VALORIZATION OF NORTHERN PIKE (*ESOX LUCIUS*) SKIN FOR COLLAGEN HYDROGELS: EXTRACTION AND CHARACTERIZATION WITH POTENTIAL APPLICATION IN WOUND HEALING

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The aim of this study was to obtain and characterize fish collagen hydrogels from Northern Pike with potential application in skin wound healing. The fish collagen hydrogels were obtained by a series of processes including acid, alkaline and alcohol treatment. The characterization of hydrogels and their freeze-dried forms was made by physical-chemical characterization, circular dichroism and FT-IR, microbiological analysis and cytocompatibility tests on human fibroblasts. The results showed good antibacterial effect on Escherichia coli and Staphylococcus aureus strains, good cell viability and proliferation, making them potential candidates for skin wound healing.

Keywords: fish collagen; wound dressing; biomaterials; hydrogel

1. Introduction

Hydrogels based on collagen are typically made with collagen as the main component. Presenting a total molecular weight of ~ 300 kDa, type I collagen is a natural protein composed of periodic polypeptide chains of 67 nm [1–3].

The most popular application for type I collagen hydrogels in 3D printing of vascularized tissues is to create an extremely conducive environment for angiogenesis. The concentration of collagen needs to be within a specified range in

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order to promote the creation of the particular vasculature [4,5]. Usually, collagen gels with concentrations between 1.2 and 1.9 mg/mL are the ones that support the stable formation of germs, allowing cell proliferation [6,7]. Nowadays, the majority of the collagen used in clinics comes from terrestrial animals. Bovine collagen is often used as a temporary dressing for extraoral wounds and also for burns on the body [8]. However, using collagen extracted from bovine or porcine is restricted due to the possibility of zoonotic disease transmission, including foot-and-mouth disease and bovine spongiform encephalopathy (BSE), as well as ethical and theological considerations [9,10]. The benefits of marine collagen, which are derived from fish or invertebrate marine species, include their high production and lack of potential for disease transmission [10]. Collagen from marine life, including their waste products, is useful for a variety of purposes [11-15]. The high-water content, excellent cytocompatibility, and physicochemical characteristics of hydrogel materials have made them perfect for use in wound dressing formulation. Collagen hydrogels are considered, according to several studies in the literature, the optimal and safest dressings, presenting a cross-linking and self-assembly capacity that leads to the formation of stable and resistant fibers [16].

The aim of this study was to obtain and characterize fish collagen hydrogels from the skin of Northern Pike (*Esox Lucius*). The novelty of our research consists in a new extraction protocol for obtaining of collagen from *Northern Pike*, in which only chemicals and mechanical treatments were used, avoiding any enzymatic treatments as in the previous study made by Kozlowska et al. The fish collagen hydrogels were obtained by a series of processes that included acid treatments for removing flesh and scales followed by alkaline treatment to remove collagen proteins and alcohol treatment for fat removal [17]. Lactic and citric acid were used in this study to eliminate the flesh, scales and fat, as they are more compatible with food and cosmetics than the acetic acid used in most studies. The properties of hydrogels were determined by physico-chemical characterization (determination of dry matter, ash, total nitrogen, protein substance contents and pH), circular dichroism, FT-IR analysis. Also, in our study the antibacterial activity and cytocompatibility properties of the samples were investigated in order to evaluate their potential for skin wound healing applications.

2. Materials and methods

2.1. Materials

All the reagents and solvents (lactic acid, citric acid, ethanol, sodium chloride, hydrogen peroxide) of analytical grade were purchased from Sigma-Aldrich and used with no further purification. Distilled water was used in all the experiments.

2.2. Methods

Northern Pike The method used for the isolation and purification of acid soluble collagen is according to Kozlowska et al. [17], with some modifications. The pike fish skin was first defrosted, then chopped into reduced samples and given a two-to-three-hour soak in a 3% detergent solution.

Then, these treatments were applied to the fish skin:

1. Acid treatment: To eliminate the flesh, scales, and some of the fat adhered to the skin, the fish skin was split into three lots and treated with an acid bath for a whole day at 4°C. 0.5% lactic acid solution was applied to lot 1, ratio skin: acid solution 1 : 8 (m/v); lot 2 was treated with a 0.5% citric acid solution, ratio skin: acid solution 1 : 8 (m/v); lot 3 was treated with a mixture of 0.5% citric acid and lactic acid in a 1 : 1 ratio, ratio skin : acid solution 1 : 6 (m/v).

