THE INFLUENCE OF THE ADDITION OF COLLAGEN ON THE RHEOLOGICAL PROPERTIES OF CHITOSAN CHLORIDE SOLUTIONS

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Abstract

Colloidal solutions of chitosan of crab origin with the addition of collagen obtained from cowhide were studied. Were presents the influence of collagen concentration and the method of preparing the sample on the obtained mechanical properties of the solutions and the observed phase transition temperature. Rheological measurements were performed to determine the viscoelastic properties and phase transition temperatures of these solutions. The study was conducted in the temperature range of 5–60°C with the use of classical techniques of rotational rheometry in the cone-plate measurement system. A significant influence of a collagen addition to chitosan chloride solutions on the viscoelastic properties of the systems was observed. The addition of collagen in all the cases increased the sol–gel phase transition temperature in comparison with the chitosan chloride solution containing β-glycerophosphate.

Key words: *chitosan, collagen, rheology, viscoelastic behaviour, thermosensitive hydrogels*

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1. Introduction

Colloidal chitosan-collagen solutions are a frequent subject of research due to their wide range of applications during the formation of fibres [1–4] or bone scaffolds in tissue engineering [5–10]. Thanks to the known features of collagen, such as improvement of mechanical properties, biocompatibility and biodegradability, as well as the ability to create scaffolds, it is often used as an addition to chitosan solutions [11]. Moreover, it constitutes a source of valuable amino acids, such as arginine and lysine, which are particularly coveted in tissue engineering [12]. Among the notable features of chitosan are the non-toxic, antimicrobial properties, low immunogenicity and support in wound healing [13]. Similar to collagen, chitosan is also biocompatible and biodegradable [14].

In the case of thermosensitive hydrogels, which are represented by chitosan-collagen systems, the parameter that initiates the phase transformation process is the appropriate temperature for the strictly defined pH of the system [15–17]. Similarly, it has been demonstrated that an increase in the concentration of collagen added to the chitosan solution causes a rise in the pH of this solution [18] and a shift in the phase transition point towards higher temperatures [19].

Chitosan solutions in organic and non-organic acids are characterised by low pH, which is dependent on the molecular weight of the polysaccharide and high phase transition temperatures [15]. In practice, the addition of a buffering substance is used to increase the pH of a chitosan salt solution. The most commonly used buffer is disodium β-glycerophosphate (Na-β-GP). Adding this substance changes the pH to the physiological value of a human body and constitutes a catalyst, causing the decrease of the sol–gel transition temperature of chitosan hydrogels to the value of ca. 37°C. Glycerophosphate is also an important source of phosphorus in cell cultures [20].

The majority of research on the properties of the discussed systems is conducted with the use of the Fourier transform infrared spectroscopy (FTIR) [7,10,21], the techniques of differential scanning calorimetry (DSC) [22] or thermogravimetric analysis (TGA) [7]. In the case of chitosan-collagen systems, the measurements of rheological properties presented in the literature are limited to rotational studies determining the flow curves and viscosity curves [1,23]. In most available literature sources, attention has been drawn to the non-Newtonian, shear-thinning characteristic of these systems [1,24]. Few authors have presented the results of measurements that would indicate viscoelastic properties of chitosan salt solutions. However, the results of oscillatory measurements have indicated viscoelastic properties of such systems with a dominance of viscous properties over elastic properties for low temperatures, and a dominance of elastic properties in the case of high temperatures [19]. It was also found that collagen significantly improves the stiffness of the obtained hydrogels. However, the presented research [19] was conducted in a narrow range of angular frequency: 1–60 rad/s. In the literature, there have been few reports presenting the changes of viscoelastic properties taking place during the heating of colloidal chitosan-collagen solutions and the accompanying structural changes evoked by the mechanical deformation of the sample in a wide range of angular frequency [25]. An undoubted advantage of the oscillatory measurements is also the non-invasiveness resulting from using very small values of mechanical deformations – the amplitude of the deformations.

The aim of this study was to determine the effect of collagen on the rheological properties and phase transition of chitosan chloride solutions to which collagen is introduced at acidic and neutral pHs (with and without the addition of disodium β-glycerophosphate-Na-β-GP).

