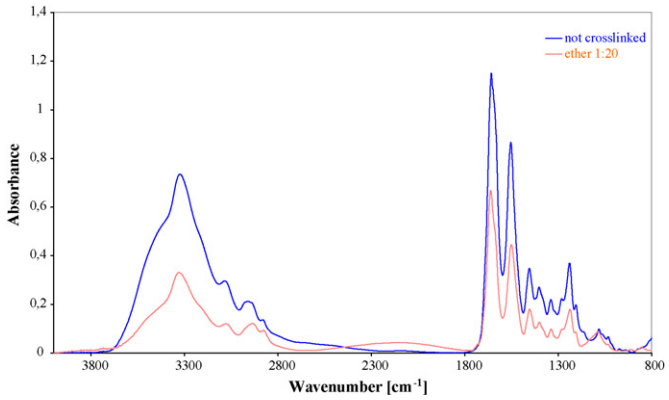

Fig. 3. FTIR spectra of collagen films after cross-linking with diepoxypropylether (DEPE).

(22 °C) using goniometer equipped with a system of drop shape analysis (DSA 10 produced by Krüss, Germany). Each contact angle is the average of maximum 10 measurements; the precision was 0.2°. The surface free energy was calculated using Owen–Wendt method [17].

Thermogravimetric analysis (TG) was performed on a TA Instruments SDT 2960 Simultaneous TGA–DTA in nitrogen and at heating rate 10 °C/min and heating programme 25–600 °C. Mechanical properties of materials have been measured by Zwick and Roell testing machine.

3. Results and discussion

The chemical structures of cross-linking agents have been shown in Fig. 1. The compounds shown in Fig. 1 have been used for chemical cross-linking of collagen. After cross-linking of collagen materials FTIR spectra have been recorded for cross-linked films. In Fig. 2, the FTIR spectra for collagen cross-linked with EDC/NHS have been presented. In Fig. 3, the FTIR spectra for collagen cross-linked with diepoxypropylether (DEPE) have been presented. In FTIR spectra of cross-linked collagen film we observe the bands typical for collagen, such N–H stretching at ~3325 cm^{-1} for the amide A, C–H stretching at ~3081 cm^{-1} for the amide B, C=O stretching at ~1657 cm^{-1} for the amide I, and N–H deformation at ~1553 cm^{-1} for the amide II. After chemical cross-linking the position of FTIR bands are nearly unchanged, and this fact may suggest that the secondary structure of collagen is not destroyed (Table 1). However, the integral absorbance (the area of the bands) is much smaller for cross-linked collagen films with EDC/NHS (1:10). Especially the area of amide A is much smaller for cross-linked specimens (Table 2). Amide A band and OH band (from water bonded to collagen) are located in the same range in FTIR spectrum (3700–3100 cm^{-1}). Much smaller integral absorbance in the above region suggests that during cross-linking reaction collagen samples lose water bonded to this biopolymer. Similar situation we have seen for collagen cross-linked with diepoxypropylether (DEPE). Integral absorbance for collagen cross-linked with DEPE in the region 3700–3100 cm^{-1} suggests that during cross-linking reaction collagen samples again lose water bonded to this biopolymer.

After thermal cross-linking of collagen the position of FTIR bands are nearly unchanged and again this fact suggests that structure of collagen is not significantly destroyed (Tables 3 and 4). However, the integral absorbance (the area of the bands) is much smaller for thermally cross-linked collagen in 60 °C (Table 5). Especially the area of amide A is much smaller for cross-linked specimens than for non-cross-linked ones. Much smaller integral absorbance in the above region suggests that during thermal cross-linking collagen samples lose water bonded to this biopolymer. Cross-linking at 100 °C leads to loss of water during short period of cross-linking (2 h), but after 24 h of heating the specimens dramatically absorb water and the area of the band in the region 3100–3700 cm^{-1} is bigger than before cross-linking (Table 6).

The alterations of band positions in FTIR spectra for collagen films containing 10% of elastin after thermal cross-linking has been shown in Table 7. Integral absorbance has been shown in Table 8. After thermal cross-linking at 60 °C the position of FTIR bands are nearly unchanged. Thermally cross-linked colla-

Table 1
The amide bands position in FTIR spectra of collagen after chemical cross-linking with EDC/NHS and DEPE.

