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PREPARATION AND CHARACTERIZATION OF NOVEL BIOMATERIALS BASED ON MIXTURES OF FISH COLLAGEN AND PEPTIDES WITH CHITOSAN FOR BIOMEDICAL APPLICATIONS

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Abstract

In the present study, novel biomaterials based on mixtures of collagen and peptides isolated from aquaculture fish residues and marine chitosan solution at pH 5 were prepared, conditioned as 3D porous sponges and characterized to select best variant for biomedical applications. The physico-chemical properties of the biomaterials were investigated by determination of their in vitro biodegradability, swelling degree and porosity, while morphology was observed by scanning electron microscopy (SEM). In addition, the biocompatibility of the prepared biomaterials was assessed by cell viability of L929 fibroblast cultures cultivated in the presence of the prepared sponges by MTT assay. The results indicated that the biomaterials prepared from mixtures of collagen and chitosan at pH 5 and mixtures of collagen, chitosan at pH 5 and peptides had lower in vitro biodegradability in the presence of collagenase, higher degree of swelling and increased porosity, compared to a sponge of fish collagen (control). The cell viability values recorded in L929 fibroblast culture cultivated in the presence of the prepared biomaterials were high, indicating a good biocompatibility with skin specific cells. In conclusion, this study demonstrated that the biomaterials prepared from fish collagen and marine chitosan at pH 5 with or without addition of fish peptides have optimal physico-chemical and structural properties and good biocompatibility, recommending their further testing and use in skin tissue engineering.

Key words: *biomaterials, chitosan, collagen, fish, peptides.*

INTRODUCTION

The fish processing industry generates a multitude of residues, which can be further valorized as raw materials for obtaining collagen or bioactive peptides (Ideia et al., 2020). The usefulness of both collagen and peptides is huge, especially in the healing of tissue injuries, and the fish residues represent a viable alternative to bovine source (Coppola et al., 2020). Thus, in the literature, evidences were found regarding a biomaterial based on sodium alginate, calcium carbonate and fish peptides that stimulated osteogenesis, indicating the possibility to be used in the restoration of bone tissue (Zheng et al., 2024). In addition, it was demonstrated the antioxidant activity of fish collagen peptides and their property to stimulate the dermal fibroblast growth (Wang et al., 2023). Other studies have confirmed that the peptides extracted from fish residues by enzymatic treatment exerted antioxidant, antihypertensive and antiproliferative activity, and had cellular biocompatibility *in vitro* (Toma et al., 2021; Tascias-Pascacio et al., 2021). It has been observed that chitosan addition in the composition of biomaterials based on collagen improved their biological and mechanical properties (Fatemi et al., 2021). *In vivo* testing of a collagen and chitosan gel covering burns made on rat skin showed an increase of angiogenesis, proliferation of fibroblasts and, finally led to the restoration of the damaged tissue (Fatemi et al., 2021). Analysis of a mixture of gelatin and chitosan solution, pH 5, indicated the formation of stable complexes due to the electrostatic interaction between constituent polymeric chains (Chen et al., 2023).
The present study aimed to prepare novel biomaterials based on mixtures of collagen and peptides isolated from aquaculture fish and marine chitosan, conditioned as porous

sponges, and to comparatively investigate their biodegradability, swelling degree, porosity and *in vitro* biocompatibility, in view of their use in biomedical applications.

MATERIALS AND METHODS

Residues (skin, bones) of aquaculture carp were kindly provided by a local fishery after fish processing (Tulcea, Romania). Chitosan from crustaceans was acquired from Sigma-Aldrich (Germany). All chemical reagents used in this study were of analytical grade and acquired from Sigma-Aldrich (Germany), unless otherwise stated.

Isolation of fish collagen and peptides

Both collagen and peptides were obtained from carp residues. The fish collagen was extracted using an enzymatic method with pepsin in acetic acid, pH 2. The peptides were prepared from fish collagen by enzymatic hydrolysis with 4% papain solution, as previously described (Toma et al., 2022).

Preparation of 3D porous biomaterials based on fish collagen

A solution of 50 mg/ml chitosan was obtained in 1 M acetic acid by magnetic stirring over-night and then it was adjusted at pH 5 with 5 M NaOH. Two variants of 3D porous biomaterials were prepared using fish collagen solution (50 mg/ml) mixed with chitosan in different weight ratios (Table 1, variants 2 and 3).

