EXTRACTION AND STUDIES ON THE PROPERTIES OF TYPE II COLLAGEN AS POTENTIAL BIOMATERIAL IN CARTILAGE REPAIR

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The aim of this study was to obtain and characterize type II collagen from bovine cartilage as a potential biomaterial that can be used in cartilage regeneration. The structure of the obtained biomaterial was investigated using Fourier-transform infrared spectroscopy, the characteristic triple-helix structure of collagen was confirmed using circular dichroism and protein characteristic was studied by isoelectric point determination. The morphology was evaluated by optic microscopy and water up-take and biological investigations were conducted by antibacterial assessments. The obtained results highlighted the performances of the extracted type II collagen as a promising biomaterial for future applications in cartilage regeneration.

Keywords: Type II collagen, cartilage, tissue regeneration.

1. Introduction

The articular cartilage is one of the most commonly used tissues daily due to its properties to decrease surface friction and joint tension. Most often, the cartilage is damaged as a result of sport injuries or accidents, genetic abnormalities and other cartilage disorders [1-3]. The articular cartilage is an avascular tissue with diminished ability of self-restoration, a great difficulty in the current clinical treatments for tissue engineering [4-5]. The extracellular cartilage matrix (ECM) is constituted almost entirely of type II collagen. Type II collagen represents approximately 90% of collagen in ECM and forms fibrils and fibers integrated with proteoglycan and glycoproteins. Supplementary, collagen types I, IV, V, VI, IX and XI are also present in ECM but only in a very small amount, aiding in the development and stabilization of type II collagen fibrils [6-8].

The field of regenerative medicine has seen a large expansion of new biomaterials based on collagen used to treat or supplant diseased or damaged tissue. Thus, for a successful implementation in clinical applications it is

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necessary to develop biomaterials and scaffolds that are compatible with the native tissues from a structural, mechanical and biological point [9]. A tissue engineering approach must offer a suitable environment for the cartilage regeneration, should exhibit high biocompatibility and must present a controlled biodegradation rate compared with the tissue growth [10]. The main function of a biomaterial used in tissue engineering is to provide architectural, biochemical and physical properties like native tissue, offering the cells a proper environment in which they are able to attach, grow and differentiate in order to form new tissue [11]. A large variation of biomaterials based on natural/synthetic polymers have been used to fabricate scaffolds for cartilage repair in a lot of forms: fibrous structures, porous matrices, meshes, particles, membranes and hydrogels [12-15].

Currently, among natural polymers, collagen is one of the most used biomaterials due to its excellent biocompatibility, biodegradability and its distinctive interaction with the human body [16]. Collagen is the major protein from conjunctive tissues: skin, bones, tendons, ligaments, cartilage, organs, basement membranes etc. It represents a larger family of several genetically distinct types. Presently, more than 28 different types of collagen have been described as part of vertebrate organisms [17-18].

Type II collagen has been identified as an important component in regeneration of articular cartilage, with a significant role in the growth and the proliferation process of chondrocytes. Thus, type II collagen or materials based on type II collagen are usually preferred in the treatment and research of the cartilage regeneration [19].

The aim of this work was to obtain and characterize type II collagen from bovine cartilage as a potential biomaterial used in cartilage regeneration. The novelty of this study consists on the fact that the technology utilized to obtain the type II collagen does not use enzymes which can affect the collagen's triple helix structure. The obtained biomaterial was characterized by Fourier-transform infrared spectroscopy (FT – IR); additional, to confirm the characteristic triple helical structure of collagen, circular dichroism (CD) study was performed. The isoelectric point was also evaluated. The morphology was analyzed by optical microscopy and water up-take and biological investigations were accomplished by antibacterial assessments. The obtained results highlighted the performances of the extracted type II collagen as a promising biomaterial for future applications in cartilage regeneration.

2. Experimental part

2.1. Materials

The type II collagen was extracted from bovine cartilage using a new technology for collagen extraction [20]. The obtained gel was freeze-dried using a Martin Christ Model Delta 2-24 LSC freeze dryer (Germany) and dried final

product was obtained as type II spongious form (matrix). Type II collagen both in the form of gel and matrix was used in further investigations.

2.2. Characterization methods

2.2.1. Fourier-transform infrared spectroscopy (FT – IR)

FT – IR spectral analysis was recorded using a Vertex 70 Bruker FTIR spectrometer on collagen matrix. The FT – IR spectrum was recorded in the ATR-FTIR mode with the following parameters: spectral region 4000 - 600 cm^{-1}, resolution 4 cm^{-1} with 30 acquisitions per each sample.