2. Alkaline treatment: Fish skin lots underwent an alkaline treatment with NaOH 0.1 M for 24 hours at 4°C to eliminate non-collagenous proteins after being first cleaned in cold water for 24 hours, until the wash water achieved a slightly acidic or neutral pH (pH=6), keeping the same skin: alkaline solution ratio as in the case of acid treatment.

3. Treatment with 3% ethanol solution: After six hours of cold water washing to achieve a slightly alkaline or neutral pH, the fish skin lots were treated with 3% ethanol to remove fat, for 24 hours, the skin: ethanol 3% ratio being 1 : 6 (m/v).

4. Treatment with hydrogen peroxide: The skin lots were cleaned for six hours, using cold water, with an hourly water change, and then treated with 1% hydrogen peroxide for 24 hours to remove pigments, the ratio of skin : hydrogen peroxide 1% being 1 : 20 (m/v).

5. Acid treatment – to solubilize collagen: The fish skin was treated with an acid treatment solution 6%, ratio skin : acid solution 1 : 8 (m/v) at room temperature, in order to solubilize the collagen.

6. Obtaining the gels: samples were crushed then filtered through filter cloth, and then mixed for 4 min at 600 rpm using a FargonLab^{TM} Pro mixer and coded G1-collagen gel obtained using lactic acid; G2- collagen gel obtained using citric acid; G3 - gel obtained using lactic acid and citric acid.

7. Lyophilization: The collagen gels were lyophilized using the same method as previously described in our research paper [18], obtaining collagen sponges.

Extraction yield was calculated using the formula (1):

$$\text{Extraction yield} = \frac{\textbf{weight of fish skin treated before solubilization, g}}{\textbf{Weight of fish skin ,g}} \text{x}100 \qquad (1)$$

2.2.2. Characterization

2.2.2.1. Characterization by physico-chemical analyses - the following analyzes were determined: dry substance content, ash content, total nitrogen content by the *Kjeldhal* method [30], protein substance content and pH.

2.2.2.2. Characterization by FT-IR analysis: Fish extracts (gels) were subjected to FT-IR spectral analysis using a Bruker Vertex 70 FTIR spectrometer. In order to record each FT-IR spectrum in ATR mode, the following settings were used: 4 cm^{-1} resolution, 4000 - 600 cm^{-1} spectral range, and 30 acquisitions for each sample.

2.2.2.3. Characterization by circular dichroism: A quartz cell with a length of 10 mm was used to acquire spectra on a Jasco J-1500 model circular dichroism spectrophotometer, made in Japan. A 500 µL aqueous solution containing 0.05% concentration of fish extracts was used for each measurement.

2.2.2.4. Characterization by microbiological analysis:

Inhibitory activity of *Escherichia coli* and *Staphylococcus aureus* strains: Two ATCC strains from the collection of the ICPI Biotechnology Laboratory were used for the evaluation of the samples, Escherichia coli (ATCC 10536) and Staphylococcus aureus (ATCC 6538). Muller Hinton Broth (MHB) was inoculated with both strains, *E. coli* and *S. aureus*, after an incubation period of 18 hours at 37°C. This tube was used to make decimal dilutions up to 10-5 CFU/mL.

200 µL of the inoculum was seeded on plates. The samples that had been prepared in this manner were then introduced and left in the incubator at 37°C for a full day. Following this, the diameter of the zone of inhibition surrounding the sample was used to determine the antibacterial activity.

The samples that were prepared in this way were then introduced and left in the incubator at 37°C for a whole day. Subsequently, the diameter of the inhibition zone surrounding the sample was used to determine antibacterial activity.

2.2.2.5. Cytocompatibility tests

Collagen samples were incubated in complete culture medium (DMEM - Dulbecco's Modified Eagle Medium) for 24 hours at 37°C, under shaking, at a concentration of 2 mg/mL. Next, the samples were centrifuged (300 g/5 min) and the supernatants were sterilized by filtration (0.2 µm pore size). The cytocompatibility of these extracts was tested on human dermal fibroblast cultures as follows. Cells were obtained as previously described [19] and cultured in complete medium as mentioned above, at 37°C and 5% CO_2.

