DYNAMIC LIGHT SCATTERING-BASED MICRORHEOLOGY OF THERMOSENSITIVE CHITOSAN HYDROGELS: PRELIMINARY STUDIES

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Abstract

This paper presents a preliminary characterisation of the local mechanical properties of thermosensitive chitosan systems using dynamic light scattering, an endeavour that has not yet been attempted. The tests included solutions of chitosan chloride with the addition of sodium glycerophosphate as well as those without a substance to support the gelation process. There was much better agreement between the data obtained from measurements on the microscopic and macroscopic scales for systems without the addition of glycerophosphate. In most cases, the addition of a pH-neutralising salt resulted in higher values of dynamic moduli than those obtained during microrheological measurements. Therefore, it seems to be possible to use dynamic light scattering to conduct microrheological studies of thermosensitive chitosan systems.

Keywords: *thermosensitive hydrogels, chitosan, DLS, microrheology*

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1. Introduction

Assessment of the rheological properties of hydrogels for biomedical applications is crucial because it allows the characterisation of the mechanical properties of the biopolymer matrix during compression (determination of Young's modulus and texture analysis [1]), tension and shear [2, 3]. The use of shear deformation allows for the determination of the mechanical spectrum under isothermal conditions [4] as well as changes in structural properties during measurements with constant deformation under non-isothermal and isothermal conditions [5–7]. The latter are particularly important when characterising thermosensitive media used as injectable biomedical scaffolds [8] that form a spatial polymer network directly in the human body after heating to the physiological temperature. These measurements, although very important at the design stage, only determine the properties of media on a macroscopic scale. From the application point of view, it seems that local mechanical properties [9] are equally important because they directly affect the environment in which the cells multiply, and they may differ from the bulk properties in the case of heterogeneous media.

Microrheological techniques using monodisperse, inert latex tracers of microscopic size represent a way to address these needs. There are passive and active microrheological techniques, which differ depending on the method of forcing the movement of these particles [10–12]. For the former group (also known as thermal), the diffusive movement of tracers is forced by the thermal energy of the system. The most common passive microrheological techniques include video particle tracking and techniques based on monochromatic beam scattering, such as dynamic light scattering (DLS) and diffusing-wave spectroscopy (DWS) [10]. In the case of active (also called forced) techniques, the beads are manipulated by an external force usually generated by a magnetic field (magnetic tweezers [MT]) or by optical beams (optical tweezers [OT]) [10]. Although they have limitations, these techniques enable the characterisation of the medium over a very wide frequency range (10^{-2} to 10^{7} Hz) as well as over a wide range of shear moduli (10^{-5} to 10^{4} Pa) [10]. The range of low shear modulus values is particularly interesting because it correlates with the range of stresses occurring on the scale of cellular/tissue interactions. DLS is a microrheological tool that uses an inverse approach relative to its classical use to determine the size of the dispersed phase. In this case, based on the scattering intensity signal on tracer particles of a known diameter, rheological properties are determined using mathematical calculations. Briefly, the correlation function on delay time is used to determine the mean square displacement of the tracers as a function of the delay time and finally, using the generalised Stokes–Einstein relation (GSER) and Fourier transform, the course of the storage modulus (G') and the loss modulus (G") as a function of frequency known as the mechanical spectrum is obtained [13].

This paper presents a preliminary characterisation of the local mechanical properties of thermosensitive chitosan systems using DLS, an endeavour that has not yet been attempted. A critical analysis of the obtained results at the macroscopic and microscopic scales revealed the real potential of using DLS to characterise the tested media, the potential limitations and future research directions.

2. Materials and Methods

2.1. Preparation of Thermosensitive Hydrogels

This study used chitosan of shrimp origin (CAS no. 9012-76-4, Sigma-Aldrich, Germany), whose average molecular weight is 862 kg/mol and degree of deacetylation is 83.4% [3]. Thermosensitive chitosan hydrogels were obtained according to the preparation proposed by Chenite et al. [14]. A chitosan solution was prepared by dissolving 0.4 g of chitosan powder in 16 ml of 0.1 M hydrochloric acid (CAS no. 7647-01-0, Sigma-Aldrich, Germany) and incubated for 24 h until the polymer had dissolved completely. Subsequently, the solution was incubated at 4°C. In parallel, a suspension of β-disodium glycerophosphate (CAS no. 154804-51-0, Sigma Aldrich, Germany) was prepared by suspending 2 g of powder in 2 ml of distilled water. After cooling, the suspension was added drop by drop to the chitosan solution, then mixed thoroughly and incubated for another 24 h at 4°C before actual measurements.

