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INFLUENCE OF FISH COLLAGEN ON VISCOELASTIC PROPERTIES AND SOL-GEL PHASE TRANSITION OF CHITOSAN SOLUTIONS

Abstract

The thermosensitive hydrogels are widely used in tissue engineering due to their non-invasive application. Special interest of researchers, due to the specific characteristics of both materials, is aimed at composites of natural origin obtained from chitosan hydrogels combined with collagen. The mechanical properties of the thermosensitive chitosan-fish collagen hydrogels and the sol-gel phase transition parameters were determined by the rotational rheometry measurement techniques. Based on comparison of the obtained storage modulus G' curves, it was found that the addition of collagen negatively affects the mechanical properties of composite scaffolds. The addition of this protein substance decreases their elasticity. Only the smallest concentration (0.25g collagen/1 g chitosan) of collagen improves the mechanical properties of composite hydrogels, from 56 kPa to 61 kPa. Conducted non-isothermal studies allowed to conclude that the addition of collagen causes an increasing temperature of sol-gel phase transition. However, the observed changes are not a monotone function of the biopolymer concentration.

Key words
Injectable scaffold, sol-gel phase transition, chitosan, fish collagen

Introduction

Hydrogels are three-dimensional, hydrophilic porous polymeric structures, capable of binding water and many biological fluids [1]. These systems are insoluble due to non-covalent interactions between the polymer chains (physical crosslinking) or secondary polymerization caused by the addition of cross-linking substances (formation of covalent interactions) [2,3]. These structures, known as scaffolds, are used in tissue engineering as temporary matrices for local delivery of cells, growth factors and drugs [4]. Hydrogels have attracted noticeable interest, e.g. in bone tissue engineering, for their structural similarity to cartilage, which is a highly hydrated tissue composed of chondrocytes deposited in type II collagen and glycosaminoglycans (GAG) [5]. Hydrogels may show thermosensitivity through the ability to undergo a sol-gel phase transition. Those systems are characterized by a relatively low viscosity and predominance of viscous properties in the sol phase and a significantly higher viscosity and the domination of elastic properties in the gel phase. The main difference between this two phases is the observed flow phenomenon characteristic for viscous liquid and no flow for the viscoelastic fluid [6]. Recently, thermosensitive hydrogels are an object of interest for many researchers with high potential due to the lower invasiveness compared to implant scaffolds [7,8]. Furthermore, due to the fluid form, the use of injectable scaffold allows to fill complex defects [8]. Another advantage is the ability to control important properties such as porosity, size, geometry and the degree of connection pores. This leads to the possibility of imitating the topological and microstructural properties of the extracellular matrix [9].

In recent years, there has been a growing interest in using polymers of natural origin for the production of scaffolds (also hydrogels) used in tissue engineering. Among the most commonly used groups the polysaccharides (chitosan) and the protein substances (collagen) should be mentioned [2,9,10]. Their most important properties are biocompatibility and biodegradability in a physiological environment [9,11]. Due to the properties of both biopolymers, chitosan-collagen composites are often used for the manufacture of implantable scaffolds [12–16]. Furthermore, collagen is the source of particularly desirable amino acids in tissue engineering such as arginine and lysine [17].

For chitosan - collagen colloidal solutions, measurements of rheological properties presented in the literature are usually limited to rotational tests with the determination of flow and viscosity curves [18,19]. In the literature, there are not many studies on the thermosensitivity of chitosan-collagen systems. Few oscillatory studies indicate viscoelastic properties of systems. At low temperatures a predominance of viscous properties

over elastic ones is observed, at high temperatures the elastic properties dominate over viscous ones [20]. Nevertheless, these studies are often conducted within a narrow measurement range of angular frequency.

The study of thermosensitivity (changes in viscoelastic properties under the influence of heating) of colloidal chitosan-collagen solutions is discussed in detail in the paper [21]. In these studies, the authors concluded unequivocally negative effect of the addition of collagen on the mechanical properties of composite hydrogels obtained. It was also found that the addition of collagen drastically increases the phase transition temperature. However, the observed change is not a monotone function of the biopolymer concentration. It is worth noticing that in the studies cited, the authors used collagen of bovine origin.

