separations

MDPI

Article

Evaluating the Degradation Process of Collagen Sponge and Acellular Matrix Implants *In Vivo* Using the Standardized HPLC-MS/MS Method

Jianping Gao [1,2], Ye Ma [1], Zhenhu Guo [1], Yang Zhang [1], Fangyu Xing [1], Tianyang Zhang [1], Yingjun Kong [1,2], Xi Luo [1,2], Liming Xu [3] and Guifeng Zhang [1,2,*]

[1] National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100190, China
[2] School of Chemical and Engineering, University of Chinese Academy of Sciences, Beijing 100049, China
[3] National Institutes for Food and Drug Control, Beijing 100050, China
* Correspondence: gfzhang@ipe.ac.cn

Abstract: The purpose of this study was to establish a collagen determination method based on an isotope-labeled collagen peptide as an internal reference via high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS), and using the established method to evaluate the degradation process of collagen-based implants *in vivo*. The specific peptide (GPAGPQGPR) of bovine type I collagen was identified with an Orbitrap mass spectrometer. Then, the quantification method based on the peptide detection with HPLC-MS/MS was established and validated, and then further used to analyze the degradation trend of the collagen sponge and acellular matrix (ACM) *in vivo* at 2, 4, 6, 8, 12, 16, and 18 weeks after implantation. The results indicate that the relative standard deviation (RSD) of the detection precision and repeatability of the peptide-based HPLC-MS/MS quantification method were 3.55% and 0.63%, respectively. The limitations of quantification and detection were 2.05×10^{-3} μg/mL and 1.12×10^{-3} μg/mL, respectively. The collagen sponge and ACM were completely degraded at 10 weeks and 18 weeks, respectively. Conclusion: A specific peptide (GPAGPQGPR) of bovine type I collagen was identified with an Orbitrap mass spectrometer, and a standardized HPLC-MS/MS-based internal reference method for the quantification of bovine type I collagen was established. The method can be used for the analysis of the degradation of collagen-based implants *in vivo*.

Keywords: *in vivo*; degradation process; collagen sponge; acellular matrix; HPLC-MS/MS

check for updates

Citation: Gao, J.; Ma, Y.; Guo, Z.; Zhang, Y.; Xing, F.; Zhang, T.; Kong, Y.; Luo, X.; Xu, L.; Zhang, G. Evaluating the Degradation Process of Collagen Sponge and Acellular Matrix Implants *In Vivo* Using the Standardized HPLC-MS/MS Method. *Separations* **2023**, *10*, 47. https://doi.org/10.3390/separations10010047

Academic Editors: Tomasz Śniegocki and Jakub Trawiński

Received: 15 November 2022
Revised: 5 January 2023
Accepted: 9 January 2023
Published: 12 January 2023

Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Collagen is a primary biopolymer and the most abundant component in the extracellular matrix of mammalian tissue. It provides a scaffold for cell attachment and migration and has specific mechanical properties [1–3]. Specifically, the excellent biocompatibility and biosafety (biodegradability and weak antigenicity) of collagen have proven it to be one of the most useful biomaterials [4–6], making it widely used as a raw material for constructing tissue-engineered medical products (TEMPs) or general surgical implants for tissue regeneration, reconstruction, or replacement [7,8]. As two kinds of important products of collagen-based biomaterials, collagen sponge, used for the development of tissue filling or hemostatic materials [9,10], and acellular matrix (ACM), used for the development of various patch materials [11,12], have attracted a lot of attention. In terms of the composition characteristics, collagen is the main component of a collagen sponge or ACM. An ACM not only contains collagen but also contains small amounts of fibronectin, laminin, and trace amounts of growth factors and cytokines [13–16]. Collagen sponges and ACMs are usually applied as hemostatic agents and prevent adhesion materials in clinical settings. Common adverse reactions, such as intestinal infarction, intestinal fistula, and

abdominal wall adhesions have been observed in clinical applications [17]. These adverse reactions may be related to the degradation rate of the materials. Therefore, the study of the *in vivo* degradation process of collagen-based materials can provide important data for their production process improvement and preclinical safety and effectiveness evaluation.

As an implantable and degradable biomaterial, the key point of the application of collagen is the controllable degradation rate and the match of the regeneration rate of new tissue. If the degradation rate is too fast, the mechanical strength will decline sharply in the early stage and lose its mechanical support. On the other hand, if the degradation is too slow, the regeneration and repair of tissues will be affected and immune reactions and fibrosis encapsulation will be triggered [18,19]. Therefore, the *in vivo* degradation dynamic evaluation of implantable degradable materials is an important means to predict and evaluate the expected clinical effects. Biodegradable metals, ceramics, polymer materials, and other materials that are difficult to degrade *in vivo* can be evaluated by traditional methods, such as micro-computed tomography (micro-CT), the mass loss method, and hematoxylin–eosin staining (HE staining) [20–22]. However, collagen-based implants are relatively easily degraded and lose their structure. Collagen is the main component of the tissue matrix, and its degraded substance will be quickly metabolized in the body. Meanwhile, the remaining materials often lose their original shape and structure. The new tissue will gradually grow in and replace the degraded implants [23]. In these cases, the traditional methods are not suitable for *in vivo* degradation evaluation.

The determination of collagen is usually regarded as an available indicator to evaluate the degradation of collagen-based implants. Collagen is a polymer with a typical triple helix structure and rich hydroxyproline. Collagen from different animal sources showed similarity in structure and properties (triple helix structure, molecular mass, and rich in hydroxyproline) [24]. Thus, it was difficult to qualify and quantify the collagen-based materials when they were implanted in experimental animals. To date, the reported quantification method of collagen or even particular collagen chains mainly includes the quantification of hydroxyproline, picrosirius red staining, and enzyme-linked immunosorbent assay (ELISA). Hydroxyproline is abundant in collagen, so the determination of the hydroxyproline content has been used to obtain the collagen content indirectly [25]. Hydroxyproline is found in different types of collagen from different animals. The traditional hydroxyproline method cannot be used to distinguish the collagen contents of different types and different animals. It has only been used to determine the total collagen by the ratio of the proportion of hydroxyproline, which might vary according to the animal source, age, and tissue [25–27]. Hydroxyproline is present both in collagen implants and host experimental animals. Thus, the hydroxyproline quantitative method cannot be used for the *in vivo* degradation of collagen-based implants. Thus, it is difficult to qualify and quantify the collagen-based materials that have been implanted in experimental animals. Picrosirius red staining was used to quantify different types of collagens by color shade and area size; this is a semi-quantitative method and showed low method accuracy [28]. The principle of the ELISA method is the antigen–antibody combination. It is sensitive and specific, but it was not suitable for tissue collagen detection and had non-specific adsorption, which easily led to false positive results [29,30]. HE staining combined with the semi-quantitative evaluation system of histopathology (ISO 10993-6 annex E) was used to evaluate host local effects while analyzing the *in vivo* degradation of collagen hydrogel and sponge in rabbit muscle [31]. Only the contour shape, size, and density of the implant could be roughly observed by HE staining. The weight of the degraded implant could not be measured. Unfortunately, the above methods could not quantify the residue of collagen or collagen-based implants accurately.