Monodisperse latex tracers (Magsphere Inc., USA) without surface modification and with a diameter of 210, 500 or 880 nm were added to the pure chitosan solutions and samples with addition of β-disodium glycerophosphate intended for microrheological tests; the final concentration was 0.1% (v/w). The pH of the obtained pure chitosan solutions and systems containing β-disodium glycerophosphate at 20°C was 5.7 and 6.9, respectively [3]. The addition of polystyrene tracers did not change the pH.

2.2. Methods

Bulk mechanical spectra were obtained using a MCR301 rotational rheometer (Anton Paar, Austria) performing a frequency sweep test ($\gamma = 1\%$, $\omega = 0.01 - 500$ rad/s) in the linear viscoelasticity (LVE) range. Microscopic evaluation was performed using the Zetasizer Nano ZS90 analyser (Malvern Panalytical, USA) using the settings proposed by Cai et al. [13]. The tests were carried out at 5, 25 and 37°C, representing the storage temperature, application temperature and human body temperature, respectively. To determine the effect of the tracers on the phase transition time, measurements were carried out at constant deformation ($\gamma = 1\%$, $\omega = 5$ rad/s) under isothermal conditions (37°C) after rapid heating from the storage temperature (5°C). There were 3–5 replicates for each sample. The microrheological test results for each measurement represent the mean of 22 scans for different measurement positions [13]. The discrepancy in the obtained data did not exceed 10%. The mechanical spectra were obtained by analysing the raw data using a code in the Python programming language developed by Krajina et al. [15].

3. Results and Discussion

According to the theory of microrheological measurements, the tracers used should be inert with respect to the medium whose rheological properties are determined. For this reason, the mechanical spectra to determine G' and G'' were compared for samples without and with the addition of tracers (Figure 1).

For the chitosan solutions without β-disodium glycerophosphate, regardless of the measurement temperature, the latex beads did not affect the dynamic modulus values. However, the addition of β-disodium glycerophosphate, which supports the sol-gel phase transition process, affected the tracers' influence on the modulus values, particularly at the lowest temperature (5°C, Figure 1b). The addition of tracers increased the G' and G'' values, indicating an enhancement of both the viscous and elastic properties of the experimental medium.

The dependence of the obtained values on the size of the tracers was non-monotonic, and the highest values were obtained for tracers with a diameter of 500 nm. This means that despite their chemically inert form, they influence the structure of the polymer medium. It should be assumed that they constitute aggregation nuclei which, despite their sol form, strengthen the structure of the medium. However, the differences observed in the G'' values are surprising because if changes are observed, then they should only concern G', which determines the elastic characteristics of the medium. At 25°C, the tracers only influenced the elastic properties. Again, the addition of latex beads increased G', with a maximum value for tracers with a diameter of 500 nm. Surprisingly, after gelation, the presence of latex beads slightly weakened the mechanical properties of the obtained gels. Moreover, it should be emphasised that the spectra obtained in the low frequency range at 37 and 25°C in the case of the medium with β-disodium glycerophosphate, although inconsistent with the course of the universal mechanical spectrum proposed by Kasapis [16], indicate progressive gelation under the influence of shear [5].