Available studies indicate that fish-derived collagen is characterized by better bioavailability than bovine-derived. This is due to the fact that the fish collagen molecules are smaller than bovine [22]. Its advantages are also a lower degree of crosslinking and higher solubility [23]. The last significant advantage of fish-derived collagen is an easier purification process, which leads to smaller damage to protein chains [24].

The aim of this study was to investigate the influence of the addition of fish-derived collagen on viscoelastic properties and condition of sol-gel phase transition of colloidal chitosan solutions.

Materials and methods
For the present study, chitosan (DD=81.8%, MW= 680 kDa) from crab shells (Sigma Aldrich Product no. 50494) and fish-derived Type 1 collagen (Kolagen NCN – Collagen Premium Fish) were used. Furthermore, as a solvent the 0.1M hydrochloric acid (Fluka Product no. 84415) was used. The disodium β-glycerophosphate (Sigma Aldrich Product no. 50020) was added as a substance neutralizing chitosan solutions to physiological pH and maintaining the biopolymer chain in solution in a hydrophilic form [25–27].

Thermosensitive chitosan hydrogels were prepared based on the commonly used method [26]. 0.4 g of chitosan was dissolved in 16ml 0.1M hydrochloric acid. After thorough mixing, the colloidal solution was left at room temperature for 24h to completely dissolve the biopolymer. The fish-derived collagen– due to its solubility – was added in an acidic environment, by dissolving appropriately 0.1g, 0.2g, 0.4g – Table 1 (sample no. 2-4). After this, a suspension of disodium β-glycerophosphate was prepared by dissolving 2g of the substance in 2ml of distilled water. Both solutions were cooled to 4 °C. Next drop by drop the glycerophosphate suspension was added to the colloidal solution of chitosan with the addition of collagen. Sample no. 1, without the addition of collagen, was a reference solution.

Table 1. Composition of tested samples.

Sample	Chitosan [g]	Hydrochloric acid [ml]	β-NaGP [g]	Collagen [g]	Collagen/Chitosan weight ratio [g collagen/1 g chitosan]
Sample no.1	0.4	16.0	2.0	0.0	0.00
Sample no.2				0.1	0.25
Sample no.3				0.2	0.50
Sample no.4				0.4	1.00

Source: Author's

Test samples were measured using rotational rheometry techniques. Rheological properties were investigated in the cone-plate measuring system (50 mm diameter, 1° slope angle, 48 μm truncation) of Anton Paar Physica MCR 301 rheometer. The sol-gel phase transition temperature and change of mechanical properties (change in the value of the storage modulus G') during heating were determined in non-isothermal measurements in the range of 5° C - 60° C, with constant deformation (ω=5 s^{-1}, γ=1%). The viscoelastic properties and thermosensitivity were determined in the frequency sweep test (ω = 0.005 s^{-1} - 500 s^{-1}) also called the

mechanical spectra. The studies were conducted for all samples at the temperatures of 5 °C, 25 °C, 30 °C, 35 °C, 40 °C.

