APPLICATION OF TEXTURE PROFILE ANALYSIS TO INVESTIGATE THE MECHANICAL PROPERTIES OF THERMOSENSITIVE INJECTABLE CHITOSAN HYDROGELS

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Abstract

In this paper, the mechanical properties of hybrid chitosan hydrogels were investigated using the texture profile analysis test. Scaffolds obtained from a polysaccharide of various molecular weights were studied, which, with the addition of glycerophosphate salt, formed a three-dimensional structure in vivo. The obtained systems were also enriched with collagen and calcium carbonate to improve the mechanical properties. The determined texture parameter values indicate that the mechanical properties of the hybrid hydrogel depend on the molecular weight of the polymer, the type of solvent and, the pH-neutralizing substance, as well as the type and concentration of the filler. Moreover, in some cases the TPA test was the only way to evaluate the mechanical properties of the obtained hydrogels due to the inability to determine the Young's modulus. Consequently, the texture analysis test is a valuable tool for selecting solutions depending on the intended application of the scaffolds.

Keywords: *chitosan, hydrogels, injectable scaffolds, mechanical properties, TPA*

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1. Introduction

The rapid development of biomedical engineering observed in recent years results from the growing demand for innovative methods of treatment and convalescence of victims of traffic accidents. As a result, in tissue engineering, a field has been distinguished whose main task is to produce biodegradable, temporary matrices called scaffolds. These scaffolds are a physical support during the process of cell proliferation, migration, and differentiation into functional tissues and organs [1]. Moreover, they should ensure mechanical stability and appropriate environmental conditions. The selection of materials from which they will be created is of key importance in the design of cell scaffolds. Materials used as cellular scaffolds combine with the tissue undergoing regeneration. For this reason, selected materials should exclude side effects during their interaction with cells, tissues, and bodily fluids. As a consequence, the materials from which the scaffolds are made should be biocompatible, biodegradable, bioresorbable, and biofunctional [1]. An ideal scaffold should also have compatible mechanical properties with the place where it is to be implanted; therefore, it must be resilient, rigid, and resistant to compression and stretching as well as exhibit high adhesiveness to regenerated tissues. Because few materials are characterized by all of these features simultaneously, recently, the tendency to combine natural and synthetic polymers with materials that support cellular interactions that promote integration into host tissue as well as improve the overall biological and mechanical properties of hybrid scaffolds has been observed [2].

The polysaccharides, such as cellulose, chitin, chitosan, and hyaluronic acid, as well as proteins, among which collagen and elastin should be mentioned, are the most commonly used, major groups of the natural polymers (biopolymers). Biodegradable biopolymers are obtained from organisms, and their degradation, accompanied by a decrease in the molar mass of the polymer, occurs as a result of biological processes [3]. In recent years, particular attention has been focused on chitosan, a natural polycationic linear polysaccharide widely used in pharmaceuticals as an implantable scaffold in tissue engineering [4], drug carrier [5] and the growth release factor [6]. Another very promising method is based on the thermo-induced sol-gel phase transition of the chitosan colloidal systems [7], which allows their use as non-invasiveness injectable cell scaffolds that in vivo form a three-dimensional spatial structure directly in the patient's body [8,9]. However, due to its relatively low mechanical resistance, the use of chitosan as a biomaterial to form scaffolds especially for bone tissue treatment requires joining it with other compounds such as collagen [10,11] as well as ceramics or other polymers [12]. So far, many studies have been carried out on the formation of chitosan scaffolds, as well as the improvement of both their biological and mechanical properties [13,14]. However, the vast majority of them concern chemical research and the influence of polymer molecules chemical structure and properties (molecular mass, deacetylation degree, etc.), as well as pH, additions of crosslinking and neutralizing substances on rheological or/and microbiological properties. The rheological properties, especially the determination of viscoelastic behaviour, quite well describe the mutual interaction of polymer chains in the structure, but do not provide sufficient macrostructure characteristics of three-dimensional cell scaffolds. Only few authors investigating the potential of using hybrid scaffolds in bone tissue engineering take into account the influence of individual scaffolding elements on macroscopic mechanical properties understood as compressive or tensile strength using typical texturometric tests [15,16]. As important as the basic mechanical properties, and thus elasticity, hardness and brittleness, are properties such as cohesiveness and adhesiveness in relation to regenerated tissue cells [14]. On this basis, the issue of developing a comprehensive mechanical test methodology to characterize the mechanical properties of scaffolds is a promising area of research.