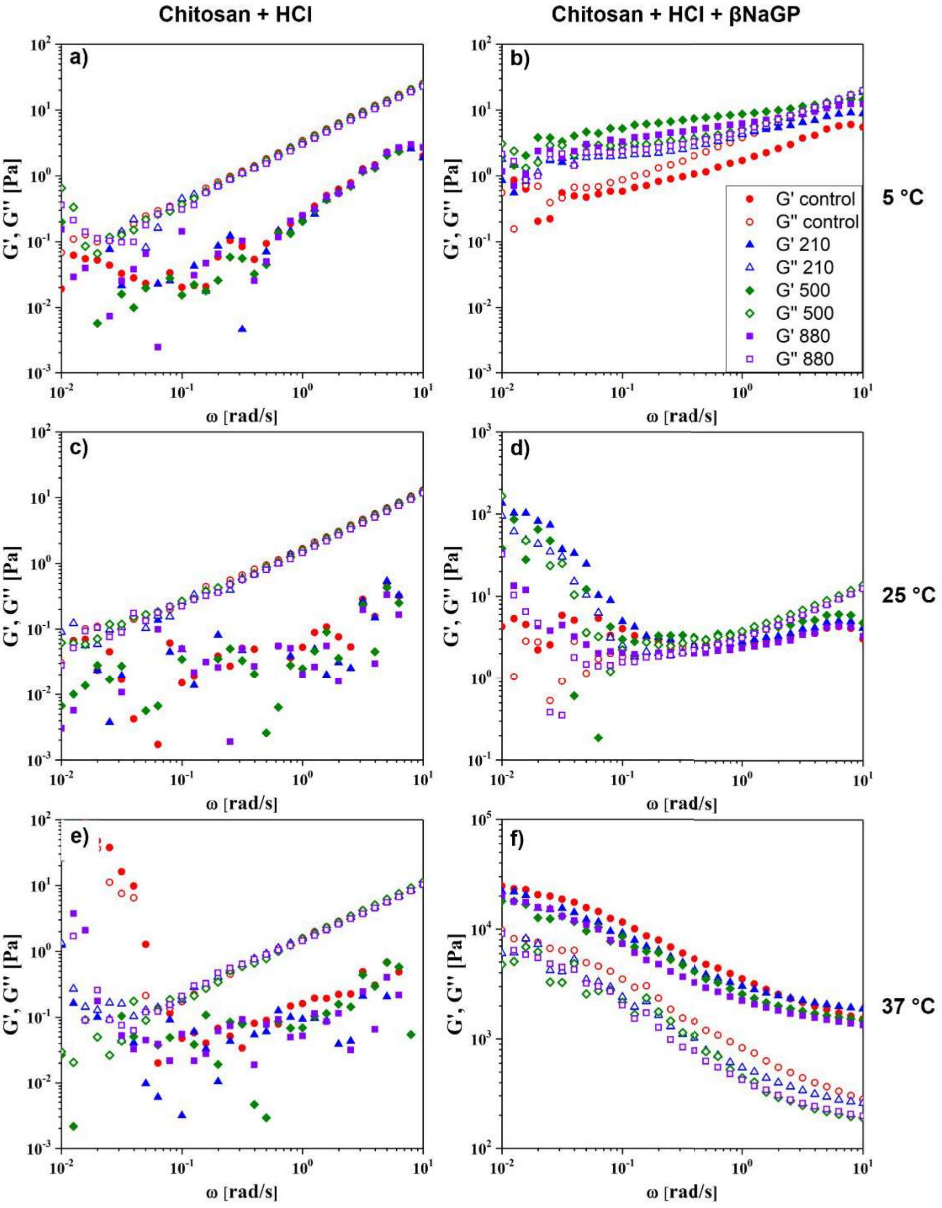

Figure 1. The effect of the tracer diameter on the mechanical spectra of (a, c and e) pure chitosan solutions and (b, d and f) systems with the addition of β-disodium glycerophosphate at 5, 25 and 37°C, respectively. Control samples contained no tracers. The markings 210, 500 and 880 refer to the size of the tracers [nm]. Abbreviations: G', storage modulus; G", loss modulus.

The actual impact of the tracers on the state of the polymer matrix was assessed by determining the kinetics of the phase transition at 37°C. Figure 2 shows the change in G' and G" over time. Applying the most frequently used method of equalising the values of dynamic moduli to determine the gelation time showed that the presence of tracers did not affect the determined gelation time. Nevertheless, for the media containing latex beads, there was a sharp increase in G' and G" that occurred much faster compared with the control samples. This proves the faster occurrence of the fast gelation area, where the primary polymer matrix is formed, which is expanded in the slow gelation area.

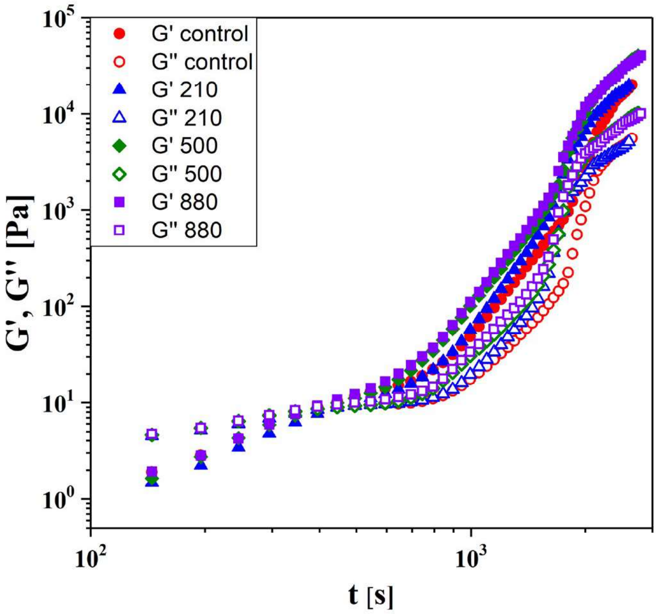

Figure 2. The change in the storage modulus (G') and the loss modulus (G") over time depending on the tracer diameter at 37°C. The control samples contained no tracers. The markings 210, 500 and 880 refer to diameter of the tracers [nm].