2. Analysis of mechanical spectra of biopolymers

The results obtained during the oscillatory measurements enabled the specification of both viscous properties, loss modulus G", and elastic properties, storage modulus G' of the studied media. Mutual relationships between the above values, presented as their quotient tan(δ) = G"/G' (damping factor), allowed the determination of changes in the structure of the studied matter [26]. The curve of both moduli obtained during the mechanical deformation of the sample or of the sample subjected to temperature changes determined the present state of the internal structure and characterised the interaction of the molecules found inside it [25,26]. Similarly, oscillation measurements are a universal technique and alloys, suspensions and solutions of polymers and biopolymers were characterised based on the obtained results.

The characteristic curve of moduli G' and G" and their relations described by the tanδ curve obtained for biopolymers were presented (Fig. 1).

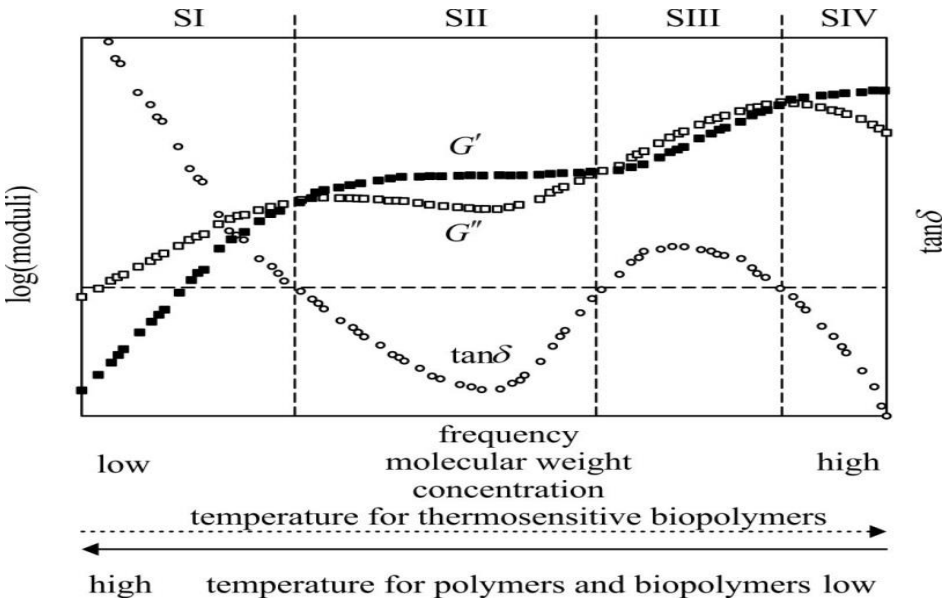

Figure 1. Change of storage modulus G' and loss modulus G" and the tangent loss angle (damping factor) in the function of temperature, frequency, molecular weight and concentration [25].

It was found that regardless of the variable being the frequency of deformations, the molecular weight, biopolymer concentration or change in temperature, the obtained results could be divided into four areas. The boundaries between these areas are designated by the subsequent points of crossing of the curves of moduli G' and G", called the relaxation times $\tau = 1/\omega$ [s] (long relaxation time for low values of oscillation frequency, relaxation time for medium values of oscillation frequency, and short relaxation time for high values of oscillation frequency) [26,27,28]. In each of the designated areas, the studied biomaterial showed different rheological properties characterising the present state of the structure and intermolecular interactions. The direction of temperature changes indicated on the graph is correct only for colloidal suspensions of biopolymers demonstrating phase transitions in accordance to the transition taking place during the melting of a polymer (e.g. starch). In the case of

biopolymers demonstrating sol–gel phase transitions induced by a temperature increase (chitosan, hydroxypropyl cellulose, proteins, etc.), the direction of temperature changes on the abscissa (x-axis) should be reversed – the dotted line in Fig. 1.

According to the denomination adopted in Fig. 1, the following areas were distinguished:

Region S I – A region of molecular flow in which the chains of the biomaterial move relative to each other due to the value of heat energy that exceeds the value of the energy of interactions. A dominance of viscous properties over elastic properties is observed here. This area is characteristic for solutions of polysaccharides and proteins. This region is also called the state of viscous liquid. In this area, no stress is observed under the influence of external forces. A disappearance of deformation is also not observed after removing the applied force.