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The aim of the work is to develop a method for investigation the macroscopic mechanical properties of cell scaffolds based on the two-cycle compression test, so-called texture profile analysis (TPA). The proposed method will allow to determine the effect of the collagen and ceramics additions on the macroscopic mechanical properties of thermosensitive hybrid colloidal chitosan systems.

2. Materials and Methods

2.1. Materials

As an experimental materials, three types of chitosan with different molecular weights and a similar degree of deacetylation were used: Chitosan from the Fluka company of unknown origin (LV), product no. 50949; chitosan from the Sigma-Aldrich® company obtained from crab shells (CRAB), product no. 50494; chitosan from the Sigma-Aldrich® company obtained from shrimp shells (SHRIMP), product no. 50494. The basic physicochemical parameters of the chitosan used are shown in Table 1.

Table 1. The physicochemical properties of chitosans.

Designation used in the text	Weight Average Molar Mass M_w (g/mol)	Number Average Molar Mass M_n (g/mol)	Polydispersity Index PDI (–)	Degree of Acetylation DA (%)
LV	463,000	79,000	5.9	16.8
CRAB	680,000	110,000	6.1	18.2
SHRIMP	862,000	145,000	5.9	16.6

The 0.1 M solutions of hydrochloric acid (Fluka Product no. 84415) and acetic acid (Sigma-Aldrich no. 695092) were used as solvents for the polysaccharide. Namely, 400 mg of the polymer was dissolved in 16 ml of hydrochloric acid or 20 ml of acetic acid. The thoroughly mixed solution was left for 24 h to allow the polysaccharide to completely dissolve. Next, a suspension of disodium β-glycerophosphate (2.0 g of NaGP in 2 ml of distilled water) or calcium β-glycerophosphate (0.5 g of CaGP in 2 ml of distilled water) cooled for 2 h was added dropwise to the chitosan acetate solution. The research material was prepared in accordance with previously published methods for NaGP [8,17] and CaGP [12], respectively.

Moreover, to improve the mechanical properties and improve the usability of chitosan scaffolds, 0.2–0.8 g of fish collagen and 0.15 g of calcium carbonate were introduced as fillers. In this case, the procedures described in the previous authors' research [12,18] were used.

After preparing the thermosensitive sol phase, the colloidal chitosan system was stored at 37°C in a water bath to form a three-dimensional gel structure.

2.2. Methods

The mechanical properties of the obtained hydrogels were determined using a Brookfield CT3 Texture Analyzer equipped with a TA18 system (d=12,7 mm). The hydrogels were two-cycle compressed at a compression rate of 0.5 mm/s, with a sample detection threshold of 0.5 g and a compression ratio of 50%. The measurements were carried out at 37 °C, in a specially designed, homemade thermostatic system. Cuvettes with the test samples were placed in the aluminium table seat, so that both the bottom and the side walls were in contact with the thermostated surface. The table was equipped with a closed thermostatic liquid container. The heat source was a liquid cryostat. Temperature regulation and measurement was based on the PT100 sensor's indication of the table temperature directly at the place in which the cuvette was located.