Figure 3 shows mechanical spectra at 5°C obtained as a result of classical bulk tests as well as microrheological tests using DLS. The chitosan solution showed consistency for G' in the low frequency range, while G" produced inconsistent results (Figure 3a). When using tracers of different sizes, there were similar G' and G" values at the macroscopic and microscopic scales. For the samples containing β-disodium glycerophosphate, there were much greater discrepancies in the data (Figure 3b). In the case of classical measurements, much higher values of dynamic moduli were obtained. Discrepancy in the results obtained using the two measurement techniques indicates significant heterogeneity of the sample. In this case, microrheological measurements using DLS cannot be used for local characterisation because, despite the use of multiple measurements at different detector positions, the final results are averaged results. It would be more reasonable to use a technique based on video multiple particle tracking.

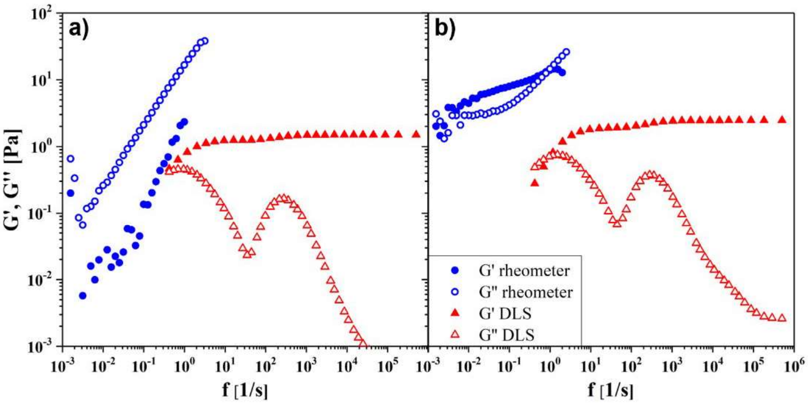

Figure 3. The mechanical spectra of (a) pure chitosan solutions and (b) systems containing β-disodium glycerophosphate at 5°C with the addition of 500 mm tracers determined using a rheometer and dynamic light scattering (DLS). Abbreviations: G', storage modulus; G”, loss modulus.

The results of classical oscillatory measurements revealed that despite the low measurement temperature, elastic properties predominated over viscous properties, which should not be the case in the case of a polymer sol. This phenomenon was not observed in the case of a control measurement for a sample without tracers. Despite the predominance of elastic features over viscous features, a gel form was not obtained due to the dependence of both moduli on frequency as well as the flow phenomenon observed during the measurements. In the case of both media, G" decreased in the frequency range above 300 s^{-1}, a finding inconsistent with theory. This may suggest an incorrect fit of the raw data recorded during the measurements. An undoubted advantage of microrheological measurements is the determination of the G_{N0} value, which is defined as the minimum value of G" in the highly elastic area in accordance with the Kasapis theory [16].

Figure 4 shows the test results at the macroscopic and microscopic scales at 25°C using tracers with a diameter of 210 nm. In the case of chitosan solutions, G" showed good compliance in the low frequency range, but G' did not.

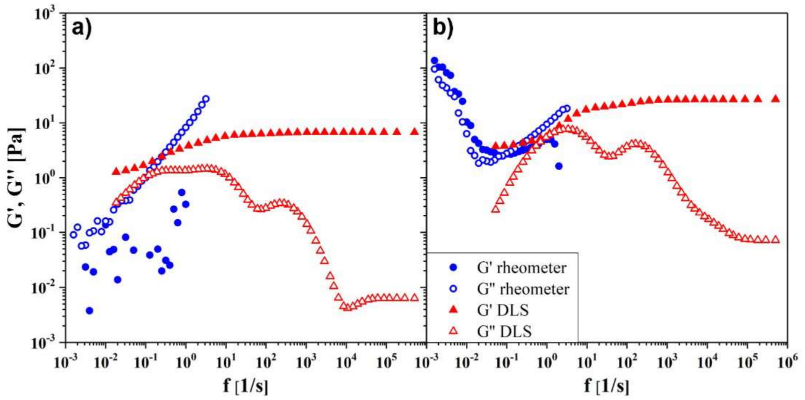

Figure 4. The mechanical spectra of (a) pure chitosan solutions and (b) systems containing β-disodium glycerophosphate at 25°C with the addition of tracers with a diameter of 210 nm determined using a rheometer and dynamic light scattering. Abbreviations: G', storage modulus; G", loss modulus.