2.2.2. Circular Dichroism (CD)

The triple helical structure preservation of the collagen extract (diluted collagen gel) was evaluated by CD. The acquisition of the spectra was done on a Jasco Model J – 1500 spectrophotometer using a quartz cylindrical cuvette with a path length of 2 mm (400 µL of 0.0625 % (w/v) collagen aqueous solution was placed into the cuvette). CD spectra were obtained by wavelength scans from 190 to 250 nm with a scan rate of 100 nm/min.

2.2.3. Isoelectric point

The isoelectric point of type II collagen was determined by measuring Zeta potential of suspensions of collagen gel with different pH values. Samples with pH values ranging between 2 to 8 were prepared and measured in the presence of 1 mL NaCl ($5x10^{-3}$ M) by DLS technique using a Zeta potential analyzer (Malvern Zetasizer Nano).

2.2.4. Optical microscopy

To analyze the morphology of the obtained type II collagen matrix a Leica Stereomicroscope S8AP0 model with 20-160x magnification capacity was used. For better evaluation, 20x magnification and incident external cold light were used.

2.2.5. Water uptake

In order to determine the water absorption, the type II collagen matrix was immersed in ultrapure water. At timetabled intervals the sample was weighed. The experiment was done in triplicate. The water uptake was calculated using the equation:

$$\%\, Water\ uptake = \frac{W_t - W_d}{W_d} \times 100 \qquad (1)$$

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

2.2.6. Antimicrobial activity

The antimicrobial tests of the extracted type II collagen were assessed using agar diffusion method. The antimicrobial activity of type II collagen was

evaluated *in vitro* against *Escherichia coli* (gram negative) and *Staphylococcus aureus* (gram positive). The volume of the gel for the lower layer without any bacteria was prepared, and a volume of about 10 mL was placed in each sterilized Petri plate. Then, the agar solution was left to solidify. After that, an amount of agar solution for the top layer was prepared and 150 mL of agarose was seeded with 1 mL of bacterial solution (1-5 x 10^8 cfu/mL). In each Petri plate, 5 mL of bacterial solution was introduced. The samples were placed on the surface of the nutrient medium and then were incubated at 37 °C for 24 hours. The bioactivity was determined by sizing the Diameter of Inhibition Zones in [mm]. Each measurement was repeated three times and the mean diameter of the inhibition areas was calculated.

3. Results and discussion

3.1. Fourier-transform infrared spectrometry (FT – IR)

FT – IR is one of the most versatile and nondestructive technique used for structural characterization of proteins. Collagen triple helical structure with a high content of glycine, proline and hydroxyproline is characterized in FT-IR by specific absorption bands at the wavelengths corresponding to the Amide A groups (3303 cm^{-1}), Amide B (3083 cm^{-1}), Amide I (1633 cm^{-1}), Amide II (1548 cm^{-1}) and Amide III (1235 cm-^{1}), presented in Fig. 1 [21, 22]. According to previous studies, the presence of these amides was related to the triple helical structure of collagen [23].

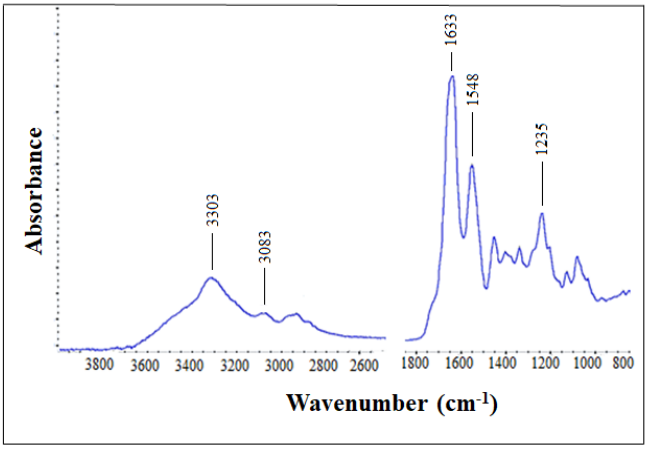


Fig. 1. FT-IR spectra for the obtained type II collagen

Thus, the FT-IR measurements of the obtained type II collagen showed that there are no alterations of triple helix after extraction protocols as there are no changes in amide II, III and pyrrolidine ring of collagen [21].

3.2 Circular Dichroism (CD)

Supplementary, to confirm the specific helical structure of the extracted type II collagen and to support the FTIR results, further CD investigation was performed. CD analysis can be used to prove the integrity of protein, a crucial requirement prior to complete structural investigations in medical applications [24]. The CD spectrum of the sample is shown in Fig. 2.