Collagen	Band position [cm^{-1}]					
	Amide A	Amide B	$=CH_2$	Amide I	Amide II	Amide III
Not cross-linked	3324	3084	2959	1657	1553	1239
EDC/NHS 1:10	3328	3073	2968	1658	1553	1237
EDC/NHS 1:19	3324	3080	2957	1658	1551	1237
DEPE 1:20	3329	3078	2935	1661	1550	1237

Table 2
The area of amide bands in FTIR spectra of collagen after chemical cross-linking with EDC/NHS and DEPE.

Collagen	Integral absorbance [cm^{-1}]				
	3702–3116	3116–3014	1722–1596	1596–1490	1303–1182
Not cross-linked	39.0	0.6	14.0	7.3	3.0
EDC/NHS 1:10	19.0	0.5	9.5	4.6	2.6
EDC/NHS 1:19	26.0	0.5	13.0	6.5	2.5
DEPE 1:20	24.0	0.5	13.0	6.5	3.0

Table 3
The amide bands position in FTIR spectra of collagen after thermal cross-linking at 60 °C.

Time of cross-linking [h]	Band position [cm^{-1}]					
	Amide A	Amide B	$=CH_2$	Amide I	Amide II	Amide III
0	3324	3084	2959	1657	1553	1239
2	3324	3079	2957	1658	1551	1237
24	3322	3080	2957	1658	1550	1237

Table 4
The amide bands position in FTIR spectra of collagen after thermal cross-linking at 100 °C.

Time of cross-linking [h]	Band position [cm^{-1}]					
	Amide A	Amide B	=CH_2	Amide I	Amide II	Amide III
0	3326	3082	2957	1661	1553	1239
2	3323	3081	2957	1660	1552	1238
24	3323	3080	2954	1657	1552	1238

Table 5
The area of amide bands in FTIR spectra of collagen after thermal cross-linking at 60 °C.

Time of cross-linking [h]	Integral absorbance [cm^{-1}]					
	3702–3116	3116–3014	3010–2896	1722–1596	1596–1490	1303–1182
0	146.00	2.38	3.71	53.02	27.06	11.47
2	91.83	2.05	4.01	50.77	24.41	10.46
24	91.83	1.84	3.71	47.18	22.74	9.51

Table 6
The area of amide bands in FTIR spectra of collagen after thermal cross-linking at 100 °C.

Time of cross-linking [h]	Integral absorbance [cm^{-1}]					
	3702–3116	3116–3014	3010–2896	1722–1596	1596–1490	1303–1182
0	178.76	3.51	5.83	72.64	38.54	18.21
2	160.75	3.32	5.98	75.67	38.11	16.99
24	198.20	3.65	6.15	80.70	40.35	16.81

Table 7
The amide bands position in FTIR spectra of collagen containing 10% of elastin after thermal cross-linking at 60 °C.

Time of cross-linking [h]	Band position [cm^{-1}]					
	Amide A	Amide B	=CH_2	Amide I	Amide II	Amide III
0	3323	3079	2958	1658	1550	1237
2	3321	3078	2956	1657	1548	1237
24	3320	3078	2957	1657	1548	1237

Table 8
The area of amide bands in FTIR spectra of collagen containing 10% of elastin after thermal cross-linking at 60 °C.

Time of cross-linking [h]	Integral absorbance [cm^{-1}]					
	3702–3116	3116–3014	3010–2896	1722–1596	1596–1490	1303–1182
0	113.03	2.16	4.53	60.25	30.03	11.00
2	102.65	2.09	4.85	63.44	30.38	10.84
24	101.11	2.02	4.78	61.11	29.11	10.46

gen/elastin materials lose less water bounded to the specimen than collagen cross-linked without elastin at the same temperature conditions.

TG and DTG plots of collagen and collagen cross-linked with ether are presented in Fig. 4. The thermograms have been obtained during heating in nitrogen. From TG curves we determined the mass decrement during the heating process. The temperature of the maximum speed of the process (T_{max}) was determined from the maximum on the DTG curve. In Tables 9 and 10 thermal parameters for collagen cross-linked with EDC/NHS, DEPE and elastin hydrolysates have been presented. In the characteristic DTG curve for collagen in nitrogen one can see two peaks. These peaks are representative for the two-stage sample destruction due to the temperature. The first stage (between 25 °C and 100 °C) is connected with the evaporation of water absorbed to collagen. In the second stage (between 280 °C and 400° C) water bound to collagen is released and small molecular products of thermal degradation of collagen are liberated. For collagen cross-linked with EDC/NHS we observed that T_{max} of first stage is almost the same like for non-cross-linked one. However, for specimens cross-linked with ether T_{max} of first stage is much higher. The loss of water absorbed and the weight loss for first stage are much smaller for cross-linked collagen than for not cross-linked collagen. In the second stage we observe bigger mass decrement after cross-linking process. T_{max} for second stage is much lower for collagen cross-linked with ether. For collagen cross-linked with EDC/NHS we observed only small alteration of T_{max} for second stage of thermal degradation (Table 10).

