

The Effect of Crosslinking Agents on the Properties of Type II Collagen Biomaterials

MARIA-MINODORA MARIN^{1,2}, MADALINA GEORGIANA ALBU KAYA²,
GEORGE MIHAIL VLASCEANU¹, JANA GHITMAN¹, IONUT CRISTIAN RADU¹,
HORIA IOVU^{1,3*}

¹Politehnica University of Bucharest, Advanced Polymer Materials Group, 1-7 Polizu Str., 011061, Bucharest, Romania

²Collagen Department, Leather and Footwear Research Institute, 93 Ion Minulescu Str., 031215 Bucharest, Romania

³Academy of Romanian Scientists, 54 Splaiul Independentei, 50085, Bucharest, Romania

Abstract: *Type II collagen has been perceived as the indispensable element and plays a crucial role in cartilage tissue engineering. Thus, materials based on type II collagen have drawn farther attention in both academic and research for developing new systems for the cartilage regeneration. The disadvantage of using type II collagen as a biomaterial for tissue repairing is its reduced biomechanical properties. This can be solved by physical, enzymatic or chemical cross-linking processes, which provide biomaterials with the required mechanical properties for medical applications. To enhance type II collagen properties, crosslinked collagen scaffolds with different cross-linking agents were prepared by freeze-drying technique. The present research work studied the synthesis of type II collagen biomaterials with and without crosslinking agents. Scaffolds morphology was observed by MicroCT, showing in all cases an appropriate microstructure for biological applications, and the mechanical studies were performed using compressive tests. DSC showed an increase in denaturation temperature with an increase in cross-linking agent concentration. FTIR suggested that the secondary structure of collagen is not affected after the cross-linking; supplementary, to confirm the characteristic triple-helix conformation of collagen, the CD investigation was performed. The results showed that the physical-chemical properties of type II collagen were improved by cross-linking treatments.*

Keywords: *type II collagen, cartilage, crosslinking agents*

1. Introduction

Collagen represents one of the most important biopolymers used in tissue engineering. It is an essential animal protein existing in extracellular matrix of vertebrates and constitutes a significant proportion of the connective tissue [1,2].

The main characteristics such as high biocompatibility, controllable rate of biodegradation, absorbability as well as low antigenicity by the host tissue, excellent cell adhesion and proliferation properties afford this biopolymer a high potential to be used for applications in cartilage, bone and dentistry regeneration [3-6].

Cartilage is a dense and fibrous connective tissue which lacks regeneration by itself because of its low vascularity. It is composed of an extracellular matrix (ECM) that contains type II collagen, proteoglycans, and water [7,8,9,10]. In the ECM, type II collagen is the highly abundant component which is produced by chondrocytes and forms interconnected fibrils and fibers with proteoglycans [11]. Thus, materials based on type II collagen have attracted a great interest in both academic and research for developing new systems with optimal properties destined for usage in cartilage tissue regeneration [12]. Mainly, type II collagen has been perceived as an indispensable new surgical material for dealing with articular cartilage defects due to its low antigenicity and cell-binding properties [11]. Also, Tamaddon and collab. [13] presented in a research study that type II collagen compared to type I collagen stimulate more chondrogenic phenotype formation, being a suitable biomaterial for cartilage damages restoration with excellent results.

*email: horia.iovu@upb.ro



However, the major drawbacks of using type II collagen as a biomaterial for tissue reparation are its low biomechanical properties and rapid biodegradation [14]. These disadvantages can be improved by using physical, enzymatic or chemical crosslinking methods, which enhance the mechanical properties making the biomaterials better suited for implantation [15]. Several cross-linking agents have been utilized in order to surpass these drawbacks.

The use of glutaraldehyde (GA) has been extensively studied as a suitable chemical crosslinker agent for proteins that can adjust the physico-mechanical properties of the material, to achieve the required characteristics for a certain application. Although it confers better thermal and mechanical properties to the biomaterial it is used on, GA has exhibited cytotoxicity both in preclinical and in clinical tests due to functional groups that do not react or are generated during the process of enzymatic degradation of the biomaterials subjected to crosslinking [16,17]. Gough and collab. [18] demonstrated in a study the cytotoxicity of using GA as cross-linking agent for collagen-based materials, showing that its use produces apoptosis. In this context, the use of natural cross-linking agents such as genipin and tannic acid, instead of glutaraldehyde, is more preferred for biomedical applications.

Genipin (GE) is a hydrophilic bi-functional crosslinking reagent, a natural aglycone derived from geniposide with an extremely low cellular toxicity and versatile crosslinking properties for amino groups in various proteins [19]. Moreover, Sung and collab. [20] illustrated in their research that the genipin used as cross-linking agent presents a considerably reduced cytotoxic effect as compared to GA.

Tannic acid (TA) is a part of the polyphenol plants, the most important constituent of hydrolysable tannins, found in a wide diversity of plants, for example in the galls of *Rhus* and *Quercus* species. Supplementary, TA is widely studied from pharmaceutical point of view due to its good antioxidant, antimicrobial, antiviral, and anti-inflammatory properties [20]. The structure of TA consists of a glucose moiety as core and hydroxyl groups of glucose which are esterified with five digallic acid units. Also, TA is presented by Brazdaru and collab. [21] as the crosslinking agent with the greatest affinity for collagen, due to its hydrogen bonding from the fifteen hydroxyl groups going to the five galloyl residues situated into the exterior shell, with the functional groups from this protein.