Results and discussion

The results of non-isothermal measurements at constant deformation of all tested samples are shown in Fig. 1. The obtained experimental curves allow to determine the sol-gel phase transition temperature. According to the theory proposed by Chambron and Winter, the point at which the tangent of the phase shift angle $\tan\delta$ reaches the value of unity determines the gelation point [28]. The temperatures of the phase change determined by this method are presented in Table 2. The obtained results indicate that the addition of collagen to the colloidal chitosan solution increases the gelation temperature in comparison to the sample without this protein. However, this increase in gelation temperature is not a monotonous function of collagen concentration. This confirms previous studies of thermosensitive hydrogels with the addition of bovine collagen [21]. Low protein concentration causes a temperature increase from 26.5 ° C to 37.4 ° C and 38.4 ° C respectively for samples 2 and 3. This may be due to the presence of large particles of biopolymer, which in the case of low concentration constitute a spatial blockage and make it difficult to bring the polysaccharide chains closer together. Moreover, the constant concentration of the glycerophosphate salt may be partially absorbed as a protein chain protecting substance [29,30]. The addition of the highest collagen concentration (sample 4) causes a decrease the gelation temperature. However, the obtained value is higher than gelation temperature of control sample. In the case of this sample, the decrease of gelation temperature, as compared to samples 2 and 3 (with lower concentration of protein), may be due to the fact that the collagen molecules are also crosslinked. Thus, the internal structure and interactions between chitosan and collagen chains are changed. As a result, there is a different crosslinking of the three-dimensional polymer matrix because of the same concentration of both polymers.
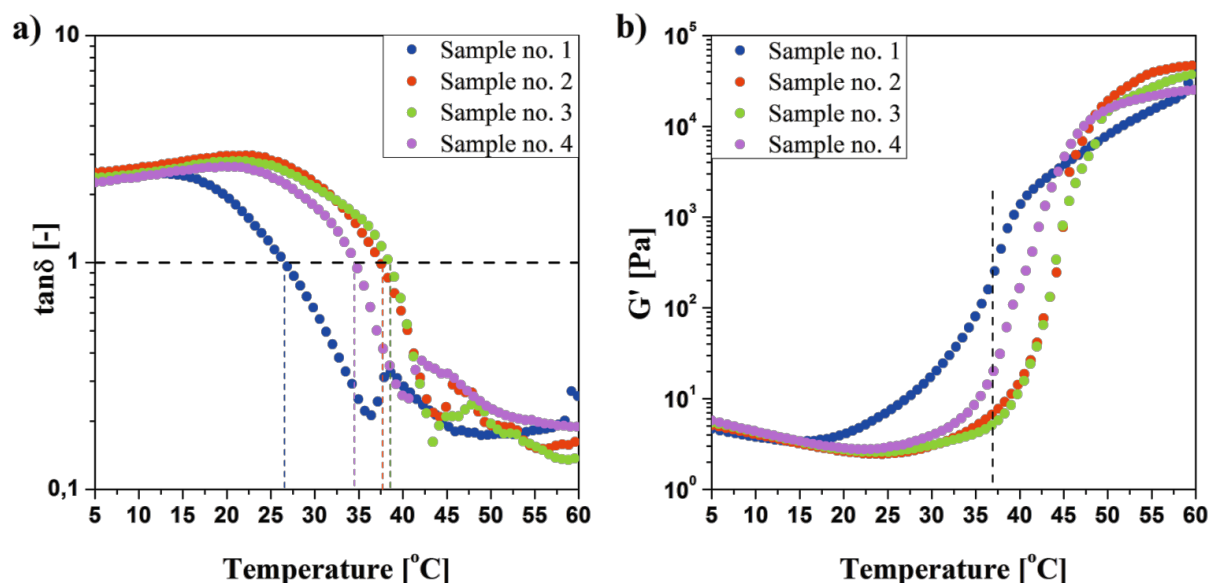

Fig. 1. Results of non-isothermal measurements with constant mechanical deformation, a) $\tan\delta$ as a function of temperature, b) storage modulus G' as function of temperature.
Source: Author's

Analysis of changes in the value of the storage modulus G' at the gelation point indicates that the solutions containing the collagen additive show less mechanical strength than the pure chitosan solution – Fig. 1b. The analysis of changes in the value of the storage modulus at 37 °C (reference temperature) was also carried out – Table 2. The reference temperature is in the region of rapid [31] gelation for all tested solutions. Nevertheless, for samples no. 2 and no. 3 the reference temperature is lower than the determined gelation temperature. It means that for both samples viscous properties dominate over elastic ones. Comparing samples no. 1 and 4, there is a significant reduction in mechanical strength for a sample containing collagen. It also results from another phase of formation of the final structure. The results of non-isothermal measurements indicate that for temperatures above 45 °C all solutions containing collagen reach higher values of the storage modulus G'. Thus demonstrating better mechanical strength. However, for the design of thermosensitive hydrogels formed in vivo, which can be used in tissue engineering, such high temperatures are not included in the analysis.