The complete medium, which supports optimal cell viability, was used as a control. After another 24 h, viability was determined by the XTT technique. To

evaluate the effect of the materials on the proliferative capacity of the cells, fibroblasts were seeded in 96-well plates at a density of 10,000 cells/cm^2, allowed to adhere for 3 hours, after which they were incubated in the test extracts. Cell viability was evaluated by the XTT technique, 24, 48 and 72 hours after seeding.

Statistical Analyses

All physico-chemical experiments were performed in triplicate (n=3). The data are expressed as mean ± Standard Deviation SD. For the biocompatibility assessment, statistical significance was determined using a One-way Anova (***p< 0.001; **p<0.01; *p<0.05; ns p ≥ 0.05).

3. Results and Discussion

3.1. Extraction

After the extraction process, it was observed that the highest yield was obtained for the samples treated with a mixture of lactic acid and citric acid (34.67 %) - G3, following by the samples obtained only by the treatment with lactic acid (31.94 %) - G1 and the low extraction yield was obtained for samples treated with citric acid (29.19 %) - G2. The difference between the extraction yield values, obtained in the present study, with the values found in the literature, indicates that intermolecular bonds are still present in collagen. By using mechanical, chemical, or enzymatic pretreatments, the yield of obtained collagen can be increased. It is crucial to apply a suitable pretreatment of the raw material to extract the insoluble collagen without changing the macromolecule in order to reconstitute collagen fibrils and fibers. From a chemical point of view, the aim is to remove intra- and intermolecular covalent bonds, which in particular involve lysine and hydroxylysine residues, ester groups and glycosidic bonds [20].

3.2. Characterization

3.2.1. Characterization by physical-chemical analyses

Table 1 presents the results for physical-chemical analyses.

Table 1.

Results for physical-chemical analyses for collagen gels.

S. No.	Name of the test	Technical specifications	Units of Measure (UM)	Determined values			The method standard
				G1	G2	G3	
1	Determination of dry matter content	Dry substance	%	1.53 ±0.30	3.60 ±0.29	2.72 ±0.28	SR EN ISO 4684:2006
2	Determination of ash content	Ash content	%	-	-	-	SR EN ISO 4047:2002

3	Determination of total nitrogen content	Total nitrogen content	%	0.26 ±0.11	0.20 ±0.10	0.20 ±0.11	SR ISO 5397:1996
4	Determination of the protein substance	Protein substance, %	%	1.46 ±1.86	1.12 ±1.87	1.12 ±1.85	SR ISO 5397:1996
5	Determination of pH	pH	pH units	2.5 ±0.1	5.51 ±0.1	2.5 ±0.1	STAS 8619/3:1990

*The results represent the mean of three independent measurements ± standard deviation.

After analyzing the information in Table 1, we concluded that a significant benefit of the extraction technique used for all fish collagen gel samples obtained was represented by an undetectable ash content. Using the suggested method, samples with pH values between 2.5 and 5.5 were obtained for collagen gels, which indicates that the obtained samples can be further used in obtaining different bioproducts, being able to adjust their pH if necessary, depending on the application.

Additional information was obtained by examining total nitrogen values, yielding approximately the same value for all samples, the low values suggesting a rather advanced degree of hydrolysis.

3.2.2. Characterization by FT-IR analysis

Fig. 1 and table 2 show the FT-IR spectrum and the location of the bands as well as their assignment for the fish collagen gel samples.

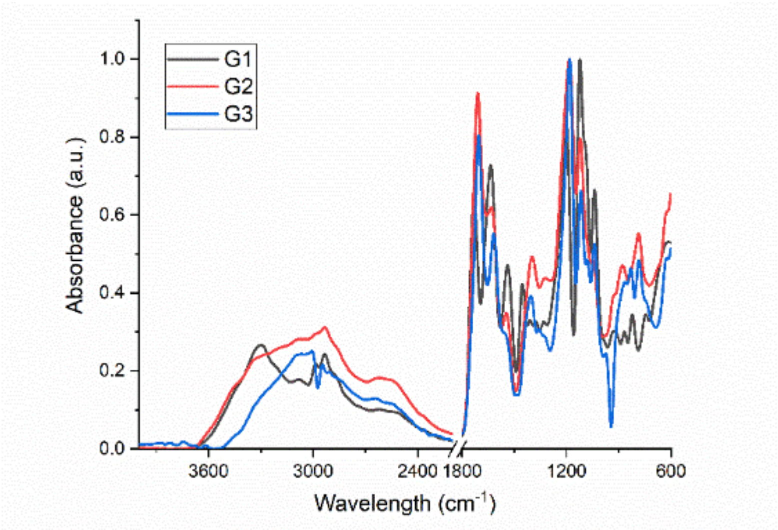

Fig. 1. Fourier Transform Infrared spectra of acid soluble collagen extracted from the fish skin.

