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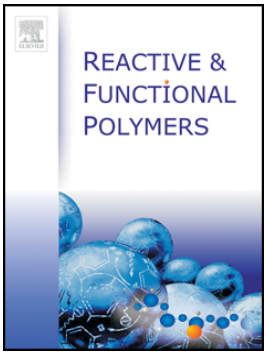

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Preparation and characterization of porous scaffolds from chitosan-collagen-gelatin composite

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Abstract

Novel porous scaffolds composed of chitosan, collagen and gelatin were prepared and characterized. For preparing scaffolds gelatin and collagen isolated from fish skins with various physicochemical properties were used. In order to reduce preparation solubility glutaraldehyde in the amount of 1%, w/w relative to the total biopolymers weight in solution was used. All obtained biomaterials showed a homogeneous porosity. Protein polymer type determined the rheology and mechanical properties of obtaining preparations. The use of protein polymers decreased swelling capacity of materials approximately 30% compared to the materials obtained from chitosan. Materials containing gelatin showed the highest solubility (approx. 30%). Scaffolds obtained in 100% of chitosan proved to be harder than collagen materials an average of 30% and less flexible more than twice. In relation to the gelatin materials were characterized by smaller values of both measured parameters on average 40% and 30% respectively. Materials containing protein polymers showed good antioxidant properties.

Keywords: Chitosan, Collagen, Gelatin, Glutaraldehyde, Porous scaffold

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1. Introduction

Natural polymers such as chitosan, collagen and gelatin exhibit excellent biocompatibility [1, 2, 3], very low antigenicity, biodegradability and similarity produced from them biomaterials to extracellular matrix [3,4].

Most of chitosan properties including biodegradability, biological functions, rheological properties of its solutions, and as a consequence of these qualities the possible applications depend on the deacetylation degree and molecular weight of this polysaccharide biopolymer [5]. The presence of hydroxyl- and amino groups in chitosan give the possibility of wide range chemical and enzymatic modifications. It is often used in designing and constructing systems for the immobilization and release of therapeutic compounds and also to obtain water-soluble chitosan derivatives [6, 7]. Because of the stability and rigidity of crystalline structure, higher molecular weight chitosan is practically insoluble in water, especially in solutions at a pH above 7 and in the common organic solvents. Instead, it is dissolved in aqueous solutions of acids such as acetic acid, citric acid, formic acid, hydrochloric acid, lactic acid. Moreover acetic acid solutions are the most commonly used, wherein it is necessary to decrease the pH below 6.2 [8]. By using such solutions can be obtained various types of biomaterials including membranes, scaffolds, fibers, microspheres and hydrogels.

An interesting solution is using collagen or gelatin in order to modify the rheological properties of the biopolymer products. Collagen is a major component vertebrates and invertebrates of tissues. It occurs in the skin, tendons, cartilage, bones and connective membranes. Collagen is estimated that in the case of mammals, it is 25-30% of the dry weight [9]. Until 2010 identified 29 types of protein, differing primarily α chain conformation and their mutual combination of the triple helix [10]. Despite identification of various types of collagen proteins, only a few of them are used to design biomaterials applicable in tissue engineering. Among them are those capable of a fiber formation: I, II, III, V and IX genetic type. Other proteins do not form fibrils, showing significant differences in the structure, their location in tissues and function in living organisms. A characteristic feature of the collagen protein is the presence of specific repeating sequence -Gly-X-Y- and significant amounts of hydroxyproline and hydroxylysine groups. It has been shown that hydroxyproline content in particular depends on the type of tissue and species origin of the animal (habitat temperature) from which collagen was isolated and affects its functional properties that are used in the construction of various kinds of products, including the thermal stability of the isolated preparation, its solubility, antioxidant and rheological properties [11, 12, 13,14]. The mature collagen in the tissues of living organisms is a protein insoluble in water, dilute acids and

buffer solutions. Only destruction of the crosslinking bonds by grinding the tissue, treating with alkali or enzymatic treatment, makes it possible the extraction of the target protein preparation [15, 16]. In turn, the isolated collagen preparation is a protein soluble in dilute acid solutions such as acetic acid and lactic acid solutions. It is not soluble in water, where the conditions do not violate its native form are provided. Conducting the thermal denaturation of collagen result in the receipt of gelatin. As a result of this process, follows the transition from helical structure to the so-called statistical hank and partial loss of its original biological properties. Yunoki et al. [17] showed that the denaturation temperature of collagen isolated from fish in most cases does not exceed 30^{o}C. In contrast, Reich [18] observed that it is possible to spontaneous partial renaturing (back to the original structure), if only the conditions and duration of exposure are not too extreme.

Gelatin is a product of partial collagen hydrolysis and it is no different in amino acid composition between them. As a result of exposure to high temperature and extreme pH conditions during obtaining of gelatin, it comes to irreversible denaturation of the tertiary structure of collagen and fragmentation of its chain [19]. Due to the two types of collagen hydrolysis process, it is also recognized two types of the gelatin, characterized by different isoelectric points [20, 21]. A specific feature of gelatins is the ability of this protein to form stable gels [22]. The main sources of gelatin obtaining are pork, bovine leathers and their bones. However, from year to year the growing demand for gelatin from alternative sources is observed. Gelatin can be obtained in a simple manner from the waste material of the fish industry. Currently, small interest of fish gelatin is caused by its less stability and a worse rheological properties of gels compared to materials prepared from gelatin derived from terrestrial animals. Recent studies indicate that the use of gelatin isolated from fish inhabiting the warm tropical and sub-tropical waters may lead to gels that have rheological properties required by the industry [23, 24].

The aim of this study was to determine the physicochemical properties of biomaterials derived from chitosan, collagen and gelatin isolated from fish skins. Although, there are many publications describing the interaction between chitosan with collagen and gelatin, but properties of the obtained preparations will be dependent upon the source of protein substrates isolation. In our research we used two different sources of collagen and gelatin differing properties and thus different affecting on the physicochemical parameters the final products. In order to design biomaterials with new properties, it is necessary to examine the impact of each of the components of these mixtures.

2. Experimental

2.1 Materials

Chitosan (low molecular weight), Chloramphenicol and Phosphate Buffer Powder, Lysozyme (70,000 U/mg), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt [ABTS (NH4)2], iron(II) chloride and ferrozine were purchased from "Sigma–Aldrich (St. Louis, MO, USA)". Acetic acid, methanol and ethanol were purchased from "POCH S.A. (Poland)". MilliQ water was used for the preparation of all aqueous solutions. All other reagents were of analytical grade or higher. Fish proteins used in this work were extracted from the skin of African catfish (*Clarias gariepinus*) from Nemex S.A. and Salmon (*Salmo salar*) collected from MorpolS.A. The skins of fresh fish were mechanically separated. The residue of adhering tissues was removed manually. After thorough mixing of the skins, samples (approximately 500 g) were prepared, and stored in polyethylene bags at -20^{o}C until used.