Region S II – A highly flexible state in which biopolymers create flexible networks. In this region, no flow was observed. The result is the dominance of elastic properties over viscous ones (G'>G"). In this area, elastic deformations are observed. Even a slight deformation leads to the destruction of the created internal structure. In this area, the heat transfer energy of the macromolecules and the energy of interaction are similar. In the result, motility of single macromolecule segments is observed, with no movement of the whole macromolecule.

Region S III – A transition area between a glassy state and a highly flexible state. In the absence of crystallisation, this area is identified with the glass transition region. This results from a gradual immobilisation of the flexible chains, the neighbouring structural nodes of the created network.

Region S IV – A glassy state in which chemical and enzymatic reactions, as well as microbiological processes rapidly slow down. This is the result of a very low thermal energy of the macromolecules.

In order to interpret the relationships between the viscoelastic functions – the interaction of the moduli G' and G", which characterises the current state of intermolecular interaction in the solution – it was necessary to introduce a dimensionless quantity, tanδ, which determines the relationship of loss modulus G" and the storage modulus G' [26,27,28]. The tanδ curve in a wide range of angular frequency or temperature [25] can also be presented in the form of its fragments, which closely correspond with the created structure [26].

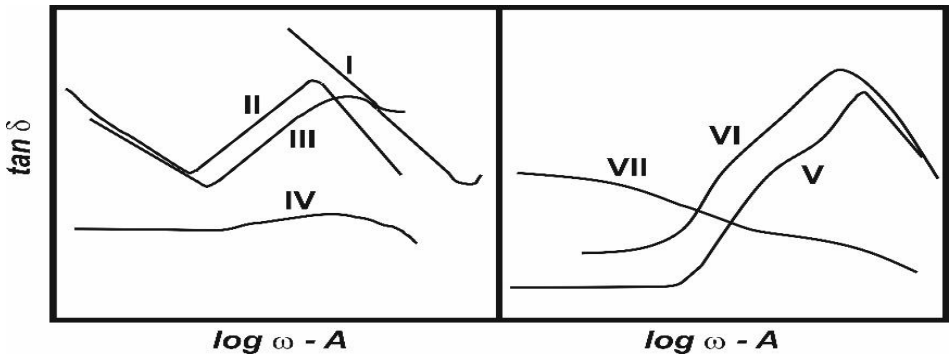

Figure 2. Change of tanδ trajectory in the function of oscillation frequency for various systems: trajectories I–IV represent non-crosslinked systems, V–VII represent crosslinked systems [26].

The tanδ curves presented in Fig. 2 correspond with the following structures:

I – an amorphous low molecular weight polymer;
II – an amorphous high molecular weight polymer;
III – an amorphous high molecular weight polymer that has long side chains. The main chain constituting a small part of its total volume is characteristic of this system;
IV – an amorphous high molecular weight polymer, the so-called glassy state;
V – a poorly crosslinked amorphous polymer whose structure has the form of filamentous network and highly flexible chains. This is the structure of the so-called soft rubber;
VI – a diluted crosslinked gel. The presence of small crystallites and the permeation of solvent molecules inside the pores of the network are characteristic of this system;
VII – a highly crystalline polymer – such a system is built from a network of crystallites linked by the permeating macromolecule chains.

3. Materials and Methods

3.1. Materials

A chitosan chloride solution was prepared by solving 0.4 g of chitosan of crab origin (Sigma Aldrich Product no. 50494-100G-F) of deacetylation degree 81.8% and molecular weight 680 kDa in 16 ml of 0.1 M hydrochloric acid (Fluka product no. 84415). The solution was left for 24 h at room temperature in order for the polysaccharide to fully dissolve. After 24 h, the sample was cooled to 4°C. Next, a cooled solution of disodium β-glycerophosphate (Sigma Aldrich product no. 50020-100G) was added drop by drop. The solution of disodium β-glycerophosphate was obtained by dissolving 2 g Na-β-GP in 2 ml of distilled water at 4°C.

In the case of solutions containing collagen, the method of preparing the chitosan solutions was analogous to that described above. Either 1 ml or 2 ml of collagen of cowhide origin (Sigma product no. C4243-20ML) was added in two ways. In the first case, it was added to chitosan solution of an acid reaction (pH ca. 6) before cooling. Next, the solution was cooled to 4°C and after 2 h, disodium glycerophosphate salt (Na-β-GP) was added. In the second case, collagen was added into a sample cooled to 4°C directly after mixing the chitosan solutions and Na-β-GP (pH of the solution ca. 7).