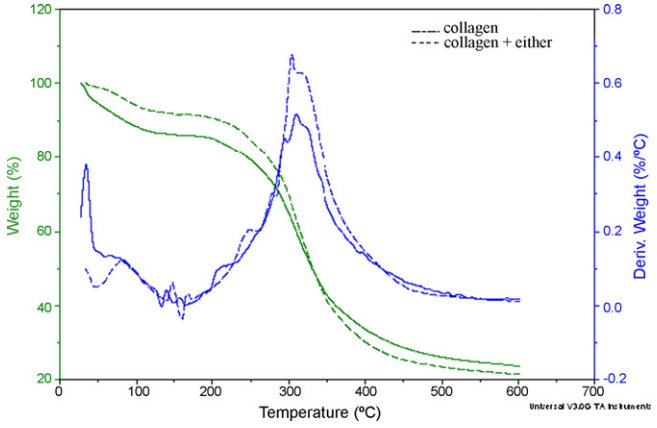

Fig. 4. TG and DTG curves for collagen films before and after cross-linking with diepoxypropylether (DEPE).

Table 9
Thermal parameters for the I stage of thermal decomposition of collagen films after chemical cross-linking with EDC/NHS and DEPE: the temperature of the maximum speed of the process (T_{max}) *and* the mass decrement during the heating process (Δm).

Specimen	I stage	
	T_{max} [°C]	Δm [%]
Collagen not cross-linked	66.82	13.87
Collagen EDC/NHS 1:10	67.95	9.11
Collagen EDC/NHS 1:19	55.52	10.65
Collagen DEPE 1:20	81.52	8.16
Collagen + 10% of elastin	77.00	7.59

Table 10
Thermal parameters for the II stage of thermal decomposition of collagen films after chemical cross-linking with EDC/NHS and DEPE: the temperature of the maximum speed of the process (T_{max}) *and* the mass decrement during the heating process (Δm).

Próbka	II stage	
	T_{max} [°C]	Δm [%]
Collagen not cross-linked	308.99	62.43
Collagen EDC/NHS 1:10	311.23	67.85
Collagen EDC/NHS 1:19	309.85	80.82
Collagen DEPE 1:20	303.52	70.36
Collagen + 10% elastin	314.85	69.27

Table 11
Thermal parameters for the I stage of thermal decomposition of collagen films after thermal cross-linking: the temperature of the maximum speed of the process (T_{max}) *and* the mass decrement during the heating process (Δm).

Specimen	I stage	
	T_{max} [°C]	Δm [%]
Collagen non-cross-linked	66.82	13.87
Collagen 2 h at 60 °C	49.87	12.40
Collagen 24 h at 60 °C	62.30	10.80
Collagen 2 h at 100 °C	52.13	14.79
Collagen 24 h at 100 °C	66.82	13.94
Collagen + 10% elastin	77.00	7.59
Collagen + 10% elastin 2 h at 60 °C	81.50	7.26
Collagen + 10% elastin 24 h at 60 °C	80.39	7.51

Thermal stability of collagen materials depends on the amount of cross-links between chains.

In Tables 9 and 10 one can see that collagen containing elastin hydrolysates has got higher T_{max} for the first and second stages of thermal degradation. It may suggest that elastin hydrolysates work as good cross-linking agent for collagen.

The mass decrement during the heating process and the temperature of the maximum speed of the process (T_{max}) for collagen samples after thermal cross-linking have been shown in Tables 11 and 12. Only for collagen thermally cross-linked together with 10% of elastin hydrolysates we observed an increase of T_{max} of the first stage. For thermally cross-linked collagen without elastin T_{max} of first stage is lower than for non-cross-linked one. Also mass decrement in the first stage is smaller for collagen thermally cross-linked with elastin. T_{max} in second stage of thermal decomposition is also bigger for collagen thermally cross-linked with elastin (Table 12). The results obtained from thermal analysis confirm that elastin hydrolysates work as good cross-linking agent for collagen.

