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I would like to take the opportunity to thank to the reviewers for insightful comments and respond in detail to each of the comments made by the reviewers in the space provided.

Reviewer #1:

In this report, the authors described the synthesis of chitosan based hydrogel that contains the collagen and alkaline phosphatase. The subsequent mineralization on these hydrogel was also evaluated. The samples were characterized by FT-IR, XPS and SEM. Moreover, the effects of collagen and alkaline phosphatase on the formation of calcium phosphate was also evaluated. This work is well conducted and organized. It is fully fulfilled the scope of Journal. Therefore, I recommend the acceptance after the following issues were completely addressed.

1. Apart from the FT-IR, XPS and SEM, other characterization data such as XRD and TGA of the hydrogel before and after mineralization should be provided.

Thermogravimetric analysis TGA were not provided due to the current lack of such equipment. Authors will include TGA characterization data in further publications.

X- ray diffraction (XRD) patterns were done for this study and obtained results and discussion are presented in revised version of this article. XRD diffraction patterns before (B) and after (A) mineralization for chitosan gel (1) and for chitosan gel containing collagen 0.5 ml (2) and 1.0 ml (3) respectively were presented in Figure 3. XRD diffraction patterns before (B) and after (A) mineralization for chitosan gel (1); chitosan-collagen 1.0 ml gel (2); chitosan-ALP gel (3) and chitosan-collagen-ALP (4) gel respectively were presented in Figure 8.

Briefly, in the gels with collagen after mineralization, two main bands are observed at an angle of 2θ 13° and 20° which are associated with chitosan, whereas 26° may be derived from a calcium-phosphate compound. In collagen and ALP gels after mineralization, these bands disappear, while the analysis of XPS spectra shows that the amount of calcium and phosphorus is significantly higher than in the sample of chitosan-collagen gels with ALP.

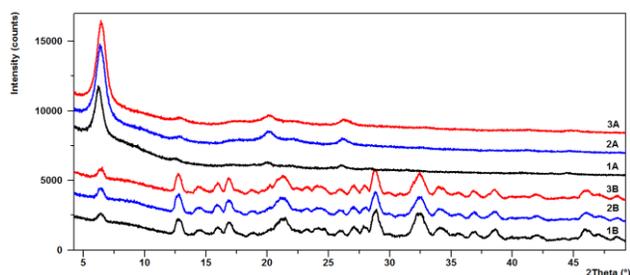


Figure 3. XRD diffraction patterns before (B) and after (A) mineralization for chitosan gel (1) and for chitosan gel containing collagen 0.5 ml (2) and 1.0 ml (3) respectively

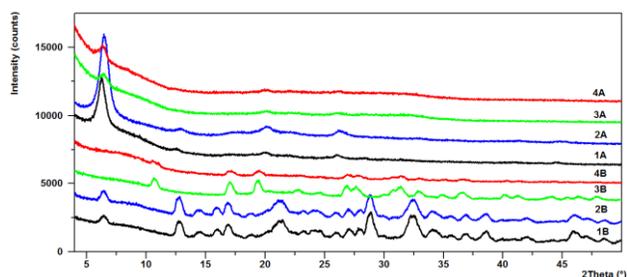


Figure 8. XRD diffraction patterns before (B) and after (A) mineralization for chitosan gel (1); chitosan-collagen 1.0 ml gel (2); chitosan-ALP gel (3) and chitosan-collagen-ALP(4) gel respectively

2. The structure and property of these hydrogel and their mineralization composites have been well characterized, however, the potential applications of these materials have not evaluated or at least discussed.

In this study hydrogels were fabricated and evaluated by determining its structural changes after mineralization for biomedical applications as a scaffolds for bone regeneration. The practical application was included in the introduction section and also in the conclusions section.

3. The quality of many figures is rather poor, all of them must significantly improved before publication.

As suggested all FTIR figures and its captures have been significantly improved and also XRD figures and captions were added.

4. The rheology analyses such as storage modulus and loss modulus of the hydrogel before and after mineralization should be determined.

According to chitosan-collagen gels with ALP after mineralization – the rheological studies are currently conducted and will be published in a separate article in the future.

The current rheological tests of chitosan gels carried out by the authors were presented in the following articles:

Rheological studies for chitosan gels with and without collagen were published in Journal Progress in the Chemistry and Application of Chitin and its Derivatives by co-author Z.Modrzejewska et.al “The influence of the addition of collagen on the rheological properties of chitosan chloride solutions” doi.10.15259/PCACD.22.18

Rheological studies for chitosan gels with and without ALP were previously studied and published by co-authors T.E.L.Douglas, A.Skwarczynska, Z.Modrzejewska et. al “Acceleration of gelation and promotion of mineralization of chitosan hydrogels by alkaline phosphatase” doi.org/10.1016/j.ijbiomac.2013.02.002

Chitosan colloidal systems, created by dispersing in aqueous solutions of hydrochloric acid, with and without the addition of disodium β -glycerophosphate were previously published by co-author Z.Modrzejewska et. al. in Polymers “Rheo-Kinetic Study of Sol-Gel Phase Transition of Chitosan Colloidal Systems” doi.org/10.3390/polym10010047

5. The toxicity of the hydrogel and their composites with calcium phosphate should be determined at least at the cellular level. The cell viability value could be based on the typical assays (e.g. Toxicology Research 1 (1), 62-68 or Toxicology Research 4 (1), 160-168).

Biological tests were carried out and published by co-authors Z.Modrzejewska et. al. in Journal of Bioactive and Compatible Polymers “Thermosensitive chitosan gels containing calcium glycerophosphate for bone cell culture” doi.org/10.1177/0883911516671150

Biological properties of the thermosensitive chitosan hydrogels prepared with the use of chitosan chloride with β -glycerophosphate disodium salt pentahydrate enriched with calcium glycerophosphate were presented and compared with chitosan hydrogels with β -glycerophosphate disodium salt pentahydrate. The study was focused on the determination of hydrogel structure and biological testing of hydrogels with human osteoblasts line Saos-2.

Moreover another biological studies of thermosensitive chitosan chloride gels with β - glycerophosphate disodium was investigated by contact with bone cells (line Saos- 2) for 7 and 21 days. The cells were encapsulated and seeded onto the gel. A live/dead test showed no cytotoxic reaction and published in Applied Polymer Science “The structural (FTIR, XRD, and XPS) and biological studies of thermosensitive chitosan chloride gels with β - glycerophosphate disodium” by co-authors A. Skwarczynska and Z. Modrzejewska et al.

Further biological studies and investigations are strictly planned in the next research steps.

6. The mussel-inspired chemistry is an important method for fabrication of functional materials for different applications especially in the biomedical field. The previous reviews and reports (e.g. Chemical reviews 114 (9), 5057-5115, Nanoscale 8 (38), 16819-16840, Biomacromolecules, 2018, 19 (6), 1858-1868) also suggested that the polydopamine could be used for preparation of hydrogel and mineralization for biomedical applications. Some related references (e.g. Nanoscale 4 (18), 5581-5584, Applied Surface Science 343, 19-27, Journal of Materials Chemistry B 3 (17), 3476-3482, Materials Science and Engineering: C 77, 972-977, Applied Surface Science 419, 35-44) about mussel-inspired chemistry should be mentioned and discussed in the revised manuscript.

Regarding to reviewer suggestions mussel-inspired chemistry was included in the text and cited.

Reviewer #2:

In the present manuscript, Skwarczynska and coworkers analyzed the influence of enzymatic mineralization induced by alkaline phosphatase (ALP) on the structural properties of thermosensitive chitosan-collagen gels, using a combination of FTIR and XPS spectra as well as SEM images. The obtained data were compared with those previously published concerning the structure of chitosan-collagen gels before mineralization. In particular, the authors investigated the effect of collagen on the structural properties of thermosensitive chitosan gels and the influence of ALP on the structural properties of chitosan and chitosan-collagen gels, before and after mineralization. It turns out that:

- * Mineralization induces similar changes in the supermolecular structure of chitosan and chitosan-collagen gels
- * Collagen and chitosan are connected through C1 carbon, amine and hydroxide groups
- * During the mineralization process, CaGP is decomposed by ALP to Ca and P
- * After mineralization, chitosan-collagen gels containing ALP contain five times more Ca than chitosan hydrogels containing ALP (8.3 % vs 1.7 %) and the Ca/P ratio is closer to that of hydroxyapatite.

The present manuscript addresses an interesting topic and the approach used by the authors is consistent with the aim of the paper. Overall, the FTIR, XPS and SEM images experiments were well performed and provided sound and interesting data. Hence, the scope and content of the manuscript match those of Journal of Molecular Structure. Nonetheless, the obtained data are

reported and discussed rather confusedly, thus making the manuscript really hard to follow. Moreover, some sentences are not clearly understandable. Therefore, in my opinion the present manuscript by Skwarczynska and coworkers requires major revision to be published in Journal of Molecular Structure.

1. The analysis of the NIR (Pages 8-10) and XPS spectra (Pages 14-15) is really hard to follow. In my opinion, both sections should be rewritten and shortened. This would greatly increase the understandability of the results obtained. As an example, the two paragraphs corresponding to Lines 14-32 of Page 9 could be merged in a single, shorter paragraph.

2. In my opinion, inclusion in the text of more frequent references to Figures 1-6 would simplify the understanding of the NIR and XPS data.

3. The text contains many sentences whose overall meaning is unclear (see for example Lines 10-12 and Lines 45-46 of Page 5; Lines 11-13, 23, 31-33, 41-47 Page 8; Lines 9-11 of Page 10, Lines 38-39 of Page 14; Lines 13-15 of Page 17).

4. Page 10, Lines 12-14, and Page 17, Lines 1-3. The authors should clearly explain which are the experimental evidences supporting their statement that "An increase of ALP concentrations causes the separation of macromolecules, thus releasing them from the strong mutual deformations".

5. Page 16, Lines 38-42. The meaning of the whole paragraph is rather unclear to me. In my opinion, the authors should explain the following statements

a. "the internal structure of ALP-containing gels that is greatly expanded"

b. "The structure of gels containing collagen and ALP is compressed compared to the structure of gels without ALP".which in the present form are rather unclear.

6. The text contains several typos that should be corrected.

Regarding to reviewer suggestions the results and discussion section was shortened and mend, unclear sentences were improved, typos were removed and corrections have been done.