Table 2. The temperatures of sol-gel phase transition of tested samples and storage modulus G' values in critical points i.e. the sol-gel phase transition point and at reference temperature 37°C

| Sample | The sol-gel phase transition (tanδ=1) | | T=37°C | |
	Temperature [°C]	G' [Pa]	tanδ [-]	G' [Pa]
Sample no. 1	26.5	9.1	0.2	226.0
Sample no. 2	37.4	7.5	1.1	6.9 (*)
Sample no. 3	38.4	7.0	1.3	5.2 (*)
Sample no. 4	34.5	7.9	0.5	19.7

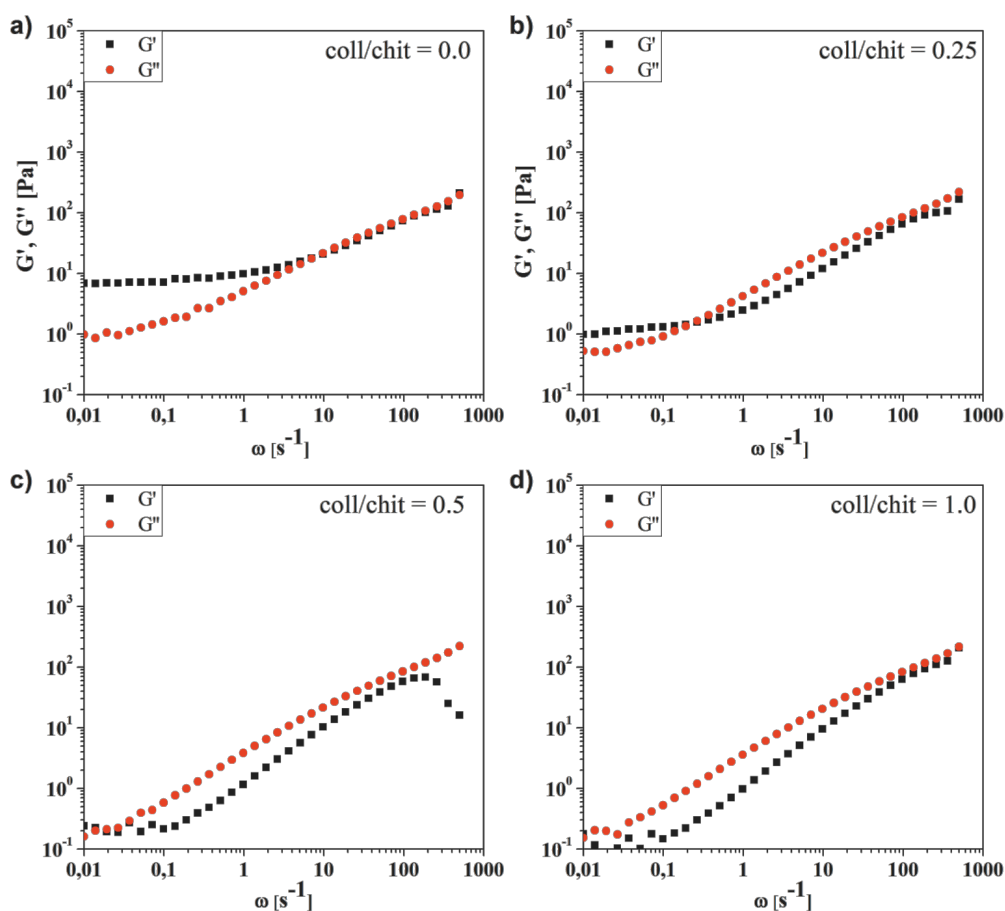(*) Gelation temperature is higher than reference temperature (37 °C). *Source: Author's*

