Converting of animal-derived protein waste into new biomaterials in a circular economy concept

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Abstract
The aim of the paper was the designing of a new biomaterial decorated with collagen and keratin nanofibers for new wound healing dressing fabrication.
The methods used to assess the *in vivo* biocompatibility of PVP/CH-PLA/PEO/KH electrospun nanofibers in white Swiss mice evaluated the effects of the nanofibers on the hematological, biochemical, and immunological profiles of the mice, as well as their impact on oxidative stress.
The use of collagen and keratin hydrolysates extracted from animal by-products and processing represents a sustainable and circular approach for added value new biomaterials and waste valorization.

Keywords: collagen, keratin, circular economy, nanofibers, electrospinning.

Introduction

The global livestock population data comes from the Food and Agriculture Organization (FAO) of the United Nations. According to the Food and Agriculture Organization (FAO)'s latest statistical yearbook, the livestock population in 2021 was estimated to be around 4.89 billion, which includes bovines (1.5 billion), goats (1 billion), ovines (1.3 billion), and swine (1.1 billion) (http://www.fao.org/faostat/en/#data/QA).

Due to advancements in animal production practices, livestock waste can now be repurposed and treated to yield fertilizers, feed, and biopolymers, resulting in a substantial reduction of pollution. Nonetheless, the development of biomaterials could present a more effective approach to managing animal waste, as it can generate innovative biomedical and pharmaceutical applications (Tarafdar et al. 2021). The biocompatible ceramic, namely hydroxyapatite (HA) (a primary and essential constituent of teeth and bone) isolated from bovine bones has found various biomedical applications. Meat industry produces a significant amount of collagen as waste from short tendons of slaughtered cattle and cattle hides. This collagen can be utilized for the production of various biomaterials for biomedical field such as for the preparation of sponges for wounds, mini-pellet for drug delivery, nanoparticle for delivery of genes, shields used in ophthalmology, support biomaterial for the formation of neo-organs, etc. Bio-ceramics made from naturally occurring biological apatites are less expensive. In a study, sheep teeth dentine HA material was used as an alternate source of bioactive biomaterial for the purpose of grafting biomaterials from animal waste are used for the fabrication of scaffolds, for promoting bone and tissue regeneration and for fibroblast cell growth.

A significant amount of proteins results from the processing of mammal skins (cattle, sheep, etc.) and sheep's wool, which can be exploited due to their bioactive potential for the regeneration of damaged human tissues. For example, these proteins can be deposited in the form of nanofibers on a cotton support through the electrospinning process. Electrospinning involves the use of an electric field to draw a polymer solution or melt into a fine fiber. The process typically involves the preparation of animal-derived protein waste solution, then its loading into a syringe, and applying an electric field to the solution causing the solution to form a fine fiber, which is collected on a grounded surface. The nanofibers have a high surface area-to-volume ratio, which enhances their ability to support cell attachment and proliferation. Additionally, the nanofibers can be functionalized with bioactive molecules such as growth factors or peptides to further enhance their regenerative properties.

Thus, collagen rabbit glue derived from the skins of rabbits has been processed into nanofibers (Rapa et al. 2020), leading to increase in its surface area, improve in the mechanical properties, making it an ideal scaffold material for promoting cell growth and tissue regenerations. By incorporating water-based dispersant of titanium dioxide nanoparticles (TiO_2 NPs) in the form of anatase, doped with nitrogen and silver nanoparticle and chitosan with high molecular weight, am excellent biocompatibility and antimicrobial activity were achieved, making it a promising material for use in tissue engineering applications (Matei et al. 2020).

The application of animal-derived proteins in electrospinning for wound dressings as compared with synthetic polymers is an environmentally friendly approach because non-toxic solvents are used for preparing solutions, and, in addition, they possess antimicrobial and biocompatibility properties (Santhanam et al. 2020, Rapa et al. 2020).

However, the method of biomaterial preparation lacks standardized protocols in terms of pre-treatment, extraction, chemical modifications and purification that restrict its continuous commercial production.

Collagen is the most abundant protein in mammals, being a major constituent of skin, bones, tendons, blood vessels, and heart tissue, and successfully used for *in vitro* and *in vivo* tissue regeneration engineering (Horbert et al., 2019). Collagen is well-known for accelerating the formation of fibroblasts and the closure of wounds.