In each case, after preparation, the samples were left for 24 h at 4°C to remove the resulting air bubbles. A summary of sample preparation is presented in Table 1.

Table 1. Summary of sample preparations

Number of sample	Presence of Na-β-GP	Presence of collagen	The order of collagen addition	
			Before Na-β-GP	After Na-β-GP
1	+			
2				
3	+	+ (1 ml)		+
4			+	
5		+ (2 ml)		+
6			+	

3.2. Methods

The study of rheological properties of the obtained solutions were conducted in a cone-plate measuring system (50 mm diameter, 1° slope angle, 0.048 mm truncation) of a rotational rheometer (Anton Paar Physica MCR 301). Two types of research were conducted.

The first was a classical oscillatory measurement conducted in a wide range of angular frequency ω from 0.005 s^{-1} to 500 s^{-1}. For all measurements, the same value of amplitude strain 10% was used. This value was determined in a previous amplitude sweep test to define the linear viscoelastic region [28,29]. The studies were conducted for all samples (1–6). A tested sample was placed in the measurement system of a rheometer at the temperature of 5°C and the frequency sweep test was conducted. The next measurements were made for the samples by subsequently heating them to the temperatures of 25°C, 30°C, 35°C and 40°C (heating rate 1 K/s). On the basis of the conducted tests for each temperature, the storage modulus G' and loss modulus G" curves and the corresponding tanδ curves were obtained.

An analysis of the obtained results was conducted on the basis of the storage modulus G' and loss modulus G" curves and based on their designated tanδ curves as a ratio G"/G'. The obtained results were compared with the characteristic curve describing these functions, which were presented in section 2 (Fig. 1). Relaxation times were determined for each sample (in the case of all examined temperatures). On the basis of comparison of the obtained curves of storage modulus G' and loss modulus G" and tanδ with the literature [25,26], characteristic regions occurring during the measurements were specified, which corresponded with the internal structure of the tested sample.

The other type of conducted research was determining the temperature of the sol–gel phase transition and the gelation time. Oscillatory tests were performed in the same measurement system for steady mechanical deformations (angular frequency $\omega = 5\ s^{-1}$ and amplitude strain $\gamma = 10\%$). In order to specify the gelation temperature, the tests were conducted at a constant heating rate 1 K/min of the sample from 5°C to 60°C. The gelation time was determined during tests conducted in isothermal conditions at 37°C. In this case, the samples were placed in the measurement system of the rheometer at 5°C and were quickly (1 K/s) heated to the required temperature of 37°C. The crossing point of the curves of storage modulus G' and loss modulus G" ($tan\delta = 1$) was adopted as the sol–gel phase transition point. At this point, the samples changed properties from viscous to elastic.

4. Results and Discussion

The changes of viscoelastic properties of a chitosan chloride solution (with and without the addition of collagen) determined during the gelation process are presented (Fig. 3). For all analysed solutions, the obtained experimental curves were analogous to those presented in the literature [25] for biopolymers (Fig. 1). Comparing the curves of moduli G' and G", it was noted that for all the studied samples, in each of the examined temperatures, regions S II and S III occurred. In the case of measurements performed for sample 2 (chitosan salt solution without the addition of Na-β-GP) at 5 and 25°C, the occurrence of the molecular flow region S I (ductile) was observed.

Region S III, which constitutes a transition range between a glassy state and a highly flexible state, occurred in all samples containing collagen. In the discussed area, the slope of the experimental curves of storage modulus G' and loss modulus G" and a local dominance of viscous properties over elastic properties (G">G' for the same value of angular frequency ω) are characteristic.

In the case of solutions containing 1 ml of collagen added after the glycerophosphate (sample 3) at 5 and 25°C, a double intersection of the curves of moduli G' and G" was observed (Fig. 3F, G). The occurrence of two relaxation times were observed. From the analysis of the moduli curves, the results showed that the second, short relaxation time took place at high values of oscillation frequency, which indicates the occurrence of a glassy structure, S IV, during the coagulation. The forces of intermolecular interaction were very high, and the external thermal and mechanical energy was too low to allow changes in the position of the macromolecules of the biomaterial [29]. At higher temperatures, when the amount of energy supplied to the system increases, this area was not observed and there was no complete immobilisation of the molecules. At these temperatures, only the occurrence of the transition region S III was observed.