2.1.1 Extraction of acid soluble collagen (ASC)

Collagen has been extracted based on the method of Sadowska et.al. [25]with slight modification. In the first step frozen samples were minced in a meat grinder using mesh diameter Ø=3 mm. Fragmented skins were mixed with 0.1 M NaOH solution (1:6, w/v) and kept at 4^{o}C to remove non-collagenous proteins. The process lasted 48 h. After that, treated skins were rinsed with cold water (4^{o}C) to remove NaOH until the rinse water reached neutral pH. The fish skins were bleached with 3% H_2O_2 solution for 24 h at 4^{o}C for the removal pigments more effectively, and rinsedwith cold water (4^{o}C) again. Lipids were removed by adding 0.5% of detergents to all solutions used for the treatment before the appropriate acid extraction. All procedures were conducted on ice. The extraction of collagen was performed with 0.5 M acetic acid for 72 h with continuous stirring at 4^{o}C. The extract was centrifuged at 2400×*g* for 30 min at the same temperature. The collagen solution obtained after extraction with acetic acid was placed in dialysis bags (Spectrapor 2 Dialysis Tubing Standard RC12000 D - 14000 D) and immersed in double-distilled water until getting 6.5 pH value. Dialysis was carried out at 6^{o}C with constant stirring. The dialysis solution was changed every 24 hours. After 7 days of dialysis, process has been completed. Such prepared samples were freeze-dried (CHRIST Alpha 2-4 LSC, Osterode am Harz, Germany). Obtained collagen was stored in an airtight container at 4°C.

2.1.2 Extraction of gelatin

Gelatin has been extracted based the method of Kołodziejska et al.[26].Minced skins were mixed with 0.1 M NaCl solution (1:6, w/v) at a temperature of 4^{o}C for 3 minutes, three times. After that treated skins were rinsed with cold water (4^{o}C) sodium chloride. After each rinse

the skins were separated from the solution with a sieve with mesh diameter ∅=5 mm. The extraction of gelatin was performed by stirring the skin in water at 45°C over 60 minutes (1:6, w/v). The extract was centrifuged at 10000 × *g* for 30 min at 15°C to remove insoluble material. The supernatant was poured into ice bags and frozen at 20°C. Such prepared samples were freeze-dried (CHRIST Alpha 2-4 LSC, Osterode am Harz, Germany). Obtained gelatin was stored in an airtight container at 4°C.

2.2 Preparation of biopolymer scaffold materials.

Scaffold materials with different biopolymer composition were obtained by dispersion of collagen and gelatin (isolated from Salmon – *Salmo salar* and African catfish –*Clarias gariepinus*) and chitosan. Each of the polymeric blends was formulated separately (chitosan and proteins). Chitosan and collagen were dissolved in 0.5 M acetic acid solution and gelatin in demineralized water using a mechanical stirrer (rotation speed adapted to the viscosity of the resulting dispersion)(IKA RW20; BIOMIX BMX 10s, Poland), at an appropriate temperature for a given polymer. Each of the polymer solutions were prepared at a concentration equal to 2%. In the case of material composed of two polymers, firstly prepared separately dispersions of each polymer and then they mixed to give a 1% concentration of each polymer in the mixture. Prepared mono- and bicomponent preparations were placed in cylindrical molds with diameter of 25 mm and height of 40 mm, frozen at -20°C (24h) and freeze-dried (CHRIST Alpha 1-4 LD plus, Germany) until dry (0.96 atm, the temperature of the sample: -21ºC, the temperature of the condenser: -84ºC). Obtained scaffold structures were stored in tightly closed containers at 4°C until the time they characterization. In order to reduce the solubility of the obtained preparation to each dispersion (before formulation. The composition and preparation conditions of biopolymer scaffold materials are shown in Tab. 1.

2.3 Amino acid composition of fish protein preparation

The dry collagen samples were hydrolyzed under reduced pressure in 6 M HCl at 110°C for 24 h. The hydrolysates were conducted by the Pico Tag method using Pico-tag HPLC system (Pico-tag, Napa, USA).

2.4 Morphological examination

The cross-sectional morphology of the scaffolds was studied by visual assessment and scanning electron microscopy (SEM, Philips-FEI XL 30 ESEM). A 1-mm piece of the scaffold was fixed in the SEM sample holder and images were collected in the water vapour atmosphere (133.3Pa,12–15 kV).

2.5 Swelling/dissolution tests

The swelling capacity of the scaffolds was evaluated by gravimetric determination the scaffolds before (W) and after (W_1) placing the scaffolds in an aqueous solution containing a salt composition similar to that of wound exudate according to EN 13726-1, prepared by adding 8.298 g of NaCl and 0.368 g of $CaCl_2 \cdot 2H_2O$ to 1 dm^3 of distilled water. The samples were incubated at 37°C for 24 h, withdrawn from the medium, and weighed after removal of the surface fluid using a filter paper. The samples were then freeze-dried (CHRIST Alpha 1-4 LD plus) and weighed again (W_2). The percentage of fluid absorption was defined as the ratio of the weight increase ($W_1 - W_2$) relative to the weight remaining after freeze-drying (W_2). Each value was calculated as the mean of three independent measurements. To show the influence of the ionicstrength of the solution, a similar test was performed using distilled water as the reference medium. The dissolution of the scaffold (in percent) is defined as the ratio of the weight decrease of the scaffold ($W - W_2$) relative to the initial weight (W).

2.6 Porosity and pore size

The porosity of the chitosan–protein scaffold was evaluated using trimmed samples of 5 $cm^2 \times$ 0.5 cm in ethanol [27], according to the following formula: $P (\%) = Vc / Vm \times 100 = (W_{24} - W_0) / Vm \times \rho \times 100$ where Vm is the total volume of the chitosan scaffold (cm^3), Vc is the pore volume of the chitosan scaffold (cm^3), W_{24} is the weight (g) of the chitosan scaffold after incubation with ethanol for 24 h, W_0 is the original weight (g) of the chitosan scaffold, and is the density of ethanol (0.808 g/cm3).

The average cross-sectional area of the pores was calculated using image - processing tools of the Photoshop CS5 software.

2.7 Mechanical properties

The mechanical properties of the scaffolds were characterized using a universal testing machine (Instron model 5543, USA controlled using the "Merlin" software V 4.42.) according to previously described methods [4, 9]. Five cylindrical samples of the test scaffolds (Ø 25 mm × 4 mm) were compressed up to 50% deformation at a test speed of 0.5 mm/s and the compression load (kPa) was measured. Based on the obtained results, the texture profile values were determined: hardness and flexibility.

2.8 Antioxidant activity

2.8.1 DPPH test

The ability of the scaffolds to react with oxygen-containing free radicals was assessed by the diphenylpicrylhydrazyl (DPPH) test adapted from [28].Briefly, different amounts of the materials tested were placed in 5 ml aliquots of a methanol solution containing DPPH (the solutions were prepared fresh, and the absorbance at 540 nm was adjusted to 1.0), and the

samples were shaken in the dark at 37°C. The change in color was monitored by spectrophotometric measurement (Spectrophotometer JENWAY 6300, U.K.) at 540 nm over a 6 h period. Measurements were performed hourly, and the scavenging effect was calculated according to the following equation:

Scavenging effect (%) = [1-(absorbance $_{sample}$ / absorbance $_{control}$)] × 100

The EC_{50} value is calculated by linear regression and is defined as the mass of sample material needed to reduce the initial absorbance at 540 nm of 1 ml of the DPPH solution by 50% after 2 h of incubation. The control consisted of the reagent solution without the material tested. Each determination was conducted in triplicate.