Keratin is a type of polypeptide molecule made up of multiple amino acids that contain both inter-molecular bonding of disulfide-cysteine amino acids and intra/inter-molecular bonding of polar and non-polar amino acids. It is both water-insoluble and resistant to a variety of weak alkaline, acidic solutions, and organic solvents. Additionally, keratin exhibits resistance to protein-digesting enzymes such as trypsin or pepsin (Athwal et al. 2022). Keratin, in particular, demonstrates reduced solubility and increased stability due to the cysteine disulfide cross-links that form within and between protein chains. The presence of keratin as a constituent provides exceptional characteristics that make it a highly effective biomaterial.

Collagen nanofibers are considered ideal for supporting cell proliferation and tissue regeneration. On the other hand, keratin promotes cell adhesion, proliferation, and migration due to the presence of Arg-Gly-Asp and Leu-Asp-Val tripeptides.

Keratin is used in wound dressing materials because it helps maintain their integrity in the epithelium. Additionally, keratin exhibits antioxidant and antimicrobial properties due to the presence of S-sulfonated cysteine groups in soluble keratin extracts. This is a significant advantage for converting valuable protein waste into new biomaterials.

In this paper, we created dual layers based on biodegradable matrices such as poly(lactic acid) (PLA), poly(ethylene oxide) (PEO) and poly(vinyl pyrrolidone) (PVP) by using mono- and coaxial electrospinning technology for potential wound dressings application (Figure 1). These matrices were loaded with keratin (KH) and bovine collagen glue (CH) in hydrolysate shapes. The advantages of creating nanofibers from these animal-derived proteins consist in ensuring the mechanical strength of dressings (due to the PLA polymer support), the controlled release of the encapsulated proteins due to the PEO and PVP, and the valorization of protein waste. PEO was used to enhance the encapsulation efficiency and accelerate the release rate of keratin. This combination (PLA/PEO/KH - 1st layer +PVP/CH - 2nd layer) may offer benefits such as the ability to keep the dressing on throughout the healing process and regulate the release of CH and KH onto the wound. The optical microscopy and spectrophotometric assays were used to examine the morphology and biocompatibility of the electrospun nanofibers.

Figure 1 Mono- and coaxial electrospinning equipment used to obtain assembled PLA/PEO/KH/PVP/CH nanofibers.

Experimental Part

Chemical enzymatic hydrolysis in alkaline conditions using calcium hydroxide and Alcalase 2.4 L (Novozymes) at 80°C and 60°C, respectively, was used to extract hydrolyzed collagen from bovine tanned leather by-products (Gaidău et al. 2009). The collagen was then concentrated to approximately 60% (w/w) at 60°C using a Hei-VAP Rotary Evaporator (Schwabach, Germany), resulting in an increase in collagen hydrolysate viscosity that made it spinnable. Intermediate processes were performed to completely remove chromium traces, which involved successive filtrations through 0.45 µm pore size media and decantation after the alkaline and enzymatic hydrolysis stages.

Sheepskin wool was first degreased using 8% NaOH rotulis (Lach-Nersro) at 80° C for 4 hours, and then solubilized to prepare keratin hydrolysate. Finally, the solution was filtered and freeze dried (Gaidău et al. 2021).

The physico-chemical properties of concentrated collagen hydrolysate and keratin hydrolysate used in this study are presented in Table 1.

Table 1. Physico-chemical characteristics of concentrated collagen and keratin hydrolysates.

Characteristics, U.M.	Value ± Standard Deviation	
	Collagen hydrolysate (CH)	*Keratin hydrolysate (KH)*
Dry matter, %	60.40 ± 0.42	9.02 ± 0.05
Ash, %	6.24 ± 0.27	13.73 ± 0.25
Total nitrogen, %	14.67 ± 0.66	14.40 ± 0.57
Protein, %	82.43 ± 2.66	80.84 ± 1.40
pH, pH units	8.54 ± 0.10	11.84 ± 0.09
Aminic nitrogen, %	1.43 ± 0.06	1.34 ± 0.06
Electrical conductivity, μs/cm	870 ± 0.1	13700 ± 20

10% (w/v) PLA pellets (NatureWorks® Ingeo™, 4032D, film grade) were dissolved in a mixture of dichloromethane (DCM) and dimethyformamide (DMF) and solvents (7:3 vol%) at a temperature of 60 °C for 6 h and 400 rpm. Then PEO as power shape (MW100000, Alfa Aesar, Kandel, Germany) was added into a PLA solution to achieve a total polymer content of 12.5 w%. The first1st layer consisting from PLA/PEO/KH nanofibers were fabricated via coaxial electrospinning technique, and served as a collected support for the encapsulated CH in PVP nanofibers.