A certain collagen type has its particular amino acid sequences in its α chains, which are different from other types with the same animal origin. The same types of collagen from different animal sources also show different sequences in their α chains. Thus, the sequential differences could be used for the characterization of its type or animal source. The enzymatic digestion of collagen coupled with various separation methods is able to achieve

some reference peptides that are particular to a specific collagen. Many specific peptides have been used for the quantitative analysis of collagens with mass spectrometer [32,33]. In order to develop a different type-specific collagen and different animal species-specific collagen determination method, Zhang et al. developed an external standard-based high-performance liquid chromatography–tandem mass spectrometer (HPLC-MS/MS) method to determine the parent ion of the reference peptide in pig type I and III collagen [34]. However, the external standard-based method using selective ion monitoring was not suitable for the analysis of samples with a complex matrix, which may interfere with HPLC-MS/MS detection. To improve the wide applicability of the method, a standardized internal reference peptide-based HPLC-MS/MS method was developed in our lab for bovine type I, pig type I, and bovine type II collagen determination, and the method was further issued in the Chinese industry standard (YY/T 1805.3-2022) [35], but the data are unpublished. The standardized method for the determination of reference peptides from bovine type I based on HPLC-MS/MS has also been approved as an international standard project and it is in the process of development (ISO AWI 6631). To back up this methodology from a scientific perspective, the methodology development and its application research are presented in this study. The establishment and validation of the HPLC-MS/MS-based internal reference method for selected peptide determination of bovine type I collagen are demonstrated. Then, the application of the established method in the *in vivo* degradation study of collagen-based implants in rabbits is presented.

2. Materials and Methods

2.1. Materials and Instruments

Experimental animals: New Zealand white rabbits (male, about 9 months old, body weight: 2.8–3.7 kg) were provided by Beijing Long'an Animal Co., Ltd., Beijing, China. Animal management was carried out in accordance with the standard operating regulations established by the Laboratory Animal Protection and Use Regulations. SPF rabbit feed (Beijing Keyao Xieli Feed Co., Ltd., Beijing, China) was provided daily, and purified water was provided in a drinking bottle. Each rabbit was bred separately, and the label information for each cage indicated the gender, animal number, and implantation date. The room temperature and relative humidity were controlled at 20–25 °C and 40–70%, respectively. The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of Beijing TongHe Litai Biotechnology (the protocol code was IACUC-C2022037 and the date of approval was 22 March 2021).

Implant materials: Collagen sponge (made from bovine type I collagen, which was extracted and purified from bovine Achilles tendon tissue) was provided by Hebei Collatech Co., Ltd., Shijiazhuang, China. The acellular matrix (ACM, derived from the bovine dermal matrix) was provided by Yantai Zhenghai Co., Ltd., Yantai, China.

Reagents: Chromatographic pure acetonitrile (Merck, Rahway, NJ, USA), chromatographic pure formic acid (Merck, Kenilworth, NJ, USA), and sequence-grade trypsin (Promega, Fitchburg, WI, USA). These three reagents are commercially available.

Bovine type I collagen reference peptide (GPAGPQGPR, simplify to G-R) and the isotope-labeled peptide ((N15) PA (N15) G (N15) PQG (N15) PR, simplified as iso/G-R) which were used as the internal reference peptides, were synthesized by China Peptides Co., Ltd., Shanghai, China. The HPLC purity values were 99.21% and 99.15% for the G-R and iso/G-R, respectively.

Bovine type I collagen reference material (380008-202001) was provided by the National Institute for Food and Drug Control, China, and used for the methodology validation.

Instruments: An Orbitrap mass spectrometer (Exploris 480, Thermo, Waltham, MA, USA) was used to identify the reference peptide of bovine type I collagen. A triple quadrupole mass spectrometer (TSQ, Quantum ACCESS MAX, Thermo, USA) was used for collagen quantification. A circular dichroism (CD) spectrometer (J-810, Jasco, Tokyo, Japan) was used to analyze the triple helix structure of the collagen sponge. Scanning electron

microscopy (SEM) (Sigma 300, Carl Zeiss AG, Oberkochen, Germany) was used to analyze the surface morphology and structural characteristics of the collagen sponge and ACM.

2.2. Characterization of Implants

The surface topography of the collagen sponge and ACM were characterized by a scanning electron microscope (SEM).

The CD spectrum was determined on a Jasco J-810 Spectropolarimeter (Jasco, Tokyo, Japan). The reference bovine type I collagen was dissolved in 0.5 mol/L acetic acid to prepare a collagen solution with a concentration of 0.5 g/L. After centrifuging at 10,000 rpm for 10 min, the collagen solution was loaded in a quartz cell of a 1 mm path length. The scan wavelength was in a range of 190–260 nm with a scan rate of 50 nm/min at 25 °C. The acetic acid solution (0.5 mol/L) was used as the blank control.

An SEM was used to compare the structural differences among the collagen sponges and ACM. The samples were glued to specimen holders using Duco cement. Images were collected using a Sigma 300 scanning electron microscope and operated in the secondary electron imaging mode.

2.3. Implantation of the Collagen Sponge and ACM

The rabbits were randomly divided into bovine collagen sponge (Group A) and ACM (Group B), which contained 7 small groups (n = 3). After anesthesia, the back skin was removed and disinfected with iodophor. Two incisions of approximately 5 cm in length were made in the skin on either side of the back spine. Then, two pieces of bovine collagen sponge and ACM with 2 cm × 2 cm area were implanted subcutaneously into the backs of the rabbits, and non-absorbable surgical sutures were used to mark around the implants (for the convenience of the end-point material collection). At last, the wounds were sutured and disinfected using iodine. To prevent infection, 50,000 U/(kg·d) of penicillin was injected for 3 consecutive days after surgery for every rabbit.

2.4. Preparation of Collagen Peptides

Three rabbits were executed by humanitarian means after anesthesia at 2-, 4-, 6-, 8-, 12-, 16-, and 18 weeks post-implantation, and the implanted material with local host tissues (containing newly regenerated tissues, which grew into the inner areas of the implants) was taken out around the signed non-absorbable surgical sutures. The fascia and other subcutaneous tissues were removed. Then, about 2 cm^2 of tissue samples containing the implant material and the rabbit skin tissue without implanted bovine type I collagen material were taken and degreased with dichloromethane and methanol (v/v, 2:1). The rabbit skin tissue without implanted bovine type I collagen material was used as the blank matrix. Then, they were mixed with 5 mL water and homogenized, freeze-dried, and weighed. The collagen sponge and ACM without implantation were also freeze-dried and weighed, and used as the 0-week control.

The samples were prepared as follows: 2 mg of the bovine type I collagen reference material, the dried samples, and the blank matrix were dissolved with 2 mL NH_4HCO_3 (0.05 M, pH 8.0) solution. Subsequently, the dissolved samples were degenerated at 60 °C for 30 min. After cooling to room temperature, the samples were mixed with trypsin solution (100 µL, 0.2 µg/µL) and incubated at 37 °C for 18 h. The enzymatic hydrolysates were centrifuged at 12,000× g for 10 min, and the supernatants were collected. Finally, the digested mixtures were analyzed by HPLC-MS/MS. The amount of collagen in the original implant material was converted from the measured amount in 2 mg to the original weight of the implanted material in 2 cm × 2 cm. The total amount of bovine type I collagen in the rabbit tissues was converted from the measured amount in 2 mg to the original weight of the freeze-dried rabbit tissues.

2.5. Preparation of Reference Peptides and Collagen Peptide Solution

The reference peptide G-R and the internal reference peptide iso/G-R of bovine type I collagen were prepared with NH_4HCO_3 (0.05 mol/L, pH 8.0) solution. The internal reference peptide solution (10 μg/mL) was mixed with bovine type I collagen reference peptide at different concentrations (0.1, 0.5, 1, 2, 5, and 10 μg/mL), and the digested sample solutions were prepared as detailed in Section 2.4 at a ratio of 1:1 (v/v). Then, they were centrifuged at 12,000× g for 10 min. The supernatant was collected for analysis.