Recently, it was found that 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) system can be used instead of GA as collagen crosslinking which allows the obtaining of biocompatible scaffolds with suitable mechanical properties [22]. The EDC/NHS system is recognized as a “zero length” crosslinking agent that is not incorporated into the amide crosslinks, leading to the formation of biomaterials with excellent biocompatibility, higher cellular proliferation and increased stability against enzymatic degradation [23,24]. Previously, Ahmad and collab. [25] demonstrated that the use of the EDC/NHS system, even in a low concentration, can develop biomaterials based on collagen with good mechanical properties, optimal degradation and superior biocompatibility.

The present research study investigated the influence of different crosslinking agents such as genipin, tannic acid and EDC/NHS system on the performances of a type II collagen-based scaffold to be used in cartilage tissue regeneration. The type II collagen-based scaffolds were crosslinked with two natural (GE, TA) and a synthetic (EDC/NHS) agent and the effect of these crosslinkers upon the morphology, mechanical and thermal properties was investigated. Moreover, the impact of the selected crosslinking agents on the secondary conformation of type II collagen, which is responsible for the therapeutic activity of the protein using circular dichroism, was investigated. Further, the swelling behavior and *in vitro* enzymatic degradation of the obtained crosslinked scaffold with natural or synthetic agents were studied.

2. Materials and methods

2.1. Materials

The type II fibrillar collagen gel with a concentration of 2.54% (w/w) was obtained from bovine cartilage using our new process for collagen extraction [26]. Tannic acid and genipin were supplied from Sigma-Aldrich, USA. EDC and NHS were provided by Sigma-Aldrich (China). All the experiments

were done using ultra-pure water.

2.2. Preparation method

The following biomaterials based on type II collagen were obtained: Col II (type II collagen - reference sample), Col II TA (type II collagen crosslinked with TA), Col II GE (type II collagen crosslinked with GE) and COL II EDC/NHS (type II collagen crosslinked with EDC/NHS). The collagen gel concentration was corrected to 1%. To obtain the Col TA and Col GE samples on the 1% collagen gel a specific amount of crosslinking agent, according to Table 1, was added and then the samples were subjected to 4 h of homogenization.

Table 1. The composition of the type II collagen hydrogels

Sample	Parameter			EDC (mM)	NHS (mM)
	Type II collagen (%) *	TA (%) *	GE (%) *		
Col II	1	0	0	0	0
Col II TA	1	0.1	0	0	0
Col II GE	1	0	0.1	0	0
Col II EDC/NHS	1	0	0	25	12.5

*the amounts of Type II collagen, TA, Tannic acid and Genipin are reported to 100 g hydrogel

To obtain Col II EDC/NHS biomaterials, the samples based on type II collagen were freeze-dried and then were immersed in a solution with crosslinking agent for 24 h. All the samples were washed three times with ultra-pure water to remove the unreacted agents and other impurities, then were subjected to lyophilization for 48 h using a Delta 2-24 LSC (Martin Christ, Germany). All the samples were used for further characterization.

2.3 Characterization methods

Fourier-transform infrared spectrometry (FTIR)

FTIR spectra were obtained using a Vertex 70 Bruker FTIR spectrometer (Billerica, MA, USA with attenuated total reflectance (ATR) module. For the obtained samples, the FTIR spectra were recorded in the ATR-FTIR mode, at a resolution of 4 cm^{-1} in 600–4000 cm^{-1} wave number region. The measurements were done in triplicate.

Circular Dichroism (CD)

The triple helical structure preservation of the collagen extract was assessed through CD. The measurements of the samples were obtained on a Jasco Model J – 1500 spectrophotometer using a quartz cylindrical cuvette, having a path length of 1 mm (500 μL of 1mg/mL collagen solution was introduced into the cuvette). CD spectra were performed (in triplicate) by uninterrupted wavelength scans ranging between 190 to 250 nm with a scan rate of 100 nm/min.

MicroCT

The porosity structure of the type II collagen samples was evaluate using Micro computed tomography (CT) utilizing a Skyscan 1272 instrument (Bruker microCT, Belgium). Uncrosslinked/crosslinked samples were cut as cylindrical forms, enclosed and fixed on the sample holder. The samples were scanned (not including any filter) with a voltage of 50 kV and having a source current of 200 μA . The results were obtained having 2 μm pixel resolution, and a rotation stage of 0.2°. The images were obtained using CT NRecon software and recreated as scientific drawings of three-dimension object using CTVox.

Mechanical studies

The mechanical studies of the samples were done using a Universal Testing Machine, Instron 3382 instrument at ambient temperature. Cylindrical porous scaffolds were used for the tests. All the measurements were obtained in triplicates, the average value was calculated and correlated, and then the curve of compressive stress (MPa) against of the applied strain (%) was constructed.