The second layer consists in the incorporation of CH into a solution of 10% PVP (K90 for molecular biology, MW = 100,000-150,000 g/mol, PanReac AppliChem, Darmstat, Germany) in ethanol, via coaxial electrospinning, using two needles with G15 and G21 dimensions, a voltage of 19.93 kV, and flow rates of 3.5 mL/h and 2.5 mL/h for shell and core solutions, respectively.

A commercial TL-Pro-BM Electrospinning machine from Tong Li Tech Co., Ltd., Bao An, Shenzhen, China was used for fabrication of these layers of nanofibers. The machine consists of a syringe pump, a high-voltage power supplier, and a grounded conductive drum collector. Each solution containing the protein, polymer, and solvent was loaded into a 10 mL Teflon syringe and was fed through a metal needle attached to the end of the tubing. The electrospun nanofibers were collected on a drum covered with aluminum foil. The nanofibers produced and the technical parameters operating at electrospinning are listed in Table 2. The experiments were conducted at a temperature of 22.2ºC and a relative humidity of 36%.

Table 2. Compositions and optimal parameters for obtaining of electrospun biomaterials

Composition	*Electrospinning*	*Flow Rate (mL/h)*	*Voltage (kV)*	*Distance between needle to collector (cm)*
PLA/PEO	Mono			
PLA/PEO/KH	1st layer, Coaxial	5.7	22.96	14
PVP/CH	2nd layer, Coaxial	3.5; 2.5	19.93	14
PLA/PEO/KH-PVP-CH	Assembled structure			
PLA/PEO/PVP	Mono			

Morphology examination

A SEM (FEI Quanta 200 Scanning Electron Microscope, Eindhoven, Netherlands) was utilized to conduct analyses on the morphology and size distribution of nanofibers. To prevent charging effects, the electrospun samples were coated with a thin Au layer measuring about 5 nm. By analyzing manual measurements of 20 identified fibers using OriginPro 7.5 (OriginLab, Northampton, MA, USA), the average diameters of the fibers were determined.

Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR FT-IR)

The INTERSPEC 200-X spectrophotometer (Interspectrum, Tartumaa, Estonia) was used to determine the chemical composition of PLA/PEO/PVP, and PLA/PEO/KH-PV/CH electrospun nanofibers. Measurements were taken in absorption mode in the wavelength range of 4000–700 cm^{-1}, at a resolution of 4 cm^{-1}.

ABTS free radical scavenging assay

An improved ABTS discoloring assay was employed in the experiment, wherein the 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) radical cation (ABTS+•) was generated by reacting a 7 mM stock solution of ABTS with 2.45 mM potassium persulphate. The resulting mixture was left in the dark for a minimum of 16 hours at room temperature before use. The ABTS+• solution was then diluted to an absorbance of 0.7 ± 0.05 at 750 nm. Subsequently, a certain amount of nanofibers were combined with 4 mL of the ABTS+• solution and kept in the dark at room temperature for 15 minutes. The capability of the proteins to quench ABTS free radicals was evaluated using the following formula:

$$\text{ABTS free radical scavenging activity (\%)} = [(A_c - A_s) / A_c)] \times 100\ \%$$

The absorbance of control and sample were denoted as A_c and A_s, respectively. Each sample was subjected to three replicates (n = 3) during the experiments, and the reported values were the mean averages with the corresponding standard deviation (±SD).