For solutions in which the collagen was added in an acid environment (samples 4 and 6), the modulus G' curves indicated a lack of possibility of the occurrence of a glassy state (Fig. 3F–O). In the case of both these solutions, as the temperature increases, one could observe a shift of moduli curves towards Region S II – highly flexible, constituting the area of a viscoelastic plateau. There may be local changes in the chain's location, with no change in the position of the entire macromolecule [29]. At 35°C, a viscoelastic plateau was observed, with a predictable intersection of the moduli (outside the assumed measurement range, both for low and high values of angular frequency ω).

Analysing the measurement results, angular frequency values ω were determined where the intersection of the curves of storage modulus G' and loss modulus G" was observed – for cases where this intersection occurred. The determined values of relaxation times, as the inverse of angular frequency $\tau = 1/\omega$, are presented in Table 2. The relaxation time characterises the structural transition occurring in the sample between regions S II and S III, and simultaneously defines the susceptibility of the system to the formation of a stiffened, elastic spatial structure.

Table 2. Comparison of relaxation times

Temperature [°C]	**Relaxation time [s]**				
	1 ml collagen	**1 ml collagen before NaGP**	**2 ml collagen**	**2 ml collagen before NaGP**	**Chitosan without collagen**
	Sample no.3	**Sample no. 4**	**Sample no. 5**	**Sample no. 6**	**Sample no. 1**
	s	s	s	s	s
5°C	7.2464	26.9542	3.7736	3.7736	0.125
25°C	3.7736	1.4085	1.0152	0.7299	0.0313
30°C	0.0275	0.0103	0.0038	0.0074	0.0025