Differential scanning calorimetry (DSC)

The changes in the denaturation temperature against the increase of crosslinking agent concentration were evaluated by differential scanning calorimetry (DSC). Assessments were performed using a Netzsch DSC 204 F1 Phoenix (NETZSCH-Gerätebau GmbH, Selb, Germany) equipment having a continuous nitrogen flow rate of about 20 mL/min and a warming rate of 5°C/min, starting after 20 to 200°C. Prior to analysis, every sample with a mass between 9–9.6 mg was put in aluminum pots and hermetically closed.

Thermo gravimetric analysis (TGA)

TGA is one of the most applied methods used for the investigation of the thermal stability of the biopolymers using a wide range of temperature. TGA analysis offers a quantitative measurement for weight variations that are correlated with thermal induced transitions. The thermal gravimeter can register the loss in mass as a function of temperature or time for transitions that involve dehydration or decomposition [27,28]. The thermal stability of the samples was assessed with a NETZSCH TG 209 F1 Libra analyzer in nitrogen atmosphere with a flow rate about 20 mL/min, scanning from 25 to 700 °C and a warming rate of 10°C/min. Before to analysis, all samples were weighed, the mass ranging between 4.4-5.4 mg and then they were introduced into aluminum pans.

Water uptake

For the water absorption determination, the collagen samples were placed in water at 20 - 25 °C temperature. At fixed time intervals, the samples were withdrawn and weighed. The water absorption was determined using the equation:

$$\%Water\ uptake = \frac{W_t - W_d}{W_d} \times 100 (g / g) \quad (1)$$

where W_d represents the weight of the dry samples and W_t is the weight of the swollen samples at immersion time t .

Enzymatic degradation

The degradation of collagen scaffold, using collagenase, was evaluated by registration of the weight loss varying on contact time to collagenase solution. At fixed time intervals, the swollen scaffold was withdrawn, and weight measurements were performed. The percentage of scaffold degradation was determined by the relation (2):

$$\%Weight\ loss = \frac{W_i - W_t}{W_i} \times 100 (g / g) \quad (2)$$

where W_i is the first measured mass and W_t is the measured mass after time t .

3. Results and discussions

After lyophilization of hydrogels, the uncrosslinked/crosslinked 3D type II collagen sponges were obtained, presenting different morphological characteristics (Figure 1).



Figure 1. Type II collagen sponges uncrosslinked/crosslinked
 The chemical structures of the crosslinking agents are shown in Figure 2.