Controlled released of protein

The quantity of proteins encapsulated onto the PLA/PEO/KH and PLA/PEO/KH-PVP/CH nanofibers was estimated via UV-Vis spectroscopy using a UV-Vis spectrophotometer. The predetermined KH and CH specific calibration curves (0–2000 ppm) were performed. The nanofibers containing encapsulated proteins were immersed into ethanol and mixed at room temperature for 60 minutes. The absorbance of supernatant was monitored at 285 nm, using a 1-cm (path width) quartz cell. The cumulative release of KH and CH was estimated according to Equation:

Cumulative Release (%) = Accumulative amount of protein released in time/Initial concentration of proteins, %

***In vivo* biocompatibility testing**

For the experimental research, the white Swiss mice that were healthy adults (3 months old) and weighed between 25-30 g were used and were purchased from the Cantacuzino Institute in Bucharest, Băneasa Resort. They had a uniform gender distribution and were not genetically modified. The mice were obtained for use at the "Grigore T. Popa" University of Medicine and Pharmacy in Iasi. The animals were brought a week before ~~to~~ the laboratory experiments for acclimatization to standard environmental conditions (constant temperature of 21°C ± 2°C, relative humidity of 50-70% and alternating light/dark lighting regime = 12 hours/12 hours), in individual cages, with granulated food (pellet type) and water available ad libitum. To prevent chronobiological influences, the experiments took place between 8-12 a.m. of each day. The research protocol was drawn up and the University's Ethics Commission's Approval (No. 5105/7.03.2022) was obtained, in accordance with international ethical standards, regarding animal studies.

On the first day of the experiment, the animals were anesthetized by intraperitoneal injection of a mixture of Ketamine 50 mg/kg and Xylazine 10 mg/kg. The integuments in the left dorsal region were shaved and a superficial incision of 0.5 cm length was made, parallel to the spine. The nanofiber-impregnated sheet (dimensions 0.8 cm/0.8 cm) was placed on the sterile textile material of a commercial patch and applied over the incision area, fixing to the skin with the help of the adhesive system. Mice in the control group were identically applied with a simple patch provided with the sterile textile material.

Figure 2 Placement of nanofibers on the adhesive system for application to the incision area.

The animals were randomly divided into 6 groups of 5 mice each, in which the test nanofibers were used according to the following notes: batch no. 1 – witness; batch no. 2 - PLA/PEO; batch no. 3 - PLA/PEO/KH; batch no. 4 - PLA/PEO/PVP; batch no. 5 - PVP/CH, and batch no. 6 - PLA/PEO/KH-PVP/CH. After making the skin incision and covering it with the test devices, the behavior of the animals was continuously monitored (respiration, spontaneous motility, motor coordination, food and water consumption,

stereotyped movements, personal hygiene), and on the 7th day, the patches were removed and the incision area was macroscopically inspected to detect local tissue changes.

The *in vivo* evaluation of the biocompatibility of nanofibers was based on studying the influence of their use on some hematological, biochemical, immunological constants and on oxidative processes.

24 hours and 7 days after the application of simple or nanofiber patches, the mice were anesthetized with 1% isoflurane and blood samples were collected from the lateral vein of the tail, for the determination of: blood count, glutamic-pyruvic transaminase (TGP), glutamic - oxalacetic transaminase (TGO), lactate dehydrogenase (LDH), urea, creatinine (Lindstrom NM et al., 2015) (Wolf MF et al., 2012). The tail of the animals was placed in warm water at 40 ºC to dilate the lateral vein. The tail was kept in a stretched position, the lateral caudal vein was identified, at a distance of 3 cm from the tip, and the respective area was antisepticized with 70% alcohol (Zou W et al., 2017) (Parasuraman S et al., 2010) (Tranquilli WJ et al., 2007). Under local anesthesia, with benzocaine 1% (sprays), the vein was punctured, and a volume of 0.3 mL of blood was extracted (Toft MF et al., 2005).

To perform the blood count, the blood was collected in vacutainers containing EDTA as an anticoagulant. The determinations were made using the automatic analyzer HEMAVET 950 (Oxford, UK), which works on the principle of flow cytometry with fluorescence. Biochemical determinations were based on the use of the ACCENT 200 biochemistry analyzer (Cormay, Warsaw, Poland) on venous blood collected on heparin.

To study the influence of nanofibers on oxidative processes, the activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) was monitored. The investigation of SOD activity in blood was based on the use of the colorimetric method with xanthine and xanthine oxidase, using RANSOD kits from RANDOX Laboratories Ltd. (Warsaw, Poland) on blood samples (0.3 mL) collected on heparin. To measure the serum values of GPx, 0.3 mL blood was collected on heparin, the activity of this enzyme was evaluated by the enzymatic method, using the RANSEL kit from RANDOX Laboratories Ltd. (Warsaw, Poland).