In the case of systems containing β-disodium glycerophosphate, there was better convergence of G' and acceptable differences in G". This could suggest an increase in homogeneity as the temperature increases. Again, for frequencies above 300 s^{-1}, the obtained data are inconsistent with the literature in the case of G". It is worth noting that at the same temperature, the use of larger tracers resulted in a greater divergence of G' and G".

Nevertheless, the results obtained using the microrheological technique are very promising because they allow for characterisation over a very wide frequency range (Figure 5), the courses of which are consistent with the theory proposed by Kasapis [16]. This makes it possible to determine all four characteristic regions: starting from the molecular flow and moving through the highly elastic, glass transition and glassy states. Additionally, it is possible to determine the short relaxation time, the relaxation time and the long relaxation time [4], which were 4.1×10^{-4}, 1.2×10^{-2} and 9.8×10^{-2} s, respectively. These times are related to the elastic dynamics of bend relaxation, the internal flexible chain relaxation as well as the total chain relaxation [13]. Consequently, this makes it possible to determine the hierarchy of molecular relaxations. A pure chitosan solution at the frequency range 200–2400 s^{-1} presented discontinuity in the obtained data and the tendency towards the value of 0 Pa. The authors of the code used in data analysis observed similar discontinuity; they interpreted it as noise occurring in the raw data [15].

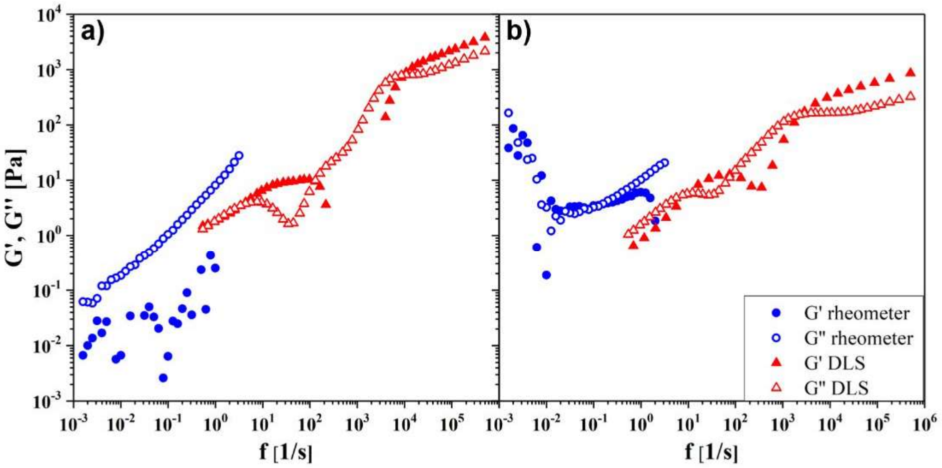

Figure 5. The mechanical spectra of (a) pure chitosan solutions and (b) systems containing β-disodium glycerophosphate at 25°C with the addition of tracers with a diameter of 500 nm, determined using a rheometer and dynamic light scattering (DLS). Abbreviations: G', storage modulus; G", loss modulus.

The results obtained at 37°C strongly depended on the medium tested. In the case of chitosan solutions without sodium glycerophosphate (Figure 6), the size of the tracers had a marked impact on the consistency of the data obtained using both measurement techniques. The use of the smallest tracer with a diameter of 210 nm led to satisfactory convergence for G". The microrheological measurements revealed discontinuity at high frequencies for both G' and G".

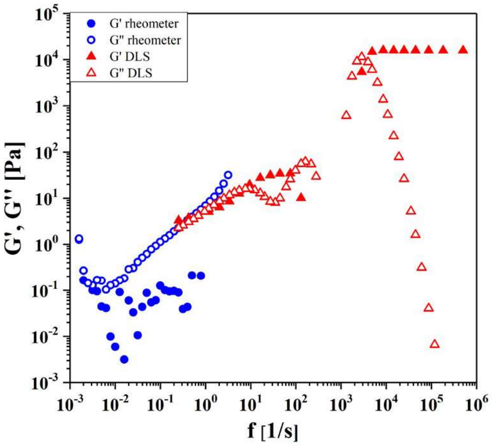

Figure 6. The mechanical spectra of pure chitosan solutions at 37°C with the addition of tracers with a diameter of 210 nm determined using a rheometer and dynamic light scattering (DLS). Abbreviations: G', storage modulus; G", loss modulus.