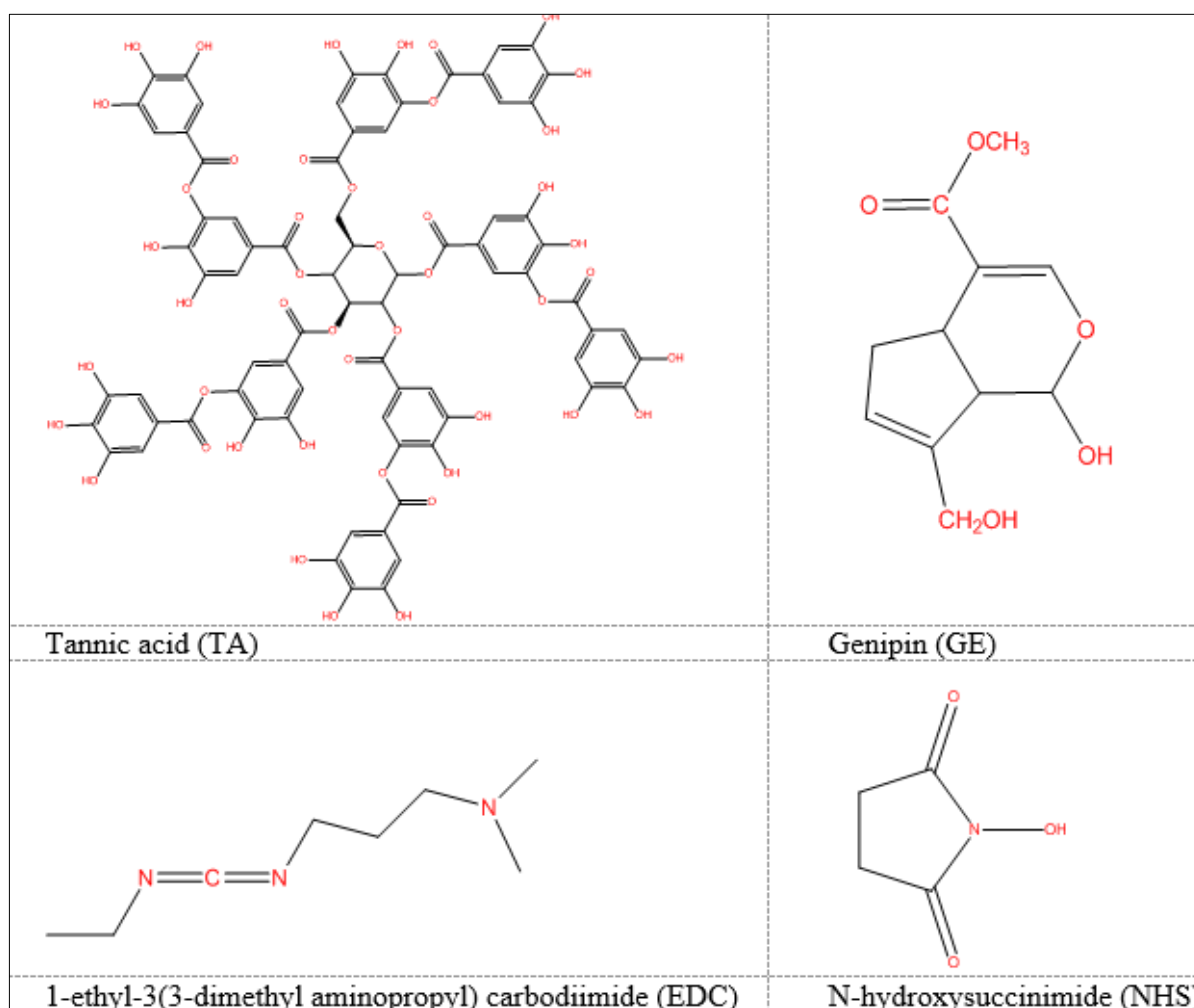


Figure 2. Chemical structures of the crosslinking agents

FT-IR

An excellent crosslinking agent should maintain the conformation and structure of collagen undestroyed after the modification process. It is crucial to investigate the structural changes in collagen after crosslinking because the triple helical structure of collagen could be altered and its bioactivity to react with other macromolecules, when acting as a biomaterial may decrease [29]. From the FT-IR spectra of the uncrosslinked/crosslinked samples (Figure 3) it can be seen the typical bands for the triple helix of type II collagen: 3312 - 3319 cm^{-1} (amide A), 3073 - 3082 cm^{-1} (amide B), 1635 - 1637 cm^{-1} (amide I, stretching tensile stresses C = O), 1552 - 1554 cm^{-1} (amide II, N-H bending vibrations and C-N stretching) and 1237 - 1238 cm^{-1} (amide III, N-H bending), the values being consistent with the literature data [30]. Also, the presence of the amide I and II confirm that the collagen existed in

polyproline type II (PPII) triple helical form in all the samples [29]. The peak at 2925 - 2935 cm^{-1} frequency is due to the C - H bends from the aliphatic chains of collagen.

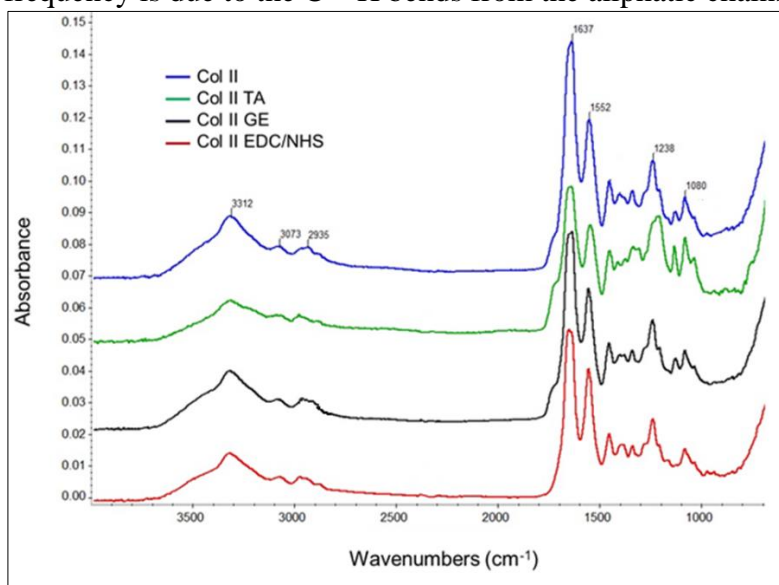


Figure 3. FT-IR spectra of the collagen samples

Some studies showed that the amide I band can be used to establish the secondary conformation of proteins [31]. Also, no differences were observed around the amide I bands of the Col II and the Col II TA matrices, which proves that the addition of TA preserves the secondary structure.

The Col II GE sample spectrum shows the same peaks as the uncrosslinked collagen. The crosslinked sample spectrum is dominated by two bands at 1080, and 1635 cm^{-1} (which are also shown in the uncrosslinked type II collagen spectra), assigned to ring C-H in-plane bond, and ring C=C double bond stretch modes of the core of the GE molecule. The absorption at 1080 cm^{-1} can also involve the C = O stretch mode of the primary alcohol on the GE molecule [16,19].

The EDC/NHS system produces crosslinks between carboxylic and amine groups, without themselves being integrated. After EDC/NHS crosslinking, the wavenumber of collagen bands is almost the same as non-crosslinked samples. This could indicate that the secondary conformation of the collagen was not altered [32].

After chemical crosslinking, the position of FT-IR bands is almost unmoved, and this fact could indicate that the secondary conformation of collagen is not damaged, and that the process leads to the modification of several properties of the material (Table 2).

Table 2. The amide frequencies in FT-IR spectra of collagen after chemical cross-linking

Sample	Frequency [cm^{-1}]				
	Amide A	Amide B	Amide I	Amide II	Amide III
Col II	3312	3073	1637	1552	1238
Col II TA	3319	3080	1637	1553	1237
Col II GE	3312	3074	1636	1554	1239
Col II EDC/NHS	3317	3082	1635	1552	1238
Assignment	VNH, VOH	VNH	VC=O, VNH	VCN, δ NH	VCN, δ NH

Thus, the use of crosslinking agents improves the physico-chemical properties of type II collagen without destroying its triple helical structure.