After chemical cross-linking we observed the gel formation during dissolving the specimen. It suggests that additional cross-links have been formed. The amount of gel is shown in Table 13. For collagen/elastin hydrolysates blend we also observe gel formation. We can confirm again our conclusion that elastin hydrolysates work as cross-linking agent in collagen matrix. After thermal cross-linking we also observed the gel formation during dissolving the specimen (Table 14).

Table 12
Thermal parameters for the II stage of thermal decomposition of collagen films after thermal cross-linking: the temperature of the maximum speed of the process (T_{max}) *and* the mass decrement during the heating process (Δm).

Specimen	II stage	
	T_{max} [°C]	Δm [%]
Collagen non-cross-linked	308,99	62,43
Collagen 2 h at 60 °C	321,40	58,76
Collagen 24 h at 60 °C	308,88	50,98
Collagen 2 h at 100 °C	306,98	70,27
Collagen 24 h at 100 °C	313,22	71,70
Collagen + 10% elastin	314,85	69,27
Collagen + 10% elastin 2 h at 60 °C	316,26	65,39
Collagen + 10% elastin 24 h at 60 °C	311,81	61,78

Table 13
The amount of gel in collagen films after chemical cross-linking with EDC/NHS and DEPE and elastin.

Specimen	Gel [%]
Collagen EDC/NHS 1:10	86.02
Collagen EDC/NHS 1:19	92.21
Collagen DEPE 1:20	93.41
Collagen + 10% elastin	98.52

Table 14
The amount of gel in collagen films after thermal cross-linking at 60 °C and at 100 °C.

Specimen	Gel [%]
Collagen 2 h at 60 °C	97.53
Collagen 24 h at 60 °C	97.63
Collagen 2 h at 100 °C	97.35
Collagen 24 h at 100 °C	86.28
Collagen + 10% elastin	98.52
Collagen + 10% elastin 2 h at 60 °C	99.47
Collagen + 10% elastin 24 h at 60 °C	99.58

Table 15
Roughness of collagen films after chemical cross-linking with EDC/NHS and DEPE.

Chemical cross-linking	Roughness [nm]
Not cross-linked	57.8
EDC/NHS 1:10	23.7
EDC/NHS 1:19	20.9
DEPE 1:20	80.2
10% Elastin	63.5

The surface of cross-linked collagen films has been observed by Atomic Force Microscopy. Surface roughness is much bigger for non-cross-linked collagen (Fig. 5) than for collagen cross-linked with EDC/NHS (Figs. 6 and 7). The surface of collagen films cross-linked with ether and elastin hydrolysates is similar to the surface of not cross-linked collagen films (Figs. 8 and 9). From AFM investigation we have got surface roughness for the specimens. Surface roughness was big for collagen before cross-linking and for collagen cross-linked with ether (Table 15). After cross-linking with EDC/NHS the specimens are rather flat. Surface roughness for collagen before cross-linking and for collagen cross-linked with elastin hydrolysates is similar. However, DEPE as cross-linking agent leads to the surface with very high roughness (Table 15). The values of surface roughness for collagen after thermal cross-linking have been presented in Table 16. The roughness of thermally cross-linked collagen films is smaller than before cross-linking. It is probably caused by partial denaturation of collagen during heating.

Contact angle is a measure of non-covalent forces between liquid and the first monolayer of material. Thus, in the case of strong interactions between phases, the liquid drop spreads on the solid and wet it. The contact angle values for two different liquids (diiodomethane – D, and glycerol – G) on collagen films after cross-