Fig. 2. Experimental curves of storage G' (■) and loss G" (●) moduli in a wide range of angular frequency ω, a) sample no. 1, b) sample no. 2, c) sample no. 3, d) sample no. 4 at temperature 5 °C.
Source: Author's

The results of the mechanical spectra obtained for the solutions tested at 5 ° C are shown in Fig. 2. The graphs show that the addition of collagen to the colloidal chitosan solution causes a change in the dominant character of the medium in the studied range of angular frequency ω. In the case of sample No. 1 (without the addition of collagen) - Fig. 2a, a significant predominance of elastic properties over viscous ones (G' > G") for low values of angular frequency is observed. For higher values ω, viscous properties dominate over elastic ones but not so significantly. The addition of collagen causes increased domination of viscous properties over elastic ones in a whole range of angular frequencies ω - Fig. 2b-d. The dominance of elastic properties over viscous is observed only for the smallest collagen concentration tested below ω=0.2 s⁻¹. It was also observed that the addition of collagen causes not only a change in the dominant properties of the solutions, but also affects the mechanical strength of the samples tested. It was determined on the basis of the decrease in the value of the storage modulus G'. The lowering of storage modulus G' value of sample no.2 for low angular frequency, from 20 Pa to 2 Pa was noticed. A further increase in the concentration of the collagen additive (sample no. 3 and 4) causes a

decrease of the storage modulus G ' value to about 1 Pa. For high values of angular frequency ω, the values of modules for individual samples are similar. This means that the samples store comparable amounts of energy in each deformation cycle. The obtained results of angular frequency sweep test are fully consistent with data presented in the literature for chitosan solutions with addition of bovine collagen.
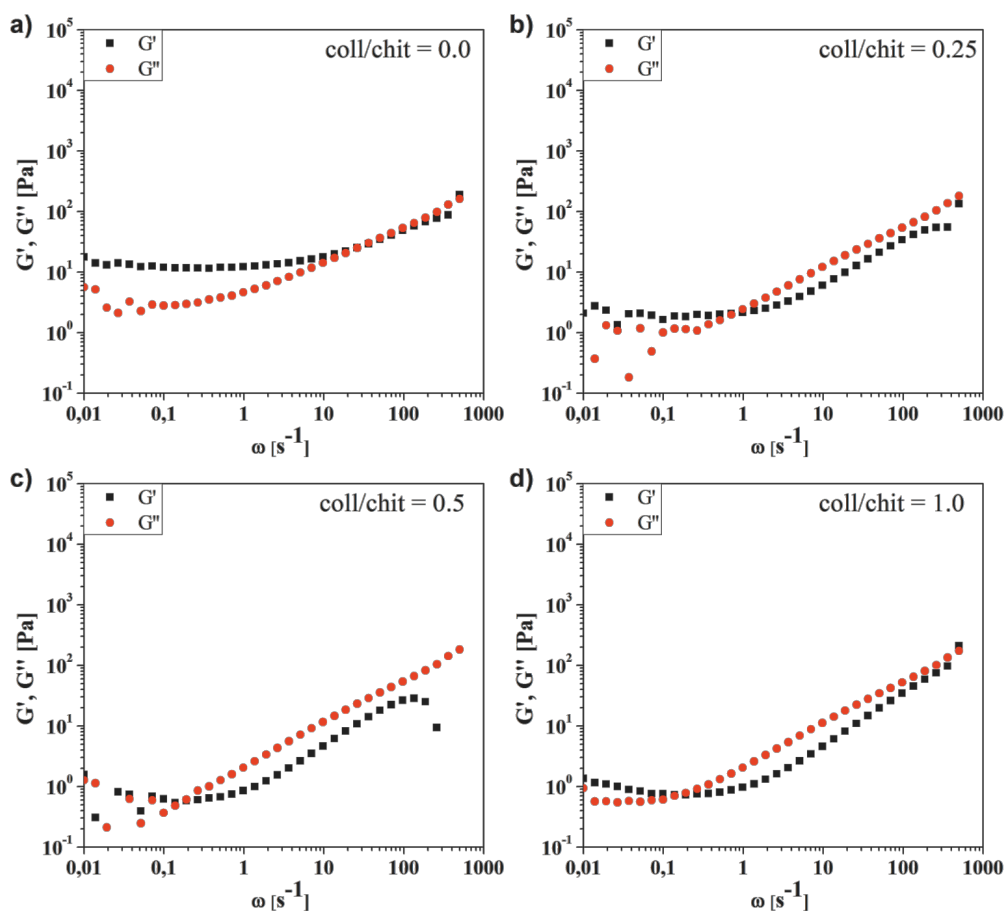