At the end of the experiment, after 7 days, serum opsonic capacity (CO) was measured using *Staphylococcus aureus* 94 cultures. The animals were then euthanized through general anesthesia using 2% isoflurane (Tranquilli WJ et al., 2007). Peritoneal macrophages were obtained from the intact peritoneal cavity by washing with 10 mL of HANKS solution, thermostated at 37 ºC. The samples were centrifuged at 1000 rotations per minute for 10 minutes, followed by incubation with *Staphylococcus aureus* 94 cultures, for 48 hours at 37 ºC. The phagocytic capacity (CF) and the bactericidal capacity (CB) of peritoneal macrophages were determined by reseeding the samples on culture media (Tartau L et al., 2012).

The euthanasia was conducted without inflicting physical and mental suffering on the animals, leading to rapid unconsciousness, cessation of heart and breathing, and eventual death. This standard procedure was carried out in dedicated necropsy rooms separate from the animals' living quarters (***Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32010L0063) (***AVMA Guidelines on Euthanasia, 2007).

The results were expressed as the arithmetic mean ± standard deviation (S.D.) of the mean values for each determined parameter and substance studied separately. Statistical analysis was performed using the unifactorial ANOVA method and the SPSS program, version 17.0 for Windows 10. This allowed for the evaluation of the significance of differences observed within the same batch of animals and between the batches that received the nanofibers compared to the control batch. Values of the probability coefficient (p) below 0.05 were considered statistically significant.

Results and Discussion

Morphology

The results show a nanofibrous structure of each layer and the assembled product demonstrated *in vivo* biocompatibility compared to the control, highlighted the potential use of animal-derived proteins in medical applications.