2.8.2 ABTS assay

The total antioxidant capacity was evaluated according to the modified ABTS assay [28]. The ABTS•+ radical cation solution [(2,20-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid))] was generated from ABTS salt (potassium persulfate (2.45 mmol) was reacted with 7 mmol of ABTS salt in 0.01 M PBS, pH 7.4, for 15 h at room temperature in the dark). The solution containing the resultant ABTS radical cations was diluted with methanol to give an absorbance of 1.0 at 734 nm. Different amounts of the material tested were suspended in 5 ml aliquots of the ABTS•+ solution and the samples were shaken in the dark at 37°C. The change in color was monitored by spectrophotometric measurement (Spectrophotometer JENWAY 6300, U.K.) at 734 nm over a 6 h period. The scavenging effect and the EC_{50} value were calculated as described above.

2.8.3 Fe (II)-chelating ability

The Fe(II)-chelating activity of the chitosan–protein scaffolds was measured according to the modified method by Yen et al. [29] where the Fe(II)-chelating ability was monitored by the absorbance of the ferrous iron–ferrozine complex at 562 nm (Spectrophotometer JENWAY 6300, U.K.). Briefly, different amounts of the material tested were suspended in 4 ml aliquots of a methanolic solution containing $FeCl_2$(2 mM) and ferrozine [5 mM, the absorbance (562 nm) of which had been adjusted to 1.0]. The samples were shaken at 37◦C for 60 min. The chelating effect and EC_{50} values were calculated as described above.

3. Biodegradation studies with lysozyme

Chitosan–protein scaffolds (Ø 25 mm × 4 mm) were placed in 50 ml of phosphate buffered saline (PBS), pH 7.4, containing lysozyme (4000 U/ml final activity) and incubated at 37°C with gentle agitation for the period of the study. The lysozyme solution was refreshed daily to ensure continuous enzyme activity. To prevent microbial colonization, chloramphenicol was

added to the medium at a concentration of 34 ng/ml. Samples were removed from the medium every 24 h for 7 days and rinsed with distilled water, dried, and weighed. The degree of in vitro degradation was calculated according to the following formula:

In vitro degradation = $(((W_0-W_t)/ W_0)_{lysosyme} - ((W_0-W_t)/ W_0)_{control}) \times 100$

where W_0 is the dry weight before the degradation test and W_t is the dry weight at time t. To discriminate between degradation and dissolution, control samples were stored for 7 days under the same conditions as the test samples, without the addition of lysozyme. Each determination was performed in triplicate.

4. Calculation

Principal Component Analysis (PCA) and chemometric computations were performed using STATISTICA software (StatSoft, Inc.,Tulsa, USA).

5. Result and discussion

5.1 Scaffold morphology

For preparation of chitosan-protein scaffolds were used collagen and gelatin isolated from the skin of two different fish species. The selection of two species living in different temperatures resulted in obtaining materials with significantly different physicochemical and rheological properties.

The differences in the properties of biomaterials obtained using protein preparations (collagen and gelatin from different sources), may often derive from disparities between their amino acid composition. Therefore, interpretation of the results was carried out with respect to such data (Tab.2).

Chitosan, collagen and gelatin scaffold materials as well as composites made from these polymers were repeatedly and detailed characterized in the available scientific literature. Due to the differences in properties of used biopolymers, the assay results of the materials obtained according to the same methodology are not clear. In case of discussed biopolymers, influence has such parameters as: molecular weight, deacetylation degree (chitosan), differences in amino acid composition (collagen and gelatin), often conditioned by their isolation source. Scaffold materials are generally obtained by freeze drying process previously frozen solutions of biopolymers or using other electrospinning, techniques, where to preparation collagen and chitosan solutions are used acid solvents (usually acetic or lactic acid solutions) and gelatin biomaterials are obtained directly from aqueous solutions. The resulting structures are subjected to many tests, the choice is conditioned by their declared purpose. Regardless of the selected application, they are in most cases characterized in terms of a few basic parameters: morphology, ability to bind liquid, solubility, parameters of texture profile and

biodegradability. For this reason, show the influence of polymer type (chitosan, gelatin and collagen) on selected properties of scaffold materials, obtained and described in different research groups is very difficult. Thanks to these experiments make it possible. Additionally, a characterization of the antioxidant properties of obtaining materials received, because it is a unique feature, rare occurrence among biopolymer materials. The resulting materials did not differ significantly in outward appearance. All were white and had a cylindrical shape, given by the used forms (Fig. 1).

Texture profile depends on the type of used biopolymer. Clearly harder materials were obtained for scaffolds containing gelatin in their composition. All materials were consistent and showed no tendency to decompose while visual evaluation. Analysis of the microstructure by scanning electron microscopy showed that all obtained materials are characterized by high porosity (Fig. 2)

Materials derived only from chitosan (100% CHI) were characterized by the smallest pores, while those composed of collagens have the largest pores with relatively regular structure. The pore size values for materials obtained from gelatin were in the range between chitosan and collagen polymers. The results also indicate that materials containing in their structure chitosan have a clearly denser network of smaller pores, as compared to other materials.

The differences in pore size caused the protein content are directly related with the total content of solute that hinders the ice crystal growth during the freezing, decreasing the pore size and porosity of the scaffolds. The increase of the solute content results in structures with smaller and almost closed pores [30]. The prepared materials showed a porosity close to 80% (chitosan) or more (Tab.3)

As a fact, the previous works reported that scaffolds with porosity higher than 80%, are ideal for uses tissue engineering, due to good conditions for nutrient supplying and gas exchaging [30].

The morphology of the material obtained by means of freeze-drying is influenced by many factors. Beside the impact with their chemical structure, effect on morphology also depends upon factors such as: polymers concentration in dispersion before freezing, cross-linking degree, type of cross-linking agent and also the conditions of the scaffold preparation process, starting from freezing temperature profile and ending the drying process parameters. Under conditions of the experiment, this variability was minimized due to the same concentration of the cross-linking agent and the polymer in the dispersion. Therefore, it can be assumed that the reported differences in morphology are related only to chemical structure of the used biopolymers and exactly the amount of amino groups. These groups are part of a cross-linking

bonds by creating bridges between free Ɛ- amino groups of the lysine and hydroxylysine residues, the free carboxylic groups of glutamic and aspartic acid residues of protein molecules, and the amino groups in the chitosan polymer. Considering the mass fraction of each polymer in the scaffolds is the same, the largest contribution to the cross-linking structure is related with the presence of chitosan, wherein the high content of amino groups due to the fact that always at least 60% of chitosan is composed of glucosamine residues and only the rest of the acetylated form. It seems to be consistent with the results recovered using SEM technique (Fig.2).