Table 1. The variants of biomaterials prepared from mixtures of fish collagen and peptides with chitosan, conditioned as 3D sponges

Variant no.	Biomaterial composition	Mixing ratio (w/w, w/w/w)	pH
1.	Collagen	-	5
2.	Collagen-chitosan	1:1	5
3.	Collagen-chitosan	1:0.5	5
4.	Collagen-chitosan-peptides	1:1:0.1	5
5.	Collagen-chitosan-peptides	1:1:0.2	5

The mixtures were homogenized by magnetic stirring for 2 h and fish peptide solution (50 mg/ml) was added to obtain variants 4 and 5 (Table 1). All mixtures were conditioned by freeze-drying in glass Petri dishes, at -35^{0}C, for 48 h using a Martin Christ lyophilizer equipment (Germany) (Craciunescu & Moldovan, 2011).

Determination of *in vitro* biodegradation degree in the presence of collagenase

The biodegradation degree of porous biomaterials was determined in the presence of collagenase, as previously described in a study performed by Craciunescu et al. (2011). Samples of biomaterial were incubated in TES buffer pH 7.4, containing collagenase and $CaCl_2$, for 5 h, at 37^{o}C. At the end of incubation period, the solution was put on ice to deactivate the enzyme and centrifuged at 5000g, for 10 min. Aliquots of supernatant were analyzed for protein content using ninhydrin-based reagent (Park et al., 2002). The absorbance was read at 660 nm using a V-650 UV-VIS spectrophotometer (Jasco, Japan).

Determination of swelling degree

Samples of biomaterial were weighed (w_i) and then, they were immersed in TES buffer pH 7.4. At predetermined periods of time, the biomaterials were removed, the excess water was absorbed on a filter paper and then, the biomaterials were weighed (w_f) (Craciunescu et al., 2011). The degree of swelling was calculated according to the formula:

$$\text{Swelling degree} = \frac{wf-wi}{wf} * 100$$

Determination of porosity

Biomaterials porosity was determined by water replacement method (Craciunescu et al., 2011). Samples of biomaterial were weighed and immersed into a graded cylinder with a known volume of water (V1). The porous biomaterial was periodically pressed to release the air from the pores and fill them with water. The total volume of water and water-impregnated matrix was measured (V2). Then, the biomaterial was removed and the volume of water remaining in the cylinder was measured (V3). The porosity of samples was calculated using the formula:

$$\text{Porosity} = \frac{V1-V3}{V2-V3} * 100$$

SEM observations

The morphology of 3D porous samples of biomaterial was observed by scanning electron microscopy (SEM) using a Hitachi SU1510 equipment. The samples were previously coated with a gold layer.

Evaluation of *in vitro* biocompatibility

A cell culture of mouse fibroblasts from the stabilized cell line NCTC (clone L929) was used to determine *in vitro* biocompatibility of the prepared biomaterials. The viability of cells cultivated in 96-well culture plates in direct contact with samples of lyophilized biomaterials was determined after 24 and 48 h by MTT (thiazolyl tetrazolium bromide) assay. The optical density (OD) of the resulting solution was measured at 570 nm at LB 940 plate reader (Berthold Mithras, Germany). Cells cultivated in culture medium without sample were used as a control and their viability was considered 100%.

$$\text{cell viability (\%)} = OD_{sample}/OD_{control} * 100$$

RESULTS AND DISCUSSIONS

Four variants of composite biomaterials were prepared from fish collagen and marine chitosan supplemented or not with fish peptides and conditioned as 3D porous sponges (Figure 1).

Figure 1. Sponge biomaterial based on fish collagen mixed with chitosan solution, pH 5 in 1:1 weight ratio

Biodegradability of fish collagen-based biomaterials

The results of the biodegradation degree of biomaterials are presented in Figure 2.