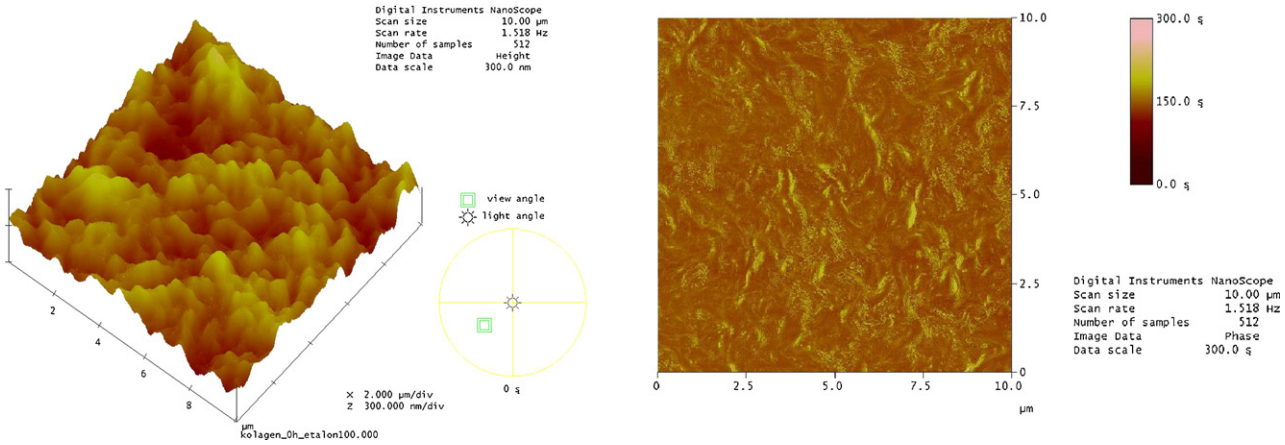

Fig. 5. AFM photograph of collagen film without chemical cross-linking.

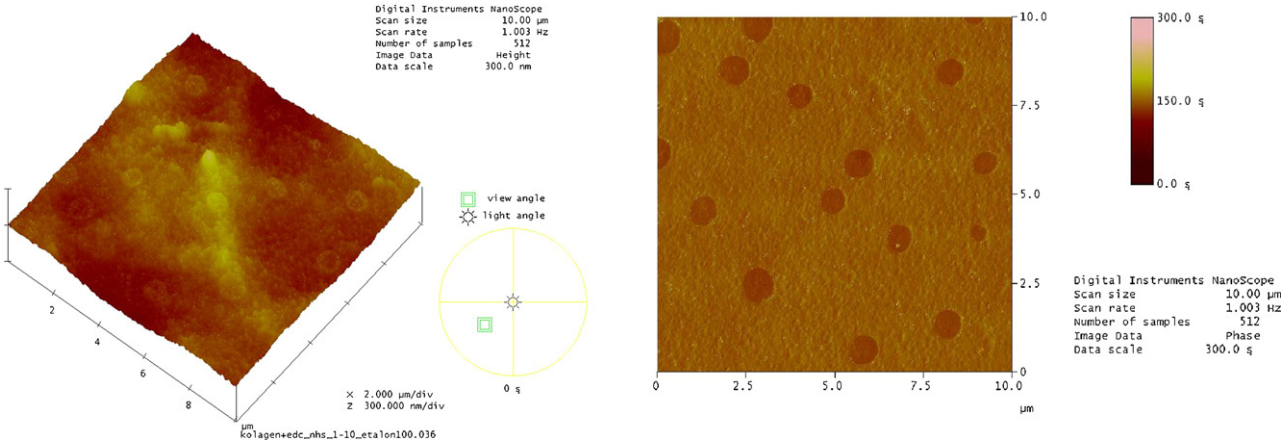

Fig. 6. AFM photograph of collagen film after chemical cross-linking with EDC/NHS (1:10).

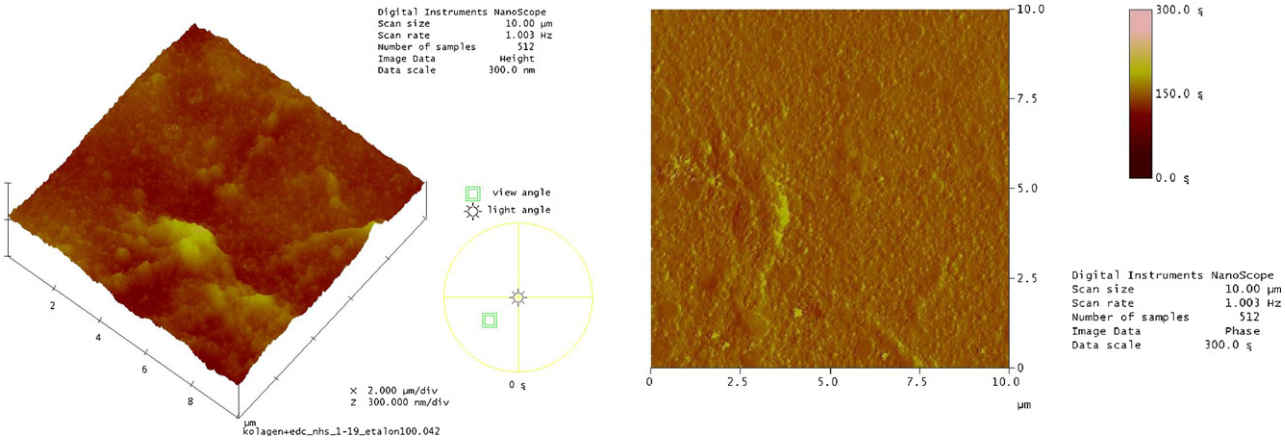

Fig. 7. AFM photograph of collagen film after chemical cross-linking with EDC/NHS (1:19).