2.6. HPLC-MS/MS Conditions

Identification of specific peptides: The specific peptides of bovine type I collagen were detected using an Orbitrap mass spectrometer (Exploris 480, Thermo Fisher, City, MO, USA) with a mass accuracy of 10 ppm and a maximum resolution of 480 k. The HPLC–MS/MS conditions were as follows.

HPLC conditions: The chromatographic separation was carried out in a Peptide BEH C_{18} column (2.1 × 150 mm, 1.7 μm) (Waters, Milford, MO, USA). The HPLC system was the Vanquish UHPLC (Thermo Fisher, Waltham, MO, USA). The mobile phases A and B were 0.1% formic acid and 60% acetonitrile with 0.1% formic acid, respectively. A linear gradient of 5~90% of mobile phase B in 100 min was used, and the flow rate was 0.1 mL/min. Then, the column was washed with 100% mobile phase B for 10 min. The injection volume was 5 μL. The column temperature was kept at 60 °C.

MS conditions: An electron spray ionization (ESI) positive mode was used. The spray voltage was 4.5 kV. The capillary temperature and vaporizer temperature were 320 °C and 300 °C, respectively. The sheath gas and aux gas were 19.8 mL/min and 5 psi, respectively. The MS scan range was set from *m/z* 300 to 2000. The MS/MS was performed in the data-dependent mode. The maximum ion injection time was 200 ms and the MS/MS collision energy was 30%. The SEQUEST algorithm in Protein Discoverer 2.4 software (Thermo Fisher, Waltham, MO, USA) was used to analyze the bovine type I collagen-specific peptides.

Quantification of bovine type I collagen: The reference peptides and digested samples were determined using a TSQ mass spectrometer (Quantum Access MAX, Thermo Fisher, Waltham, MO, USA). The HPLC–MS/MS quantification conditions were as follows.

HPLC conditions: The chromatographic separation was carried out in a Zorbax C_{18} column (2.1 × 150 mm, 5 μm) (Agilent, Santa Clara, CA, USA). The HPLC system was the U3000 (Thermo Fisher, Waltham, MO, USA). The mobile phases A and B were 0.1% formic acid and 60% acetonitrile with 0.1% formic acid, respectively. A linear gradient of 5% phase B was used to equilibrate the column in the first 0.5 min. Then, the ratio of phase B was adjusted to 25% in 6 min. After that, the column was washed with 100% phase B for 2 min. Finally, 5% phase B was used to equilibrate the column for 2 min. The flow rate was 0.3 mL/min, the injection volume was 5 μL, and the column temperature was held at 30 °C.

MS conditions: The ESI positive mode was used to perform TSQ mass spectrometry with a mass accuracy of 0.7 FWHM and a maximum resolution of 140,000 FWHM. The spray voltage was 3.5 kV. The capillary temperature and vaporizer temperature were 320 °C and 300 °C, respectively. The sheath gas was 19.8 mL/min. The aux gas was 5 psi. The selected reaction monitoring (SRM) was used. The monitored target ion of bovine type I collagen reference peptide G-R and the internal reference peptide iso/G-R were *m/z* 418.74→611.27 and *m/z* 420.71→613.27, respectively.

2.7. Quantitative Methodological Validation

The bovine type I collagen reference material was used for the quantitative methodological validation. Each parallel sample was digested separately.

Precision verification: Samples of bovine type I collagen were prepared with concentrations of 0.5 mg/mL (Group A) and 1.0 mg/mL (Group B). Each group contained 10 samples. They were digested separately and used for precision analysis.

Repeatability verification: Three parallel samples of bovine type I collagen were prepared with concentrations of 0.5 mg/mL. Each sample was analyzed 3 times.

Spiking recovery validation: The reference peptides (0.2, 0.4, 1.0 μg) were added to the hydrolysate of the bovine type I collagen sponge. Each sample was detected three times in parallel, and the recovery rate was calculated.

Determination of detection and quantitation limits: The limits of detection and quantitation for the bovine type I collagen reference peptide were determined from the calibration curves of 3 S/N and 10 S/N, respectively, where S is the signal of the target peak and N is the noise.

2.8. Analysis of Data

The concentration of the reference peptide was calculated with Formula (1).

$$Y = aX + b; R^2 \quad (1)$$

where X: the concentration of the reference peptide, μg/mL; Y: the ratio of the sample peak area to the internal reference peptide peak area; a: a constant; b: a constant; R^2: the correlation coefficient.

The weights of the bovine type I collagen in the samples were calculated with Formula (2).

$$M_i = \frac{X \times V \times M_1}{M_2 \times 2 \times 1000} \quad (2)$$

where M_i: the weight of the bovine type I collagen in the sample, mg; *X*: the concentration of the reference peptide in the sample calculated by Formula (1), μg/mL; V: the final volume of the sample, mL; M_1: the molecular weight of the bovine type I collagen, Da; M_2: the molecular weight of the reference peptide, Da; 2: the conversion coefficient (there were 2 α1 chains in type I collagen).

3. Results

3.1. Characterization of Implants

The collagen sponge implant made from bovine type I collagen was characterized with a CD spectrogram as shown in Figure 1, with a positive extreme at 220 nm and a negative peak at 197 nm. The ratio of the positive peak to the negative peak (RPN) was 0.104, which is within the RPN ranges of collagen (0.09–0.15) [36]. Therefore, the bovine type I collagen-based collagen sponge implants contained the typical triple helix structure.

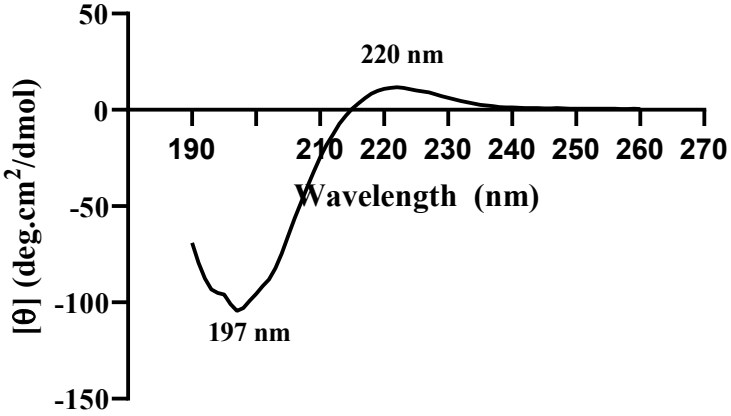


Figure 1. CD spectrum of collagen sponge implant.

Figure 2 illustrates the SEM images of the collagen sponge and ACM. The collagen sponge was composed of collagen-based membrane and fibrous components. It had an irregular and relatively fluffy structure with varying pore sizes. The ACM was mostly

composed of fibers and had a denser structure. The porosity or density of implants may directly relate to the degradation *in vivo*.