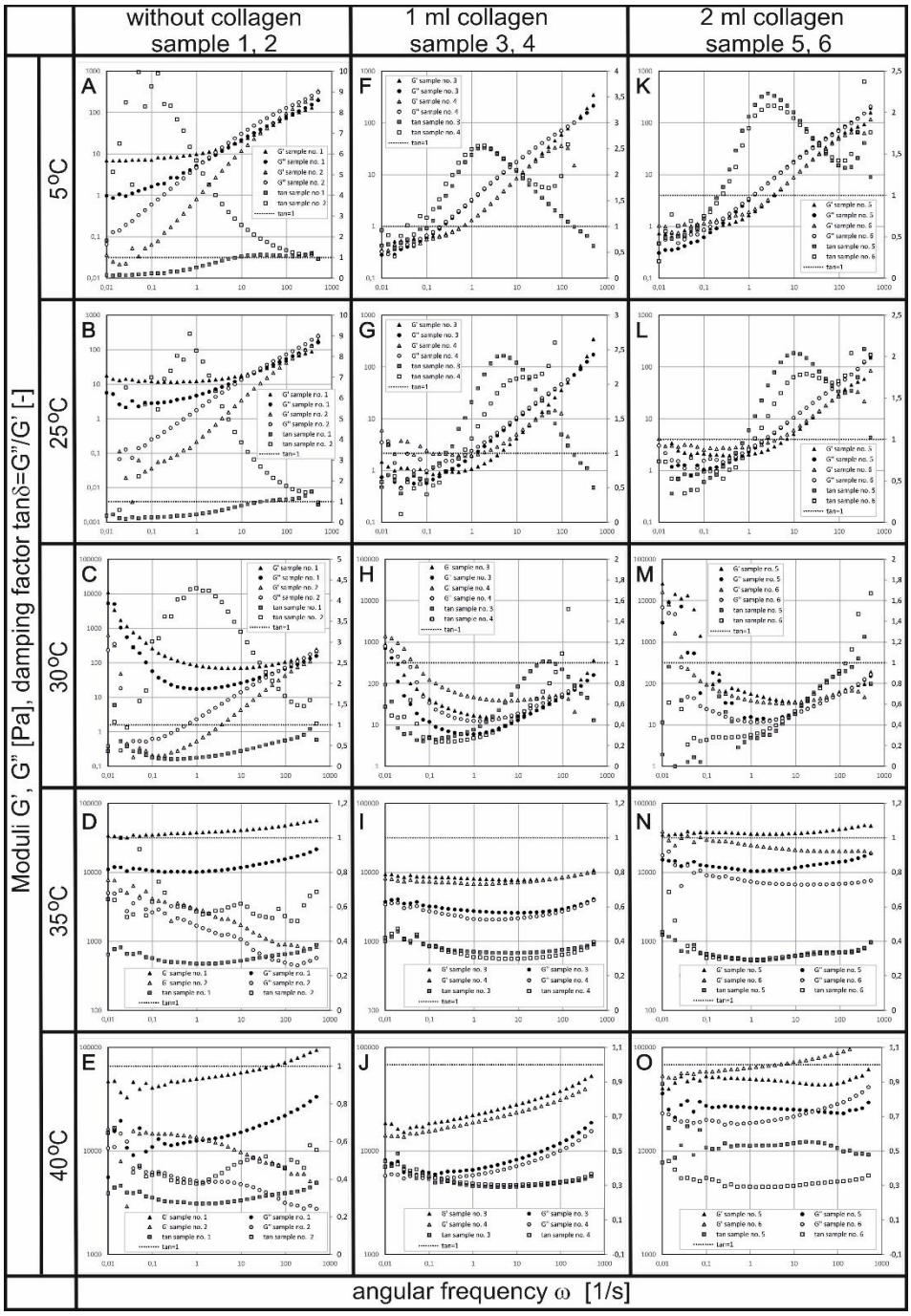

Figure 3. Storage modulus – G', loss modulus – G" and damping factor – tanδ curves obtained during the frequency sweep test conducted at 5, 25, 30, 35 and 40°C (for samples 1, 3, 5: ▲ – G', ● – G", ■ – tan δ for samples 2, 4, 6: Δ – G', ○ – G", – tanδ).

Significant differences in the values of the designated relaxation times were observed, which are identified with the time necessary for the structure to return to its original form. When stationary, a very unstable resilient structure is formed in the liquid, which is destroyed by even slight mechanical deformations.

The greatest differences were observed in samples containing 1 ml of collagen (3 and 4), only differing in the order in which components were added. In the case of sample 3, when the collagen was added in a neutral environment (after glycerophosphate), the intersection of moduli G' and G" was observed for oscillation frequency value $\omega = 0.138$ s^{-1} (relaxation time $\tau = 7.2464$ s). For collagen added in an acid environment (sample 4), the angular frequency ω, for which an intersection of moduli was observed, was 0.0371 s^{-1} (relaxation time $\tau = 26.4542$ s). For sample no. 3 containing 1 ml of collagen, added after Na-β-GP, the time necessary for the relaxation of the arisen stresses was over three times shorter than in the case of adding collagen in an acid environment (sample no. 4). At higher temperatures, the situation was different. Longer relaxation times were observed for the samples in which the collagen was added after Na-β-GP (Fig. 3G–H). Regardless of the conditions for the addition of collagen and glycerophosphate, a decrease in relaxation time was observed with the increase in temperature (Fig. 3F–H).

In the case of chitosan solutions with an addition of 2 ml of collagen (samples 5 and 6), a similar trend of experimental curves was observed, regardless of the order in which the components were added. For the systems already in a low temperature 5°C (Fig. 3K), Regions S II and S III (viscoelastic and highly flexible) occurred. The designated relaxation times had approximate values, ca. $\tau = 3.77$ s ($\omega = 0.265$ s^{-1}). At 25 and 30°C, the relaxation times also had similar values (compare: Table 2). As in the case of lower collagen concentration, the formed structures were characterised by similar curves of moduli G' and G" – characteristic for a viscoelastic plateau state. In each of these cases, it was not possible to determine the relaxation time due to the assumed measurement range. From the experimental curves of the moduli, it can be deducted that their intersection point, i.e. the relaxation time, will be close to zero. This means that the system immediately returns to its initial state after taking away the external stress.

In the case of samples no. 1 and 2 – solutions of chitosan chloride without collagen (Fig. 3A–E) – the occurrence of Regions S II and S III was also observed. An important difference in comparison with the previously discussed system is a significant shift in the course of the experimental curves towards Region S II, which was already visible at 5°C (Fig. 3A). The obtained curves of storage modulus G' and loss modulus G" intersected at an angular frequency of $\omega = 8$ s^{-1}. For these curves, the calculated relaxation time amounted to $\tau = 0.125$ s. Together with a temperature increase, similar to the case of samples 3–6, a shift of the experimental curves towards a stronger dominance of elastic properties over viscous ones and an increase of the highly elastic region relative to the viscoelastic region was observed. This means that in a wider angular frequency range, the system obtains the state of a viscoelastic plateau. At 30°C, the structure in the whole tested range corresponded with the characteristics of Region S II described in the literature [29]. Similar to the case of solutions containing an addition of collagen, an increase in temperature caused an increase in frequency at which the crossing of moduli curves and thus a shortening of the relaxation time took place (the designated value $\tau = 0.03125$ s). The moduli curves, designated at the temperature 30°C enabled the