THE MINERALIZATION EFFECT ON CHITOSAN HYDROGEL STRUCTURE CONTAINING COLLAGEN AND ALKALINE PHOSPHATASE

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Abstract

Introducing collagen the basic ingredient of bone tissue into the structure of chitosan gels that are formed at physiological body temperature, aims to create so-called biomimetic structures, which are close in composition to the natural composition of bone tissue. The aim of this study was to determine the influence of mineralization on the structural properties of thermosensitive chitosan-collagen gels containing alkaline phosphatase (ALP) by SEM, XRD FTIR and XPS analyses, compared to the previously presented structure of chitosan-collagen gels before mineralization.

Keywords: *chitosan, hydrogels, collagen, alkaline phosphatase.*

1. Introduction

Chitosan, as a biomaterial, has unique properties (biocompatible, biodegradable) and is used to form scaffolds for cell culture. They are produced as either dried forms or gels [1-7].

Hydrogels are especially interesting materials for scaffolds which are formed by increasing the temperature. They are characterized by the presence of water in the structure, increased flexibility (creating a structure similar to tissue) and the possibility of injecting the scaffold into diseased tissue [1].

In order to give the chitosan scaffolds osteoinductive properties, it is necessary to introduce calcium phosphate compounds into the structure, creating composite systems (i.e. where the so-called mineralization is conducted). The method of creating mineralized scaffolds relies mainly on introducing hydroxyapatite micro- or nanopellets into a chitosan salt, and then obtaining a 3D structure by phase separation or freezing and lyophilisation, or both processes one after the other.

For the ~~creation~~ preparation of chitosan structures containing hydroxyapatite, the process of ion exchange is also used. Chitosan scaffolds are created at the same time as hydroxyapatite during the formation of the scaffold. In this case, reactions of phosphoric acid H_3PO_4 and calcium hydroxide $Ca(OH)_2$ are applied. The phosphoric acid is added to a solution of chitosan salt (chitosan acetate) and introduced later into a suspension of calcium hydroxide. Hydroxyapatite may be also obtained in the structure by introducing calcium nitrate $Ca(NO_3)_2$ and ammonium (V), hydrogen phosphate $(NH_4)_2HPO_4$ or calcium nitrate $Ca(NO_3)_2$ and phosphoric acid H_3PO_4 or potassium dihydrogen phosphate K_2PO_4 into a solution of chitosan salt. In order to obtain a

scaffold, a solution must be frozen, lyophilized and in some cases neutralized in NaOH and in ally washed [8-32].

Moreover the mussel-inspired chemistry is an important method for fabrication of different functional materials e.g hydrogels for different applications especially in the biomedical field. Biom mineralization was adopted for the fabrication of hydroxyapatite termed polydopamine-assisted hydroxyapatite formation, to integrate hydroxyapatites within scaffold materials [33-39].

In recent years, alkaline phosphatase has often been used in research into mineralization of scaffolds intended for osteoblast culture because ALP is the enzyme which participates during the bone mineralization processes.

A new trend in the process of mineralization of chitosan scaffolds is the introduction of ALP into the structure. During the incubation of the gel in a solution of calcium glycerophosphate (CaGP), ALP incorporated into the structure of the hydrogel induces the formation of the mineral calcium phosphate. The first research on introduction of ALP into the structure of chitosan gels formed under the influence of temperature were patented and presented in scientific papers [40-42].

Composite chitosan-collagen gels, including those formed at physiological body temperature are known in literature [43-45], whereas there is far less literature available on chitosan-collagen hydrogels containing ALP [46]. The structure of chitosan-collagen gels containing ALP after mineralization has not been published. Introducing collagen, the basic ingredient of bone tissue, into the structure of chitosan gels which are formed at physiological body temperature, aims to create so-called biomimetic structures (i.e. close to the composition of natural bone tissue).

The current research concerns the evaluation of the structure of composite chitosan-collagen gels containing ALP after mineralization. The study references a previously published study on chitosan and chitosan-collagen gels containing ALP before mineralization by the same authors [46]. In this study, the following topics were investigated:

- The influence of collagen on the structural properties of thermosensitive chitosan gels before [46] and after mineralization
- The influence of ALP on structural properties of :
 - chitosan gels before [46] and after mineralization,
 - chitosan-collagen gels before [46] and after mineralization.

2. Materials and methods

Thermosensitive chitosan gels were prepared according to the method described by Chenite [47]. To prepare the hydrogels, shrimp chitosan (Sigma Aldrich Product No. 50494) of deacetylation degree SD ~ 79.5 % and molecular weight 860 kD were used. 0.4 g of chitosan powder was dissolved in 16 ml of 0.1 M HCl (Sigma Aldrich). After preparing the chitosan solution in this way, it was left on a mechanical shaker for approximately 12 h to ensure complete dissolution. At the same time, 2 g sodium beta-glycerophosphate (Na-β-GP) (Sigma Aldrich Product No. 50020) was dissolved in 2 ml of deionized water (18 mS). The resulting chitosan salt solution was cooled to 4°C. Na-β-GP solution was added and mixed to the chitosan solution (sol) drop by drop.

Thermosensitive chitosan gels containing ALP (Sigma Aldrich Product No. P7640) were created by adding 1 ml of ALP dissolved in deionized water (at the concentrations: 2.5, 10, and 25 mg/ml) to the 16 ml chitosan chloride solution containing Na-β-GP. The final concentration of ALP in each hydrogel was 0.156; 0.625; 1.562 mg/ml, respectively.

Thermosensitive chitosan-collagen gels were created by adding 0.5 and 1 ml of collagen (Sigma Aldrich Product No. C4243) to the 16 ml chitosan chloride containing Na- β -GP.

Thermosensitive chitosan-collagen gels containing ALP were created by adding 1 ml ALP dissolved in deionized water at concentrations of 2.5, 10, and 25 mg/ml into a chitosan solution containing Na- β -GP with collagen. The final concentration of ALP in each hydrogel was 0.156; 0.625; 1.562 mg/ml respectively.

All resulting solutions were incubated at 37°C for 24 h in order to complete their gelation.

The mineralization was conducted in static flow conditions. The gels containing ALP and gels with ALP and collagen were incubated in a solution of CaGP (Sigma Aldrich Product No. 50043) for 36 h. 50 ml 0.1 M CaGP solution was changed twice per day. After that, the samples were lyophilized.

The structural properties of the gels were determined by infrared spectroscopy with Fourier transformation (FTIR) using a Nicolet 6700 spectrometer from Thermo Nicolet equipped with a snap Photoacoustics MTEC model 300. Room temperature powder X-ray diffraction patterns (XRD) were collected using a PANalytical X'Pert Pro MPD diffractometer in the Bragg–Brentano reflection geometry. Copper CuK α radiation was used from a sealed tube (40 kV, 30 mA). Data was collected in the 2 θ range 3–50° with a step of 0.0167° and an exposure per step of 20 s. The samples were spun during data collection to minimize preferred orientation effects. Photoelectron spectra (XPS) were measured using the ESCALAB-210 spectrometer (VG Scientific). A non-chromatized source of x-ray radiation at a voltage of 15 kV producing a radiation beam Al Ka (1486.6 eV) at an intensity of 20 mA was used. The sample was measured after being pressed into a tablet, while maintaining a vacuum of 8*10⁻⁹ mbar in the analytical chamber of the spectrometer. A survey spectrum was made at pass energy in the hemispherical analyzer (pass energy, CAE) = 75 eV and energy jump of 0.4 eV. High-resolution spectra were recorded at an analyser pass energy of 20 eV with jump of 0.1 eV. For SEM imaging FEI QUANTA FEG 250 scanning electron microscope was used.

3. Results and Discussion

3.1 Influence of collagen on the structural properties of thermosensitive chitosan gels

The influence of collagen on the structural properties of thermosensitive chitosan gels before mineralisation are shown in Figure 1 (FTIR spectra), and after mineralization in Figure 2 (FTIR spectra) and Figure 3 (XRD diffractogram).

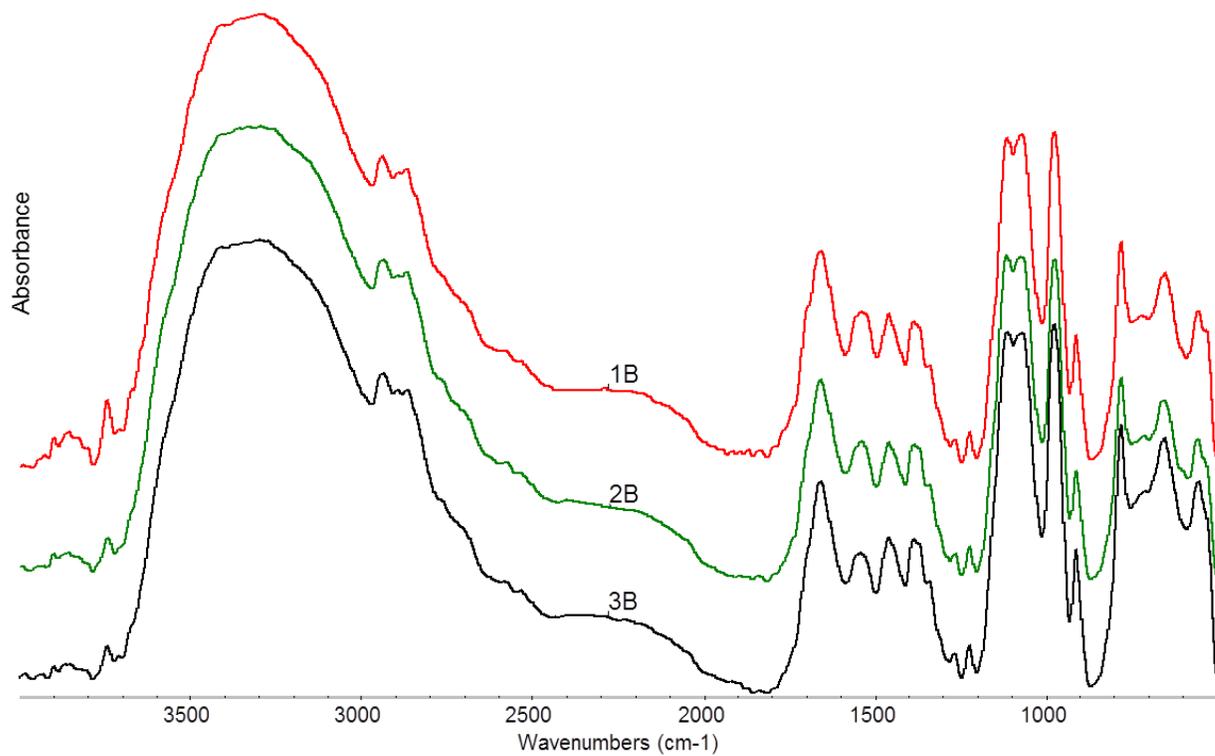


Figure 1. FTIR spectra for chitosan gels without collagen (1B) and containing collagen 0.5 (2B) and 1.0 ml (3B) before mineralization [46]