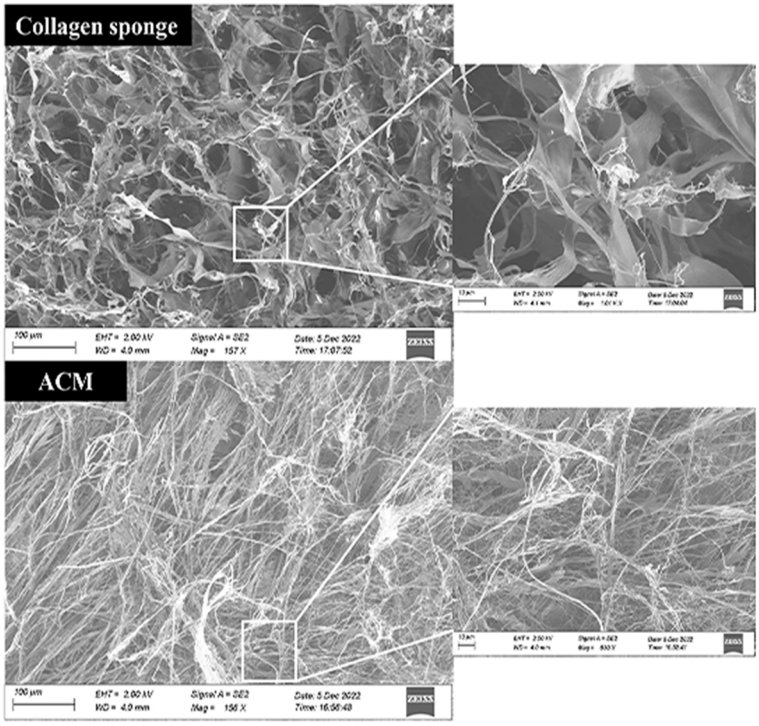


Figure 2. SEM images of collagen sponge and ACM.

3.2. Specific Peptide Identification of Bovine Type I Collagen

The main components of a collagen sponge and ACM are bovine type I collagen. In this study, in order to specifically detect the residues of bovine type I collagen-based implants in rabbit, it was necessary to select a bovine type I collagen-specific peptide, while this peptide must not exist in rabbit type I collagen. To achieve this goal, the theoretical sequence of the α1 chain of bovine type I collagen was used to mimic trypsin digestion, and then GPAGPQGPR (simplified as G-R) was selected from all peptides obtained by searching the Uniprot database. Then, using the same method to search and compare the G-R sequence from the α1 chain of rabbit type I collagen, it was not found, indicating that the selected G-R sequence is bovine-specific and not found in rabbit.

To further verify whether the G-R peptide was specific to bovine type I collagen and if the mass spectrum peak had sufficient signal strength and resolution in the real collagen sample, a bovine type I collagen reference material was used. The peptides resulting from the trypsin-digested bovine type I collagen reference material were analyzed by HPLC–MS/MS, and a total of 14 specific peptides of bovine type I collagen were found (as shown in Table 1). Figure 3 shows the total ion chromatogram of the digested bovine type I collagen reference material. BLAST multi-sequence alignment and Protein Discoverer software were used to analyze the mass spectrometric information of the collagen peptides. Figure 4 shows the mass spectrum of *m/z* 418.721. The isotopic peak distribution indicates that this ion had two charges. The MS/MS spectrum (Figure 5) illustrates the product ions of *m/z* 418.721. The product ions highly corresponded to the peptide G-R. This demonstrates that the digested mixture of the bovine type I collagen contained the G-R sequence, while the G-R mass spectrum peak had sufficient signal strength and resolution. On the other hand, the rabbit skin tissue (without implanted bovine type I collagen material), as prepared in Section 2.4, was analyzed, and the G-R peptide was not found in it, indicating that the G-R peptide is bovine type I collagen-specific, and not found in rabbit.

Table 1. The identified specific peptides of bovine type I collagen.

m/z	Charge State	Sequences	Rt/Min	Confidence
604.96722	3	VGPPGPSGNAGP*P*GP*PGPAGK	15.98	High
580.82599	2	GVPGPPGAVGPAGK	20.73	High
418.72323	2	GPAGPQGPR	2.18	High
844.74567	3	GNDGATGAAGPPGPTGPAGP*P*GFPGAVGAK	38.70	High
1012.81787	3	GLPGPP*GAP*GPQGFQGPPGEPGEPGASGPMGPR	46.34	High
520.94293	3	GETGPAGPAGPIGPVGAR	29.13	High
924.44672	2	GEPGPTGIQGPP*GPAGEEGK	22.28	High
1067.49597	2	GEPGPPGPAGFAGP*P*GADGQP*GAK	27.99	High
702.35028	2	GEPGPAGLPGPP*GER	23.45	High
764.38611	3	GDAGPPGPAGPAGPPGP*IGNVGAP*GPK	39.42	High
691.67938	3	GAPGADGPAGAPGTP*GPQGIAGQR	24.60	High
558.75189	2	EGAP*GAEGSP*GR	2.27	High
781.40356	2	DGLNGLPGP*IGP*P*GPR	36.60	High
845.89221	2	DGEAGAQGPPGPAGPAGER	15.89	High

P* was the site of proline hydroxylation.

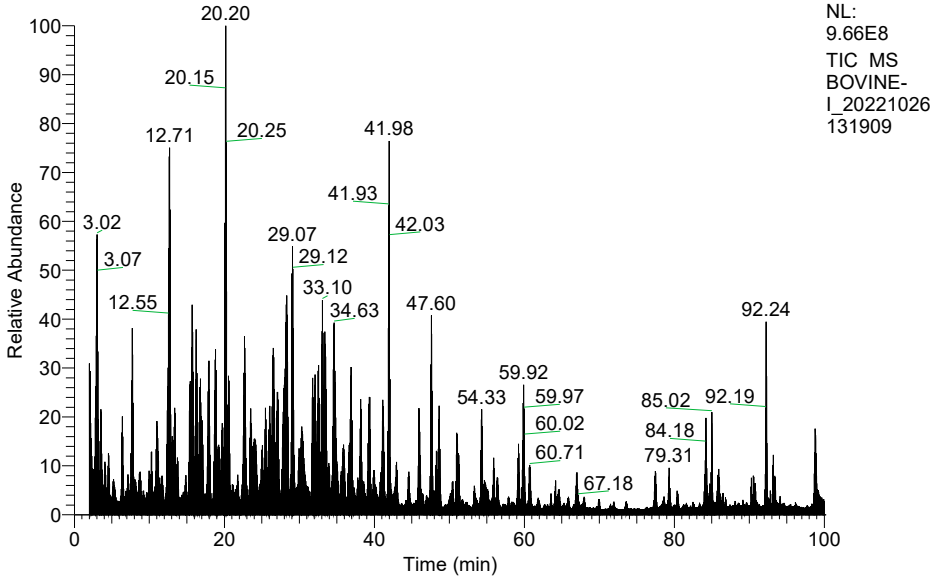


Figure 3. Total ion chromatogram of digested bovine type I collagen reference material.

The peptides selected as specific peptides also had to meet the following criteria: (a) They were absolutely digested by trypsin and had only one trypsin digestion site (K or R). (b) The proline in the specific peptides was not hydroxylated. (c) Their abundance was relatively high and stable. (d) They had to be a short-peptide sequence. The peptide G-R fulfilled all of the above conditions. Thus, peptide G-R was selected for the quantitative analysis of bovine type I collagen.