specifying of both a short ($\tau = 2.5 \cdot 10^{-3}$ s) and long relaxation time ($\tau = 62.5$ s). The values of the relaxation times determined for these samples were significantly different from the values determined for the solutions with the addition of collagen (Table 2).

Solutions of chitosan chloride without the addition of collagen were more resistant to mechanical deformation – they easily formed an internal structure. They are characterised by significantly higher values of storage modulus G' obtained for low angular frequency values ω at 5°C (Fig. 3A) in comparison with solutions containing an addition of collagen. For high temperatures, the G' modulus achieved lower values than solutions containing collagen. This may be the result of collagen coagulation initiated by a temperature rise.

The structural changes occurring in the colloidal chitosan salt solutions described above, which were defined based on the oscillation tests performed at various temperatures, was confirmed by the determined values of gelation temperature points and the kinetics of the process. Fig. 4 shows the tanδ = G"/G' curves for all tested samples obtained during heating. The tanδ = 1 value assumed in the literature [30] as the gelation point determines the change in the nature of the medium from a liquid of viscous properties into a system characterised by a dominance of elastic properties.

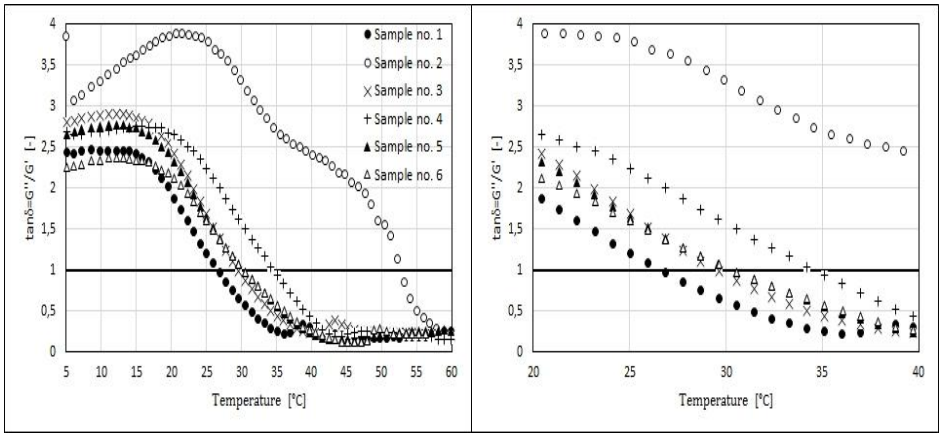

Figure 4. The curves tan δ= G"/G' designated for samples 1–6 during the heating of the system at the speed 1 K/min. A – the whole range and B – detail of the curves between 20 and 40°C repeated for easier observation of differences near the gelation point. The gelation point temperature is determined by the temperature at which the curve crosses the value tan δ = 1.

The obtained gelation temperature range for samples containing an addition of Na-β-GP (sample no. 1, 3–6) reached between 26 and 34°C. In the case of sample no. 2, the gelation point was reached at 52°C. The obtained results confirm the influence of Na-β-GP addition on a significant decrease in the gelation temperature of chitosan chloride [15]. This shows that an addition of collagen (samples 3–6) increased the temperature at which the phase transition occurred, in comparison with a solution of chitosan chloride containing glycerophosphate (sample no. 1). In the case of solutions containing a low concentration of collagen (samples no. 3 and 4), an influence of the pH of the solution

into which the collagen was added was observed on the values of the gelation temperatures. For sample no. 3, at a neutral pH, gelation temperature reached 29°C; for sample no. 4 of an acidic pH, the gelation temperature was -34°C. In the case of greater collagen concentration – 2 ml (samples no. 5 and 6), no influence of the pH of the solution into which the collagen was added was observed on the temperature of the phase transition. In both cases, the gelation temperature reached ca. 29°C.