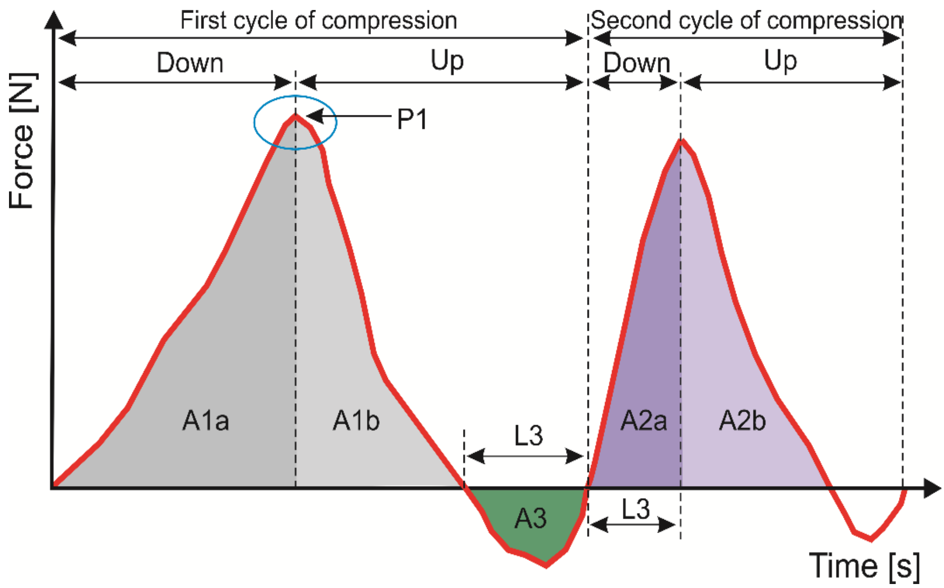

Figure 1. The theoretical force-to-time curve obtained during two cycle compression test [19]

Based on the force-to-time experimental curves (Fig. 1), the following TPA parameters were determined:

- Hardness is the peak load of the first compression cycle (P1). It determines the force required to attain a given deformation.
- Adhesiveness expresses the work necessary to overcome the attractiveness forces between the surface of the sample and the probe. This parameter is calculated as the area (A3) under the negative peak as probe withdraws after the first compression.
- Cohesiveness defines structural reformation after shear stress during application [20]. This parameter indicates the attraction force of molecules in the gel. This parameter is determined as the ratio of the area of the second compression cycle A2 to the area of the first compression cycle A1.
- Resilience indicates how the sample recovers from deformation. In other words, it is the elastic recovery of the sample [21]. Resilience is calculated as the ratio of upstroke energy of the first compression (A1b) to the downstroke energy of the first compression (A1a).

The above parameters are most often considered when characterizing hydrogels for biomedical applications [16]. Moreover, the Young's modulus was calculated from the initial linear slope of the force–time curve.

3. Results and Discussion

As a result of texture measurements, a number of experimental curves (Fig. 2) were obtained that were consistent with literature [19]. Fig. 1 shows an example of the experimental data. Preliminary analysis of raw data indicates a significant effect of the applied mechanical load on the properties of the scaffolds obtained. This is observed as a reduction in the value of the second peak.

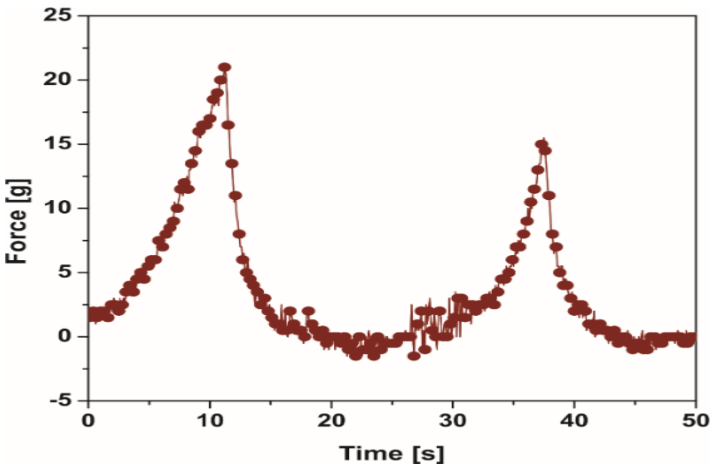

Figure 2. Experimental curve of the double compression test

The results obtained for colloidal chitosan systems with the addition of glycerophosphate salts are shown in Fig. 3 and Fig. 4. Based on the determined parameters, there is a significant effect of the composition (solvent and neutralizing salt) on the mechanical and textural properties of the obtained scaffolds. However, it is worth paying attention to the determined values of the Young's modulus (Fig. 3A). In the case of more than half of the tested solutions, it was not possible to determine this parameter, marked as "x". This is most likely due to insufficient mechanical properties and the lack of a unified linear stress–strain dependence of experimental curves. Thus, limiting the analysis only to the value of Young's modules does not provide valuable information. The use of the two-cycle compression test (TPA) significantly improved the analysis of the considered experimental material, among others, by providing information on scaffolds most frequently studied in the literature obtained by dissolved chitosan of crab origin in hydrochloric acid with addition of disodium glycerophosphate.