5.2 Swelling/dissolution tests

The largest swelling capacity was characterized by systems containing chitosan in its structure, the smallest systems derived only from protein polymers (Fig.3),wherein the addition of protein polymer to the chitosan dispersion caused a decrease in absorbency parameter an average of 30%, in relation to the materials obtained only with chitosan.
In case of solubility analysis, biomaterials obtained in 100% of collagen proved to be the least soluble and the most those derived from gelatins. Loss of weight chitosan materials was 11%. This represented a three times lower value relative to both gelatin materials, but two and four times higher value in relation to the collagen materials isolated from salmon and African catfish, respectively. For both groups of material obtained from gelatin and collagen isolated from the same fish species, the difference in solubility was 28% for preparations isolated from salmon and 30% for preparation isolated from African catfish. As the both proteins did not differ with respect to amino acid composition, a clear difference in solubility between the materials derived from gelatin and collagen isolated from the same source arise out of their spatial structure. Collagen is a protein with a specific structure of the triple helix stabilized by a number of short-range interactions, including hydrogen bonds between groups of amino acid residues, so that the protein is insoluble in water under conditions which do not violate its native structure. In contrast, gelatin is a protein obtained by the denaturation of collagen, which is the process in which there is total or partial destruction of interactions stabilizing its structure. During the denaturation of collagen occurs also its partial fragmentation, resulting presence of gelatin in the form of statistical hank. This also causes its solubility in water. The addition of glutaraldehyde (crosslinking agent) to the isolated biomaterial limited dissolution of all the structures. Without the addition of glutaraldehyde gelatin materials would be completely dissolved. Chitosan – collagen and chitosan – gelatin materials were characterized by intermediate values of both measured parameters. Further analysis of the obtained results suggests marked differences both in terms of absorbency as well as the solubility of materials

derived from proteins isolated from different fish. It also applies chitosan-protein materials. Scaffold material containing collagen or gelatin isolated from Salmon was characterized by a lower absorbency and a higher solubility compared to the materials containing collagen or gelatin isolated from African catfish. Visible differences may be the result of differences in amino acid composition of both collagen preparations. Higher content of lysine residues in the preparation isolated from African catfish (31/1000 residues) compared to Salmon (20/1000 residues) (Tab. 2) can be the cause that scaffold derived from African catfish could be more cross-linked with glutaraldehyde. A higher level of crosslinking may also explain the lower solubility of materials derived from African catfish preparation compared to those obtained using the Salmon preparations. In case of materials containing collagen in their structure, an additional effect on the rate of the dissolution process have denaturing process, which intensity depends on the experiment conditions. Dynamics of denaturation process for different collagen preparations is dependent on the number of interactions stabilizing its helical structure, which is conditioned by the content of hydroxyproline residues. Higherhydroxyproline content in the preparation isolated from African catfish (70/1000 amino acid residues) relative to its content in Salmon preparation (65/1000 residues) (Tab. 2) may also be the reason of lower solubility of materials obtained with the use of this biopolymer. The higher content of hydrophilic residues (lysine, hydroxylysine, arginine, aspartic acid, glutamic acid, asparagine, serine, threonine) in African catfish preparation(278/1000 amino acid residues) relative to the amino acid residues for preparation of Salmon (267/1000 amino acid residues) (Tab. 2) promotes a higher absorbency of materials containing protein polymers from this source.

5.3 Mechanical properties

Materials containing gelatin in their composition are harder than those containing collagen and simultaneously slower recover their shape after compression. They are less flexible regardless of the protein polymer isolation source. This observation applies both to materials constructed exclusively from protein preparations, as well as chitosan – protein composites. The materials obtained in 100% of chitosan proved to be harder than collagen materials with an average of 30% and more than twice less flexible. However, compared to the gelatin materials they were characterized by smaller values of both measured parameters on average by 40% and 30%, respectively (Fig. 4).

Distinct differences are also seen between the materials derived from collagens isolated from the skins of both studied species. Scaffold containing African catfish collagen was characterized by higher hardness and less flexibility compared to scaffolds containing

collagen isolated from Salmon (by 50% and 37%, respectively). This relationship was also observed for chitosan-collagen materials. However, in this case the differences were smaller (the difference in hardness - 36%, the difference in elasticity - 25%). The values of texture profile parameters are the result of many factors. Besides the effect related to the chemical structure of each polymer, it is influenced by the crosslinking degree and the morphology of the tested preparations, in particular the pore structure. During compression of the porous material, a substantial part of the deformation is associated with deformation of the pores filled with air. That determines the lower force value of the first peak during mechanical testing, describing the hardness of the material. Differences in the pore structure of the individual materials visible on the Fig. 2 confirm the higher flexibility and less hardness of collagen scaffolds compared to gelatin and chitosan materials. Moreover, the differences in the texture profile between collagen and gelatin materials (as in the case of differences in solubility), can be explained by differences in the structure of the two biopolymers. Occurrence of gelatin in the form of a statistical hank provides greater availability of free amino groups of lysine residues to cross-linking agent than the regular structure of collagen. It is one of the reasons for a greater hardness of materials obtained from gelatin preparations. The flexibility depends on the type and strength of bonds that occur between the protein chains building the scaffold. Covalent bonds in a gelatin statistical hank have a stiff character. The collagen triple helix is dominated by electrostatic interactions, giving flexibility to the protein, which is reflected in the flexibility results for tested materials. The differences between the materials derived from proteins isolated from African catfish and Salmon skins may also be explained by differences in their amino acid composition. The higher content of lysine residues (which might affect the higher degree of cross-linking) and higher content of hydroxyproline (affecting the strength of interactions between the chains of collagen helix) in the African catfish preparations (Tab. 2) may also explain the higher hardness of materials obtained by their use. The differences between the materials containing in their composition gelatin isolated from various sources are much smaller than those observed for the collagen materials, because the tertiary structure of the protein has a much greater effect on the mechanical properties than the amino acid composition. Denaturation and fragmentation of collagen following during the manufacturing process cause adoption by gelatin the statistical hank structure, namely its averaging which minimizes impact of this factor on texture profile parameter values.

5.4 The antioxidant properties

For the evaluation of the antioxidant properties of derived biopolymer systems three common tests were used: DPPH, ABTS and the ability to chelate Fe^{2+} ions. The ability of free DPPH radical scavenging consists in measuring the change in absorption of purple DPPH radical solution. The disappearance of the violet color informs about the level of radical neutralization contained in the DPPH solution. In the case of ABTS assay mechanism is identical, with the only difference that the solution has a green color. The ability to chelate iron ions is observed by the disappearance of the pink color of the solution which is the result of interactions of Fe^{2+} with ferrozine. This interaction is specific with respect to iron (II). Stronger interaction of these ions, e. g with chitosan or protein results in the destruction of iron-ferrozine complexes and the disappearance of the pink color. Radical reactions progress in time, causing visible loss of solution color, and their speed depends on the concentration of the test material. The disappearance of the color caused by the stronger complexing properties of the tested materials in the case of iron (II) reaches equilibrium. After two hours the test run there were no further changes in the color of the solutions. In order to compare the antioxidant activity the EC_{50} values for all measured parameters were determined (Fig. 5).