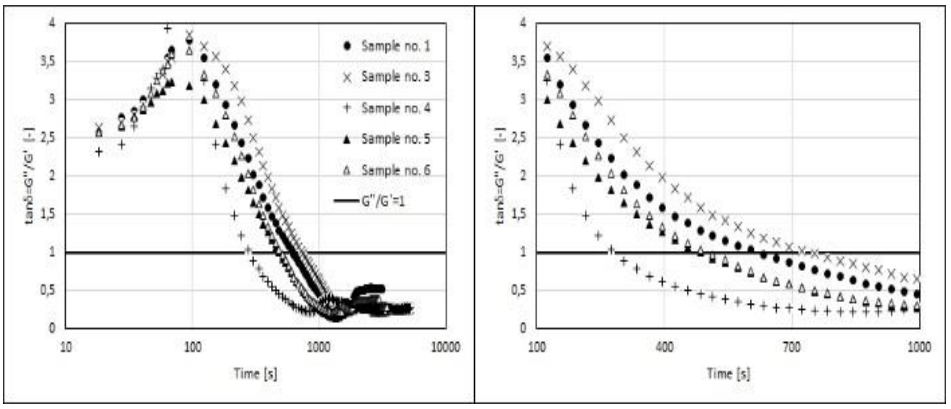

Figure 5. The curves tan δ = G”/G’ designated for samples 1–6 obtained in an isothermal test at the temperature 37°C. A – the whole range and B – detail of the curves between 100 and 1000 s repeated for easier observation of differences near the gelation point. The kinetics of the gelation process is determined by the time after which the curve crosses the value tan(δ) = 1.

Figure 5 presents the curves of changes in the tanδ = G”/G’ value as a function of time, obtained in isothermal tests at 37°C. The experimental curves allowed the determination of the kinetics of the structural changes occurring in the tested solutions (samples 1, 3–6) during the gelation process at a constant temperature. As in the case of the previously obtained results, for samples no. 3–4 the greatest differences were observed in the designated values of time after which the phase transition occurred. For a greater collagen concentration – 2 ml (samples no. 5 and 6), similar to the case of tests with heating, no influence of the preparation method of the samples (pH of the solution into which collagen was added) on the phase transition time was observed. It was stated that an addition of collagen (samples 4–6) accelerated the phase transition in comparison with a solution of chitosan chloride containing glycerophosphate. Only sample no. 3 demonstrated a later sol–gel phase transition. Due to the designated high gelation temperature of 52°C (Fig. 4A), no measurement of the gelation kinetics for a solution of chitosan chloride without the addition of Na-β-GP (sample no. 2) was performed.

5. Conclusions

A significant influence of a collagen addition to chitosan chloride solutions on the viscoelastic properties of the systems was observed. Chitosan chloride solutions without this addition are characterised by a greater stability of the formed structure (shorter relaxation times) than the systems containing collagen. A greater impact of the

preparation conditions, the pH of the solution into which collagen was added, was observed on the obtained structure in the case of the lower collagen concentration (1 ml – samples 3 and 4). For solutions containing 2 ml of collagen (samples 5 and 6), no significant changes resulting from the conditions of the pH of the solution were observed. Taking into account the obtained experimental data, it can be assumed that with a higher collagen concentration, the interaction between chitosan and collagen molecules formed the structure. For the tested temperature range, the majority of the systems was characterised by the occurrence of a structure corresponding with the regions of highly flexible S III and the viscoelastic S II [25]. The occurrence of a region of molecular flow was observed only in the case of chitosan chloride solutions without the addition of Na-β-GP (sample no. 2). In the case of solutions containing 1 ml of collagen added after glycerophosphate (sample 3), at 5 and 25°C, a double intersection of the curves of moduli G' and G" was observed (Fig. 3F, G). The occurrence of two relaxation times and the analysis of the curves of the moduli indicate that during the coagulation a structure of glassy S IV was formed. The addition of collagen in all the cases increased the sol–gel phase transition temperature in comparison with the chitosan chloride solution containing β-glycerophosphate. Gelation temperatures between 26 and 34°C were obtained. In the case of sample no. 2, a solution containing neither collagen nor Na-β-GP, the gelation point was obtained at 52°C. It was found that the presence of collagen in the solution accelerated the gelation process conducted in isothermal conditions at the physiological temperature of 37°C.

6. Acknowledgement:

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