The determined hardness values are shown in Fig. 3B. On this basis, it can be found that the applied chitosan (more specifically, its molar mass) has a significant impact on the hardness of the obtained scaffolds. Regardless of the composition, it is evident that scaffolds obtained from crab chitosan show higher hardness than those obtained from shrimp-origin polysaccharide. However, the interpretation for low molecular weight chitosan with the addition of NaGP is unclear. Namely, when dissolved in hydrochloric acid, it reaches the highest hardness values, whereas dissolution in acetic acid leads to a reduction in the hardness of the scaffold. In addition, the results clearly indicate an improvement in hardness when using acetic acid as a solvent. In this case, the effect of the salt neutralizing the pH is also visible.

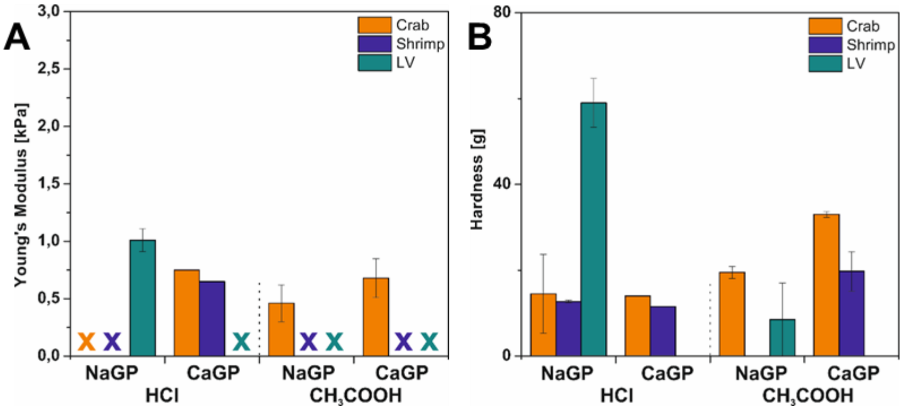

Figure 3. Effect of solvent and neutralizing agent on (A) Young's modulus value and (B) hardness.

The characteristic parameters of the TPA test are shown in Fig. 3. Analysis of the cohesiveness values allows us to state that the strength of internal bonds constituting the structure of gels characterized by higher hardness and Young's modulus is lower compared to other systems. As a consequence, when the given scaffold exhibits a more rigid structure, the higher probability of its destruction under the influence of a locally applied load is observed. Regarding the adhesiveness parameter, it was found that its increase occurs when using calcium glycerophosphate as a salt neutralizing the pH. The influence of these parameters seems to be crucial in the case of tissue engineering, due to its strong association with the best clinical response [20,22,23]. The higher adhesiveness value, the greater adhesion at tissue surface and increase the retention time is observed. Ambiguous results were noted in the case of resilience, but certainly the influence of the scaffolds composition used affects the ability of the hydrogel to return to the original shape after subtracting the stress factor.