Table 2.

FTIR absorption characteristic bands for fish collagen gel samples

Characteristic band	Peak Location, Wavenumber (cm^{-1}) G1	G2	G3	Assignment [21,22,23]
Amide A	3298	3072	3007	hydrogen-bond coupled N-H stretching vibrations
Amide B	2933	2933	2946	asymmetric stretching vibrations of the -CH_2 group
Amide I	1634	1630	1618	asymmetric stretching vibrations of the C=O group
Amide II	1538	1545	1550	deformation vibrations of the -N-H group coupled with stretching vibrations of the -C-N group
Amide III	1201	1183	1182	C-N stretching and N-H in-plane bending from amide linkages

Similar values have been presented in the literature, which are associated with triple helix conformation of intact fibrillar collagen. Each peak in the FTIR spectra corresponds to the vibration of the functional groups of the molecules. The amide A commonly associated with N-H elongation vibrations typical of intermolecular hydrogen bonding and is observed in the wave number ranging from 3400 to 3500 cm^{-1}. In this case the vibration appears at lower values, which suggests a denaturation of the triple helix during the hydrolysis processes [24].

3.2.3. Characterization by circular dichroism

Fig. 2 presents the CD spectra of analyzed pike collagen gels.

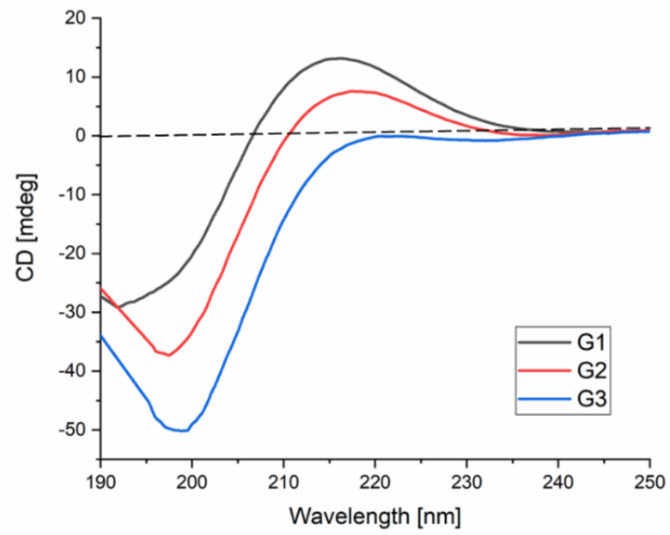

Fig. 2. Circular dichroism spectrum of the analyzed fish collagen gels.

Every fish collagen gel sample that was examined had a distinct negative band between 192 and 197 nm, which is indicative of a randomly coiled structure, and a positive maximum only for G1 and G2, between 220 and 217 nm, and at 216 and 217 nm, respectively, which is indicative of a triple helix structure [25]. The absence of a positive signal in sample G3 indicates that it has a certain structure that is typical of denatured collagen, or gelatin-based collagen [26]. Also, the quantitative secondary structure of fish collagen gels was assessed using CD Multivariate Secondary Structure Estimation (SSE) program (Table 3).

Table 3.

Secondary structure estimation of fish collagen gel samples.

Sample	α-helix, %	β-sheet, %	β-turn, %	Other, %	Total Sum, %
G1	23.0	14.8	17.3	44.9	100
G2	22.5	12.9	17.7	46.9	100
G3	22.0	11.6	18.0	48.4	100

The SSE analysis confirmed that sample G1 presents the highest α-helix, β-sheet, and β-turn content, compared with samples G2 and G3.

According to these results it was decided that further tests (microbiological and cytotoxicity tests) should be performed only for samples G1 and G2.