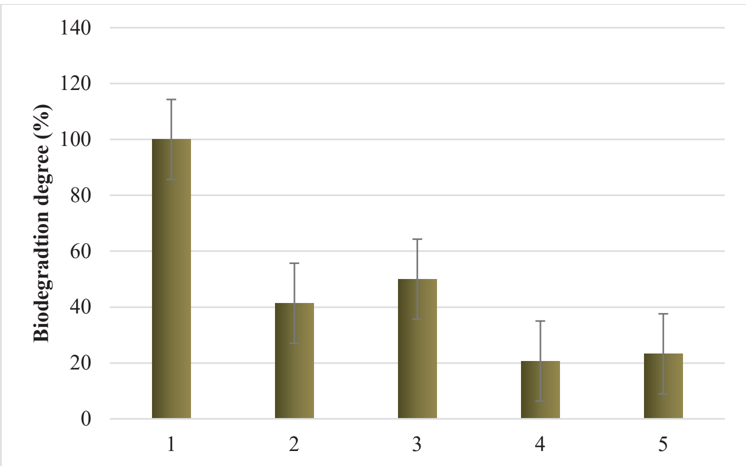

Figure 2. *In vitro* biodegradation degree of biomaterials based on fish collagen

The biodegradation degree of the collagen sponge (variant 1, control) was 100% after 5 h of incubation in the presence of collagenase. Mixing collagen with chitosan solution, pH 5 increased the resistance of the biomaterial to collagenase action *in vitro* with 58.63% in variant 2 and 50% in variant 3, compared to collagen sponge. The lowest biodegradation degree of approx. 20% was observed in the case of variants 4 and 5 obtained by mixing collagen with chitosan solution pH 5 and fish peptides. It was observed that the addition of peptides in the composition of the biomaterial based on collagen and chitosan pH 5 increased the resistance to the action of collagenase *in vitro* with 79.31%, compared to collagen sponge, and with 28.26%, compared to variant 1. The increased resistance to enzyme action of biomaterials based on collagen and chitosan compared to collagen biomaterial was also observed in Ressler's study (Ressler et al., 2023).

Swelling degree of fish collagen-based biomaterials

The results of the swelling degree of biomaterials are presented in Figure 3.

The swelling degree of the composite biomaterials was lower than that of the fish collagen sponge, except for the variant 3 consisting of a mixture of collagen and chitosan pH 5 in a ratio of 1:0.5. The lowest swelling degree was recorded for variants 4 and 5 that contained fish collagen, chitosan solution pH 5 and fish peptides. It was observed that the addition of peptides decreased the degree of swelling of the biomaterials by 50.67% in variant 4 and by 39.08% in variant 5. Still, a value of 900-1000% swelling degree registered for variants 4 and 5 is mostly due to the presence of a polysaccharidic constituent (chitosan) in the composition of the biomaterial and represent an optimal parameter, indicating the capacity to absorb the exudate at the wound situs. Yang observed that the degree of swelling is not proportional to the amount of chitosan added to the collagen-based biomaterial, a result similar to the result obtained in the study presented in this article (Figure 3) (Yang et al., 2021).

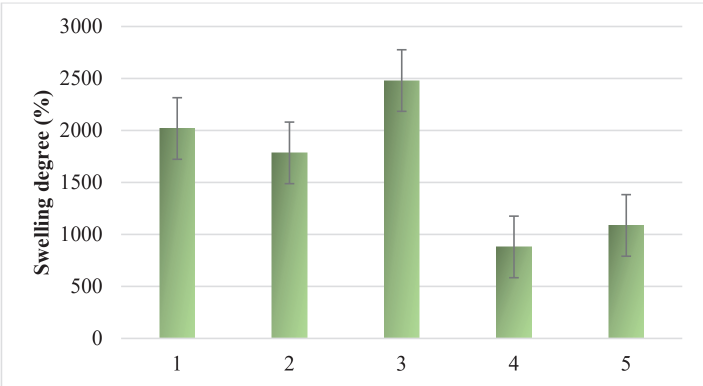

Figure 3. Swelling degree of biomaterials based on fish collagen

Porosity of fish collagen-based biomaterials

The results of biomaterials porosity are presented in Figure 4.

The porosity of composite biomaterials was higher than that of the fish collagen sponge, except for variant 3. The values varied between 54.42-61%. The highest porosity value (61%) was found in the case of variants 4 and 5 containing in addition fish peptides. Variant 2 of collagen and chitosan pH 5 in a ratio of 1:1 had a porosity of 58.8%, higher than that of variant 3 (54.4%), containing less chitosan (1:0.5).