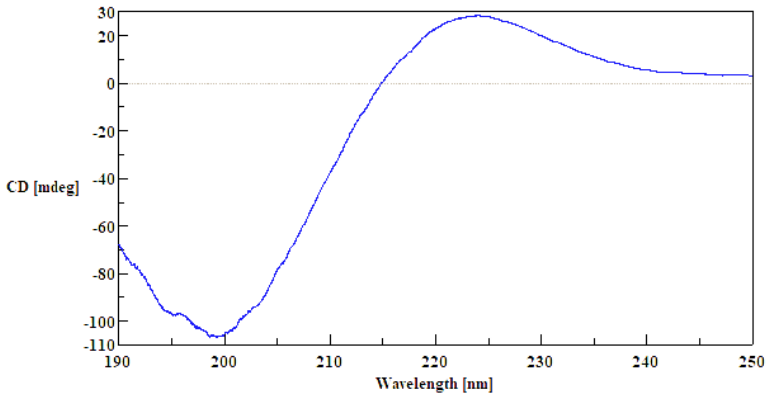


Fig. 2. CD spectrum of type II collagen

Collagen is one of the optically active proteins composed of more than 20% proline and hydroxyproline that also forms a related type of helical structure. Many researchers also describe this structure as having the polyproline II helix conformation with an intersection point at 214 nm, a pronounced negative minimum absorption band around 190 – 200 nm and a positive maximum absorption band at 210-230 nm [25].

Unlike other ordered conformations such as the α helix and β structures, the triple helix is a compact conformation [25].

The obtained type II collagen had a cross point at 214 nm, a positive maximum peak at 223 nm and a negative minimum peak at 199 nm, suggesting a characteristic triple helical configuration [26]. Based on the obtained findings and FTIR results it can be concluded that the secondary structure of protein, the most important from therapeutically point of view, was not significantly altered during the extraction process.

3.3. Isoelectric point (pHi)

Furthermore, the isoelectric point, one of the most important characteristics of proteins, associated with the percentage of acid amino residues in protein, was determined. The isoelectric point is defined as the pH at which the total charge of the protein is zero [27,28]. This physicochemical factor is very important for different techniques of solubility determination, an important stage in the development of collagen biomaterials with medical applications. Usually, proteins present several isoelectric points with large limits variations based on the

number of acidic or alkaline groups that dominate the surface of the macromolecule [28].

Fig. 3 shows a measurement of the Zeta potential (pZ) as a function of the pH. Zeta potential is applied in assessing colloidal dispersions; a high zeta potential will give high stability and the ability to resist aggregation. Opposite, when the zeta potential is close to zero, attractive forces can be bigger than the repulsion ones, and the colloid can form aggregates [29].

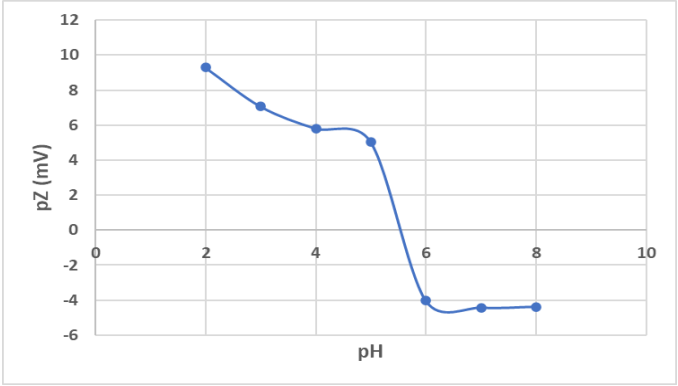


Fig. 3. Isoelectric point of type II collagen

From the Fig. 3 it can be observed that at low pH values, the pZ of the suspension was positive. The increase of the pH value leads to a negative reversion of the pZ which has a cross-point at 5.8 and was corelated with pHi value, this behavior being in concordance with the literature data [30].

3.4. Optical microscopy

The optical microscopy was performed to evaluate the morphology of the obtained type II collagen sample. The pore size distribution and surface areas are generally regarded as essential factors for a biomaterial used in tissue engineering [30]. The optical microscopy image of the obtained sample revealed a homogenous structure (Fig. 4).

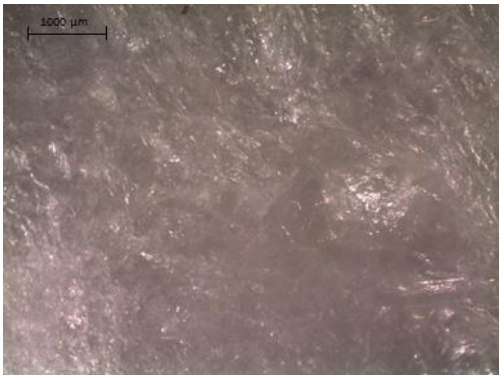


Fig. 4. Optical microscopy for the obtained type II collagen sample (20x)

The type II collagen sample has showed a highly porous structure with interconnected pores presenting various dimensions and shapes which is beneficial to the cell adhesion and proliferation. This conformation is a characteristic structure for collagen after the freeze-drying process. Biomaterials obtained through freeze-dry processes are usually used for tissue regeneration [31].