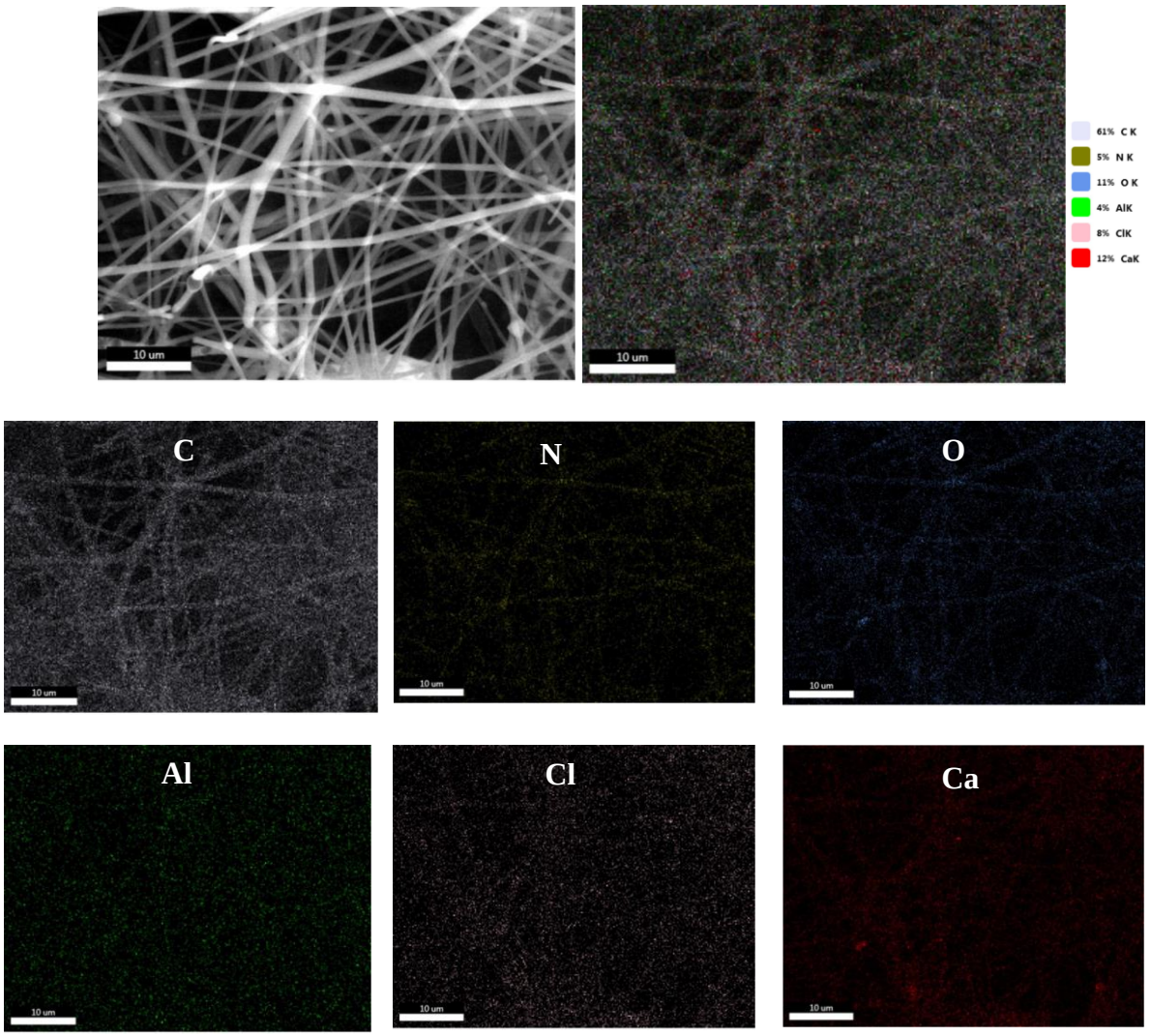

Figure 3 Morphology and the mapping of the assembled PLA/PEO/KH-PVP/CH nanofibers structure.

ATR-FT-IR

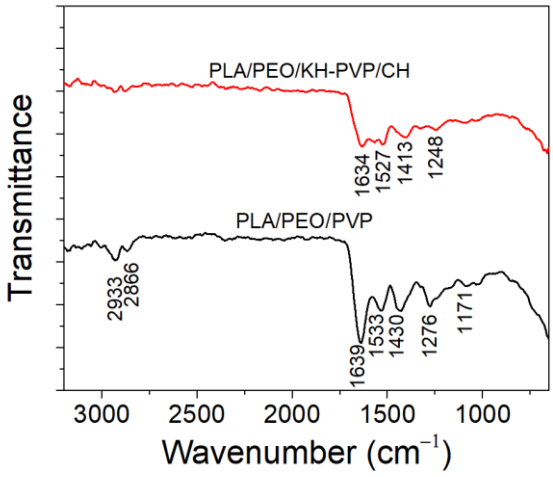

Figure 2: ATR-FT-IR for PLA/PEO/PVP and PLA/PEO/KH-PVP/CH

Antioxidant activity

Antioxidant activity of PLA/PEO/KH-PVP/CH nanofibers measured by ABTS radical scavenging bioassay was 97% ± 5.4%.

Controlled released of proteins

4.5 ± 0.3% at 5 min
5.8 % ± 0.2% at 10 min
7.6 % ± 1% at 60min

***In vivo* biocompatibility tests**

During the monitoring period, no behavioral changes were detected in the animals to which the studied nanofibers were administered. Mice performed specific movements of exploring the environment, feeding, watering and personal hygiene. On the 7th day of the experiment, the patches were removed and the incision area was inspected macroscopically. It was observed that, both in the animals in the control group and in those that received PLA/PEO, PLA/PEO/KH, PLA/PEO/PVP, PVP/CH, and PLA/PEO/KH-PVP/CH electrospun nanofibers, the integuments had normal appearance, no signs of inflammation, and the incision area was completely cicatrized.

Leukocyte formula

Table 3 shows the proportion of the various components (neutrophil polymorphonuclear (PMN), lymphocytes (Ly), eosinophils (E), monocytes (M), and basophils (B)) in the leukocyte formula of animals who received electrospun nanofibers compared to the control group (designated as witness), both after 24 hours and 7 days.