EC_{50} parameter defines the weight of the sample material, which causes neutralization of free radical, or chelation of Fe^{2+} in 50% after two hours from the start of the test. Values for this parameter is determined by linear regression of the results obtained from the dependence of the scavenging degree (or chelating Fe^{2+} degree) obtained for the various weights of tested materials. The larger EC_{50} value indicates that the material has less ability to neutralize free radicals or chelate iron ions. All prepared materials have the ability to neutralize the DPPH radical, ABTS cation - radical and also ability to chelate Fe^{2+} ions. Materials containing in its structure collagen isolated from both the fish species are characterized by a significantly lower values of the three measured parameters in respect to materials containing gelatin. Collagen materials received an average two, four, and three times lower EC_{50} values appropriate for testing DPPH, ABTS and Fe^{2+} respectively, compared to materials constructed solely of gelatin. Materials constructed in 100% from chitosan are characterized by the highest EC_{50} values for all tested parameters. Chitosan materials have on average more than four- and two times lower abilities to scavenge DPPH radicals and nine- and three times lower abilities to chelate iron (II) ions in comparison to materials constructed from collagens and gelatins, respectively. The exception is the comparison with the gelatin material for the variable determining the ability to sweep the ABTS cation – radical, in which these materials have achieved similar EC_{50} values in respect to materials derived from the gelatin isolated from the African catfish. EC_{50} values of other materials reached 23% lower results compared

to materials obtained from the Salmon gelatin preparations (higher ability to sweep the ABTS cation - radical) and an average of 70% higher compared to both collagen preparations (lower ability to sweeps ABTS cation - radicals).In the scientific literature, little attention was paid to the study of antioxidant properties of scaffold biomaterials. The results indicate that these materials retain in solid form antioxidant properties characteristic of biopolymer solutions from which they were derived. Antioxidant properties of natural polymers, in particular proteins, are influenced by a number of factors, such as chemical structure, tertiary structure and molecular weight. Clear differences between the materials obtained from the gelatin and collagen isolated from the same source, are not fully explained. Both preparations do not differ significantly in terms of chemical composition. However, the difference is in the spatial structure of the constituent particles of biopolymers. It may affect the level of visibility of functional groups in individual amino acid side chains, and thus the frequency of interaction with DPPH radical and ABTS cation-radical molecules can differ. Improved antioxidant properties of materials containing collagen may also result from differences in the processes of both protein preparations. During collagen isolation takes a place decolorization step, in which minced fish skins have been treated with 3% hydrogen peroxide solution for 48 hours. It can cause changes in the functional groups of amino acids as a result of oxidation. The newly formed group may be characterized by a greater capacity to free radicals scavenging and explain the higher activity of materials containing collagen. The mechanisms underlying the antioxidant activity of chitosan are associated with the presence and activity of hydroxyl- and amino groups, which can neutralize free radicals by transferring hydrogen atom to free radical molecules, e.g. reactive oxygen form. However, tests have shown that the antioxidant properties of chitosan scaffolds are much weaker than those which occur for materials derived from collagens and gelatins. There was also a significant difference in the EC_{50} values for materials obtained from proteins isolated from different fish species. These differences are clearly visible in the case of materials obtained from different gelatins.EC_{50} parameter values for DPPH, ABTS and Fe^{2+} test materials obtained from the gelatin isolated from African catfish were 18%, 34% and 13% lower compared to the values assigned to the materials derived from Salmon gelatin, respectively. Aleman et al. [31]comparing the antioxidant properties of the gelatin solution isolated from squid and sole, explains the observed differences the different amino acid composition for both preparations. In that work it was suggested that the higher content of the amino acids such as valine, alanine, leucine, isoleucine, histidine, methionine, proline and hydroxyproline should promote increased antioxidant properties. The higher content of the above mentioned amino acid residues in the

preparation isolated from the skins of African catfish, could therefore explain the higher antioxidant properties of biomaterials containing the gelatin from this source. Collagen isolated from African catfish has 386/1000 amino acid residues that influence the free radicals scavenging ability relative to 337/1000 amino acid residues present in preparation of Salmon (Tab. 2). Tests performed by Saiga et al.[32] indicate that protein hydrolysates ability to chelate transition metal ions increases with increasing the content of amino acids such as histidine, proline and hydroxyproline. The higher content of these amino acid residues in preparations isolated from African catfish can therefore explain higher ability to chelate Fe^{2+} ions by obtained from these scaffolds. The ability to chelate iron ions by the preparation of chitosan has not been fully explained. There are known literature reports suggest that divalent metal ions are chelated by interaction with the hydroxyl group on C6 atom and an amino group on the C2 atom of chitosan subunits. Other studies have reported dependency to the deacetylation degree showing an almost complete reduction of the ability to chelate divalent metal ions in the case of a completely N-acetylated chitosan preparations. Regardless the mechanism, the antioxidant properties of chitosan are significantly weaker related to that occurring in materials obtained from protein polymers.

5.5 Biodegradation with lysozyme

Information about the biodegradation kinetic together with information about solubility of materials are the basis for the design systems for the controlled release of immobilized or incorporated therein active compounds. Lysozyme is the only enzyme found in human serum, capable to depolymerization of chitosan [33, 34].This enzyme in a highly specific manner hydrolyzes glycosidic bonds inter alia connecting glucosamine and acetylglucosamine subunits each other, building chitosan chains. The lack of such bonds in collagen and gelatin, suggests that these polymers should not be hydrolyzed under the influence of its operation. Nevertheless, many authors conducting characteristics of obtained by them the protein preparations, show their susceptibility to degradation based on the hydrolytic activity test of lysozyme [35].In this study performing the test with lysozyme the lack of biodegradation of the materials obtained from protein polymers was observed, both for the first and the last day of the experiment (Fig.6).

No differences were also observed in the case of chitosan-collagen and chitosan-gelatin materials. Therefore it can be concluded that the collagen and gelatin materials are not biodegradable under the hydrolytic action of lysozyme. Perhaps the results presented by the authors of the work cited above do not indicate biodegradability of obtaining materials by lysozyme, but may be associated with matrix degradation as a result of the processes related

with their dissolution. In conducting test, the weight loss of materials obtained in 100% of chitosan increased from about 3.5% after 24 h exposure to the lysozyme to more than 8% after 120 hours of incubation. The increase in weight loss was also observed in the case of scaffolds obtained from two chitosan copolymers with each selected protein polymer. Regardless the type of used protein , and also the source of its isolation, the weight loss was 2% and 6%, respectively. Susceptibility to matrix enzymatic degradation may be important in the design of systems for the controlled delivery of active compounds, because this is an additional mechanism next to erosion processes associated with the dissolution, which affects to the release kinetics of incorporated therein active compounds.In the case of chitosan-gelatin material, weight losses caused by the lysozyme activity in relation to the mass loss as a result of dissolution processes, seems to be so small to consider this degradation mechanism as negligible. In the case of chitosan-collagen materials situation is different, because erosion being a result of hydrolytic activity of lysozyme constitutes about 40% weight loss related with dissolution processes. Probably degradation of chitosan with lysozyme affected to the loss of material homogeneity.

6. Chemometrics

Chemometric approach was applied to compare all received biopolymer systems. The data were normalized to give all variables the same importance by subtracting the variable average form each variable and dividing the difference by the standard deviation. The number of principal components was chosen on the basis of the magnitude of the eigen values which is a measure of the amount of information conveyed by each principal component. In the instance of biopolymer scaffolds data set were applied the first two PCAs for which eigen values above unity were kept. The clustering of the scaffolds was done manually. The data of the nine tests described in Sections 5.1–5.5 were used to compare the nine biopolymer scaffolds. Principal component analysis indicates that the nine tests correlate to some extent, with the maximum correlation factor of biodegradation with lysozyme day one to biodegradation with lysozyme day five approaching r ∼1.0 and between the variable describing swelling ability and the two variables describing the level of enzymatic degradation (r ~ 0.95). Both observations can be reasonably explained by referring to the methodology of the test. A high level of correlation between the swelling ability and the level of enzymatic degradation is related to the intensity of the exposure of the sample to the lytic action of lysozyme. In the test run, only the outer surface of the materials was equally exposed to it. The differences resulted from different amounts of lysozyme, which was able to penetrate into the sponge, and this is correlated with the ability of each scaffold to absorb liquids (Section 5.2).The two principal components

explain about 87% of variability observed in the biopolymer scaffold set. Principal component analysis of the analyzed data set has shown that materials obtained on the basis of pure chitosan, collagens, and gelatins as well as materials obtained by combining chitosan with protein preparations may be divided into five clusters shown in Fig. 7.