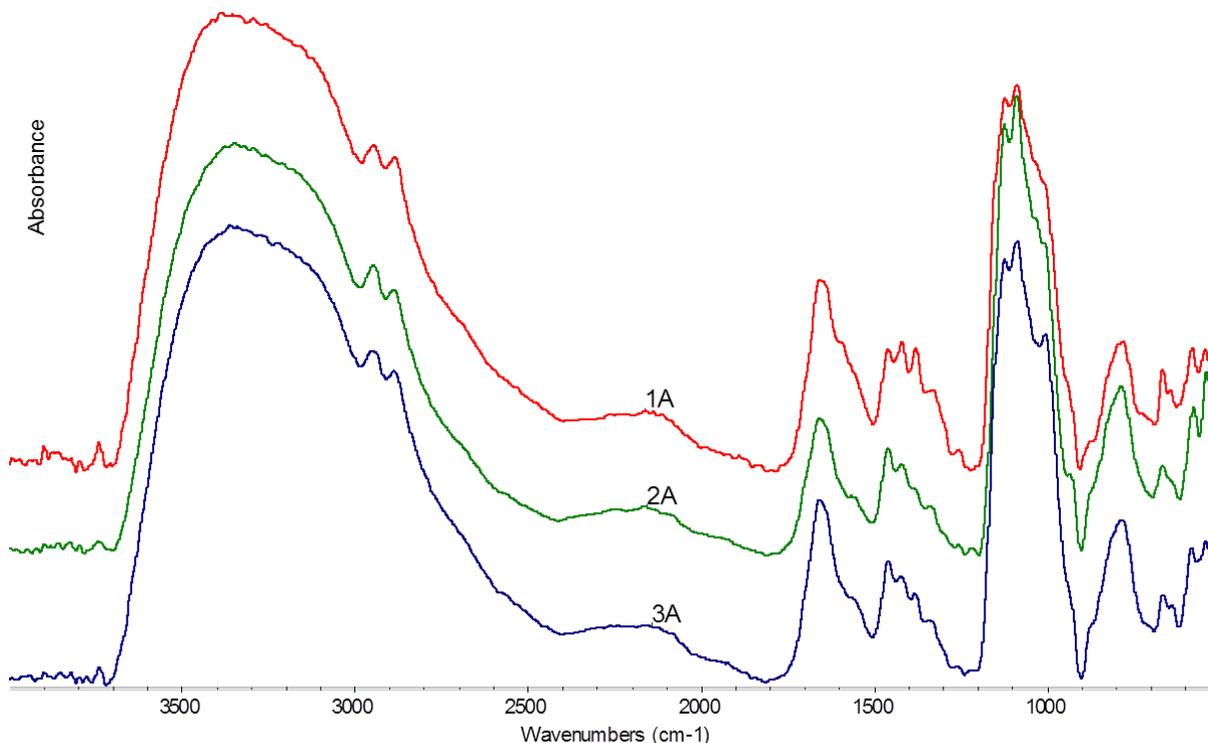


Figure 2. FTIR spectra for chitosan gels without collagen (1A) and containing collagen 0.5 (2A) and 1.0 ml (3A) after mineralization

The presence of collagen in the chitosan gels does not cause any significant changes to the oscillations of chemical groups. After the introduction of collagen, we noticed a lower intensity of the arm of amide I band occurring at wavenumber 1700 cm^{-1} and the amide II band of secondary amides at wavenumber 1560 cm^{-1} [46].

Mineralization of chitosan and chitosan-collagen gels has a similar effect on the change of gel structure. Mineralization of chitosan gels shows a very strong influence of this process on the resonant frequencies of grouping vibrations in the whole spectral range, **observed in We observe a deformation of oscillators -O-H and N-H band at approximately ca. 3400 cm^{-1} . Also, oscillation bands of C-H groupings after the mineralization process increase the wavenumber of vibrations. This indicates significant changes in the conformation of chains and changes taking place in the supermolecular structure of the obtained gels.**

In the spectra of chitosan and chitosan-collagen gels a band is visible next to the wavenumber 2450 cm^{-1} . After mineralization, it moves towards the lower wavenumber 2150 cm^{-1} .

The main changes are observed for wavenumbers corresponding to amide I band and amide II band of secondary amides, the saccharide structure and in the range of wave numbers connected with the presence of phosphate ions.

In all cases the asymmetry of the band at 1660 cm^{-1} (arm at 1770 cm^{-1}) and amide II band of secondary amides at 1540 cm^{-1} disappears, the intensity of bands at 1460 , 1380 , 1270 and 1225 cm^{-1} change, proportionally declining together with the reduction of the wavenumber; the band at 1420 cm^{-1} appears, which may also be the effect of moving the band at 1440 towards the lower wavenumbers.

In the saccharide band in spectra of chitosan gels and chitosan-collagen gels is observed similar change. In the spectra of chitosan gels, bands at 1110 cm^{-1} and 1080 cm^{-1} are visible after the mineralization, the intensity of band at 1050 cm^{-1} increases in comparison with the intensity before the mineralization and the shape of the band changes and becomes visibly asymmetrical; this is probably connected with the disappearance of the band at 980 cm^{-1} . Also the band at 910 cm^{-1} disappears, the band at 780 cm^{-1} becomes wider, the band at 650 cm^{-1} disappears and is visible with low intensity for wave number 670 cm^{-1} with an arm 650 cm^{-1} ; band 550 cm^{-1} after mineralization, it is divided into two bands: 580 and 520 cm^{-1} .

In the spectra of chitosan-collagen gels the shape of the band at 1050 cm^{-1} is less asymmetrical; the band at 980 cm^{-1} is still clearly visible in comparison to the spectrum of chitosan gels, and the remaining changes are close to those in the spectrum of the chitosan gel.

~~Tensions in the polymer chains are visible through the increase of resonant frequencies of C-O groups occurring both in the heterocyclic ring, and in the oxygen bridge at ca. 970 cm^{-1} for the gel. They all shift towards higher vibration energies after mineralization.~~

The mineralization of chitosan gels with the addition of collagen take a place without any major impact of the collagen concentrations on the observed changes in the molecular and supermolecular structure.

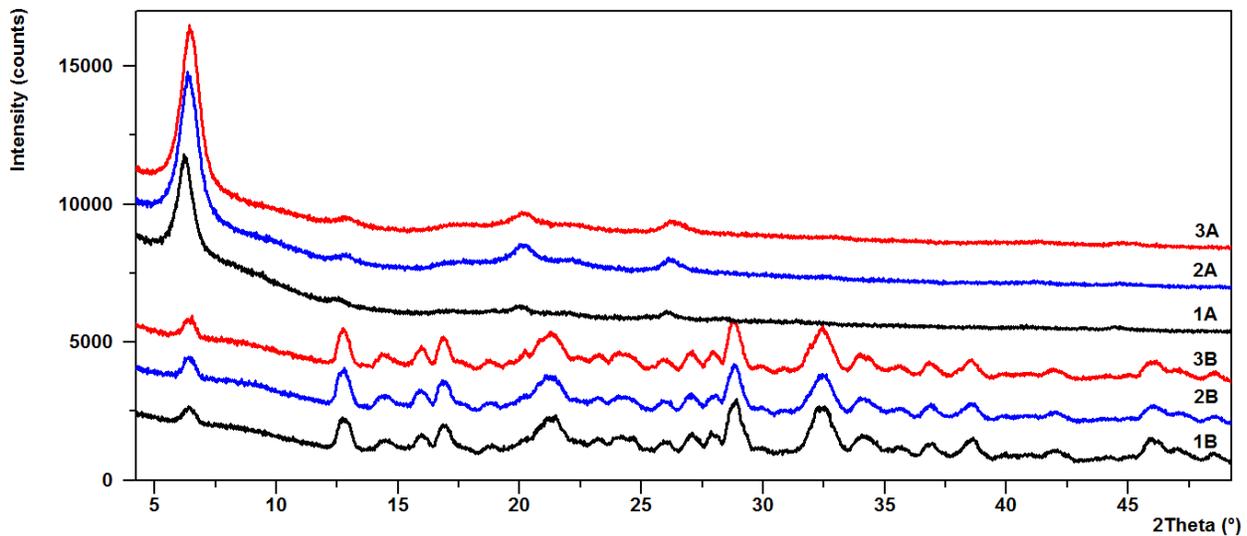


Figure 3. XRD diffraction patterns before (B) and after (A) mineralization for chitosan gel (1) and for chitosan gel containing collagen 0.5 ml (2) and 1.0 ml (3) respectively

XRD diffraction patterns before and after mineralization of chitosan and chitosan gels containing collagen (0.5 ml and 1.0 ml) samples are shown in Figure 3. The crystalline structure of chitosan gels due to the presence of collagen does not change since the collagen-free and collagen-chitosan gels diffractograms are quite similar. They are characterized by a number of peaks that are particularly noticeable at an angle of 2θ about 6.4° , 12.8° , 16.9° , 21.5° , 28.9° and 32.4° . The variation of collagen concentration does not affect the crystalline structure. After mineralization, we observed significant changes in the crystalline structure of study samples. The diffraction pattern of chitosan gel contains a strong, wide peak at an angle of 2θ about 6.5° and several small broad peaks at an angle of 2θ about 12.8° , 20.1° and 26.1° . Similar changes we observe for chitosan gel containing collagen samples. This indicates the semi-

crystalline nature of the study samples. In addition to the increase in collagen concentration, the intensity of these peaks decreases.

3.2 Influence of ALP on the structural properties of chitosan and chitosan-collagen gels

The FTIR spectra of chitosan and chitosan-collagen hydrogels containing 1 ml of ALP in the concentration of 0, 2.5, 10 and 25 mg/ml before mineralization are presented in Figure 4 and 5 and after mineralization in Figure 6 and 7 (changes with the addition of 0.5 ml of collagen are the same). Figure 8 shows XRD diffraction patterns before and after mineralization.