Fig. 3 Experimental curves of storage G' (■) and loss G" (●) moduli in a wide range of angular frequency ω, a) sample no. 1, b) sample no. 2, c) sample no. 3, d) sample no. 4 at temperature 25 °C.
Source: Author's

Interpretation of the results of measurements carried out for a higher temperature (Fig. 3) indicates that the dependences presented above are maintained. More precisely, the addition of collagen causes a stronger domination of viscous properties over elastic ones in a wider range of angular frequency and reduction the mechanical strength. However, for all experimental curves, a shift toward a stronger domination of the highly elastic region on the theoretical curve [21,32] is observed. For all samples, the dominance area of elastic properties over viscous ones is stronger noted. These shifts result from a change in the temperature at which the measurement is carried out and is characteristic for thermosensitive polymers and biopolymers systems. The obtained mechanical spectra indicate shortening the characteristic relaxation times. This parameter was determined as the inverse of the angular frequency ω, for which the intersection of the storage G' and loss G" curves was observed. Moreover, for samples no. 2 and 3, structure instabilities for low values of angular frequency, defined as discontinuities of the obtained experimental data were observed.
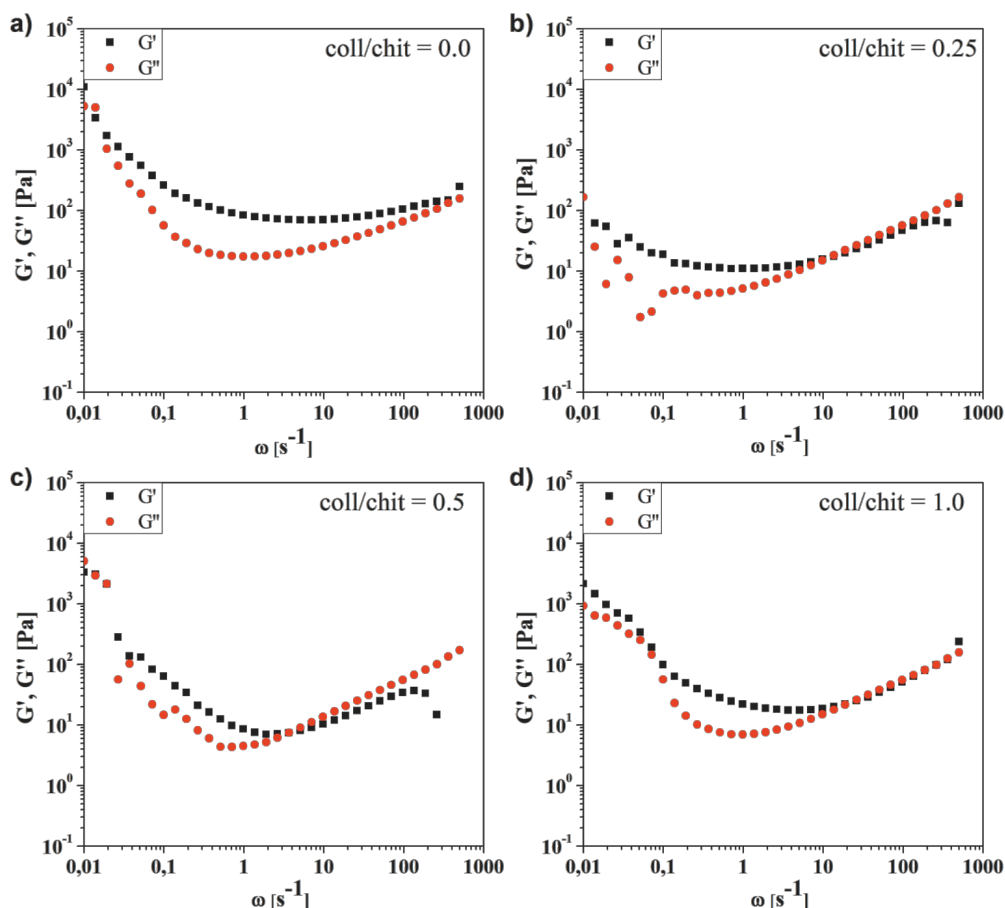
Fig. 4. Experimental curves of storage G' (■) and loss G" (●) moduli in a wide range of angular frequency ω, a) sample no. 1, b) sample no. 2, c) sample no. 3, d) sample no. 4 at temperature 30 °C.
Source: Author's