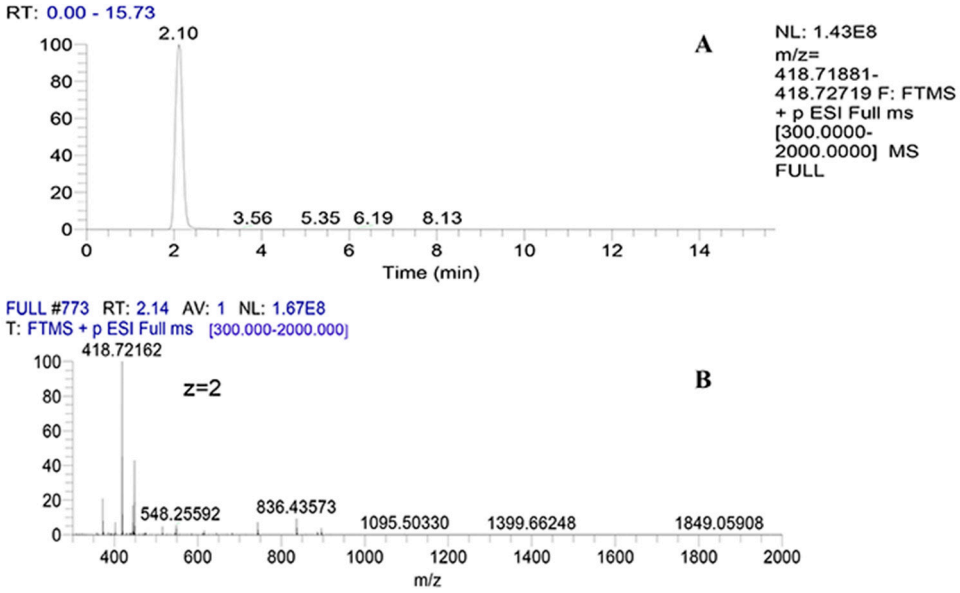


Figure 4. The extracted ion chromatogram (**A**) and MS spectrum (**B**) of *m/z* 418.723 detected in the digested bovine type I collagen reference material.

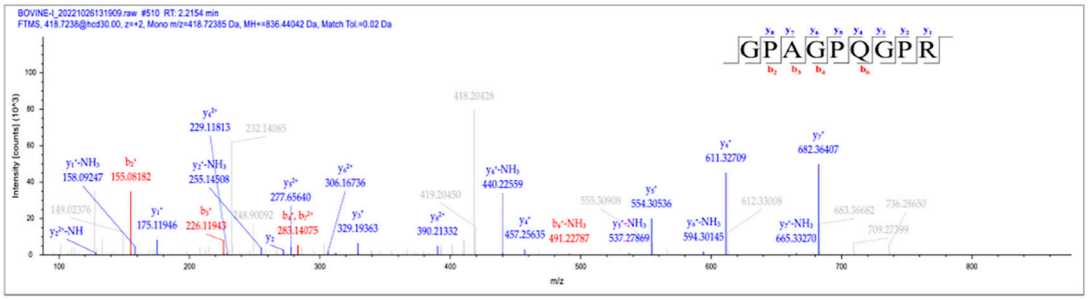


Figure 5. MS/MS spectrum of *m/z* 418.721 detected in digested bovine type I collagen reference material.

3.3. Quantification of Bovine Type I Collagen

Compared to purified collagen, the implants (collagen sponge and ACM) and the implants combined with animal tissues are relatively more complex in composition. In order to decrease the interference of the matrix in animal tissues on the quantitative results, the isotopic internal reference method was used for the quantitative analysis of the *in vivo* degradation of collagen-based implants. The reference peptide, which was the specific peptide G-R identified in Section 3.2, and the internal reference peptide iso/G-R were synthesized and used for quantification. The signal of the internal reference peptide iso/G-R is comparable to the reference peptide G-R. Figure 6 shows the extracted ion chromatogram and the MS/MS spectra of the reference peptide G-R and internal reference peptide iso/G-R. The monitored target ions of the bovine type I collagen reference peptide G-R and the internal reference peptide iso/G-R were *m/z* 418.74 (2+)→611.27 (1+) and *m/z* 420.71 (2+)→613.27 (1+), respectively. The concentration of the reference peptide G-R was prepared from 0.1 to 10 μg/mL. The peak area ratio between the reference peptide G-R working solution and the internal reference peptide iso/G-R was used as the ordinate. The different concentrations of the reference peptides were used as the abscissa (as shown in Figure 7). The regression equation and correlation coefficient were y = 0.0218706 + 0.272941x and R^2 = 0.9993, respectively.

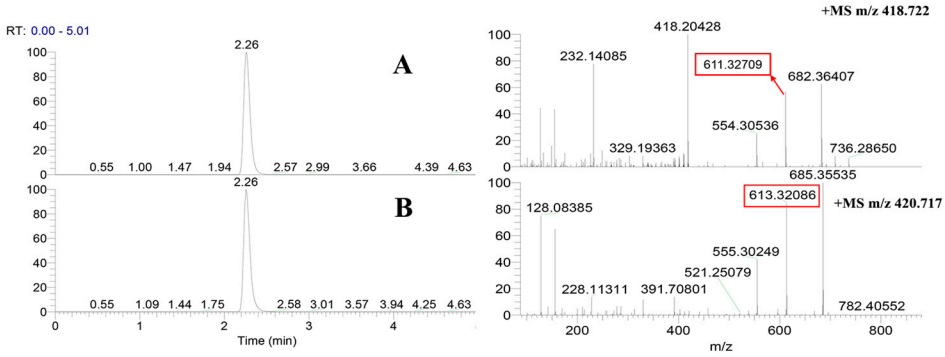


Figure 6. The MS/MS extracted ion chromatograms and the MS/MS spectrum of the reference peptide G-R (**A**) and the internal reference peptide iso/G-R (**B**).

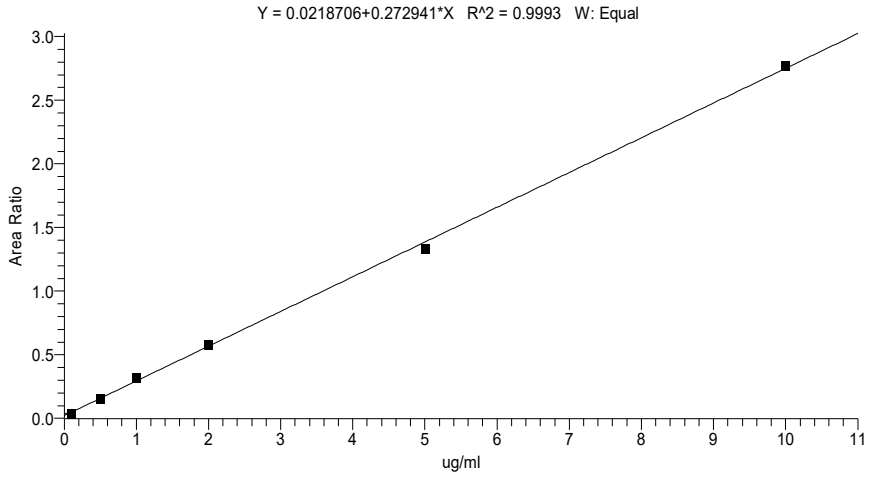


Figure 7. Standard curve. A correlation curve of concentration of the reference peptide and area ratio of the reference peptide and internal reference peptide.

3.4. Quantitative Methodological Validation

3.4.1. Method Precision

The method precision was validated by the samples that were digestion products of the bovine type I collagen reference material with concentrations of 0.5 mg/mL (Group A) and 1 mg/mL (Group B). Every group contained 10 parallel samples, which were digested and analyzed. The average peptide G-R concentration and the relative standard deviation (RSD) were calculated. Table 2 shows that the RSD values of Group A and Group B were 1.70% and 1.79%, respectively. The RSD between the two groups was 3.55%, which indicates that the experiment results are accurate and the method is reliable.

Table 2. Precision of quantification method for peptide G-R of bovine type I collagen.