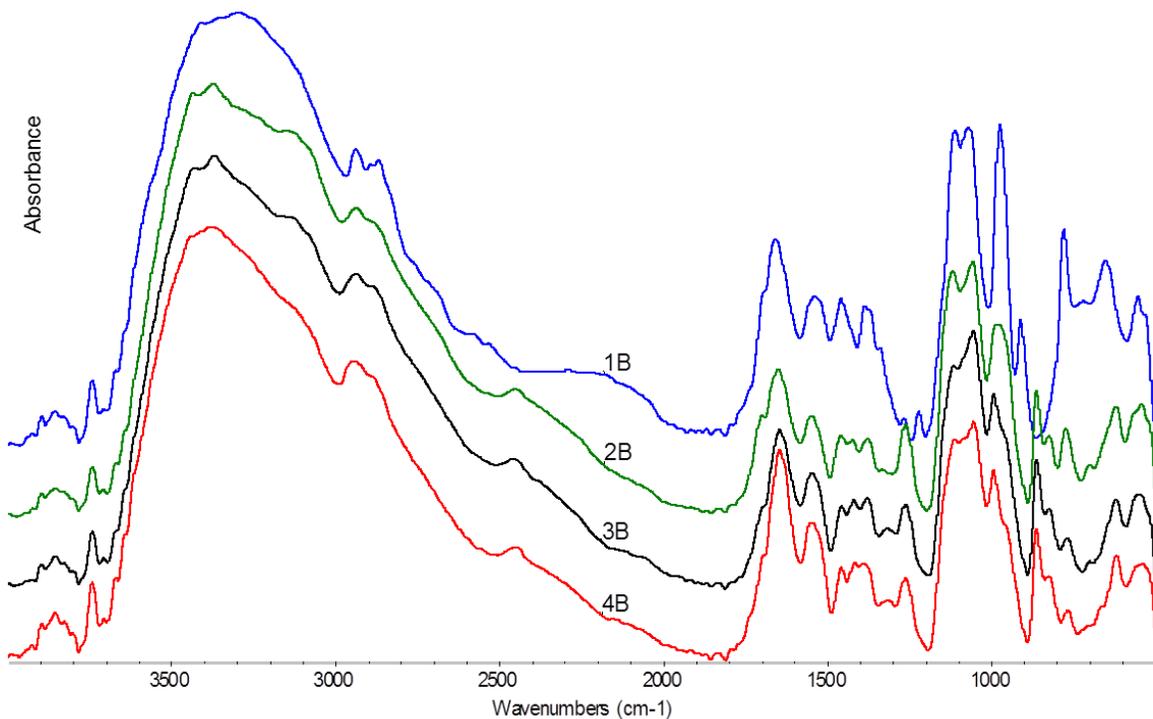


Figure 4. FTIR spectra for chitosan gel before mineralization (B) containing ALP - 0 mg/ml (1B), 2.5 mg/ml (2B), 10 mg/ml (3B) and 25 mg/ml (4B) [46]

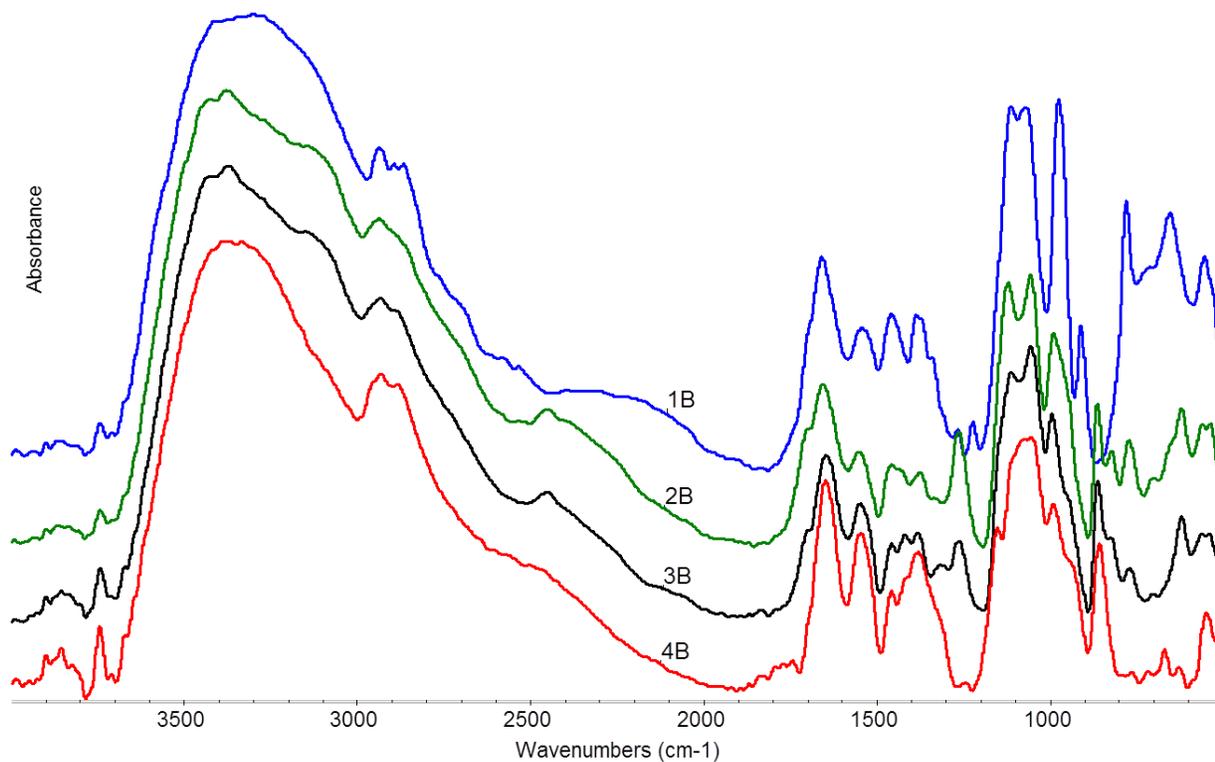


Figure 5. FTIR spectra for chitosan-collagen gel 1.0 ml before mineralization (B) - containing ALP 0 mg/ml (1B), 2.5 mg/ml (2B), 10 mg/ml (3B) and 25 mg/ml (4B) [46]

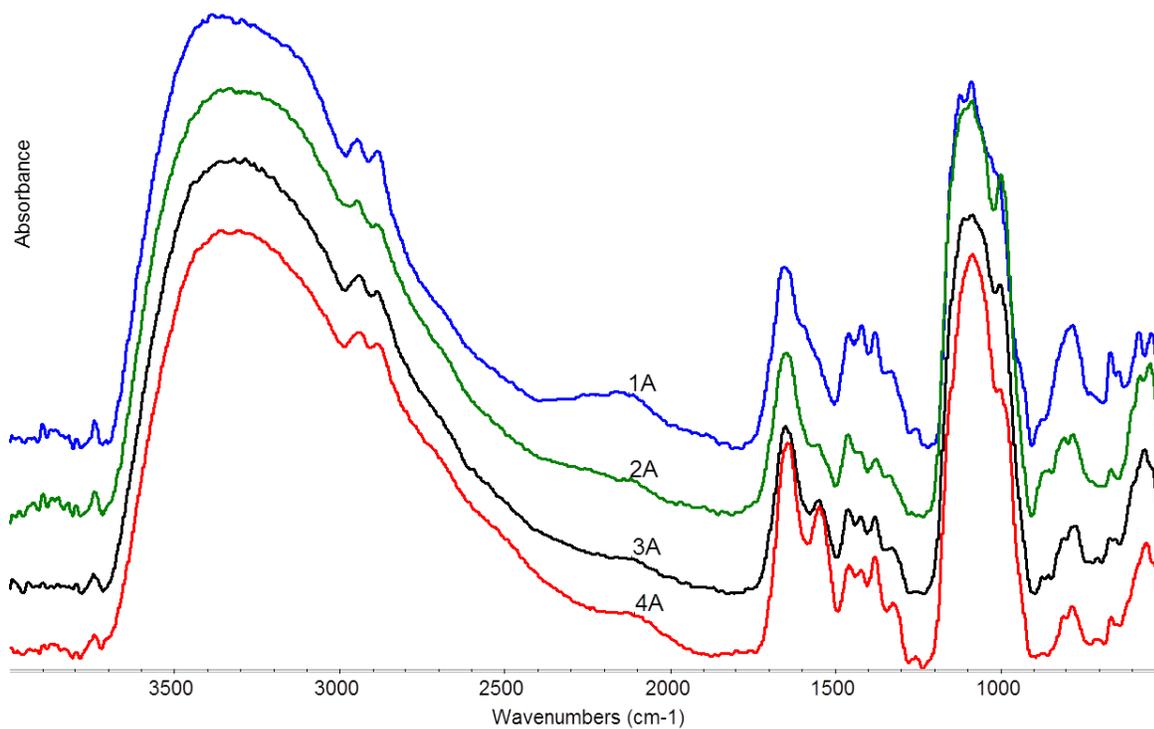


Figure 6. FTIR spectra for chitosan gel containing ALP after mineralization (A) - 0 mg/ml (1A), 2.5 mg/ml (2A), 10 mg/ml (3A) and 25 mg/ml (4A)