Table 16
Roughness of collagen films after thermal cross-linking at 60 °C.

Time of cross-linking [h]	Roughness [nm]
0	63.5
2	51.5
24	29.2

linking were measured. For chemically cross-linked collagen films we have measured the contact angle and calculated the surface free energy. The results have been presented in the Table 17. As one can see the surface of collagen films cross-linked with ether and EDC/NHS (1:10) is more polar than for not cross-linked collagen. When we used bigger concentration of EDC/NHS (1:19) the distribution of polar groups is irregular and the surface is less polar. Contact angle and surface free energy for collagen films containing 10% of elastin after thermal treatment at 60 °C have been presented

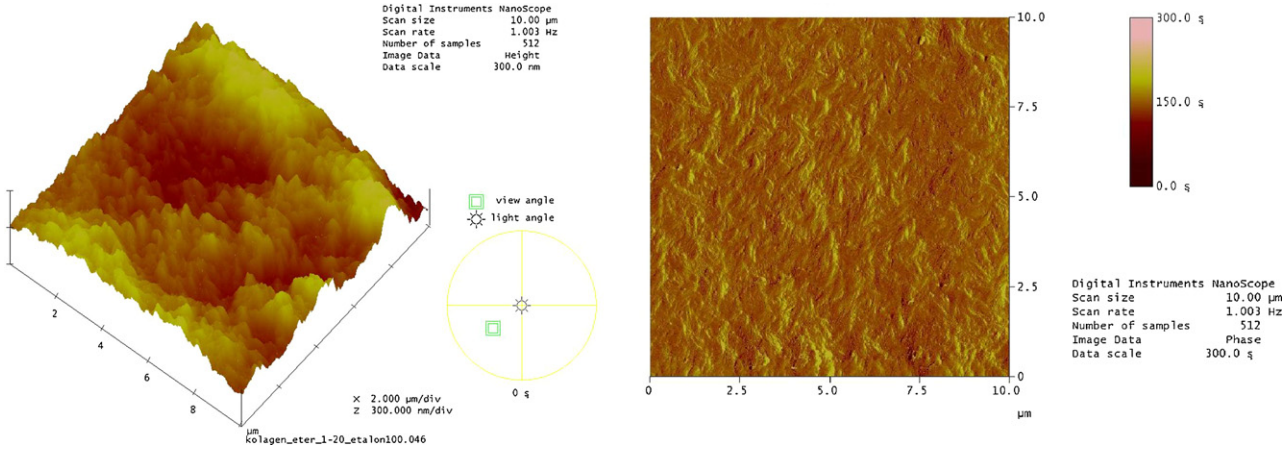

Fig. 8. AFM photograph of collagen film after chemical cross-linking with diepoxypropylether (DEPE) (1:20).