3.5. Water uptake

Moreover, to demonstrate the hydrophilic ability of the obtained type II collagen, which allows it to be used as an absorbable biomaterial, the swelling behavior was evaluated.

Fig. 5 shows the water uptake capacity of the collagen sample in the first 6 hours; the experiment was performed in ultrapure water at pH = 7.4 to simulate pH the biological environment.

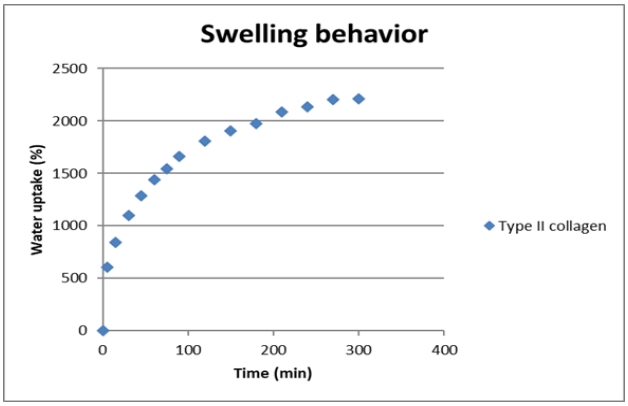


Fig. 5. Water uptake for type II collagen sample

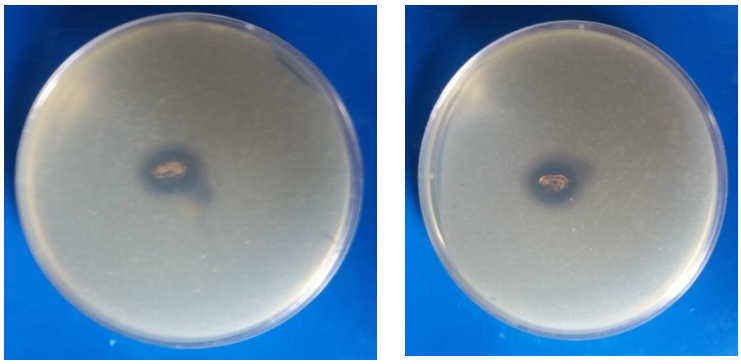
From Fig. 5 it can be observed that the type II collagen sample absorbs a high quantity of water in only 6 hours. The sample revealed a high-water absorption capacity due to the porous structure, this type of structure promoting the swelling behavior. A high-water absorption capacity is recognized as an essential parameter for an implantable collagen biomaterial which promotes the development of an environment for cells adhesion and proliferation [32].

3.6. Antimicrobial activity

Therapeutic activity of type II collagen is reflected in the high content of hydroxyproline specific for the undenatured collagen [33].

The type II collagen sample was tested for microbial activity against *Escherichia coli* and *Staphylococcus aureus* according to SR EN ISO 20645/2005 – Control of the antibacterial activity.

The evaluation is based on the absence or presence of bacterial development in the contact area between the inoculum and the sample and on the occurrence of a possible inhibition zone around the samples (Fig. 6).



a) b)

Fig. 6. The antimicrobial activity of type II collagen
a) *Staphylococcus aureus*; b) *Escherichia coli*

From the picture it can be observed the presence of inhibition zones without any bacterial growth around each tested sample, suggesting that collagen sample exhibits a sufficient antibacterial activity.

Table 1.

The evaluation of antimicrobial activity of type II collagen against *Escherichia coli/ Staphylococcus aureus*

Bacterial strain	Sample	Inhibition area diameter (mm)	Total number of aerobic germs (cfu/ cm^2)	Evaluation
Escherichia coli ATCC 11229	Type II collagen	15	0	Satisfactory effect
Staphylococcus aureus ATCC 6538	Type II collagen	14	0	Satisfactory effect

Table 1 illustrated that the extracted type II collagen showed antimicrobial activity against both microorganisms with inhibition areas equal to 14 and 15 mm, respectively, indicating that the type II collagen has a certain antibacterial effect without the use of any additional antibacterial drug. The antimicrobial activity of collagen was exhibited most likely due to the proline-rich content, some studies suggesting that the triple helix structure of collagen noticeably influenced the antimicrobial potential [32 -33].

4. Conclusions

Type II collagen from bovine cartilage was obtained and characterized. FT-IR measurements of the obtained type II collagen showed that there are no alterations of the triple helix because there are no changes in amide II, III and pyrrolidine ring of collagen. Also, the CD spectrum confirms a characteristic triple helical configuration for collagen. The samples revealed a porous structure with a high-water absorption capacity and an isoelectric point around 5.8.

Samples tested from the point of view of microbial activity showed good results due to the proline-rich content which promotes antimicrobial activity. The obtained results highlighted the performances of the extracted type II collagen as a promising biomaterial for future applications in cartilage regeneration.

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