In order to highlight the relationship between the obtained materials, centers of the areas characteristic for materials obtained in 100% of the each biopolymer were joined together to form a triangle of relationship. This action showed that the materials obtained by combining two selected biopolymers, are close to the line connecting areas of biomaterials made only from one of these biopolymers, e.g. the location of chitosan-collagen material is on the straight line joining the area of occurrence of material of pure chitosan and pure collagens (the same observation was made for chitosan-gelatin scaffolds).The result of the above analysis shows that by combining each two of three polymers, it is likely to obtain a material with properties which are resultant of the properties of the materials made with each of them separately.

7. Conclusions

The use of biopolymer mixtures of fish collagen, fish gelatin and chitosan had a significant impact on the rheological properties of obtaining scaffold materials. The application of biopolymer mixtures allows to modify in a simple way (without additional factors) such properties as: swelling capacity, solubility, biodegradation and mechanical properties of designing materials. The source of collagen and gelatin isolation is also important. It is less important in the case of using collagen or gelatin isolated from homeothermic organisms. For usage collagen or gelatin from ectothermic organisms it becomes crucial. Scaffold materials containing collagen or gelatin isolated from Salmon skin were characterized by a lower swelling capacity and a higher solubility compared to the materials composed form collagen or gelatin isolated from African catfish skin. Importance is also the method of protein polymer isolation. Gelatin-containing materials are harder than those containing collagen and slowly recover the original shape after compression, regardless the source of the protein polymer isolation. In this case, the impact on the rheological properties is dictated by the tertiary structure of the protein. In the scientific literature, little attention to the study of antioxidant properties of scaffold made from used by us biomaterials was given. Because of the potential possibilities of using these materials in tissue engineering, it turns out that this property becomes important. Oxidative stress is one of the factors affecting the elongation rate of wound regeneration. This condition results from an imbalance between the action of reactive oxygen forms and mechanisms of organism antioxidant defense. Natural polymers, especially

collagen, gelatin and chitosan are a group of macromolecules that for most of the commonly used low molecular weight antioxidants, shows a much lower activity, while retaining much greater stability. However, high stability provides a long term activity. Clear differences between the materials obtained from the gelatin and collagen isolated from the same source, are not fully explained. Both preparations do not differ significantly in terms of chemical composition. On the other hand, they differ in the spatial structure of the constituent particles of biopolymers, which may affect the exposition level of individual amino functional groups of side chains, and thus the frequency of interaction with radical molecules. In conclusion, it has been shown that the physicochemical properties of the biopolymer mixtures are affected by many factors and depending on the biopolymers origin, is possible to create materials with specific properties. Future research will be conducted to assess the suitability of the obtained preparations as a wound dressing material attractive for biomedical uses, notably in tissue engineering or as a specific active compounds or drug delivery system.

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Figure captions

Fig. 1 Biopolymer scaffold materials obtained from chitosan and protein (gelatin and collagen isolated from skin of African catfish and Salmon) solutions (* descriptions of materials accordance with Tab. 1 labels).

Fig. 2 Structure of biopolymer scaffold materials obtained using Scanning Electron Microscopy (* descriptions of materials accordance with Tab. 1 labels).

Fig. 3Swelling capacity and dissolution of obtaining chitosan and chitosan- protein scaffolds.

Fig. 4 Texture profile parameters of obtaining chitosan and chitosan- protein scaffolds.

Fig. 5 The antioxidant properties of obtaining chitosan and chitosan- protein scaffolds.

Fig. 6 Susceptibility of chitosan and chitosan-protein scaffolds to degradation by lysozyme.

Fig. 7Results of Principal Component Analysis. Location of direction vectors (B) for the variables: swelling capacity (a), solubility (b), EC_{50} ABTS (c), EC_{50} DPPH (d), EC_{50} Fe^{2+} (e), hardness (f), flexibility (g), enzymatic degradation – 1st day (h), enzymatic degradation – 5th day (i) and linear map of objects (A) in the area of the first and the second main component for materials obtained from the dispersion: collagen from Salmon (1), collagen from African catfish (2), gelatin from Salmon (3), gelatin from African catfish (4), chitosan and collagen from Salmon (5), chitosan and collagen from African catfish (6), chitosan and gelatin from African catfish (7), chitosan and gelatin from Salmon (8), chitosan (9).

Tab.1 The composition and preparation conditions of biopolymer scaffold materials.

No.	Sample	Biopolymer	Biopolymer concentration in disperion [%, w/w]	Solution	Dissolution temperature [^{0}C]
1	100% $COLL_S$	collagen *Salmon*	2.0 %	0.5M CH_3COOH	5^0C
2	100% $COLL_{AC}$	collagen *African catfish*	2.0 %	0.5M CH_3COOH	10^0C
3	100% GEL_S	gelatin *Salmon*	2.0 %	H_2O	60^0C *
4	100% GEL_{AC}	gelatin *African catfish*	2.0 %	H_2O	60^0C *
5	50% $COLL_S$ 50% CHI	chitosan; collagen *Salmon*	1.0 %; 1.0 %	0.5M CH_3COOH	5^0C
6	50% $COLL_{AC}$ 50% CHI	chitosan; collagen *African catfish*	1.0 %; 1.0 %	0.5M CH_3COOH	10^0C
7	50% GEL_{AC} 50% CHI	chitosan; gelatin *African catfish*	1.0 %; 1.0 %	0.5M CH_3COOH; H_2O	Room temperature
8	50% GEL_S 50% CHI	chitosan; gelatin *Salmon*	1.0 %; 1.0 %	0.5M CH_3COOH; H_2O	Room temperature
9	100% CHI	chitosan	2.0 %	0.5M CH_3COOH	Room temperature

* gelatin were pre-swelled at room temperature (20 min) and then heated at 60°C for 1 minute.

Tab. 2. Amino acid composition of fish skin collagen isolated from African catfish and Salmon (residues per 1000 total amino acid residues)

Aminoacid	African catfish	Salmon
Hydroxyproline	70	65
Aspartic acid	47	56
Threonine	29	20
Serine	33	51
Glutamic acid	78	72
Glycine	322	371
Alanine	110	102
Cysteine	0	0
Valine	22	14
Methionine	14	17
Isoleucine	12	10
Leucine	28	21
Tyrosine	6	2
Phenylalanine	19	12
Lysine	31	20
Histidine	7	12
Arginine	49	56
Proline	123	96
Hydroxylysine	0	3
Tryptophan	0	0

Tab.3 Porosity and pore size of biopolymer scaffold materials.