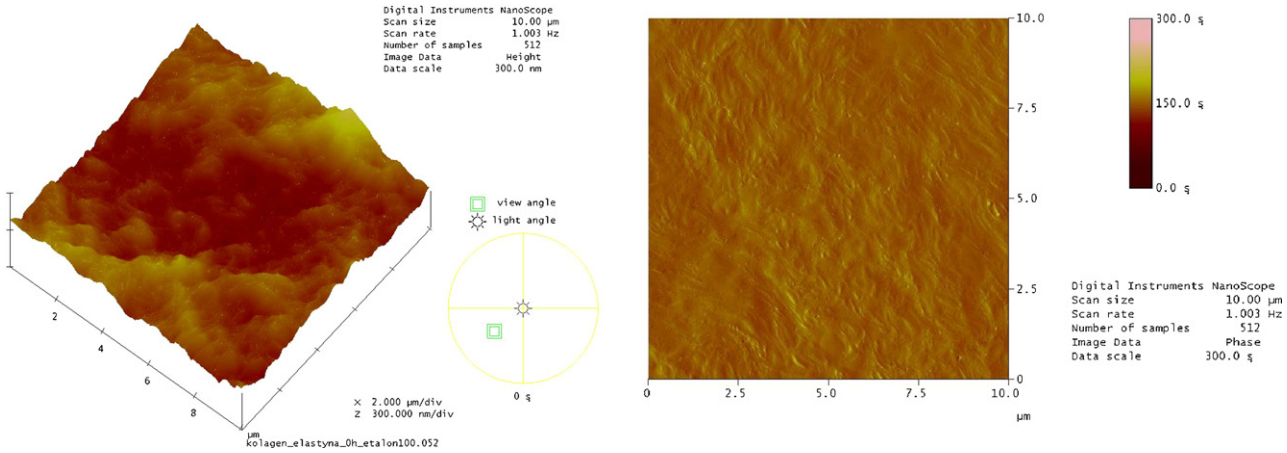

Fig. 9. AFM photograph of collagen film after chemical cross-linking with 10% of elastin hydrolysates.

Table 17
Contact angle and surface free energy for collagen films after chemical cross-linking with EDC/NHS and DEPE.

Chemical and thermal cross-linking	Contact angle		Surface free energy [mN/m]		
	Glycerol	Diiodomethane	Total energy	Dispersive part	Polar part
Not cross-linked	70.7	51.6	35.46	29.94	5.51
EDC/NHS 1:10	67.1	54.5	35.69	27.48	8.20
EDC/NHS 1:19	76.0	52.3	33.83	30.49	3.35
DEPE 1:20	63.7	49.3	38.75	30.12	8.63
Cross-linking at 60 °C					
2 h	70.1	44.1	38.67	34.46	4.21
24 h	70.8	51.1	35.63	30.28	5.35
Cross-linking at 100 °C					
2 h	74.9	50.0	35.09	31.74	3.35
24 h	72.9	54.0	33.85	28.82	5.03

Table 18
Contact angle and surface free energy for collagen films containing 10% of elastin after thermal at 60 °C.

Time of cross-linking [h]	Contact angle		Surface free energy [mN/m]		
	Glycerol	Diiodomethane	Total energy	Dispersive part	Polar part
0	76.0	51.0	34.43	31.32	3.11
2	71.2	48.6	36.53	31.92	4.62
24	70.9	48.3	36.73	32.05	4.69

in Table 18. As one can see the surface of collagen film after thermal cross-linking is more polar than non-cross-linked ones. In cross-linked films we can see that the polar and non-polar groups have been distributed in very irregular way. Irregular distribution was confirmed by AFM. Using AFM method one can investigate the surface roughness, which has been discussed above.

Mechanical properties of materials have been measured only for thermally cross-linked collagen films as chemically cross-linked

Table 19
Values of ultimate tensile strength (σ_r), ultimate percentage of elongation (ε_r) and Young's Modulus (E) of collagen films after cross-linking at 60 °C.

Time of cross-linking at 60 °C [h]	Values of mechanical parameters		
	E [MPa]	σ_r [MPa]	σ_r [%]
0	2040	92.5	8.4
2	3610	91.4	11.2
24	3380	77.9	6.4

Table 20
Values of ultimate tensile strength (σ_r), ultimate percentage of elongation (ε_r) and Young's Modulus (E) of collagen films after cross-linking at 100 °C.

Time of cross-linking at 100 °C [h]	Values of mechanical parameters		
	E [MPa]	σ_r [MPa]	σ_r [%]
0	2040	92.5	8.4
2	1910	72.8	12.3
24	2470	77.9	10.8

collagen films were very fragile and it was not possible to cut them for measurement of mechanical properties.

Ultimate tensile strength UTS, ultimate percentage of elongation UPE an Young's Modulus (E) of collagen films after thermal cross-linking has been shown in Tables 19 and 20. The ultimate tensile strength of thermally cross-linked collagen film decreases after 2 h and 24 h of thermal treatment at both 60 °C and 100 °C. The ultimate percentage of elongation of the collagen film increases after 2 h and 24 h of thermal treatment at100 °C. The ultimate percentage of elongation of collagen decreases after 24 h of thermal treatment at 60 °C. The thermal cross-linking of collagen films leads to an increase in Young's modulus of the collagen films. The results of mechanical measurements confirm that additional cross-liked have been formed after chemical and thermal treatment.