CD

Circular dichroism (CD) is a spectroscopic method, whose principle consists in the differential absorption of circularly polarized left-right light of chiral macromolecules. The CD technique is typically

used to evaluate the secondary protein structure [33]. CD investigation was performed to confirm the characteristic triple helix structure of type II collagen, which is one of the optically active proteins. Collagen is a chiral macromolecule and present the polyproline II-like helical structure with a negative minimum absorption band at 190-200 nm, a weak positive maximum absorption band around 220-230 nm and an intersection point (at which the ellipticity is annulled) at approximately 212-215 nm [34].

CD spectra of the uncrosslinked/crosslinked samples are shown in Figure 4.

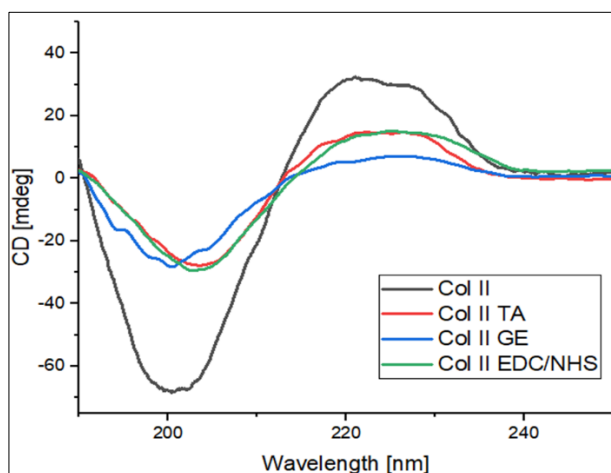
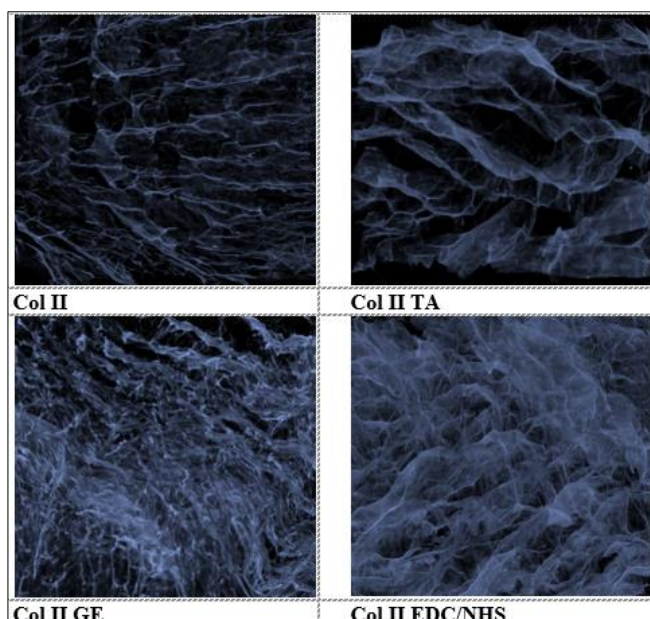


Figure 4. CD spectra of type II collagen samples

For uncrosslinked Col II sample, it is showed a negative absorption band at around 199 nm, a maximum at 223 nm and an intersection point at 213 nm, which normally describes a set of attributes characteristic to the native (triple helical) structure of collagen [34]. Also, Figure 4 shows that negative and positive bands from type II collagen samples decrease with the addition of crosslinkers, but they do not disappear, and crossing points suffer some small variation according to their size and location, respectively. Thus, considering the results for all the analyzed samples, it may be concluded that the crosslinking process does not alter, in a significant way, the native collagen conformation.

MicroCT

The main characteristics of the scaffolds with potential biomedical applications are the great number of interconnected pores and the significant surface area/volume percentage in order to support the cells proliferation and the development of new extracellular matrix [35]. The microarchitecture of the obtained biomaterials based on type II collagen was investigated from the point of view of the uniformity, pores dimension and shape. The reconstructed MicroCT images highlighted a high interconnected porous structure, regardless of the addition or not of the crosslinking agents (Figure 5).



In Figure 6, the graphical distribution of total porosity is presented. As it can be observed, the micromorphological characteristics (MicroCT images, Figure 5) and the calculated total porosity (Figure 6) are in good agreement. The Col II samples showed almost fully interconnected micro-porosity.