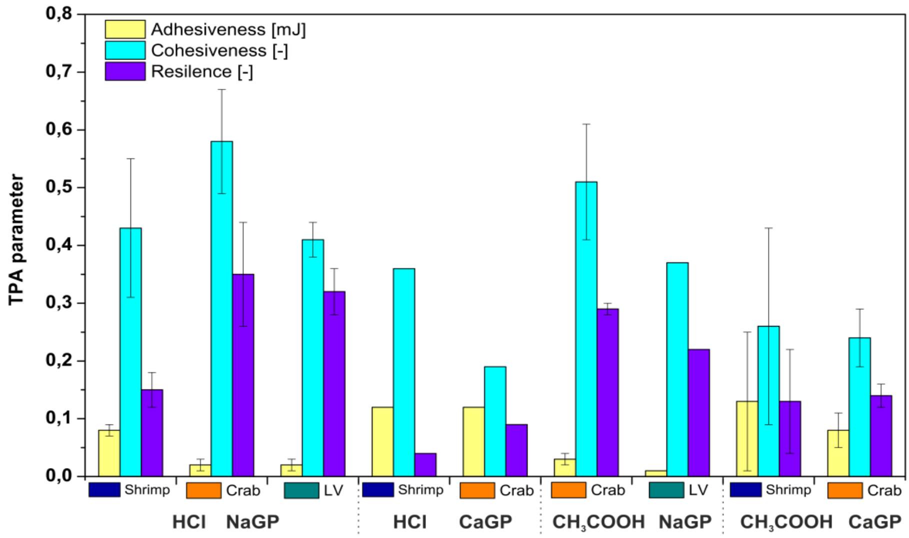

Figure 4. Effect of solvent and neutralizing agent on TPA parameters

3.1. Effect of Collagen Addition

Hybrid chitosan–collagen scaffolds are widely studied in the literature [10,11,14]. However, as it was mentioned, most often these studies concern implant scaffolds. In addition, they discuss the broadly understood mechanical properties of obtained scaffolds, which often leads to unequivocal conclusions.

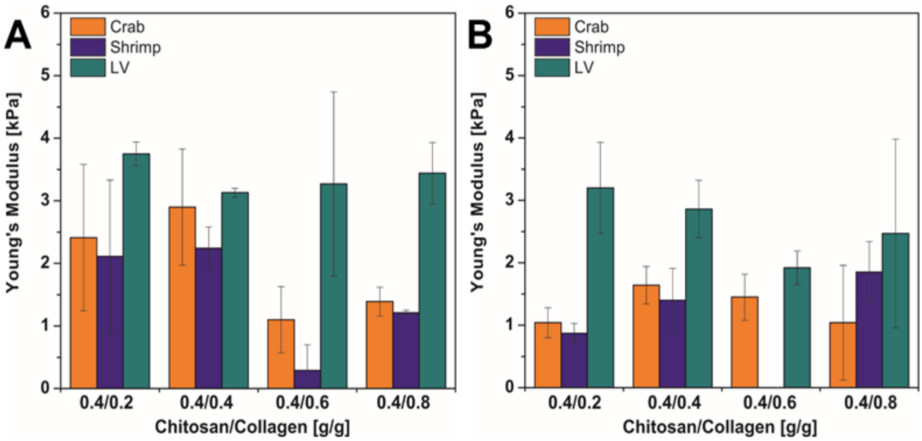

Figure 5. Effect of collagen concentration on Young's modulus for chloric (A) and acetate (B) chitosan/NaGP/collagen hybrid hydrogels.

The first step of the analysis included assessing the effect of collagen concentration on the Young's modulus value depending on the solvent used and the neutralizing salt, as well as the molar mass of eh polysaccharide. The results are shown in Fig. 5. The obtained results allow us to conclude that there is a critical concentration of collagen, above which there is a significant decrease in mechanical strength. This phenomenon is particularly observed in the case of a hybrid obtained from high molecular mass polymers dissolved in chloric acid with the addition of NaGP, which occurs when the ratio of collagen to chitosan is higher than 1:1. However, when using hydrochloric acid and NaGP, the addition of fish collagen led to an improvement in mechanical strength.

An interesting observation is the effect of the polymer molecular weight on the sensitivity to the applied concentration of collagen. Analysis of the hardness values (Fig. 6) of the biopolymer gels with the lowest molecular weight tested indicates that they are characterized by the highest sensitivity to collagen concentration, particularly in the case of systems prepared with the use of acetic acid (Fig. 6B).