4. Conclusions

Chemical and thermal cross-linking of collagen materials lead to loss of water bounded to the macromolecule. Collagen films cross-linked by EDC/NHS become fragile with poor mechanical properties. Thermally cross-linked collagen films get slightly better mechanical properties. Collagen films cross-linked by DEPE are thermally less stable and contained much less water bonded than collagen films before cross-linking. Elastin hydrolysates can work as good cross-linking agent for collagen. Thermal and chemical cross-linking of collagen films lead to alteration of polarity of the surface.

Acknowledgement

Financial support from the Ministry of Science (MNII, Poland) Grant No. N N507 3495325 is gratefully acknowledged.

References

[1] A. Sionkowska, A. Kamińska, Christopher A. Miles, BaileyF Allen J., Wpływ promieniowania UV na strukturę i właściowości kolagenu, Polimery 6 (2001) 379–452.
[2] C.A. Miles, A. Sionkowska, S.L. Hulin, T.J. Sims, N.C. Avery, A.J. Bailey, Identification of an intermediate state in the helix-coil degradation of collagen by ultraviolet light, Journal of Biological Chemistry 275 (42) (2000) 33014–33018.
[3] W.F. Daamen, J.H. Veerkamp, J.C.M. van Hest, T.H. van Kuppevelt, Elastin as a biomaterial for tissue engineering, Biomaterials 28 (2007) 4378–4398.
[4] J. Skopinska-Wisniewska, A. Sionkowska, A. Kaminska, A. Kaznica, R. Jachimiak, T. Drewa, Surface charakterization of collagen/elastin based biomaterials for tissue regeneration, Applied Surface Science 255 (2009) 8286–8292.
[5] N. Bonzon, X. Carrat, C. Daminiere, G. Daculsi, F. Lefebvre, M. Rabaud, New artificial connective matrix made of fibrin monomers, elastin peptides and type I + III collagens: structural study, biocompatibility and use as tympanic membranes in rabbit, Biomaterials 16 (1995) 881–885.
[6] L. Debelle, A.M. Tamburro, Elastin: molecular description and function, The International Journal of Biochemistry and Cell Biology 31 (1999) 261–272.
[7] W.Q. Sun, P. Leung, Calorimetric study of extracellular tissue matrix degradation and instability after gamma irradiation, Acta Biomaterialia 4 (2008) 817–826.
[8] D.T. Cheung, N. Perelman, D. Tong, M.E. Nimmi, The effect of γ-irradiation on collagen molecules, isolated α-chains, and crosslinked native fibers, Journal of Biomedical Materials Research 24 (1990) 581–589.
[9] R.S. Davidson, The photodegradation of some naturally occurring polymers, Journal of Photochemistry and Photobiology Part B: Biology 33 (1996) 3–25.
[10] A. Sionkowska, J. Skopinska, M. Wisniewski, A. Leznicki, J.J. Fisz, Spectroscopic studies into the influence of UV radiation on elastin hydrolysates in water solution, Journal of Photochemistry and Photobiology Part B: Biology 85 (2006) 79–84.
[11] H. Cao, S.-Y. Xu, EDC/NHS cross-linked type II collagen–chondroitin sulfate scaffold: characterization and in vitro evaluation, Journal of Materials Science 19 (2008) 567–575.
[12] K. Nam, T. Kimura, F. Tsuyoshi, A. Kishida, Physical and biological properties of collagen–phospholipid polymer hybrid gels, Biomaterials 28 (2007) 3153–3162.
[13] K. Nam, T. Kimura, F. Tsuyoshi, A. Kishida, Preparation of a collagen/polymer hybrid gel designed for tissue membranes. Part I. Controlling the polymer–collagen cross-linking process using an ethanol/water co-solvent, Acta Biomaterialia 6 (2010) 403–408.
[14] K. Nam, T. Kimura, F. Tsuyoshi, A. Kishida, Preparation of a collagen/polymer hybrid gel for tissue membranes. Part II. In vitro and in vivo biological properties of the collagen gels, Acta Biomaterialia 6 (2010) 409–417.
[15] A. Kaminska, A. Sionkowska, Photochemical transformations in the collagen at the presence of β-carotene, Journal of Photochemistry and Photobiology Part A: Chemistry 96 (1996) 123–127.
[16] N.T. Soskel, T.B. Wolt, L.B. Sandberg, Isolation and characterisation of insoluble and soluble elastin, Methods in Enzymology 144 (1987) 196–214.
[17] A.W. Adamson, Physical Chemistry of Surfaces, 5th ed., Wiley-Interscience, New York, 1990.