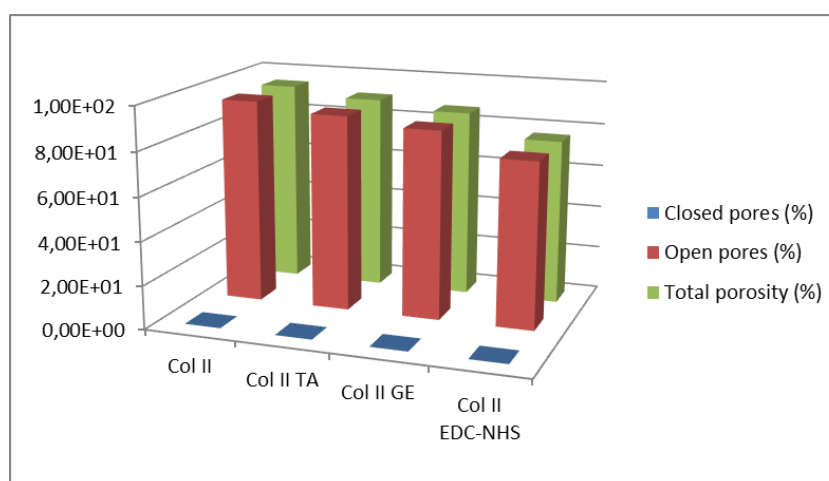


Figure 6. Porosity value of the crosslinked/uncrosslinked samples

MicroCT images presented a structure of regular interconnected pores for type II collagen modified with crosslinking agents. The lowest porosity was registered for the Col II EDC/NHS comparative with the others crosslinked samples. Moreover, by comparing the micromorphology of uncrosslinked and crosslinked type II collagen-based samples it can be deduced that crosslinking process of the samples induce pore size modification in the crosslinked biomaterials based on type II collagen. Accordingly, the morphology of all scaffolds can be considered proper for their application in tissue engineering.

Figure 5. MicroCT images of the porous scaffolds

of

Mechanical studies

The mechanical characteristics of all the obtained scaffolds based on type II collagen were then evaluated by compression tests. The value of compressive modulus is related on the structure of the compounds [36]. In Figure 7 is shown the compressive stress versus strain curves of the uncrosslinked/crosslinked biomaterials.

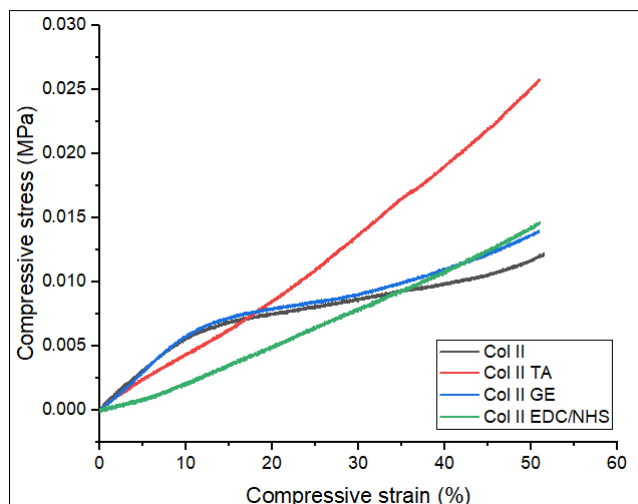


Figure 7. Effects of crosslinking agents on mechanical properties of collagen scaffolds

Mechanical evaluation of uncrosslinked type II Col and crosslinked type II Col matrices by TA, GE and EDC/NHS revealed a nonlinear stress-strain behavior which is roughly comparable to that of many native tissues, the only exception being observed in the case of the Col II TA sample which presents a linear stress-strain behavior characteristic for elastic materials. Also, the Col II TA exhibited maximum compressive stress of 0.028 MPa that was determined at a 50 % compression strain ratio value.

DSC

The thermal stability of biomaterials based on type II collagen is directly correlated with its ordered structure and triple helical conformation. In this context, an important parameter related to thermal performances of these biomaterials is to investigate the denaturation temperature of the obtained biomaterials [37]. Figure 8 shows the DSC curves of uncrosslinked/crosslinked type II collagen samples, the values for the denaturation temperatures of the samples being illustrated in Table 3.

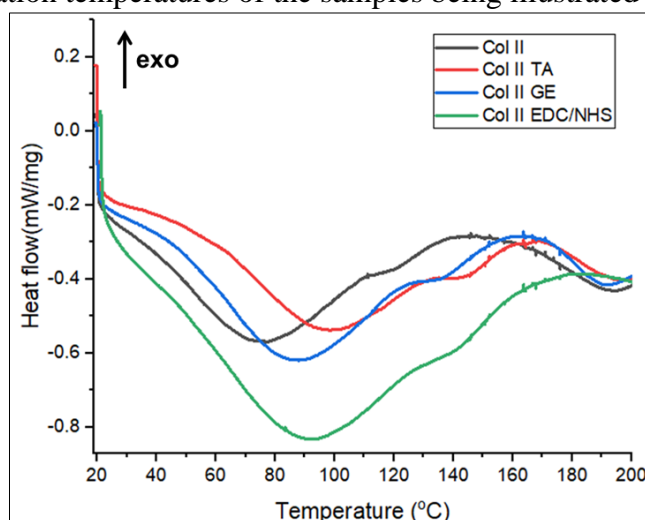


Figure 8. DSC curves of the uncrosslinked/crosslinked type II collagen

From Figure 8 it may be seen the influence of crosslinking agents on the thermal stability of biomaterials based on type II collagen.