No.	Sample	Porosity [%]	Poze size [µm]
1	100% $COLL_S$	86.70 ± 2.65	758 ± 1.2
2	100% $COLL_{AC}$	88.67 ± 2.02	832 ± 2.5
3	100% GEL_S	86.91± 1.99	630 ± 3.5
4	100% GEL_{AC}	87.28 ± 2.06	620 ± 2.1
5	50% $COLL_S$ 50% CHI	85.85 ± 2.55	435 ± 3.7
6	50% $COLL_{AC}$ 50% CHI	86.09 ± 2.12	428 ± 3.6
7	50% GEL_{AC} 50% CHI	85.46 ± 2.10	278 ± 2.3
8	50% GEL_S 50% CHI	85.22 ± 2.19	288 ± 1.7
9	100% CHI	77.43 ± 1.14	189 ± 1.9

The data are presented as the means ± SD (n=3).

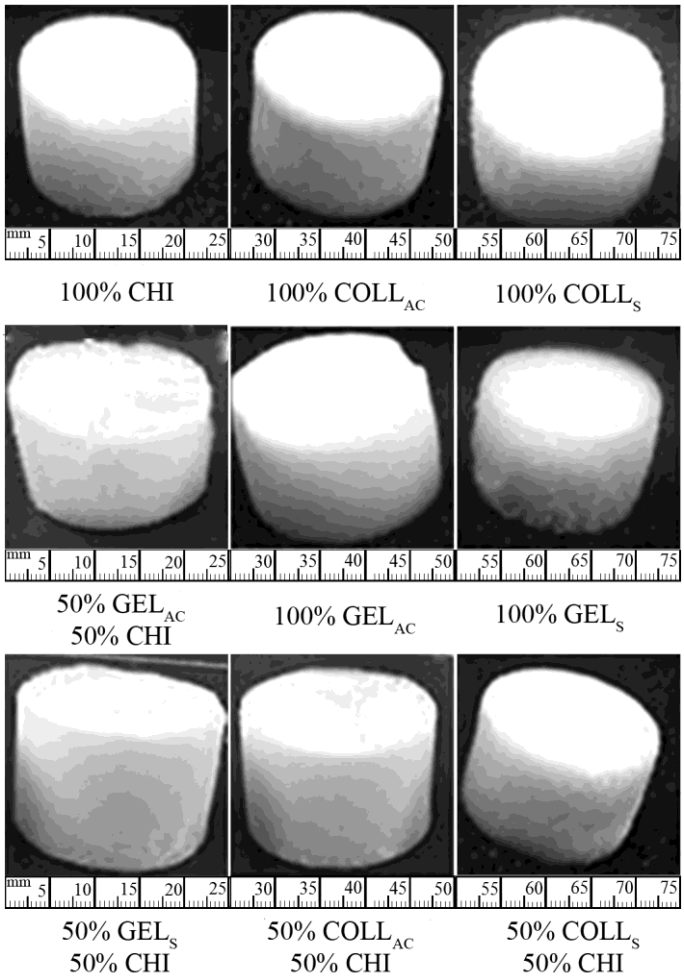
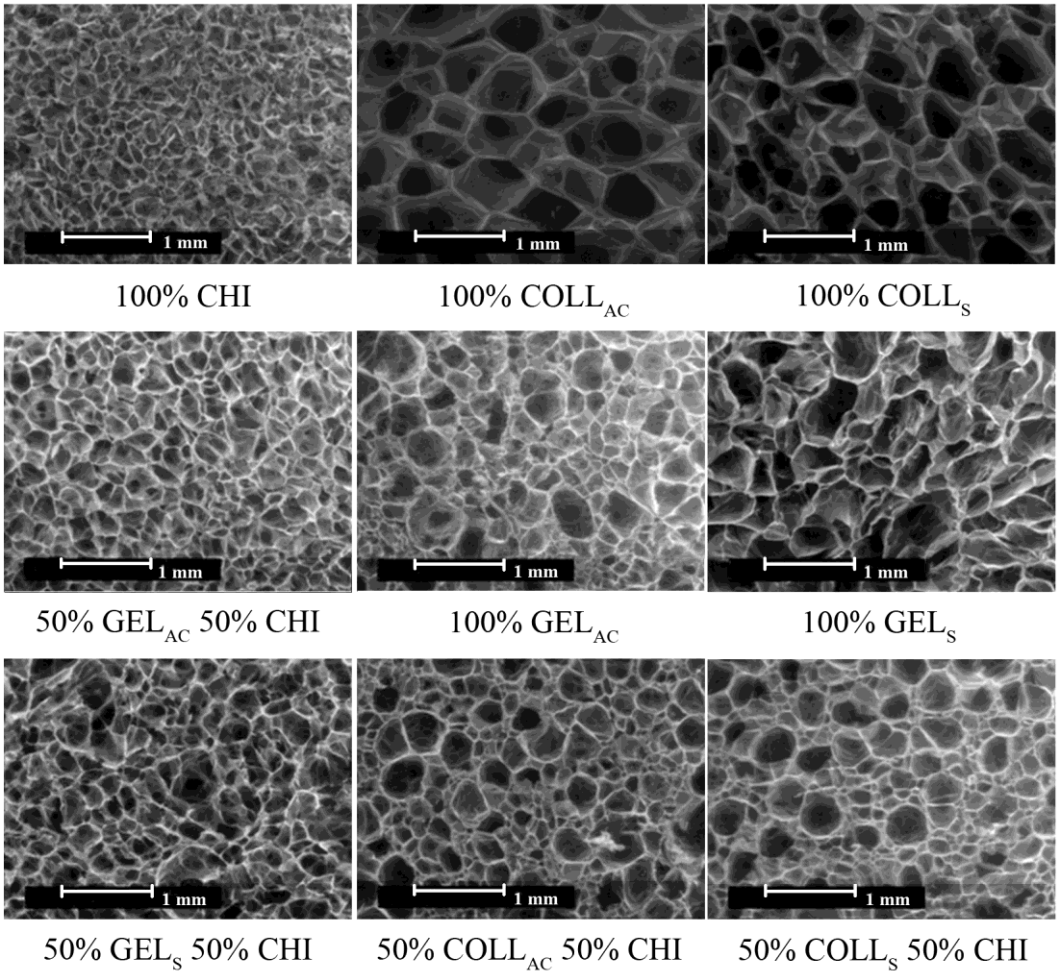


Fig. 1



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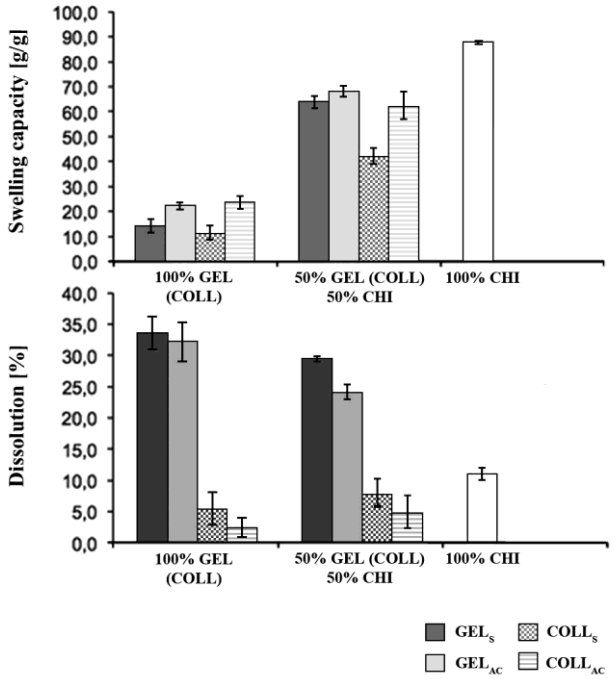