Groups	Sample No.	Peptide G-R (μg/mg)	Average (μg/mg)	SD	RSD%
A	1	5.57	5.63	0.10	1.70
	2	5.64			
	3	5.52			
	4	5.66			
	5	5.58			
	6	5.52			
	7	5.74			
	8	5.80			
	9	5.70			
	10	5.56			
B	1	5.84	5.92	0.11	1.79
	2	5.90			
	3	5.86			
	4	5.97			
	5	5.80			
	6	5.96			
	7	5.93			
	8	5.89			
	9	5.86			
	10	6.18			
Average			5.77		
SD			0.21		
RSD %			3.55		

3.4.2. Method Repeatability Verification

The digestion products of the bovine type I collagen reference material (0.5 mg/mL) were used for repeatability verification. Three samples were prepared for the verification process, and every sample was detected three times independently. Table 3 shows that the average concentration of the reference peptide was 5.55 μg/mg. The RSD between the different experiments was 0.63%, indicating that this method has high repeatability.

Table 3. Repeatability of quantification method.

Groups	Sample No.	Peptide G-R (μg/mg)	Average (μg/mg)
1	1-1	5.45	5.52
	1-2	5.53	
	1-3	5.59	
2	2-1	5.46	5.55
	2-2	5.77	
	2-3	5.43	
3	3-1	5.52	5.59
	3-2	5.66	
	3-3	5.58	
Average concentration (μg/mg)		5.55	
SD		0.04	
RSD %		0.63	

3.4.3. Detection Recovery Rate Based on Reference Peptide Spiking Study

The results of the reference peptide spiking study at different spiking levels are shown in Table 4. It is shown that the spiking recovery rate was between 94% and 118.3%, which meets the general requirements for spiking recoveries (80–120%). When the spiking amount was 0.2 ug, the spiking recovery rate was more than 110%. The main reason was that the less the amount added, the greater the error caused. The average spiking recovery rate

was 106.78%. The RSD between the different spiking levels was 8.63%. This meets the requirements for spiking recoveries in methodological validation.

Table 4. Spiking recovery rate of quantification method.

Sample No.	Spiking Amount of Reference Peptide (μg)	Peptide G-R Concentration of Test Sample (μg/mg)	Peptide G-R Concentration of Spiking Samples (μg/mg)	Spiking Recovery Content (μg)	Spiking Recovery Rate %
1-1	0.20	5.37	7.57	0.24	118.28
1-2	0.20	5.37	7.55	0.23	116.79
1-3	0.20	5.37	7.48	0.22	112.12
2-1	0.40	5.37	9.42	0.43	107.45
2-2	0.40	5.37	9.34	0.42	105.34
2-3	0.40	5.37	9.48	0.44	109.15
3-1	1.00	5.37	14.24	0.94	94.27
3-2	1.00	5.37	15.01	1.02	102.36
3-3	1.00	5.37	14.34	0.95	95.25
Average spiking recovery rate %			106.78		
SD			9.23		
RSD %			8.63		

To verify the interference effect of the matrix on the determination of the implant samples, the reference peptide with different concentrations was spiked in the blank matrix, and the spiking recovery rate is shown in Table 5. The spiking recovery rate was between 93% and 112%, and the average spiking recovery rate was 103.6%. The RSD between the different spiking levels was 5.38%. This indicates that this method accurately determined the bovine type I collagen implanted in rabbits.

Table 5. Spiking recovery rate of the quantification method in the blank matrix.

Sample No.	Spiking Amount of Reference Peptide (μg)	Spiking Recovery Content (μg)	Spiking Recovery Rate %
1-1	0.5	0.468	93.6
1-2	0.5	0.497	99.4
1-3	0.5	0.503	100.6
2-1	1.0	1.102	110.2
2-2	1.0	1.030	103
2-3	1.0	0.987	98.7
3-1	2.5	2.712	108.5
3-2	2.5	2.683	107.3
3-3	2.5	2.780	111.2
Average spiking recovery rate %		103.6	
SD		5.58	
RSD %		5.38	

3.4.4. Detection and Quantification Limits

The reference peptide was gradually diluted and detected. The detection concentration with a signal-to-noise (S/N) of 10 was set as the limit of quantification (LOQ) in the method, and an S/N of 3 was set as the limit of detection (LOD). The LOQ and LOD were 2.05×10^{-3} μg/mL and 1.12×10^{-3} μg/mL, respectively.

3.5. Degradation Analysis of Implants

The residual content of bovine type I collagen in the sample from the *in vivo* experiment study was analyzed with the established bovine type I collagen peptide determination-based HPLC–MS/MS. The results are shown in Figure 8, indicating that the whole degra-

dation process of the collagen sponge was fast. It was essentially completely degraded at 10 weeks. In the collagen sponge group, the degradation rate reached 66.67% 2 weeks after implantation. Additionally, only 0.3% of the collagen remained at 6 weeks after implantation. The degradation of the ACM was relatively slow, and the complete degradation period needed 18 weeks. The degradation rate of the ACM in the first 8 weeks was relatively fast, and the degradation rate of the ACM reached 57.62% at 6 weeks. After 6 weeks, the degradation of the ACM began to slow down. At 16 weeks after implantation, only 2.38% of the ACM residue remained.

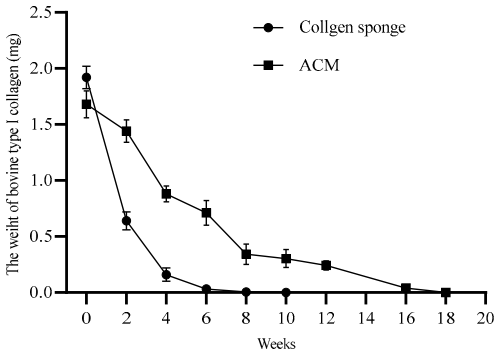


Figure 8. Degradation trend chart of collagen sponge and ACM *in vivo*.

4. Discussion

In this study, a standardized internal reference peptide-based HPLC-MS/MS method was established for bovine type I collagen determination. To ensure the accuracy of the quantification method, three different spiking levels of a reference peptide were used to verify the spiking recovery rate. The spiking recovery rate was between 94% and 119%, and the RSD between the three different spiking levels was 8.63%. The spiking recovery rate of the quantification method in the blank matrix was between 93% and 112%. The LOQ and LOD in the established method were 2.05×10^{-3} μg/mL and 1.12×10^{-3} μg/mL, respectively. All of the verification results comply with the provision of the *Pharmacopoeia of the People's Republic of China* [37]. The methodological studies indicate that the established collagen quantification method based on HPLC–MS/MS and the reference peptide had good precision, repeatability, and stability. Furthermore, combined with an isotope-labeled peptide as an internal reference, the interference of the matrix in the complex (tissue) samples was overcome. This standardized method was issued in the China industry standard (YY/T 1805.3-2022) for the quantitative determination of bovine type I collagen and published in 2022 [35], and it also has been approved as an international standard project that is under development (ISO AWI 6631).

In this study, the established internal reference peptide-based HPLC-MS/MS method was applied for determining the content of type I collagen in the implants at different periods after implantation. The peptide G-R as a detection target was found in bovine type I collagen-based materials but not in the experimental animal (rabbit) host tissues, effectively avoiding the interference of collagen from animal tissues. In this *in vivo* experiment, we could not see any collagen sponge and ACM residues at 6 weeks and 10 weeks with visual observation. However, the results of the collagen peptide G-R show that no peptide G-R was detected until 10 weeks for the collagen sponge, and in the ACM group, 0.2 μg/mL of peptide G-R could be detected at 16 weeks. The degradation rate of the purified bovine type I collagen-based sponge was faster than that of the bovine skin-derived decellularized ACM. The main reason may be that the ACM implant contained collagen and small amounts of fibronectin, laminin, and so on [15,16], and it had more fibril and a relatively dense structure, as shown in Figure 2, but the collagen sponge implant had a loose and porous structure. The loose and porous structure facilitated the conducive growth of host cells and the entry of blood, various enzymes, and biological factors, thus accelerating the

degradation of implants [38,39]. Compared to the collagen sponge, the ACM had a stable three-dimensional network and dense structure, as a result, the ACM took a longer time to degrade *in vivo*. This suggests that more complete data on the degradation period of collagen-based materials *in vivo* could be obtained using the HPLC–MS/MS method. Therefore, the HPLC–MS/MS method has high accuracy and can be used to evaluate the *in vivo* degradation analysis of collagen-based materials.