Table 3. The values for the denaturation temperatures of the samples

Sample	Parameter	
	Weight [mg]	Td [°C]
Col II	9,4	77.3
Col II TA	9.5	98.7
Col II GE	9.5	88.0
Col II EDC/NHS	9.2	92.5

For uncrosslinked type II collagen, it can be observed a peak assigned to the denaturation temperature at 77.3 °C. Type II crosslinked collagen with TA exhibits the highest denaturation temperature (Td = 98.7 °C). The high thermal stability for Col II TA is attributed to the formation of additional hydrogen bonds in the crosslinking process due to the presence of hydroxyl groups from the crosslinking agent [38]. Also, the crosslinked sample with GE offers a considerable increase in thermal stability (Td = 88.0 °C) compared to the blank sample. This increase in denaturation temperature is associated with the reaction that occurs between genipin and the collagen amino groups with the formation of interhelical and intrahelical crosslinks [39]. Col II EDC/NHS sample shows a mean thermal stability (Td = 92.5 °C) attributed to the endothermic process from the temperature range 40-120 °C, compared to the previously analyzed samples. EDC interacts with the carboxyl group of the aspartic and glutamic acid residues from the collagen structure leading to the formation of activator which is an unstable product of urea. The addition of the NHS enhances the crosslinking efficiency of carbodiimides by producing a further stable ester [34,40]. Also, in the EDC/NHS crosslinking process, the carboxyl and amino groups present in the structure of type II collagen can interact to generate hydrogen bonds. Thus, the stable ester production and formed hydrogen bonds increase the thermal stability of the crosslinked collagen compared to the uncrosslinked sample [34]. The obtained thermograms and the registered thermal characteristics of the investigated samples, suggested that the crosslinking process with the agents used in this study can improve the physico-chemical properties of the biomaterials based on type II collagen without altering their helical structure.

TGA

Supplementary, to confirm that the crosslinking process achieved with the agents used in this study can improve the thermal stability of type II collagen, further TGA investigation was performed. The TGA thermograms of the uncrosslinked/crosslinked type II collagen are shown in Figure 9.

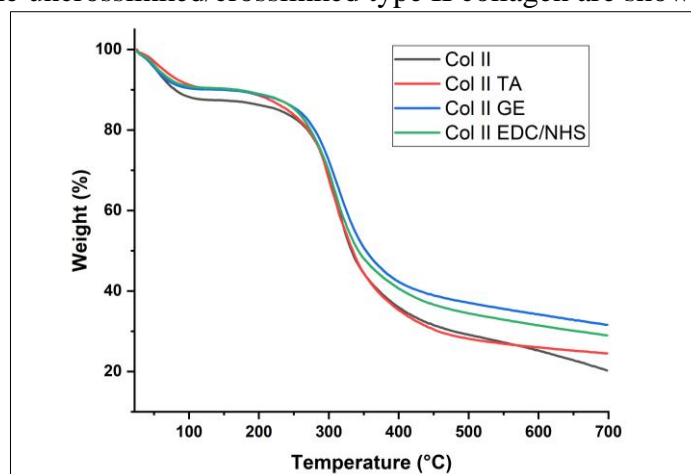


Figure 9. TGA thermograms of the uncrosslinked/crosslinked type II collagen

According to the thermograms, the pattern of decomposition for the samples reveals that the crosslinking process promoted an increase of their thermal stability. TGA thermograms of the uncrosslinked/crosslinked type II collagen samples showed multistep degradation for all samples. The first thermal transitional change occurs in the range 40-120°C and may be linked to the loss of water in

the material. The stability of the triple helix conformation of collagen macromolecules is related with intra- and inter-molecular hydrogen bonds as well as hydrogen-bound water [27,28,41,42]. Thus, the obtained results present some partial alterations in the secondary structure of type II collagen macromolecule. Also, a significant increase of the temperature at which significant weight loss occurs can be observed for the crosslinked samples. The higher stability in this stage was presented by the sample Col II TA. The second thermal event was recorded in the range 120-300°C which is associated with a gradual decomposition stage, respectively to the irreversible denaturation process. After the second thermal event, the gelatin and hydrolysate are obtained. The most stable sample from this stage was Col II EDC/NHS. The third stage can be observed in the range 300-550 °C which is correlated with a rapid decomposition of type II collagen and evaporation of the residual groups from all the samples [27,28,41,42].

In table 4 are shown the values of weight loss temperatures for the uncrosslinked/crosslinked type II collagen.

Table 4. Weight loss temperatures for the obtained samples

Sample	Parameter		
	Td _{3%} [°C]	Td _{5%} [°C]	Td _{10%} [°C]
Col II	43	53	80
Col II TA	50	63	153
Col II GE	45	54	150
Col II EDC/NHS	46	56	170

As it can be observed in table 4, the enhanced thermal stability along with the increase of the weight loss temperature confirms the chemical crosslinking of the type II collagen, being in concordance with DSC results.

Water uptake

The swelling behavior of the designed scaffolds based on type II collagen represents another key parameter which should be thorough investigated when the biomaterials for tissue regeneration are envisaged. Moreover, the capacity of the biomaterial to absorb water is a critical element which influences on the one hand the mechanical properties and the degradation rate of the scaffold and, on the other hand, its potential to deliver and release a therapeutic agent [36,43].

Swelling is the mechanism where a biomaterial, being immersed in a fluid absorbs the liquid and the volume suddenly expands [40]. It may be seen from Figure 10 that the presence of crosslinking agents in the biomaterials based on type II collagen decreased the swelling tendency when correlated with the Coll II scaffold as reference.