Further heating of the tested samples leads to the largest changes in the course of the experimental curves – Fig. 4. At 30 ° C, only the control sample (without the addition of collagen) in the whole range of the angular frequency shows the dominance of elastic properties over viscous ones (G' > G"). The addition of collagen leads to a change in the dominant properties of the tested solutions depending on the applied deformation. It is worth noting that the most similar shape, compared to sample no. 1, has a sample with the highest concentration of collagen – Fig. 4d. Although the shape of the experimental curves is similar, especially for low angular frequency ω, the storage G' and loss G" modules of sample no. 4 reach lower values by a decade compared to sample no. 1. In the case of samples no. 2 and no. 3 for low values of the angular frequency, the discontinuity of the obtained experimental data, which may indicate structural disorders, is again observed. It was also found that as the temperature of the measurement increases, the characteristic relaxation times for each solution are shortened. Their determination was possible only for samples containing a collagen additive. For those systems the unambiguous intersection of the experimental curves of the storage G' and loss G" modulus was observed.
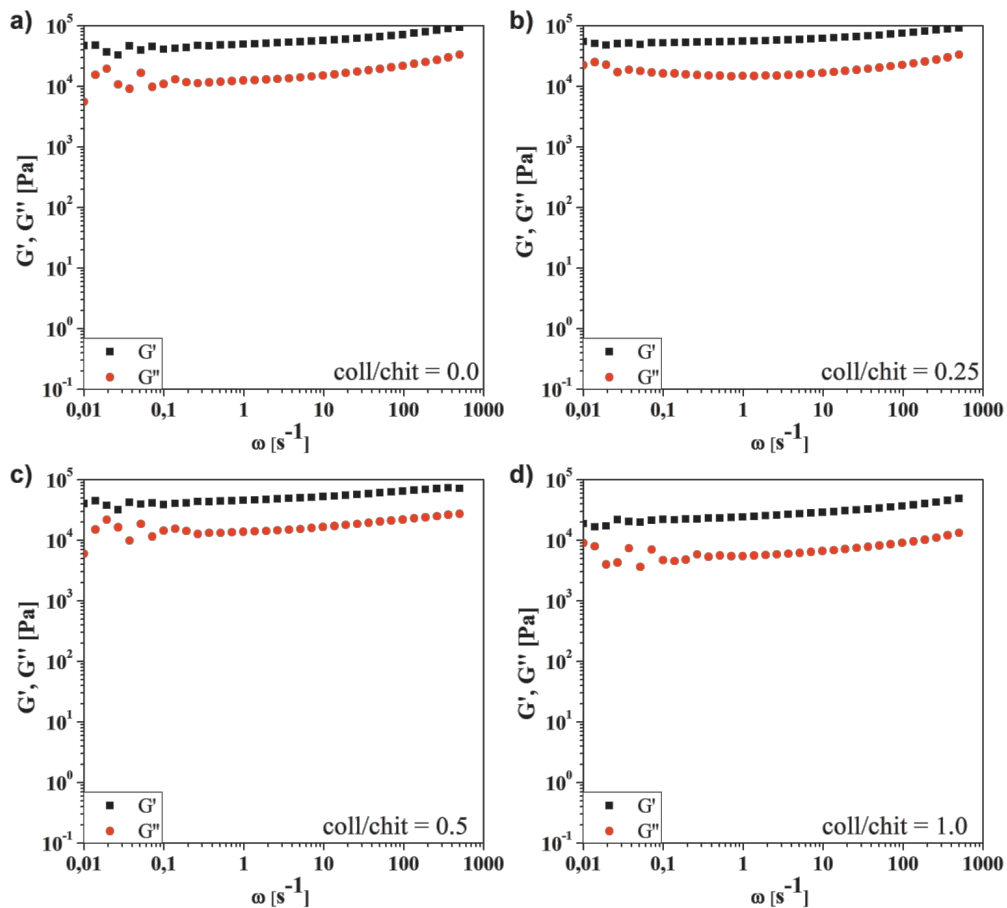
Fig. 5. Experimental curves of storage G' (■) and loss G" (●) moduli in a wide range of angular frequency ω, a) sample no. 1, b) sample no. 2, c) sample no. 3, d) sample no. 4 at temperature 40 °C.
Source: Author's