3.2.4. Characterization by microbiological analysis

Inhibition of growth of Staphylococcus aureus and Escherichia coli strains

Depending on the diameter of the formed inhibition zones, the results are interpreted as follows:

- diameter of 0-10 mm, inactive - marked "-";
- diameter of 10-14 mm, weak activity - noted "+";
- diameter of 15-19 mm, moderate activity - marked "++";
- diameter of ≥20 mm, definite activity - marked “+++”.

Table 4 presents the antibacterial effect of collagen sponges:

Table 4.

Results for the antibacterial effect of collagen sponges

Samples	Bacterial strains	
	Escherichia coli ATCC 10536	*Staphylococcus aureus* ATCC 6538
G1	+++	+++
G2	+++	+++

Microbial contamination determination

Determination of the total number of aerobic microorganisms

The results for G1 and G2 samples are presented in Table 5:

Table 5.

Results for G1 and G2 samples for Total number of aerobic microorganisms (TAMC) and Total number of yeasts and filamentous fungi (TYMC)

Microbiological characteristics	Admissibility conditions according to The European Pharmacopoeia (Ph. Eur.) 10th Edition [27]	Results for G1	Results for G2
Total number of aerobic microorganisms (TAMC), CFU/g	Up to 100 CFU/g for topical products Up to 1000 CFU/g for pharmaceutical products	2 CFU	3.33 CFU
Total number of fungi and filamentous fungi (TYMC), CFU/g	Up to 100 CFU/g for topical products Up to 100 CFU/g for pharmaceutical products	0 CFU	2.33 CFU
Staphylococcus aureus	absent	absent	absent
Escherichia coli	absent	absent	absent
Pseudomonas aeruginosa	absent	absent	absent

Inhibition of growth of Staphylococcus aureus and Escherichia coli strains

From the Table 5 it can be seen that each sample showed antibacterial activity against the two strains that were tested. The study of the activity on the two strains showed a more intense activity on *Escherichia coli* than on *Staphylococcus aureus*, which proves the efficiency of these biomaterials for the intended applications. Unlike *Staphylococcus aureus*, which is a Gram-positive bacterium with a more resilient cell wall because of its fundamental unit, peptidoglycan, *Escherichia coli* is a Gram-negative bacterium.

Determination of microbial contamination

The results for Total Aerobic Microbial Count (TAMC) for collagen samples presented in Table 5 are acceptable, according to the European Pharmacopoeia (Ph. Eur.) 10th Edition. On the Sabouraud agar medium, the average CFU is represented by The Total Yeast and Mold Count (TYMC). In calculating the total number of CFU produced on this medium, CFU bacteria that are found on it are included. The Total Yeast and Mold Count (TYMC) presented in Table 5 for collagen samples are in accordance with the values required by The European Pharmacopoeia (Ph. Eur.) 10th Edition [27].

3.2.5. Cytocompatibility tests

The results were calculated as a ratio of live cells to the total number of cells, expressed as a percentage of viable cells, obtained in the case of the control incubated in complete culture medium, at 24 h and are presented below (Fig. 3).

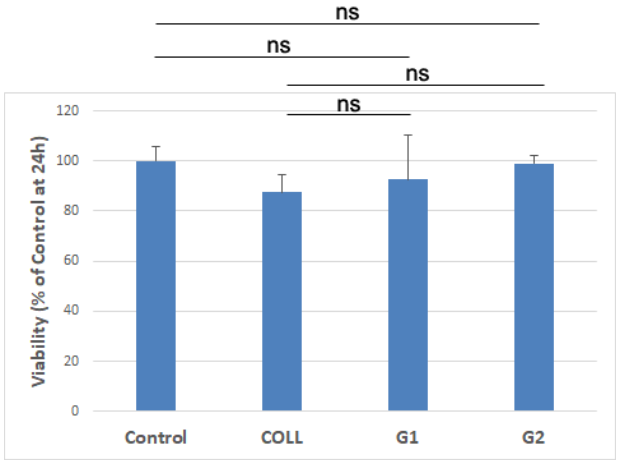

Fig. 3. Cell viability after 24 h of human fibroblasts using XTT technique for fish collagen gel samples (G1 and G2) compared with bovine collagen (COLL) (ns – non significant) by incubating a pre-confluent culture of human fibroblasts in the presence of the extracts for 24 hours, a good compatibility was observed for all tested samples. There were no statistically significant differences between any of the assessed extracts in comparison to the control, which was complete medium only, or to the collagen extract.