Table 3. Percentage values of leukocyte formula elements measured in animals that received nanofibers. The results are presented as the mean ± standard deviation of percentages for each element, based on data from 5 mice per batch

	Period of time	**Leukocyte formula (%)**				
		PMN	**Ly**	**E**	**M**	**B**
Witness	24 h	29.4±7.7	63.9±18.3	0.1±0.01	6.1±1.1	0.2±0.05
	7 d	28.6±8.9	64.8±19.5	0.2±0.05	6.2±1.1	0.2±0.05
PLA/PEO	24 h	28.5±8.5	65.0±17.5	0.2±0.05	6.1±1.3	0.2±0.05
	7 d	28.7±9.1	64.6±18.7	0.2±0.01	6.3±1.3	0.2±0.1
PLA/PEO/KH	24 h	29.2±8.3	64.1±18.9	0.1±0.01	6.4±0.5	0.2±0.1
	7 d	28.9±9.3	64.5±18.3	0.1±0.01	6.3±1.1	0.2±0.05
PLA/PEO/PVP	24 h	29.3±7.9	64.3±17.9	0.1±0.05	6.1±1.1	0.2±0.1
	7 d	29.1±8.7	64.4±19.1	0.1±0.01	6.2±1.3	0.2±0.1
PVP/CH	24 h	28.6±8.5	64.8±18.5	0.2±0.01	6.2±1.1	0.2±0.1
	7 d	27.8±8.9	64.5±18.7	0.1±0.05	6.3±1.3	0.2±0.05
PLA/PEO/KH-PVP/CH	24 h	28.6±8.7	65.6±18.9	0.1±0.01	6.3±1.1	0.2±0.05
	7 d	28.8±9.1	64.4±17.7	0.2±0.05	6.4±0.5	0.2±0.1

Hematological investigations revealed no significant variations in the percentage values of the leukocyte formula elements (PMN, Ly, E, M, and B) in animals that received nanofibers, compared to the control group, at 24 hours and 7 days in the experiment. These results suggest that covering the incision area with simple patches, or patches containing nanofibers, prevented the occurrence of a local or systemic inflammatory reaction.

Liver enzymes

The serum values of glutamic-oxaloacetic transaminase (TGO), glutamic-pyruvic transaminase (TGP), and lactate dehydrogenase (LDH) for mice that were administered electrospun samples are presented in Table 4.

Table 4. Changes in serum levels of TGP, TGO and LDH in animals that received nanofibers. Values are expressed as arithmetic mean ± standard deviation of mean TGO, TGP and LDH values for 5 mice per batch

	Period of time	TGP (U/ml)	TGO (U/ml)	LDH (U/l)
Witness	24 h	39.6±10.3	158.6±31.5	328.28±64.33
	7 d	40.2±10.5	160.4±30.7	333.56±70.67
PLA/PEO	24 h	40.5±10.9	161.7±32.9	332.34±71.13
	7 d	40.7±10.7	163.2±33.7	335.83±66.83
PLA/PEO/KH	24 h	39.4±9.7	159.8±35.3	330.45±69.45
	7 d	40.3±10.5	161.3±32.7	334.32±58.83
PLA/PEO/PVP	24 h	40.6±11.3	158.5±33.5	331.27±72.13
	7 d	39.8±9.9	162.7±31.7	335.19±71.33
PVP/CH	24 h	39.5±10.7	160.6±34.1	332.53±67.67
	7 d	39.8±10.3	163.8±33.5	336.13±69.27
PLA/PEO/KH-PVP/CH	24 h	39.7±10.5	160.4±35.3	330.67±71.33
	7 d	40.5±11.1	161.3±30.9	333.21±70.67

The results from Table 4 showed that no substantial differences in TGP, TGO, or LDH activity were detected between mice treated with PLA/PEO, PLA/PEO/KH, PLA/PEO/PVP, PVP/CH, PVP/CH/ PLA/PEO/KH, compared to those of witness, after 24 day and after 7 days in the experiment. These results constitute arguments on the fact that the use of nanofibers did not produce disturbances in liver function.

Table 5. Changes in blood urea and creatinine levels observed in animals receiving nanofibers. The values are expressed as the arithmetic mean ± standard deviation of the mean urea and creatinine levels for 5 mice per batch.