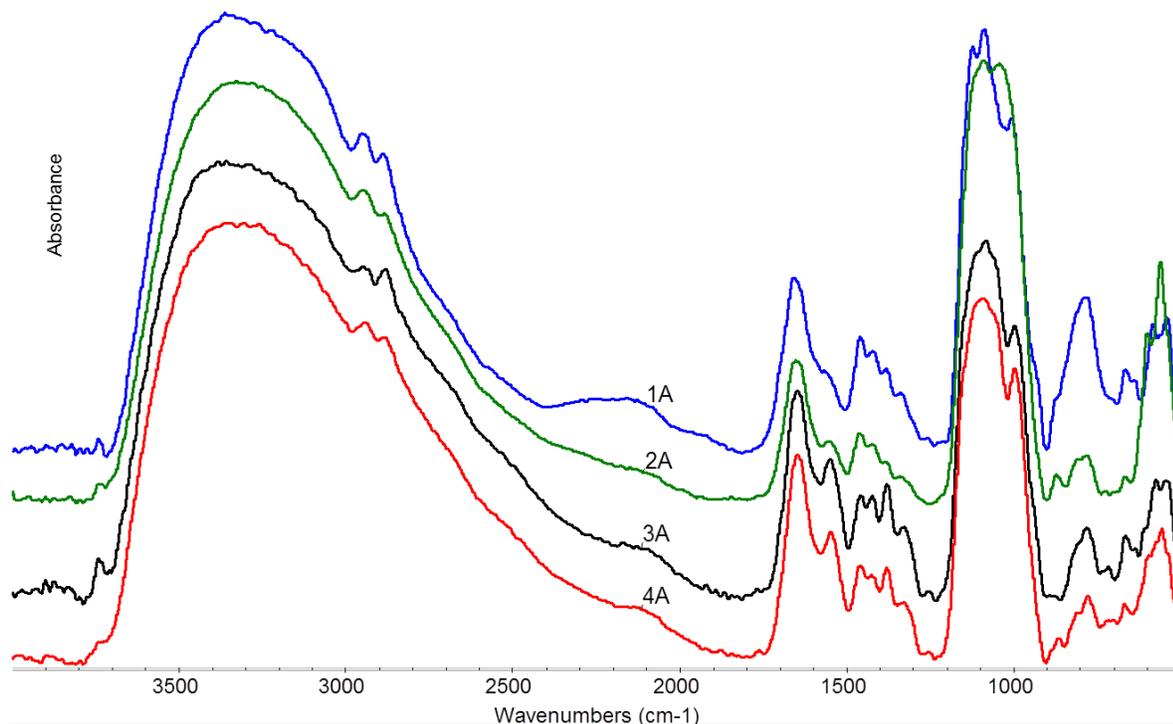


Figure 7. FTIR spectra for chitosan-collagen gel 1.0 ml containing ALP after mineralization (A) - 0 mg/ml (1A), 2.5 mg/ml (2A), 10 mg/ml (3A) and 25 mg/ml (4A)

The spectra before and after mineralization of chitosan hydrogels (Figure 4 and 6) and chitosan collagen- hydrogels containing ALP (Figure 5 and 7) indicate changes that can be observed in the wavenumber range 3000-3500 cm^{-1} and in the range of the saccharide structure, and in connection with the presence of phosphate ions in the range 1100-700 cm^{-1} [48-50].

Changes in the spectra in asymmetrical band corresponding to O-H vibrations (3600-3100 cm^{-1}) of chitosan and chitosan-collagen hydrogels before mineralization containing ALP and without ALP ~~in asymmetrical band corresponding to O-H vibrations (3600-3100 cm^{-1})~~ are similar. The asymmetry is visible while moving towards lower wavenumbers, and is higher in the spectrum of hydrogels with ALP which indicates the presence of strong hydrogen-oxygen bonds and amine groups N-H in the structure. As a result of introducing a low amount of ALP 2.5; 10 mg/ml ~~in proportion to the weight of chitosan gel produced~~ a specific effect of a significant change in the spectra was observed. In the range of oscillation of stretching groups OH and N-H at circa 3400 cm^{-1} sharp peaks are formed. This phenomenon can be explained of OH and N-H groups taking part in intermolecular bonds in various places of the polymer chain. This means that the supermolecular structure of chitosan is more organised. After the mineralization, no changes were observed in the spectra of chitosan and chitosan-collagen gels containing ALP in the range of the bands corresponding to O-H vibrations, nor does the asymmetry connected with the presence of amine groups N-H. ~~change~~

Stretching vibrations of aliphatic groups -CH₂ and -CH₃ of the spectra for chitosan and chitosan-collagen hydrogels at the wavenumbers 2800-2970 cm^{-1} are divided into three bands which peaks at wavenumbers 2870 cm^{-1} , 2890 cm^{-1} and 2940 cm^{-1} . For hydrogels containing

ALP, the two maximum bands can be observed at wavenumbers 2940 cm^{-1} and 2890 cm^{-1} . After mineralization in all cases, the spectra are observed two bands at 2890 cm^{-1} and 2940 cm^{-1} . In the wavenumber stretching vibrations of aliphatic groups (CH_2 and CH_3), ($2800\text{--}2970\text{ cm}^{-1}$) - the spectra of chitosan and chitosan-collagen hydrogels are divided into three bands which peak at wavenumbers 2870 cm^{-1} , 2890 cm^{-1} and 2940 cm^{-1} . For hydrogels containing ALP, the wavenumbers at 2870 cm^{-1} disappears, the two maximum bands can be observed at wavenumbers 2940 cm^{-1} and 2890 cm^{-1} . After mineralization in all cases, the wavenumbers at 2870 cm^{-1} disappears, the spectra are observed two bands at 2890 cm^{-1} and 2940 cm^{-1} .

In spectra of chitosan-collagen hydrogels before mineralization a very wide band was also observed at $2500\text{--}2050\text{ cm}^{-1}$, but after introducing ALP this band disappears (Figure 5). ~~with decreases together with an increase in ALP concentration in the gel.~~ In the spectra of the hydrogels after mineralization the band appears at wavenumber 2150 cm^{-1} and with an increase of ALP concentration it moves towards lower wavenumbers and disappears.

In spectra before mineralization of chitosan and chitosan hydrogels containing ALP (Figure 5), changes in their structure can also be observed in this wavenumber range, mainly in the range of amide I band and amide II band of secondary amides. In the spectra of chitosan and chitosan-collagen gels before mineralization bands at wavenumbers 1660 cm^{-1} were observed with a slight asymmetry towards higher wave numbers 1700 cm^{-1} and a number of bands for wavenumbers $1540, 1460, 1380, 1340, 1270, 1220\text{ cm}^{-1}$. ~~Spectra in the presence of ALP in the structure of~~ for chitosan and chitosan collagen gels with ALP ~~it varies~~ depending on the concentration of ALP. ~~The presence of ALP in the structure~~ for low ALP concentrations 2.5 mg/ml makes the band at 1700 cm^{-1} more visible in the spectra; however for the concentration of ALP 25 mg/ml the band disappears. The intensity of band 1540 cm^{-1} increases for the largest addition of ALP, i.e. 25 mg/ml . In the range of low ALP concentrations a very intensive band is present at 1260 cm^{-1} ; however the intensity decreases together with the increase of ALP concentration (at high ALP and collagen concentrations the band disappears). After mineralization the amide I band at 1660 cm^{-1} remains unchanged, both in the spectra of chitosan gels and chitosan-collagen gels, while the amide II band of secondary amides occurring at wave number 1550 cm^{-1} becomes more visible as ALP concentration increases.

Before mineralization in spectra of chitosan and chitosan-collagen with and without ALP (Figure 5) major changes in the band in the saccharide structure at the wavenumber range $1200\text{--}900\text{ cm}^{-1}$ can be observed. In spectra of chitosan and chitosan-collagen without ALP at wave numbers $1110, 1070$ (stretching vibrations P-O-C), 975 ($-\text{PO}_4^{3-}$), 910 ($-\text{HPO}_4^{2-}$), $780, 650$ and 554 cm^{-1} were observed. These are bands deriving from both the saccharide structure and resulting from the presence of Na- β -GP.

- ~~○ bands at wavenumbers 1070 cm^{-1} and 780 cm^{-1} are characteristic of Na- β -GP and correspond to stretching vibrations P-O-C;~~
- ~~○ a band at wave number 975 cm^{-1} is characteristic of ($-\text{PO}_4^{3-}$)~~
- ~~○ a band at wavenumber 910 cm^{-1} indicates the presence of ($-\text{HPO}_4^{2-}$).~~

~~Before mineralization in the range of~~ For low ALP concentrations in chitosan-collagen (Figure 5) gels spectra, bands at 1115 cm^{-1} and 1055 cm^{-1} are separated and at high ALP concentrations they combine; at high ALP concentrations a new band for wavenumber 1150 cm^{-1} appears; the intensity of the band at wavenumber 975 cm^{-1} definitely decreases, and together with the increase of ALP concentration it becomes more asymmetrical. A band appears at wavenumber 860 cm^{-1} with an arm at 820 cm^{-1} , the arm disappears at high ALP concentrations. The band at wavenumber 910 cm^{-1} disappears, the band at wavenumber 780 cm^{-1} decreases

intensity, and the band at wavenumber 650 cm^{-1} moves towards lower wavenumber 610 cm^{-1} . After the introduction of ALP in chitosan hydrogels, the spectrum in this wavenumber range changes: the intensity of the band at 1110 cm^{-1} decreases, the band at 1070 cm^{-1} shifts towards the lower wavenumber 1055 cm^{-1} , the intensity of band at wavenumber 975 cm^{-1} decreases and, for the large additions of ALP, moves towards the higher wavenumber 994 cm^{-1} ; the bands at wavenumbers 910 and 780 cm^{-1} decrease, and the band at wavenumber 650 cm^{-1} moves towards the lower wavenumber 610 cm^{-1} . Also, after adding ALP, bands appear at wavenumbers 860 cm^{-1} and 820 cm^{-1} .

After mineralization in the spectra for chitosan hydrogels with ALP (Figure 6) in the range of the saccharide band, the bands at 1110 cm^{-1} , 1080 cm^{-1} and 1050 cm^{-1} combine, a band is formed at 980 cm^{-1} , the wide asymmetrical band at wavenumber 800 cm^{-1} divides into two bands at 820 cm^{-1} and 780 cm^{-1} , the band at 660 cm^{-1} loses intensity and its arm at 650 cm^{-1} disappears. The bands at 590 cm^{-1} and 540 cm^{-1} combine. After mineralization, in the spectra of chitosan-collagen gels with ALP (Figure 7) the range of the saccharide band, also all bands 1110 cm^{-1} , 1080 cm^{-1} and 1050 cm^{-1} combine. In gels of higher concentrations of collagen, the band at 980 cm^{-1} becomes more visible (than in the gels without collagen), similarly to mineralized chitosan gels, a band at 870 cm^{-1} appears, a wide asymmetrical band at wavenumber 780 cm^{-1} decreases its intensity after the addition of ALP and divides into two bands at 820 cm^{-1} and 780 cm^{-1} , the intensity of band 660 cm^{-1} decreases and its arm 650 cm^{-1} disappears; bands 590 cm^{-1} and 540 cm^{-1} combine.

The FTIR spectra of chitosan-collagen gel samples with and without the addition of ALP show a strong influence of ALP addition on the conformation and the intermolecular form of gels. This is probably triggered by blocking of the polar groups by ALP. This influence is, in turn, stimulated by collagen. In the spectra deformation oscillations of groups containing C-O and C-O-C bonds can be observed in a wide range of wavenumbers depending on the presence of a given group. The spectra of chitosan gel after addition of ALP shows very significant changes in the supermolecular construction, regardless of the amount of ALP added. Even the smallest amount of ALP has a strong impact on the conformation of chitosan, and, as a result, its ability to organise molecules during binding. This can be explained by a direct and strong impact of -OH and N-H, which, at the same time, enables an unconstrained migration of molecules during binding. The result of this phenomenon is, for instance, a band moving from 910 cm^{-1} to 864 cm^{-1} . Increasing the amount of ALP has no further impact on the changes in conformation, probably due to earlier saturation of groups forming intermolecular bonds.