Implants based on biological materials, which are different from metal, ceramic, and polymer materials, are easy to degrade *in vivo* when used without special treatment, such as cross-linking [40]. Due to the rapid loss of their original shape and structure in the process of degradation, and because the appearance of degraded implants is similar to the color of the host tissue, it is difficult to identify the remaining implants with the naked eye. Moreover, collagen materials from biological sources are degraded to form fragments, short peptides, and even amino acids, which can be used by the animal body or in the blood circulation and be metabolized. Therefore, there is a lack of effective analytical methods to study the degradation kinetics of biodegradable materials *in vivo*. The method established in this study solves this problem and provides an indirect quantitative method for determining the residue of implants through the determination of the collagen, which is the main component of implants, via the reference peptide detection method so as to analyze the degradation period and the dynamic changes of implants.

5. Conclusions

In this study, we first identified the specific peptides of bovine type I collagen using HPLC–MS/MS. Then, an isotope-labeled internal reference peptide method based on product ions was established. It was proven that this method had high precision, repeatability, a good spiking recovery rate, and a low LOQ. The complete degradation periods of the collagen sponge and ACM were 10 and 18 weeks after implantation, respectively. The degradation rate of the collagen sponge was faster, while the degradation rate of the ACM was relatively slow. The proposed method is useful for accurately analyzing the degradation of collagen-based materials *in vivo*.

Author Contributions: Conceptualization, G.Z. and L.X.; Methodology, J.G., Y.M. and T.Z.; Software, J.G. and F.X.; Validation, Y.Z. and F.X.; Data Curation, J.G.; Writing—Original Draft Preparation, J.G.; Writing—Review & Editing, G.Z., X.L. and Z.G.; Supervision, Y.K.; Project Administration, F.X.; Funding Acquisition, G.Z. and X.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Key Technology R&D Programs of China (2021YFC2400801), the Science and Technology Program of Guangzhou, China (201803010086), the Open Funding Project of the State Key Laboratory of Biochemical Engineering (2021KF-04), and the Independent Research Project of the State Key Laboratory of Biochemical Engineering (2021ZZ-03).

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by Chinese Research Hospital Association (CRHA). The protocol number was IACUC-C2022037 and the date of approval was 22 March 2021.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Xu, H.F.; Bihan, D.; Chang, F.; Huang, P.; Farndale, R.; Leitinger, B. Discoidin domain receptors promote a1b1- and a2b1-integrin mediated cell adhesion to collagen by enhancing integrin activation. *PLoS ONE* **2012**, *7*, e52209. [CrossRef] [PubMed]
2. Kadler, K.; Baldock, C.; Bella, J.; Boot-Handford, R. Collagens at a glance. *J. Cell Sci.* **2007**, *120*, 1955–1958. [CrossRef] [PubMed]
3. Zha, Z.; Han, Q.; Huo, S. The protective effects of bexarotene against advanced glycation end-product (AGE)-induced degradation of articular extracellular matrix (ECM). *Artif. Cells Nanomed. Biotechnol.* **2020**, *48*, 1–7. [CrossRef] [PubMed]
4. White, J.F.; Werkmeister, J.A.; Bisucci, T.; Darby, I.A.; Ramshaw, J.A. Temporal variation in the deposition of different types of collagen within a porous biomaterial implant. *J. Biomed. Mater. Res. Part A* **2014**, *102*, 3550–3555. [CrossRef]
5. Blackburn, N.J.; Sofrenovic, T.; Kuraitis, D.; Ahmadi, A.; McNeill, B.; Deng, C.; Rayner, K.J.; Zhong, Z.; Ruel, M.; Suuronen, E.J. Timing underpins the benefits associated with injectable collagen biomaterial therapy for the treatment of myocardial infarction. *Biomaterials* **2015**, *39*, 182–192. [CrossRef]