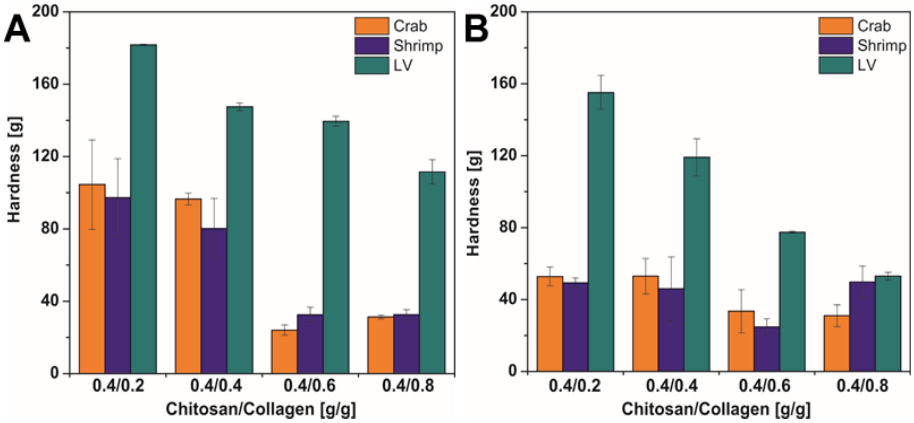

Figure 6. Effect of collagen concentration on hardness for (A) chloric and (B) acetate chitosan/NaGP/collagen hybrid hydrogels.

Regardless of the solvent used, the increase in collagen concentration leads to a decrease in the resilience value (Fig. 7). However, for the other parameters, the change in their value is closely related to the solvent used. In the case of hydrogels obtained from chitosan dissolved in hydrochloric acid (Fig. 7A), increasing the concentration of collagen also leads to a decrease in the adhesiveness value, as well as a weakening of the hydrogel structure.

On the other hand, the use of acetic acid as the solvent led a decrease in the value of cohesiveness (Fig. 7B). At the same time, a slight improvement in adhesion with an increase in collagen concentration was observed.

In the case of hybrids using a calcium glycerophosphate salt, regardless of the solvent used, it is evident that the addition of collagen leads to a reduction of all considered parameters (Fig. 8). It unambiguously allows us to state that the presence of collagen in calcium systems reduces both the adhesive properties and weakens the internal structure of the scaffolds. Strength improvement was observed only in the case of scaffolds obtained from chitosan acetate, where the concentration of collagen was not greater than the concentration of polysaccharide. In other cases, the increase in collagen concentration prevented the formation of a stable gel structure.

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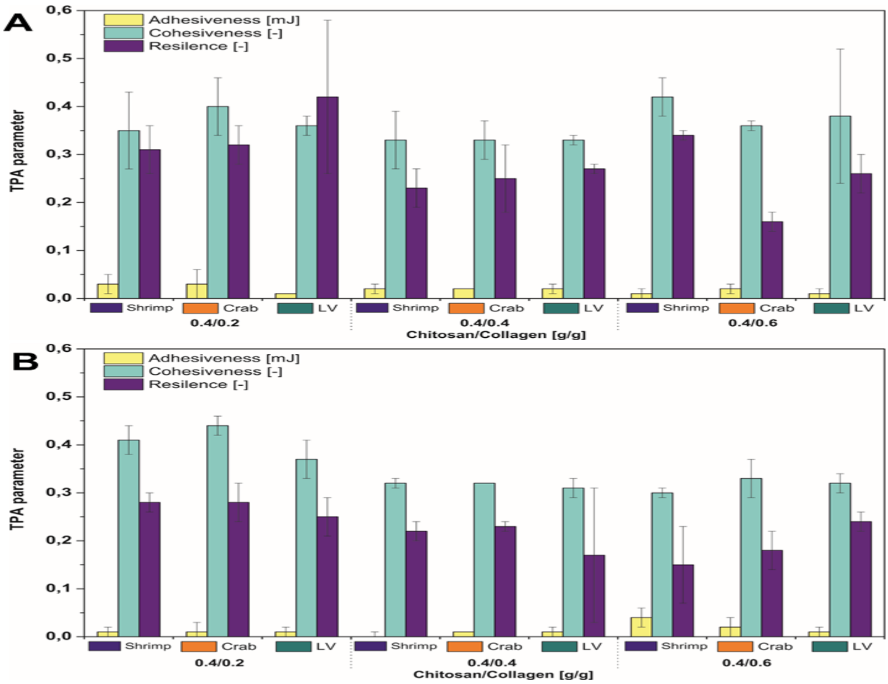

Figure 7. Effect of collagen concentration on TPA parameters for (A) chloric and (B) acetate chitosan/NaGP/collagen hybrid hydrogels