~~After mineralization in the spectra for chitosan hydrogels with ALP in the range of the saccharide band, the bands at 1110 cm^{-1} , 1080 cm^{-1} and 1050 cm^{-1} combine, a band is formed at 980 cm^{-1} , the wide asymmetrical band at wavenumber 800 cm^{-1} divides into two bands at 820 cm^{-1} and 780 cm^{-1} , the band at 660 cm^{-1} loses intensity and its arm at 650 cm^{-1} disappears. The bands at 590 cm^{-1} and 540 cm^{-1} combine.~~

~~After mineralization, in the spectra of chitosan-collagen gels with ALP the range of the saccharide band, also all bands 1110 cm^{-1} , 1080 cm^{-1} and 1050 cm^{-1} combine. In gels of higher concentrations of collagen, the band at 980 cm^{-1} becomes more visible (than in gels without collagen), similarly to mineralized chitosan gels, a band at 870 cm^{-1} appears, a wide asymmetrical band at wavenumber 780 cm^{-1} decreases its intensity after the addition of ALP and divides into two bands at 820 cm^{-1} and 780 cm^{-1} , the intensity of band 660 cm^{-1} decreases and its arm 650 cm^{-1} disappears; bands 590 cm^{-1} and 540 cm^{-1} combine.~~

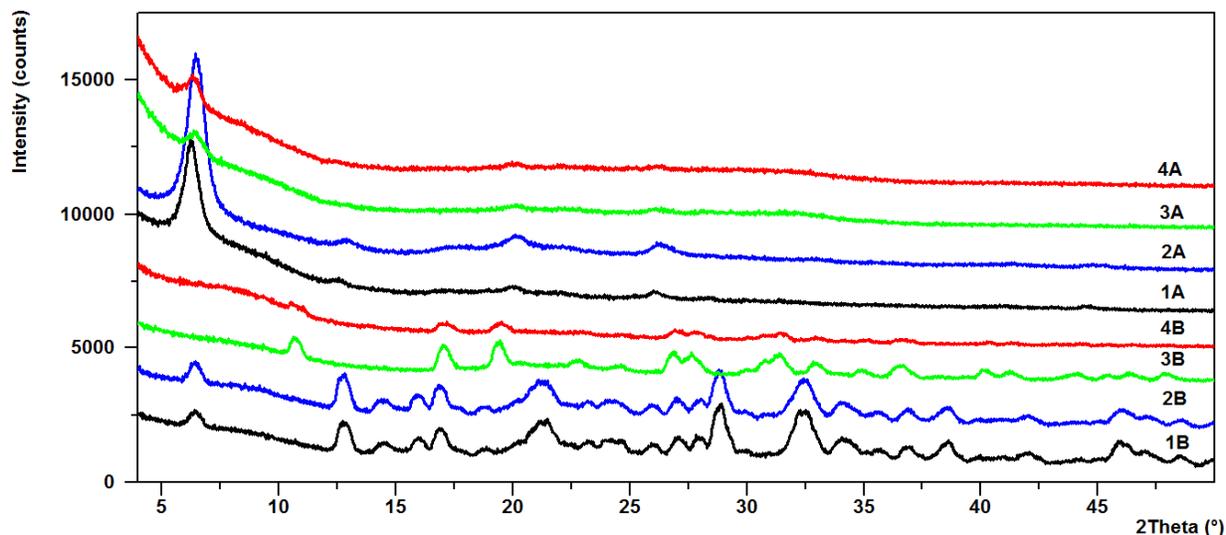


Figure 8. XRD diffraction patterns before (B) and after (A) mineralization for chitosan gel (1); chitosan-collagen 1.0 ml gel (2); chitosan-ALP gel (3) and chitosan-collagen-ALP (4) gel respectively

Significant changes in the crystalline structure are observed after the introduction of ALP into the chitosan gel (Figure 8). Before mineralization, there are several maxima observed at an angle of 2θ about 10.7° , 17.0° , 19.4° , 26.9° , 27.6° , 31.4° , 32.9° and 36.6° . In chitosan-collagen-ALP gels these peaks are also present but of less intensity. After mineralization, the structure of both chitosan and chitosan-collagen-ALP containing gels becomes more amorphous, with only a wide peak at an angle of 2θ of about 6.4°

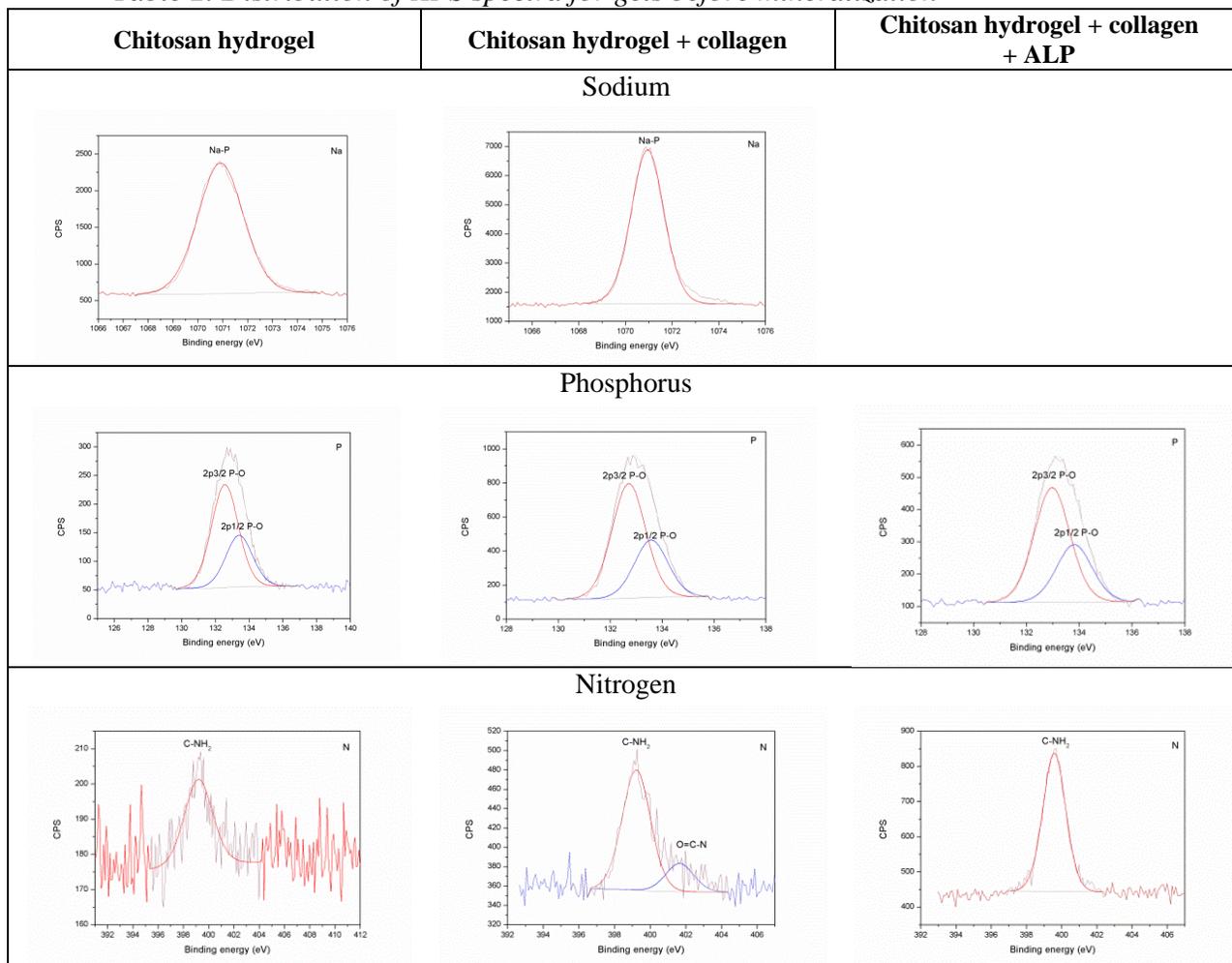
A change of the structure of chitosan-collagen hydrogels before and after the mineralization process was determined on the basis of FTIR spectra and confirmed by XPS analysis. The XPS results are presented in Table 1, Table 2 and Table 3.

Table 1. Analysis of XPS spectra

Groups		Chitosan hydrogel		Chitosan hydrogel + collagen		Chitosan hydrogel + collagen + ALP		Chitosan hydrogel + ALP		Chitosan hydrogel + collagen + ALP			
		Before mineralization						After mineralization					
		Binding energy (eV)	% atm contribution of groups	Binding energy (eV)	% atm contribution of groups	Binding energy (eV)	% atm contribution of groups	Binding energy (eV)	% atm contribution of groups	Binding energy (eV)	% atm contribution of groups		
Na		1070.8	16.5	1070.9	17.7	1071.00	8.89						
			16.5		17.7		8.89						
O 1s	NH(C=O)-C	530.16	14.1	530.46	20.4	530.87	14.35	531.55	7.95	530.72	26.11		
O 1s	C-OH	531.91	19.3	532.28	19.8	532.48	18.17	533.21	30.60	532.58	21.32		
O 1s	C-O-C	535.65	9.6	535.61	6.9	535.98	4.43						
	$\beta(1\rightarrow4)$		43		47.1		36.95		38.55		47.43		
N 1s	C-NH ₂	399.18	0.9	401.65	1.2	399.58	3.21	399.67	2.94	399.21	2.61		