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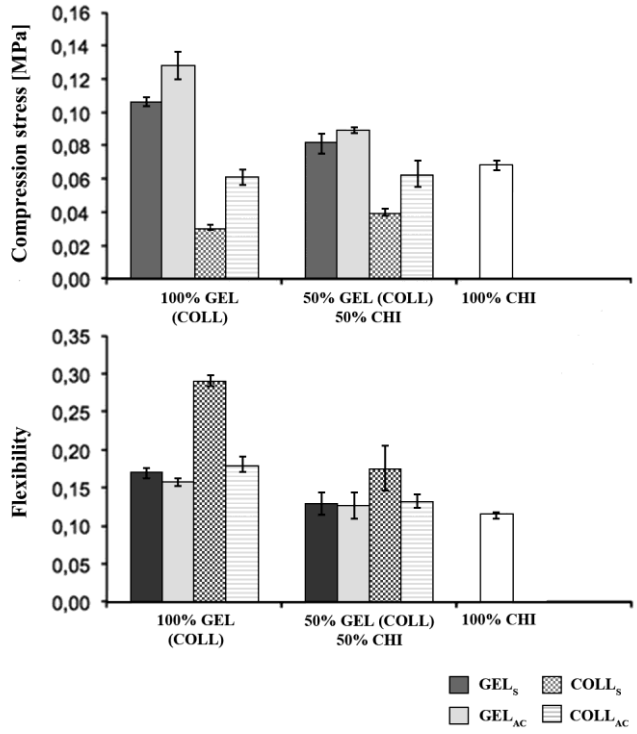

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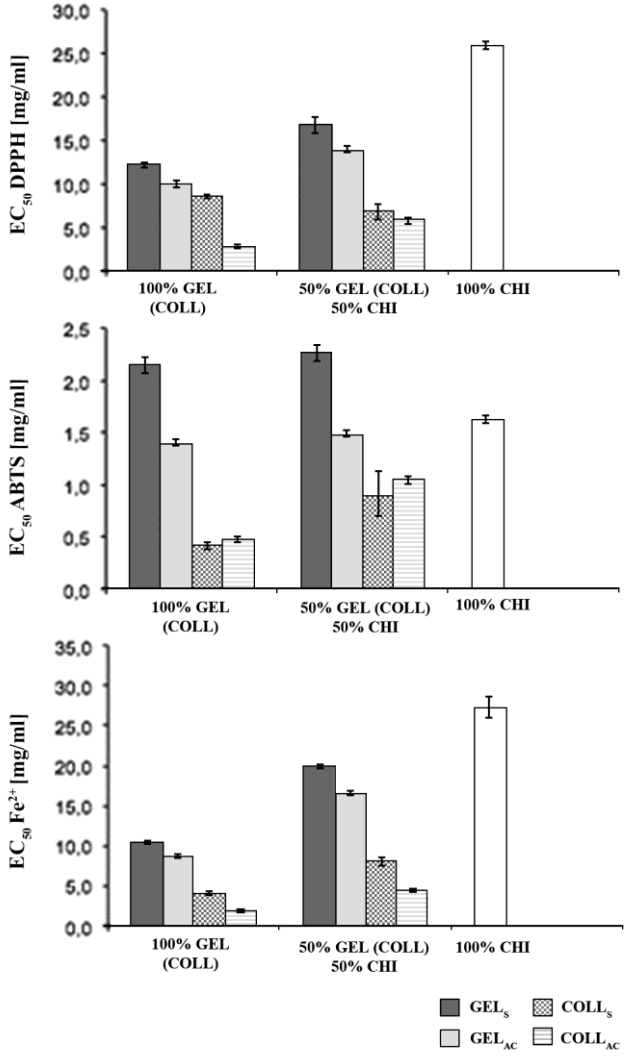

Fig. 5

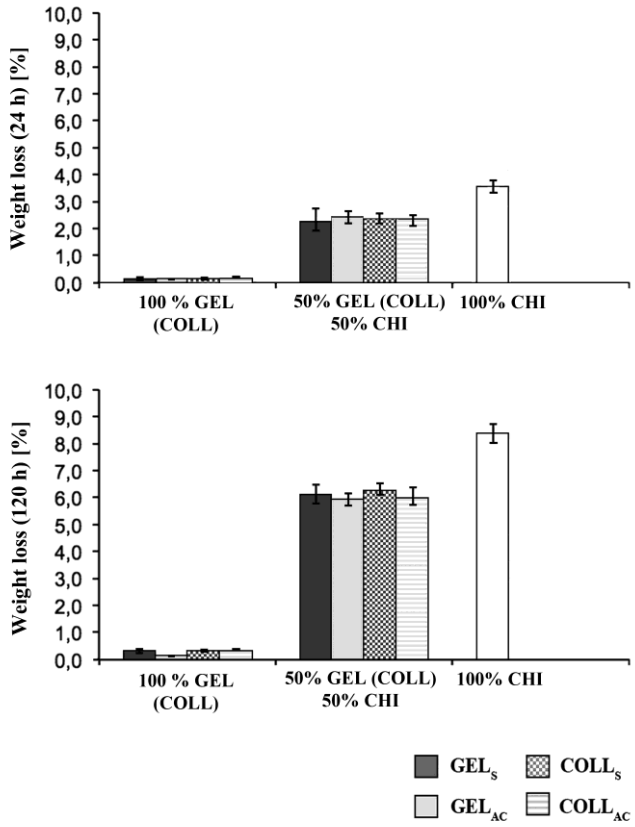

Fig. 6

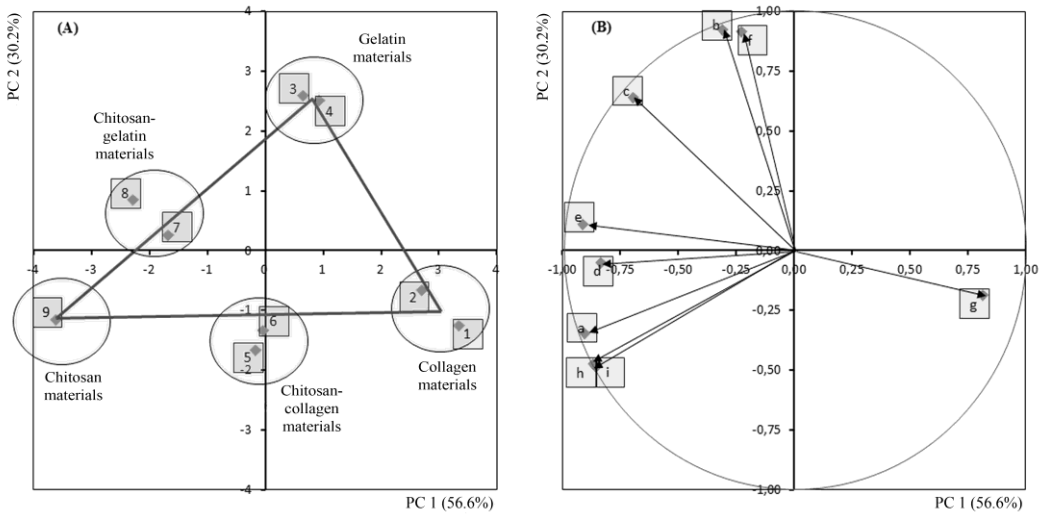

Fig. 7