	Period of time	Urea (mg/dL)	Creatinine (mg/dL)
Witness	24 h	27.7±3.7	0.7±0.01
	7 d	28.3±4.3	0.7±0.03
PLA/PEO	24 h	26.9±4.7	0.8±0.03
	7 d	28.5±4.5	0.9±0.05
PLA/PEO/KH	24 h	29.1±5.3	0.8±0.01
	7 d	29.5±4.9	0.8±0.01
PLA/PEO/PVP	24 h	29.1±5.1	0.7±0.01
	7 d	28.9±3.7	0.9±0.03
PVP/CH	24 h	29.3±4.5	0.8±0.03
	7 d	29.7±4.9	0.8±0.05
PLA/PEO/KH-PVP/CH	24 h	29.5±4.7	0.9±0.01
	7 d	28.7±5.3	0.8±0.03

The application of patches containing nanofibers over the incision area did not result in noticeable changes in the serum levels of urea and creatinine, compared to the use of simple patches, at the two time points of the determination. These findings suggest that the use of nanofibers did not cause disturbances in renal function.

The blood levels of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in mice that were administered electrospun samples are shown in Table 6.

Table 6. Changes in SOD and GPx activity observed in animals that received nanofibers. The results are presented as the arithmetic mean ± standard deviation of mean SOD and GPx values for each batch of five mice

	Period of time	SOD	GPX

		(U/mg protein)	(μm/mg protein)
Witness	24 h	103.6±16.5	13.1±2.3
	7 d	105.2±19.7	12.3±1.7
PLA/PEO	24 h	103.5±18.3	12.7±1.3
	7 d	104.6±18.5	13.3±1.9
PLA/PEO/KH	24 h	104.7±17.7	13.5±2.1
	7 d	104.4±19.3	12.7±1.5
PLA/PEO/PVP	24 h	104.2±19.1	12.9±1.7
	7 d	105.8±20.3	12.3±1.1
PVP/CH	24 h	103.1±18.7	13.1±2.5
	7 d	105.5±17.9	13.3±1.7
PLA/PEO/KH-PVP/CH	24 h	104.8±19.1	12.7±1.5
	7 d	105.4±19.5	13.5±1.3

No significant differences were observed in the serum SOD and GPx values of the nanofiber-treated mice tested, compared to the control mice, after 24 hours and 7 days. Therefore, the use of nanofibers did not significantly influence the oxidative processes during the experiment.

Immunological tests

The study examined the incision areas coated with PLA/PEO, PLA/PEO/KH, PLA/PEO/PVP, PVP/CH, PLA/PEO/KH-PVP/CH nanofibers, was not accompanied by substantial variations in serum opsonic capacity (OC), phagocytic capacity (PC), and bactericidal capacity (BC) levels in compared to witness group animals after 7 days. These evidences reveal that the use of nanofibers did not induce changes in the immune defense capacity of the tested animals.

Table 7. Changes in OC, PC, and BC values in animals that received nanofibers. The values for each parameter were expressed as the arithmetic mean ± standard deviation of the mean values for a batch of 5 mice

	Period of time	OC (colonies/ml)	PC (colonies/ml)	BC (colonies/ml)
Witness	7 d	769.83±65.27	529.37±41.17	713.56±62.21
PLA/PEO	7 d	773.13±61.43	531.41±39.33	718.34±65.37
PLA/PEO	7 d	781.67±58.67	519.55±36.67	720.43±49.55
PLA/PEO/PVP	7 d	794.33±70.21	522.13±40.21	714.21±53.67
PLA/PEO/KH	7 d	785.45±67.45	525.29±38.25	717.55±60.33
PLA/PEO/KH-PVP/CH	7 d	788.29±62.83	530.67±33.83	721.82±57.17

All studies demonstrated that, in our laboratory conditions, the use of nanofibers containing bovine glue and keratin hydrolysate, incorporated in biodegradable polymers, did not produce significant hematological, biochemical, immunological changes, and did not significantly influence some specific stress parameters oxidative, compared to the use of patches with a textile support. These findings suggest a good biocompatibility *in vivo* and allow us to appreciate that the tested systems may have biomedical applications.

Conclusions

Overall, these tests are important to assess the safety and potential health effects of new biomaterials developed, such as the PLA/PEO/KH-PVP/CH electrospun nanofibers, before they are used in medical applications.

The results showed a nanofibrous structure of each layer and a high degree of *in vivo* biocompatibility of the assembled product. The research will continue with *in vitro* and *in vivo* investigations on healing ability of new biomaterials based on animal byproduct extracts.

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