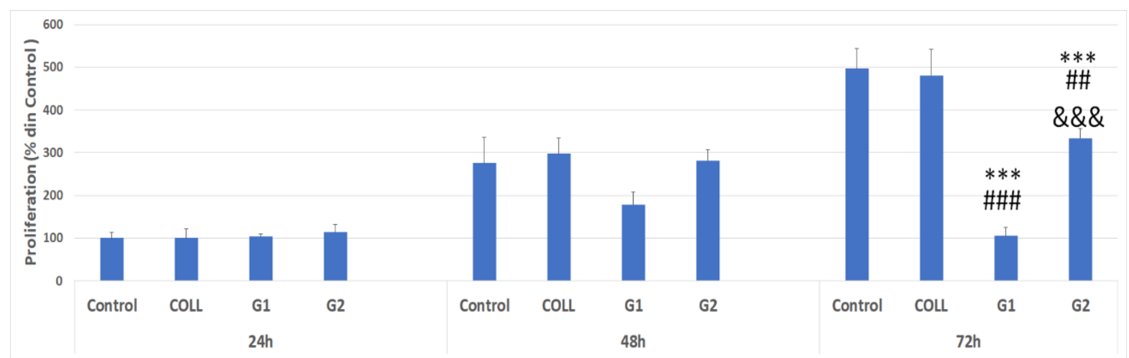

Fig. 4. Adult human fibroblast proliferation after 24, 48 and 72 hours of incubation determined by the XTT assay in the presence of collagen samples (** $p<0.01$, *** $p<0.001$ vs. Control at each time point, # $p<0.05$, ### $p<0.001$ vs. COLL, &&& p,0.001 G2 vs. G1 at each time point).

Our results showed a decrease in the proliferation capacity of fibroblasts in the presence of extracts of matrices G1 and G2. Thus, the matrix obtained with lactic acid (G1) had a higher impact on this cell process, leading to an 80% decrease in proliferation capacity in comparison to the collagen only matrices. On the other hand, the extract obtained from citric acid-based matrix (G2) induced a decrease of proliferation capacity of fibroblasts of 30% in comparison to the same control. However, it has been shown that citric acid could enhance the collagen synthesis process, which is essential in the wound healing process. Thus, the citric acid added to the fish skin collagen matrices could boost its healing properties by stimulating the endogenous collagen production by the local dermal fibroblasts [28-29].

4. Conclusions

The proposed method for obtaining hydrogels from fish skin is a new one, which uses a combination of acids (lactic and citric, both individually and together). This method presents numerous advantages like having low costs, using two acids which are more compatible with skin and is efficient in the extraction and solubilization of collagen, compared to other methods presented in the literature (which generally use acetic acid or enzymes).

For the new collagen hydrogels extracted from fish skin, the most efficient yield was obtained for sample G3 extracted using the mixture of lactic acid and citric acid.

Regarding the physico-chemical characterization, the results show that all the samples present a low content of protein and an undetected value of ash content, proving the efficiency of the extraction method. The pH of all the samples is acidic with values between 2.5-5.5.

The structural characteristics of the collagen were validated by FT-IR and CD investigations, except for sample G3 which indicated the presence of a certain

structure typical of denatured collagen (gelatine), according to CD spectra. The study of the microbiological activity on the two strains for the tested samples demonstrated a good antibacterial activity on *Escherichia coli* and *Staphylococcus aureus*, especially for sample G2, proving the efficiency of these biomaterials in fulfilling the purpose for which it was intended.

All the samples presented a good cell viability, also, the best results of the cytocompatibility test after 72 h were obtained for the sample G2, which was obtained using citric acid.

The results demonstrated that the newly obtained fish collagen extracts can be used as the main component in the design and manufacture of innovative hydrogels with potential for application in skin wound healing, as a type of biomaterial for wound dressing. In the future, additional tests will be performed to demonstrate this potential.

Acknowledgments

This work was supported by a grant from the Ministry of Education and Research, Romania, UEFISCDI Agency, project number PN-IV-P7-7_1-PED-2024-2326 and by the Romanian Academy. The biocompatibility was financially supported by the Romanian Academy and by UEFISCDI through project number PN-III-P1-1.1-TE-2021-1344.

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