The mechanical spectra obtained at 40 ° C are shown in Fig. 5. For all samples tested, the independence of both experimental curves from the applied deformation, characteristic for gel phase, is observed. This demonstrates the formation of infinite, three-dimensional structure. Based on the values of storage modulus G', which represents the stored energy as well as strength or mechanical rigidity, the mean value was calculate – see Fig. 6. It has been found that the addition of collagen in the lowest concentration (0.25g collagen/1 g chitosan) leads to improvement mechanical strength. However, increasing the concentration causes a decrease in mechanical strength – Fig. 6. The negative effect of collagen addition on mechanical properties of chitosan hydrogels is consistent with the results of studies with bovine-derived collagen [21]. The obtained mechanical spectra at 35 °C were almost no different from the curves shown in Fig. 5. Samples at 35 °C were characterized by lower mechanical strength, both modules have reached lower values.
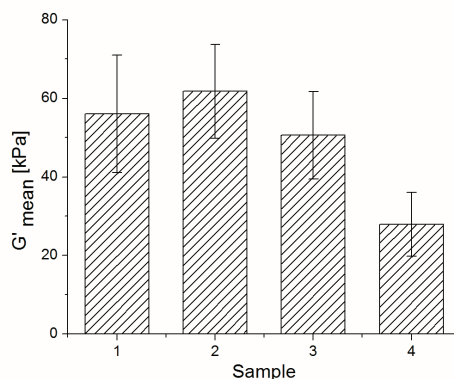

Fig. 6. The mean value of the storage modulus G ' with error bars for the full range of deformation at temperature 40 ° C.
Source: Author's

Conclusions

The conducted studies indicate a significant effect of fish collagen addition on the viscoelastic properties of colloidal chitosan solutions. Different viscoelastic properties cause different sol-gel phase transition temperatures. Regardless of the collagen concentration, an increase in gelation temperature was always observed. Moreover, it was shown that the dependence of sol-gel phase transition temperature is not a monotonous function of collagen concentration. Furthermore, the conducted research indicates that the presence of fish collagen negatively affects the mechanical strength of the obtained scaffolds, the lower values of the storage modulus G' was observed. Only the smallest concentration (0.25g collagen/1 g chitosan) of the collagen additive has improved the mechanical properties. The obtained results of viscoelastic properties as well as temperatures of sol-gel phase transition are consistent with the studies presented in literature for thermosensitive hydrogels chitosan-collagen of bovine origin.

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