N 1s	O=C-N			399.22	0.3			400.64	2.52	401.24	0.56
N 1s	C-NH ₃ ⁺							402.31	0.76		
Ca 2p3				0.9	1.5		3.21		6.22		3.17
Ca 2p1				346.98	0.1			347.65	1.13	346.90	5.56
				350.48	0.0			351.21	0.57	350.44	2.78
					0.1				1.70		8.34
C 1s	C-C C-H	284.60	10.0	284.60	8.8	284.60	17.71	284.60	7.62	284.60	9.31
C 1s	C-NH ₂	285.97	9.0	286.06	12.5	285.83	17.22	285.89	20.94	286.32	9.81
C 1s	C-OH		6.9	287.62	3.3	286.55	5.93	286.78	12.75	287.62	4.56
C 1s	C-O-C	288.12									
C 1s	NH(C=O)			289.03	0.8	287.70	4.25	287.69	6.70	285.82	7.97
C 1s	O-C-O							288.83	2.52	288.72	1.23
	(C=O)-O								50.52		32.88
			49.48		25.3		45.11				
Cl 2p3		198.49	3.8	198.35	0.5	197.38	0.24				
Cl 2p1		200.09	1.9	199.95	0.2	198.98	0.12				
Cl 2p3						199.24	0.69				
Cl 2p1						200.84	0.34				
			5.8		0.7		1.39				
P 2p3		132.56	4.7	132.71	5.1	132.97	2.97		2.00	132.76	5.46
P 2p1		133.40	2.4	133.57	2.5	133.81	1.48		1.01	133.60	2.73
			7.1		7.6		4.45		3.01		8.19
Ca/P									0.56		1.018

Table 2. Distribution of XPS spectra for gels before mineralization



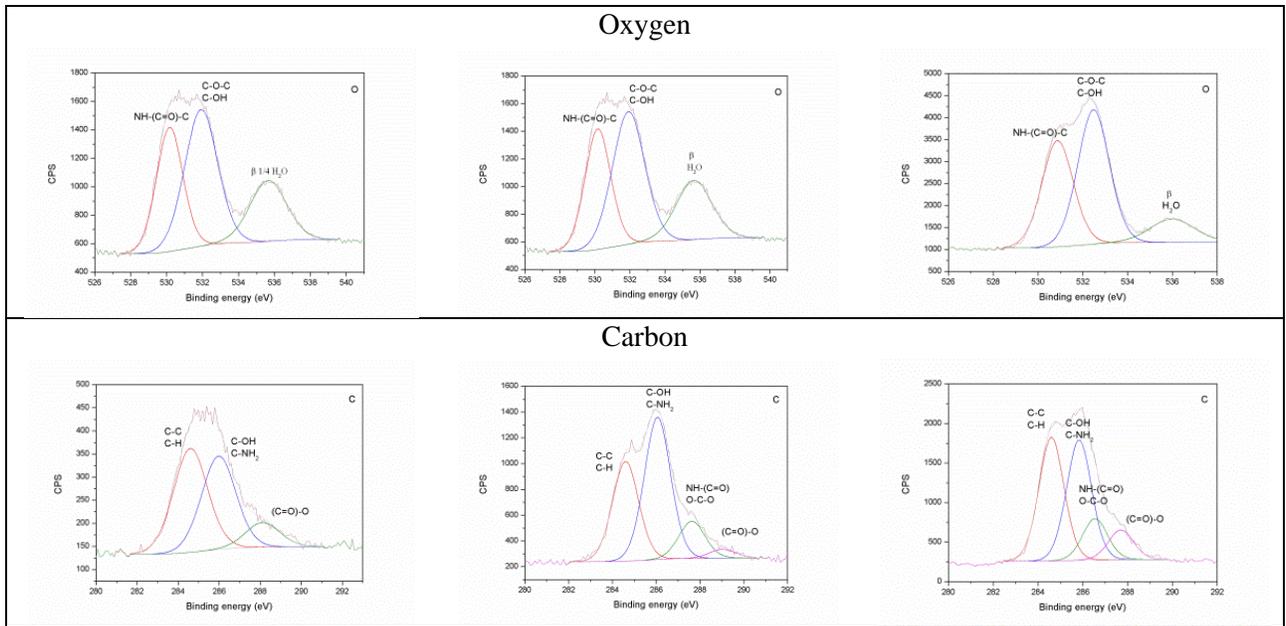
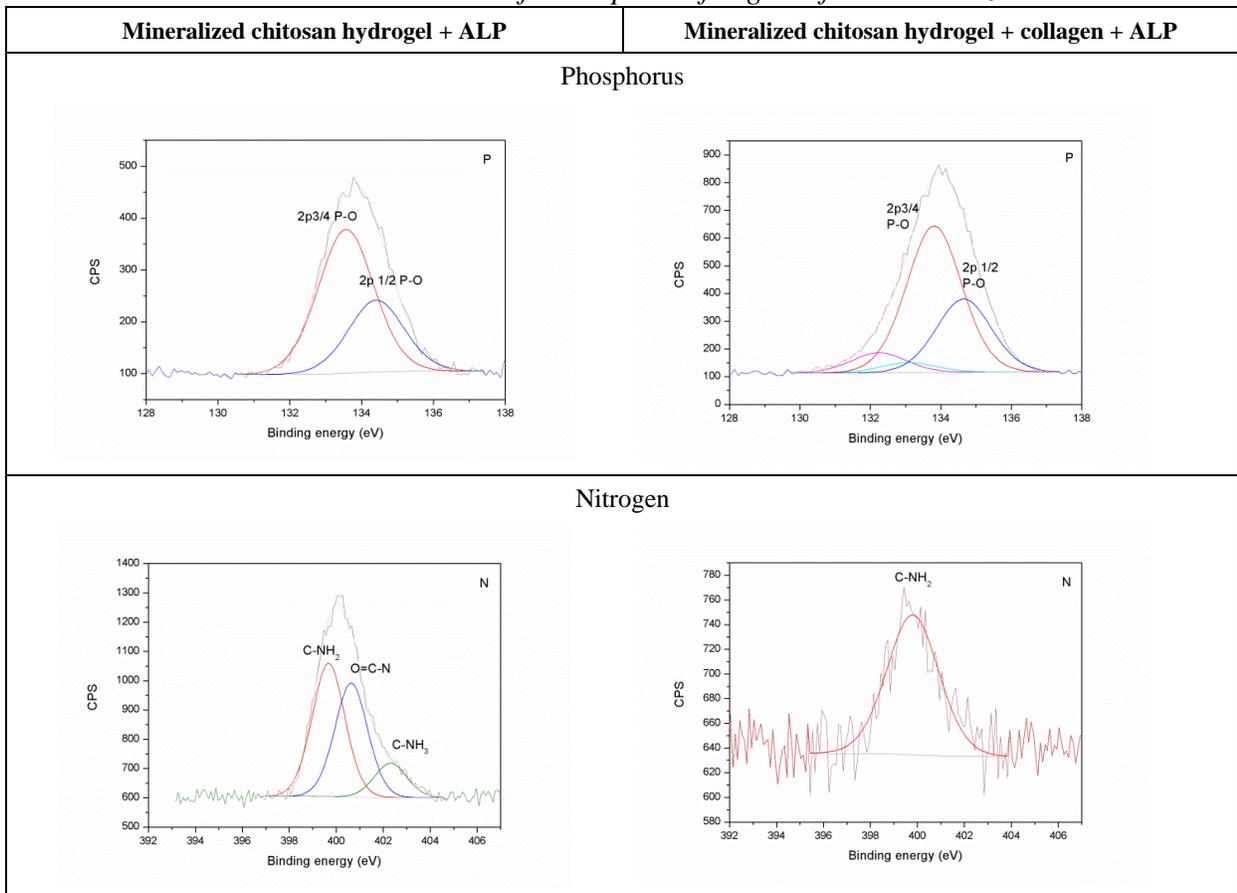
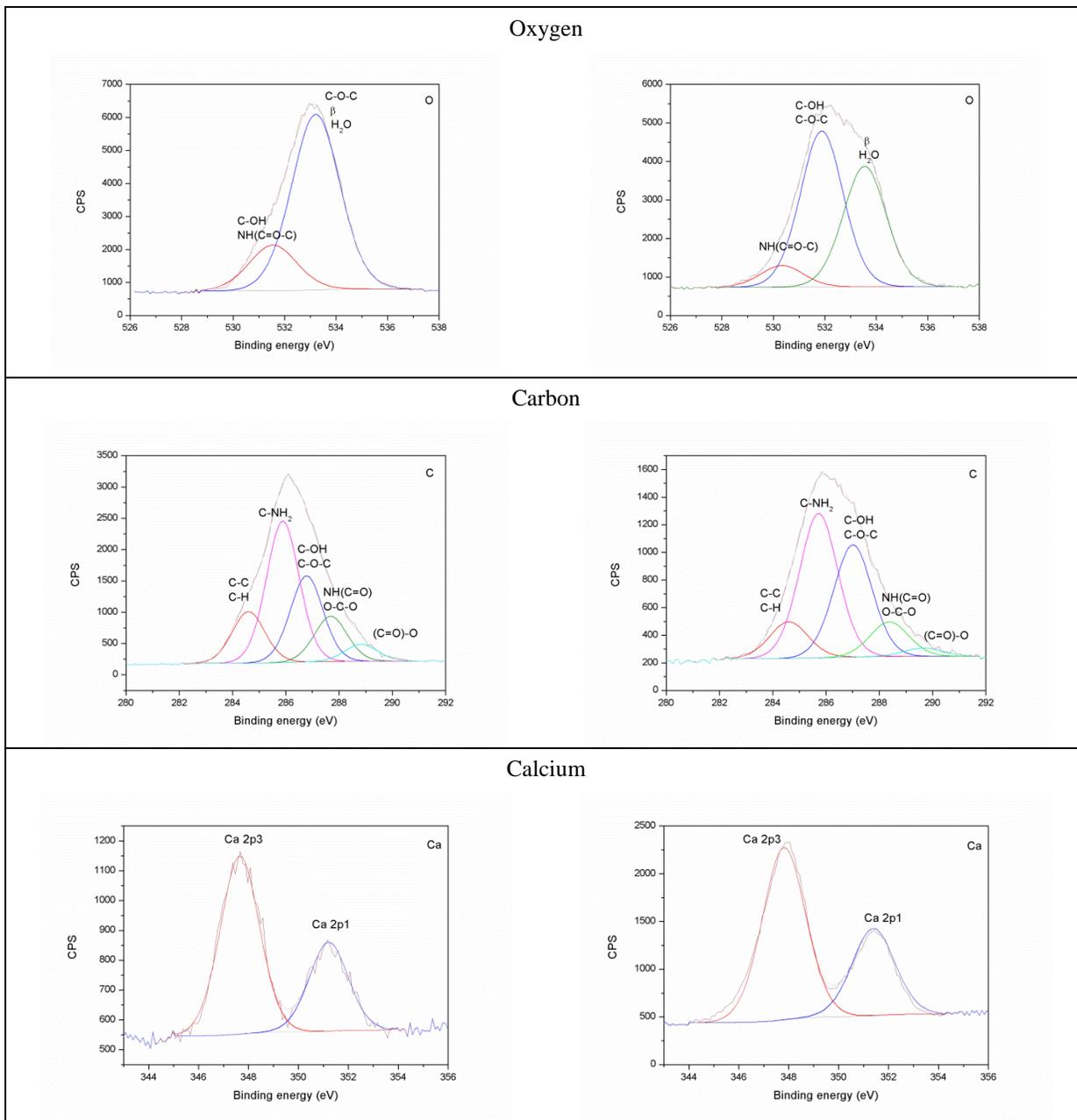