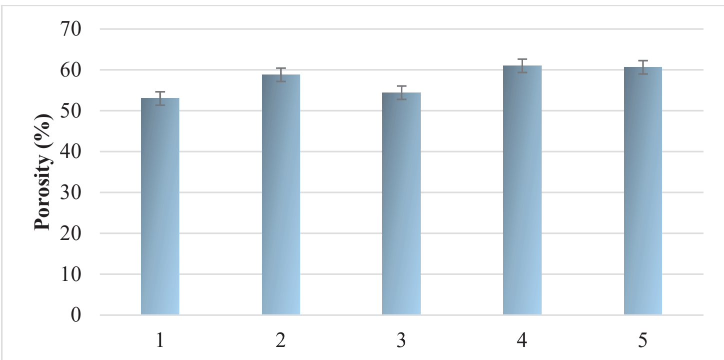

Figure 4. Porosity of biomaterials based on fish collagen

These results confirmed previously observations reported in the case of a hydrogel made of collagen and chitosan, i.e. the porosity increased for higher amount of chitosan in the composition of the biomaterial (Valentino et al., 2023).

Morphology of fish collagen-based biomaterials

Through SEM visualization, the morphology of composite biomaterials was observed (Figure 5). The presence of interconnected pores with variable sizes was apparent and characteristic to biomaterials used as scaffolds for tissue regeneration.

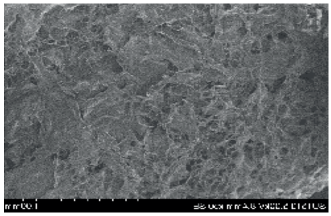

Figure 5. Representative SEM image of composite biomaterial consisting of fish collagen and chitosan at pH 5 in a ratio of 1:1

***In vitro* biocompatibility of fish collagen based biomaterial**

The results of cell viability testing are presented in Table 2.

Table 2. Cell viability of L929 cells cultivated in the presence of composite biomaterials based on fish collagen, chitosan and fish peptides, determined by MTT assay

Incubation time	Variant 1	Variant 2	Variant 3	Variant 4	Variant 5
24 h	108.77 ± 2.52	92.03 ± 2.54	93.51 ± 3.54	107.08 ± 2.71	115.44 ± 1.91
48 h	91.47 ± 2.32	80.08 ± 2.45	85.08 ± 1.79	94.84 ± 2.45	103.16 ± 2.34

The cell viability values after cultivation in the presence of composite biomaterials were high, exceeding 90% in direct contact for 24 h, and above 80% after 48 h. In the case of composite biomaterials supplemented with fish peptides, it was highlighted a stimulation of fibroblast metabolism (107-115%), similar (variant 4) or higher (variant 5) than in the case of fish collagen sponge (variant 1). Te results of cell viability are presented in Table 2. Similar studies showed that a hydrogel based on collagen and chitosan mixtures stimulated the adhesion and cell proliferation of human dermal fibroblasts *in vitro* (Valentino et al., 2023). They reported that higher cell proliferation was found in the case of samples with lower amount of chitosan. In the specialty literature, biomaterials based on collagen and chitosan did not show cytotoxicity in the case of treating cell cultures with them (Valentino et al., 2023; Yang et al., 2021; Ressler et al., 2023).

CONCLUSIONS

By mixing the collagen solution extracted from aquaculture fish with a solution of chitosan pH 5, a biomaterial with an increased resistance to collagenase action *in vitro* was obtained. Fish peptides addition to the polymeric composition increased even more the resistance to collagenase action *in vitro*. The degree of swelling was lower for composite biomaterials containing fish collagen, chitosan pH 5 and fish peptides, compared to that of fish collagen sponge. The porosity of biomaterials increased in variants containing chitosan and fish peptides. The novel biomaterials based on fish collagen did not show cytotoxicity in L929 fibroblast culture and, in addition, the biomaterials containing fish peptides stimulated cell proliferation. All these results demonstrated optimal physico-chemical and structural properties, and good biocompatibility of 3D porous biomaterials resulted from mixing collagen with chitosan at pH 5 or with chitosan and peptides extracted from fish, recommending their use in biomedical applications. Future work will investigate their interaction with skin cells in terms of mechanism of action, to better solve tissue engineering issues.

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