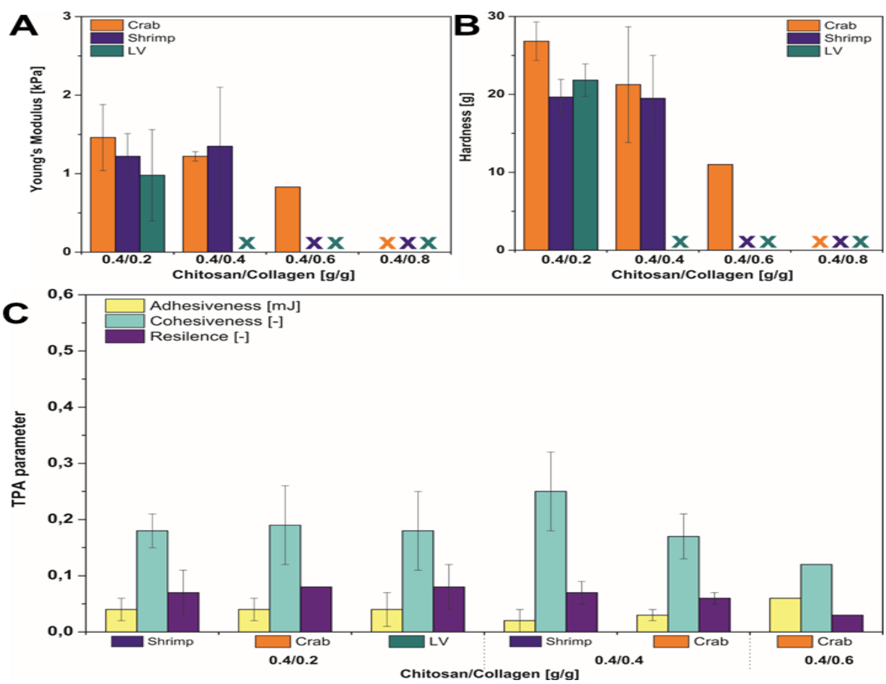

Figure 8. Effect of collagen concentration on Young's modulus (A), hardness (B), and TPA parameters (C) for acetate chitosan/CaGP/collagen hybrid hydrogels

3.2. Effect of Calcium Carbonate Addition

As mentioned above, chitosan hydrogels are mainly enriched with calcium phosphate ceramics to improve their mechanical properties. The most commonly used materials are calcium phosphate, β-tricalcium phosphate. and hydroxyapatite. Another compound that can be used as a filler is calcium carbonate [12], which, in an acidic environment, dissociates to calcium ions, carbon dioxide, and water.

The analysis of the effect of the addition of calcium carbonate in the presence of the calcium salt of glycerophosphate allows us to state that this additive contributes to the improvement of the mechanical strength of the tested gels (**Fig. 9A**). This is particularly noticeable in the case of chitosan acetate gels. Moreover, it has been found that the addition of calcium carbonate increases the stability of the gels structure obtained from polymers with higher molecular weights. Simultaneously, gels formed from low molecular weight chitosan have not been subjected to tests due to the lack of a sufficiently developed polymer structure.