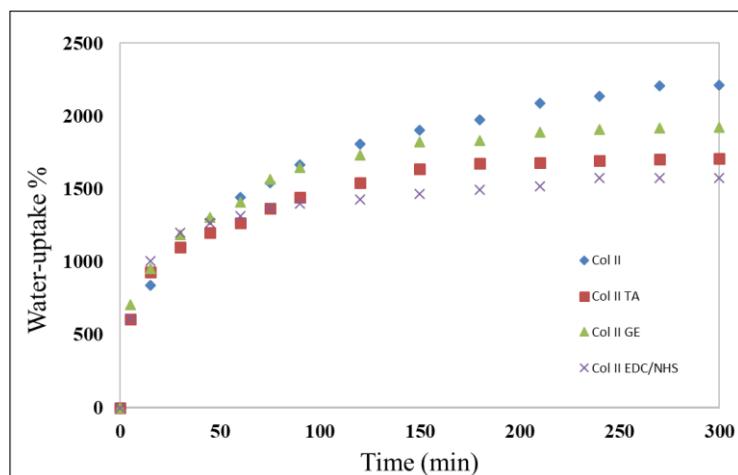


Figure 10. Water uptake for the uncrosslinked/crosslinked type II collagen

The sample crosslinked with EDC/NHS shows the lowest water absorption, followed by Col II TA, COL II GE and Col II. The results are also in concordance with the morphological features previously presented by MicroCT scanner and graphical distribution of total porosity. It can be observed that the high interconnected porous structure (Col II) increased the water absorption in the biomaterial, thus promoting the swelling process while the compact micromorphology of samples (Col II EDC/NHS) reduced water absorption capacity, exhibiting the lowest swelling response.

Enzymatic degradation

An important aspect for the scaffolds with biomedical applications is represented by the control of the degradation rate, as the *in vivo* resorption influences tissue regeneration ability. Type II collagen can be completely degraded only by collagenases treatment which are specific enzymes as they are able to destroy collagen triple helical region under biological conditions of pH and temperature. Another purpose of crosslinking step is to enhance the resistance of the type II collagen-based scaffolds under collagenase enzymatic degradation [36]. As it is highlighted in Figure 11, a considerable enhancement of the resistance to enzymatic degradation of the crosslinked samples based on type II collagen was observed.

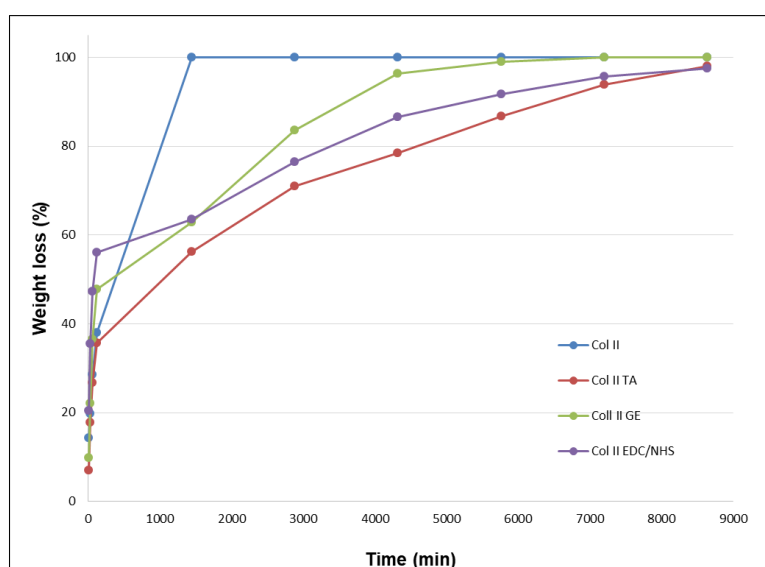


Figure 11. Enzymatic stability of collagen scaffolds

Even a small quantity of crosslinking agents was capable to noteworthy decrease the degree of enzymatic degradation by conserving the matrix ultrastructure under a period-collagenase process. Uncrosslinked collagen sample showed complete degradation after a short time (2 h of collagenase action), whereas Col II EDC/NHS presented a partial degradation (of up to about 97%) after six days of collagenase treatment; also, Col II TA showed a partial degradation (of up to about 98%) after six days of collagenase treatment, and Col II GE exhibits a total enzymatic degradation after 2 days. An explanation can be that the crosslinking is a better and effective method to prevent the alteration of the triple helical conformation by collagenase and improve the characteristics of type II collagen as biomaterial. The treatment with collagenase activates a hydrolytic separation in type II collagen structure, that produces a destabilization of the triple helical conformation followed by its destruction [34,40]. These results were in good agreement with the micro-porosity and the swelling results.

4. Conclusions

Type II collagen scaffolds were successfully stabilized by crosslinking treatment with TA, GE and EDC/NHS. The results of the physico-chemical characterization of the crosslinked scaffolds indicate that crosslinking treatment has not significantly destroyed the triple helical structure of the porous

scaffolds but improve the structural stability for short time and thermal properties, a necessary behavior in materials for tissue engineering applications. All these results suggest that this study could be useful for the development of a collagen-based biomaterial for tissue engineering applications having suitable properties and reduced antigenicity with all crosslinking agents. The findings of this study are limited to the *in vitro* behavior. Furthermore, they are a starting point and the *in vivo* performance will be evaluated.

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