When using larger tracers (i.e. a diameter of 500 or 880 nm), the experimental data were similar, especially regarding G'. This points to the important issue of selecting tracers for a specific type of measurement. Selecting a tracer that is too small may result in its free diffusion, unrestricted by polymer chains, resulting in a much more reliable measurement of viscous properties during microrheological measurements. On the other hand, the use of larger tracers provides a better characterisation of the elastic properties. Although the tracer diameter does not directly affect the obtained values of the dynamic moduli – because it is considered when analysing the raw data – latex beads with different diameters will interact with polymer chains differently, which results directly from the diffusion rate. This was schematically presented by Li and Mooney [17] when analysing the release rate of active substances from the polymer matrix.

The largest discrepancies between the data obtained from classical rheological measurements and microrheological measurements were obtained for the systems containing β-disodium glycerophosphate at 37°C (Figure 7).

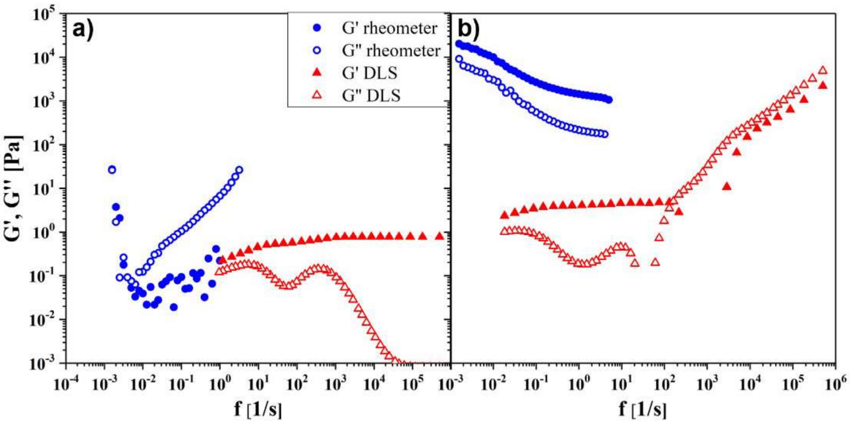

Figure 7. The mechanical spectra of (a) pure chitosan solutions and (b) systems containing β-disodium glycerophosphate at 37°C with the addition of tracers with a diameter of 880 nm determined using a rheometer and dynamic light scattering (DLS). Abbreviations: G', storage modulus; G", loss modulus.

The nature of the observed changes was independent of the size of the tracer used. For the same frequency range, there were much higher G' and G" values based on classic rheological measurements. This indicates much weaker local properties of the polymer network, which are strengthened throughout the volume, for example, by numerous junction zones. Although measurements were made at several detector positions, the observed discrepancies may result from the non-ergodic state of the medium in which the scattering intensity signal strongly depends on the detector position. In the case of microrheological measurements, two areas are observed on the universal mechanical spectrum curve: a highly elastic area and a glassy transition area. Ultimately, the value of the characteristic time was determined to be 6.7×10^{-3} s. As in the case of measurements at lower temperatures, discontinuities in the courses of both dynamic modules from measurements using the DLS technique are again visible.

4. Conclusions

The results demonstrated much better agreement between the data obtained from measurements at the microscopic and macroscopic scales for systems without β-disodium glycerophosphate. In most cases, the addition of a pH-neutralising salt resulted in higher G' and G" values than those obtained during microrheological measurements. Moreover, although the polymer tracers are inert from a chemical point of view, they affect the viscoelastic properties, changing the dominant characteristics from viscous to elastic at low temperatures. Moreover, specific crystallisation/aggregation nuclei slightly affect the gelation kinetics by shortening the time after which the rapid gelation area. Ultimately, latex beads of different sizes results in different properties of the tested media. Therefore, it is possible to use DLS to conduct microrheological studies of thermosensitive chitosan systems. However, further research is necessary, including microscopic optical assessment of the state of the polymer matrix and attempts to characterise the tested media using other microrheological techniques such as video multiple particle tracking.

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