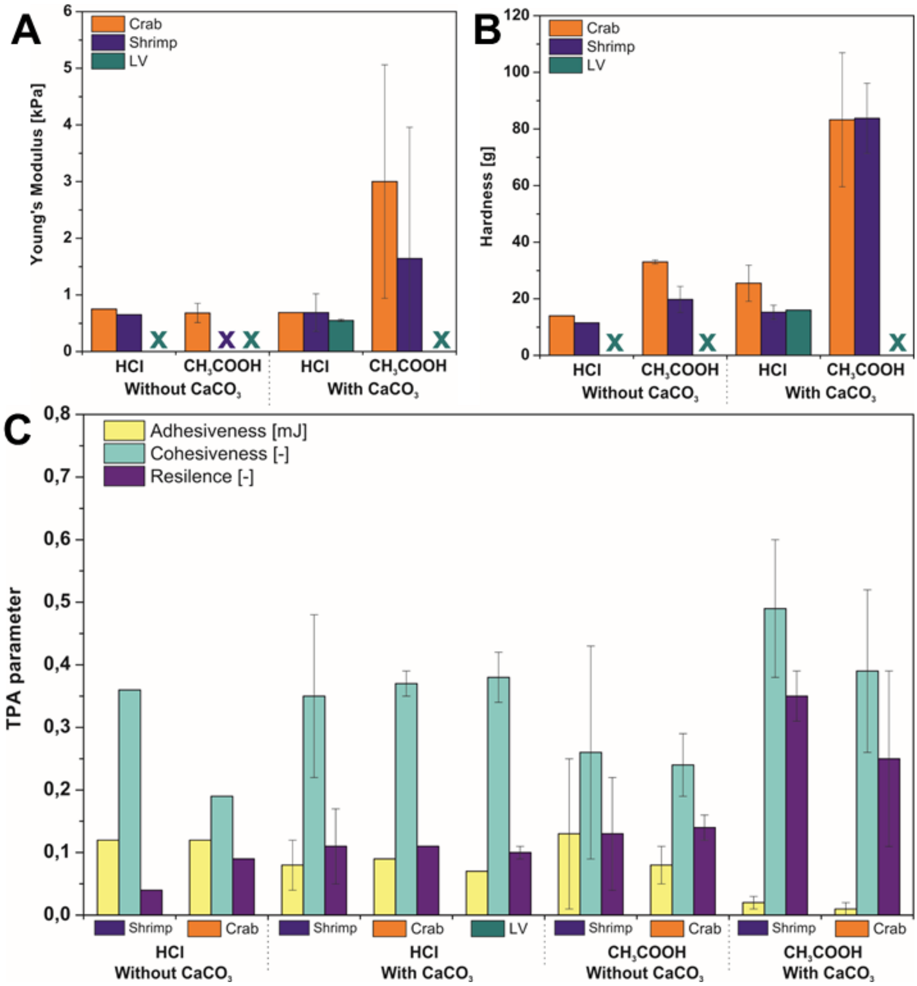

Figure 9. Effect of calcium carbonate addition on Young's modulus (A), hardness (B), and TPA parameters (C) for chitosan hydrogels with disodium glycerophophate

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The values of the texture parameters (Fig. 9C) indicate the relationship between the mechanical strength of the gels and their adhesiveness. With the increase in strength, the decrease of adhesiveness is observed. However, these systems provide physiological composition of the substrate, namely the ratio of calcium ions to phosphorus, which is an additional advantage in the case of scaffolds dedicated to bone tissue engineering applications. Regardless of the solvent used, an improvement in the cohesiveness parameter is observed as a measure of the strength of internal bonds constituting the structure of the sample. At the same time, the type of solvent used affects the resilience of the sample. Clearly, when acetic acid is used, an increase is observed, whereas for hydrochloric acid a decrease in the resilience value has been noted.

4. Conclusions

The conducted structural strength tests of colloidal chitosan systems confirmed the possibility of using standard texture profile analysis (TPA) measurements, commonly used in the food industry, to characterize the mechanical properties of these hydrogels. The determined texture parameter values indicate that the mechanical properties of the hybrid hydrogel depend on both the molecular weight of the polymer, the type of solvent, and the pH neutralizing substance, as well as the type and concentration of the filler. It was found that in the case of commonly used disodium β-glycerophosphate, the best texture parameters were characterized by hybrids obtained from low molecular weight chitosan. In the case of the use of calcium β-glycerophosphate, the hybrids obtained from the high molecular weight polymer have been found to be much more mechanically resistant. Moreover, it has been found that the pH-neutralizing substance has a greater impact on the mechanical properties of the colloidal chitosan gels compared to the solvent used. In the case of the addition of collagen, its effect is strongly dependent on the concentration used and the basic composition of the scaffold. The enrichment of chitosan solutions with calcium carbonate in the presence of calcium β-glycerophosphate leads to improved mechanical properties.

The conducted research indicates the possibility of controlling mechanical properties depending on the intended use by changing the composition of the scaffolds.

Moreover, it was found, that the use of the TPA test enables a more comprehensive analysis of mechanical properties than in the case of applying a single compression leading to the determination of the Young's modulus. Furthermore, in some cases it was the only way to evaluate the properties of the obtained hydrogels. Consequently, the texture analysis test is a valuable tool for selecting solutions depending on the intended application of scaffolds.

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