6. Mayer, S.; Decaluwe, H.; Ruol, M.; Manodoro, S.; Kramer, M.; Till, H.; Deprest, J. Diaphragm repair with a novel cross-linked collagen biomaterial in a growing rabbit model. *PLoS ONE* **2015**, *10*, e0132021. [CrossRef]
7. Otake, T.; Toriumi, T.; Ito, T.; Okuwa, Y.; Honda, M. Recovery of sensory function after the implantation of oriented-collagen tube into the resected rat sciatic nerve. *Regen. Ther.* **2020**, *14*, 48–58. [CrossRef]
8. Ahtzaz, S.; Waris, T.S.; Shahzadi, L.; Chaudhry, A.A.; Yar, M. Boron for tissue regeneration-it's loading into chitosan/collagen hydrogels and testing on chorioallantoic membrane to study the effect on angiogenesis. *Int. J. Polym. Mater. Polym.* **2020**, *69*, 525–534. [CrossRef]
9. Yang, Y.; Zhang, Y.Y.; Min, Y.P.; Chen, J.H. Preparation of methacrylated hyaluronate/methacrylated collagen sponges with rapid shape recovery and orderly channel for fast blood absorption as hemostatic dressing. *Int. J. Biol. Macromol.* **2022**, *222*, 30–40. [CrossRef]
10. Sun, L.L.; Li, B.F.; Jiang, D.D.; Hou, H. Nile tilapia skin collagen sponge modified with chemical cross-linkers as a biomedical hemostatic material. *Coll. Oid. Surface. B* **2017**, *159*, 89–96. [CrossRef]
11. Zhou, Y.J.; Zhang, Z.H.; Chen, H.Q.; Liu, J.; Lin, R.Y. Application of acellular dermal matrix to reconstruct the defects after hypopharyngeal carcinoma resection. *Am. J. Otolaryngol.* **2021**, *42*, 102847. [CrossRef] [PubMed]
12. Sbi, L.J.; Wang, Y.; Yang, C.; Jiang, W.W. Application of acellular dermal matrix in reconstruction of oral mucosal defects in 36 cases. *J. Oral. Maxillofac. Surg.* **2012**, *70*, e586–e591.
13. Pang, K.; Du, L.; Qu, X. A rabbit anterior cornea replacement derived from acellular porcine cornea matrix, epithelial cells and keratocytes. *Biomaterials* **2010**, *31*, 7257–7265. [CrossRef]
14. Paulo Roberto, G.A.; Hueliton, W.K.; Fernandes, K.R.; Fortulan, C.A.; Muniz Renno, A.C. Effects of bioinspired bioglass/collagen/magnesium composites on bone repair. *J. Biomater. Appl.* **2019**, *34*, 261–272.
15. Yao, Q.; Zheng, Y.W.; Lin, H.L.; Lan, Q.H.; Huang, Z.W.; Wang, L.F.; Chen, R.; Xiao, J.; Kou, L.F.; Xu, H.L.; et al. Exploiting crosslinked decellularized matrix to achieve uterus regeneration and construction. *Artif. Cell Nanomed. B* **2020**, *48*, 218–229. [CrossRef]
16. Li, Z.Q.; Kong, W. Cellular signaling in Abdominal Aortic Aneurysm. *Cell. Signal.* **2020**, *70*, 109575. [CrossRef]
17. Gilbert, T.W.; Stewart-Akers, A.M.; Badylak, S.F. A quantitative method for evaluating the degradation of biologic scaffold material. *Biomaterials* **2006**, *18*, 128–130.
18. Kishore, V.; Uquillas, J.A.; Dubikovsky, A.; Alshehabat, M.A.; Snyder, P.W.; Breur, G.J.; Akkus, O. In vivo response to electrochemically aligned collagen bioscaffolds. *J. Biomed. Mater. Res. Part B Appl. Biomater.* **2012**, *100*, 400–408. [CrossRef]
19. Krawetz, R.J.; Taiani, J.T.; Wu, Y.E.; Liu, S.; Meng, G.; Matyas, J.R.; Rancourt, D.E. Collagen I scaffolds cross-linked with beta-glycerol phosphate induce osteogenic differentiation of embryonic stem cells in vitro and regulate their tumorigenic potential in vivo. *Tissue Eng. Part A* **2012**, *18*, 1014–1024. [CrossRef]
20. Hou, Z.P.; Chen, S.; Hu, W.R.; Guo, J.; Li, P.; Hu, J.S.; Yang, L.Q. Long-term in vivo degradation behavior of poly (trimethylene carbonate-co-2, 2-dimethyltrimethylene carbonate). *Eur. Polym. J.* **2022**, *177*, 111442. [CrossRef]
21. Guo, Y.W.; Chen, Z.C.; Wen, J.C.; Jia, M.H.; Shao, Z.Z.; Zhao, X. A simple semi-quantitative approach studying the in vivo degradation of regenerated silk fibroin scaffolds with different pore sizes. *Mat. Sci. Een. C* **2017**, *79*, 161–167. [CrossRef] [PubMed]
22. Chen, Z.G.; Kang, L.Z.; Meng, Q.Y.; Liu, H.Y.; Wang, Z.L.; Zhongwu Guo, Z.L.; Fuzhai Cui, F.Z. Degradability of injectable calcium sulfate/mineralized collagen-based bone repair material and its effect on bone tissue regeneration. *Mater. Sci. Eng. C-Mater.* **2014**, *45*, 94–102. [CrossRef] [PubMed]
23. Rathod, L.; Bhowmick, S.; Patel, P.; Sawant, K. Calendula flower extract loaded collagen film exhibits superior wound healing potential: Preparation, evaluation, in-vitro & in-vivo wound healing study. *J. Drug. Deliv. Sci. Tec.* **2022**, *72*, 103363.
24. Bhagwat, P.K.; Dandge, P.B. Collagen and collagenolytic proteases: A review. *Biocatal. Agric. Biotechnol.* **2018**, *15*, 43–55. [CrossRef]
25. Hofman, K.; Hall, B.; Cleaver, H.; Marshall, S. High-throughput quantification of hydroxyproline for determination of collagen. *Anal. Biochem.* **2011**, *417*, 289–291. [CrossRef]
26. Silva, C.M.L.; Spinelli, E.; Rodrigues, S.V. Fast and sensitive collagen quantification by alkaline hydrolysis/hydroxyproline assay. *Food Chem.* **2015**, *173*, 619–623. [CrossRef]
27. Langrock, T.; Hoffmann, R. Analysis of hydroxyproline in collagen hydrolysates. *Methods Mol. Biol.* **2019**, *2030*, 47–56.
28. Brown, S.R.; Cleveland, E.M.; Deeken, C.R.; Huitron, S.S.; Aluka, K.J.; David, K.G. Type I/type III collagen ratio associated with diverticulitis of the colon in young patients. *J. Surg. Res.* **2017**, *207*, 229–234. [CrossRef]
29. Song, H.D.; Zhang, S.Q.; Zhang, L.; Li, B. Effect of orally administered collagen peptides from bovine bone on skin aging in chronologically aged mice. *Nutrients* **2017**, *9*, 1209. [CrossRef]
30. Liu, G.Y.; Kawaguchi, H.; Ogasawara, T.; Asawa, Y.; Kishimoto, J.; Takahashi, T.; Chung, U.; Yamaka, H.; Asato, H.; Nakamura, K.; et al. Optimal combination of soluble factors for tissue engineering of permanent cartilage from cultured human chondrocytes. *J. Biol. Chem.* **2007**, *282*, 20407–20415. [CrossRef]
31. Yuan, T.; Xiao, Y.M.; Fan, Y.J.; Liang, J.; Zhang, X.D. The degradation and local tissue effects of collagen hydrogel and sponge implants in muscle. *Polym. Test.* **2017**, *62*, 348–354. [CrossRef]
32. Zhang, G.F.; Sun, A.M.; Li, W.J.; Liu, T.; Su, Z.G. Mass spectrometric analysis of enzymatic digestion of denatured collagen for identification of collagen type. *J. Chromatog. A* **2006**, *1114*, 274–277. [CrossRef]

33. Zhang, G.F.; Liu, T.; Wang, Q.; Chen, L.; Lei, J.D.; Luo, J.; Ma, G.H.; Su, Z.G. Mass spectrometric detection of marker peptides in tryptic digests of gelatin: A new method to differentiate between bovine and porcine gelatin. *Food Hydrocoll.* **2009**, *23*, 2001–2007. [CrossRef]
34. Zhang, Y.; Chen, Y.; Zhao, B.; Gao, J.P.; Xia, L.L.; Xing, F.Y.; Kong, Y.J.; Li, Y.C.; Zhang, G.F. Detection of type I and III collagen in porcine acellular matrix using HPLC-MS. *Regen. Biomater.* **2020**, *7*, 577–582. [CrossRef]
35. *YY/T 1805.3-2022*; Chinese Industry Standard, Tissue engineering medical device products—Collagen protein—Part 3: Quantification of collagen based on marker peptide detection—Liquid chromatography—mass spectrometry. Standardization Administration of China: Beijing, China, 2022.
36. Holmes, R.; Kirk, S.; Tronci, G.; Yang, X.B.; Wood, D. Influence of telopeptides on the structural and physical properties of polymeric and monomeric acid-soluble type I collagen. *Mat. Sci. Eng. C* **2017**, *77*, 823–827. [CrossRef]
37. Wang, X.; Wang, F.; Wang, Z.J.; Wang, X.J.; Shi, S.M.; Shen, M.R.; Bai, X.J. *Pharmacopoeia of People's Republic of China*; Chian Medical Science Press: Beijing, China, 2020; pp. 480–483.
38. Agren, M.S.; Schnabel, R.; Christensen, L.H.; Mirastschijski, U. Tumor necrosis factor-alpha-accelerated degradation of type I collagen in human skin is associated with elevated matrix metalloproteinase (MMP)-1 and MMP-3 ex vivo. *Eur. J. Cell Biol.* **2015**, *94*, 12–21. [CrossRef]
39. Klinger, A.; Asad, R.; Shapira, L.; Zubery, Y. In vivo degradation of collagen barrier membranes exposed to the oral cavity. *Clin. Oral Implants Res.* **2010**, *21*, 873–876.
40. Shanmugam, K.; Subha, V.; Renganathan, S. Type 1 collagen scaffold functionalized with ciprofloxacin loaded gelatin microspheres– fabrication, In vitro & In vivo evaluation, histological and biochemical analysis. *Drug Des. Dev. Ther.* **2019**, *3*, 1–10.

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.