Table 3. Distribution of XPS spectra for gels after mineralization





The results of the XPS analysis suggest that, after the introduction of collagen into the chitosan gel structure, the number of NH-(C=O)-C bonds increases, while the number of bonds $\beta(1 \rightarrow 4)$ decrease, the percentage of nitrogen slightly decreases, the percentage of carbon falls dramatically, and also the number of C-C, C-H, C-N and C-OH bonds decrease, while phosphorus remains unchanged. ~~This suggests the possibility of combining collagen with chitosan and C1 carbon, as well as with amine groups and hydroxide.~~

In the case of the addition of ALP to the chitosan-collagen gel a clear decrease of NH-(C=O)-C can be observed which is connected with the participation of acetylamine groups in the reaction with ALP. This is confirmed by FTIR spectra where the arm at wavenumber 1700 cm^{-1} of amide band 1660 cm^{-1} disappears. After the addition of ALP, in the XPS spectra an increase of amine groups takes place – they are revealed and phosphorus disappears, so ALP

probably conjugates the phosphate residue interacting with electrostatic interactions with amine groups; the number of C-C, C-H increases, which confirm the breaking of glycosidic bonds.

After the mineralization of both chitosan gels and chitosan-collagen gels the bonds most likely break because oxygen bands corresponding to bonds $\beta(1\rightarrow4)$ disappear.

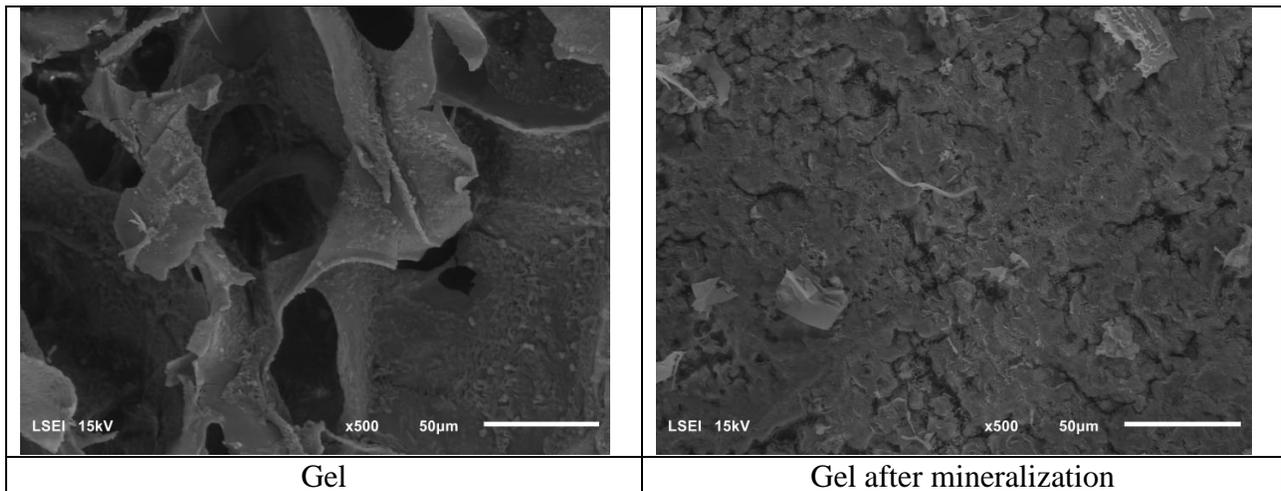
After the mineralization of chitosan gels containing ALP, significant changes were observed in relation to chitosan gels before the mineralization; the proportion of NH-(C=O)-C groups decrease, while the proportion of C-OH C-O-C bonds increase, also nitrogen and the number of assigned bonds C-N C-OH, (C=O)-O increase.

After the mineralization of chitosan-collagen gels containing ALP, significant changes in comparison with pure chitosan-collagen gels take place at the following bonds: the proportion of NH-(C=O)-C groups increases and C-OH slightly increases, the percentage of C-C, C-H C-N C-OH, (C=O)-O groups remains at the same level and the proportion of nitrogen rises; if those changes are compared with chitosan-collagen hydrogel containing ALP before mineralization, the number of C-C, C-H C-N C-OH bonds significantly decrease, whereas nitrogen remains at the same level. Thus, ALP in chitosan-collagen gels is bound by NH-(C=O)-C groups while during the mineralization process CaGP was decomposed by ALP to Ca and P and disconnected from NH-(C=O)-C groups. Due to the fact that the spectrum of O and C of chitosan-collagen gel containing ALP after mineralization is close to the pure spectrum of chitosan-collagen gel before mineralization, the compounds of Ca and P were created in the hydrogel pores.

24-hour mineralization of chitosan-collagen gels containing ALP allows compounds of Ca and P with Ca/P atomic ratio close to 1.0 to be obtained, while mineralization of chitosan gels containing ALP results in an atomic ratio of Ca/P equal to approximately 0.6.

After the mineralization in the gels containing ALP, 1.7 % of Ca was obtained and 8.3 %

SEM images of freeze-dried hydrogels after incubation in mineralization solution after 24 hours can be seen in Figure 9.



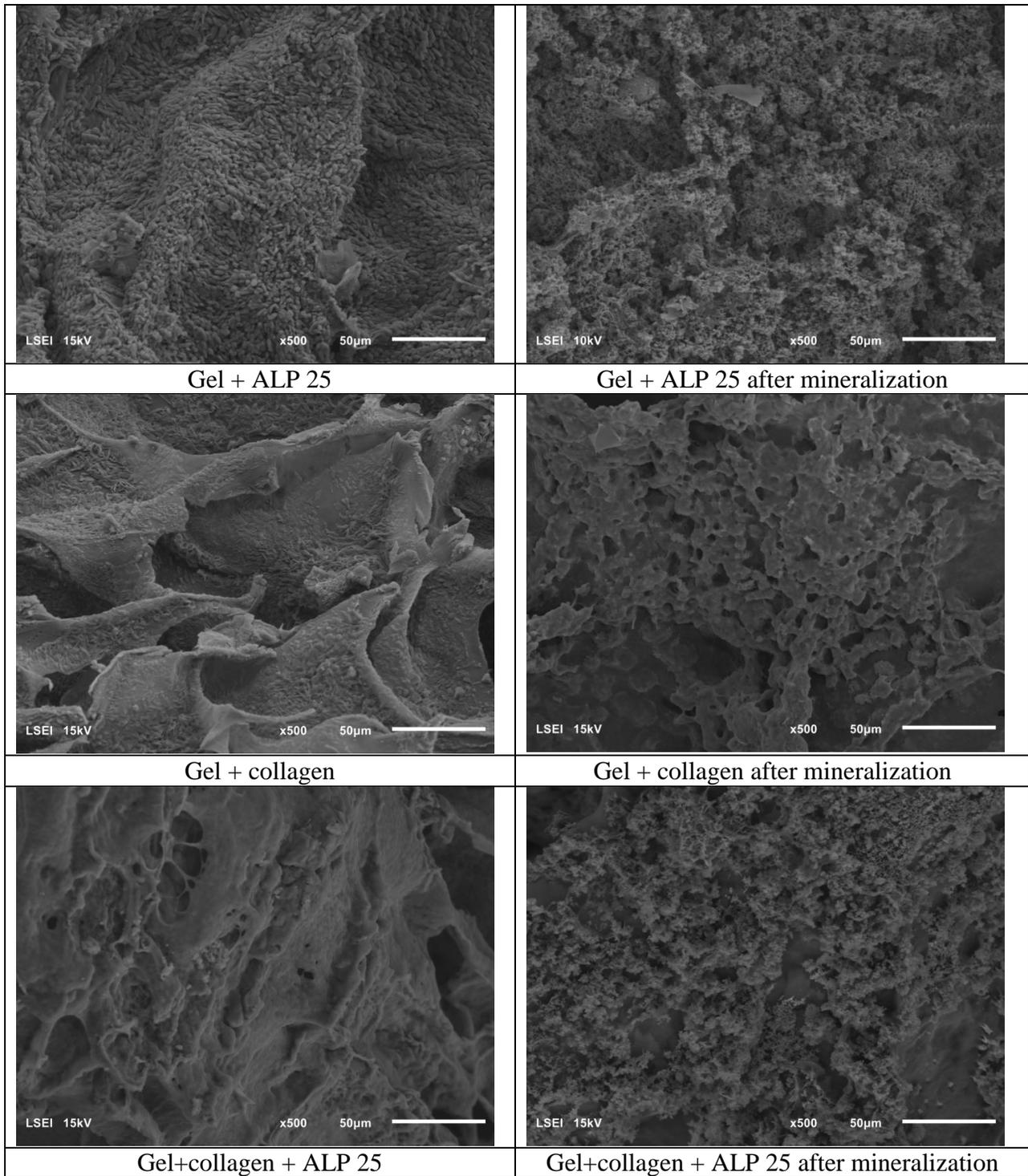


Figure 9. SEM images of the gels before and after mineralization

All samples, both containing ALP and collagen contained platelet-shaped structures. ~~The structure of ALP-containing gels is greatly expanded.~~ However, such structures displayed different, rougher morphologies in samples containing ALP and collagen after mineralization in

~~The structure of gels containing collagen and ALP are more compressed~~—comparison to the structure of gels without ALP. After mineralization especially, in gels containing ALP the mineral-like deposits were present.

Conclusions

Mineralization of chitosan gels and chitosan-collagen gels have a similar effect on the changes of the gel structure. The spectra indicate a very strong influence of this process on the resonant frequencies of the grouping vibrations in the whole spectrum. We observed a deformation of the band of oscillators -O-H and N-H and a change for bands connected with C-H groups. This proves that significant changes in the conformation of chains and changes in the supermolecular structure of the obtained gels are taking place. Tensions in the polymer chains are revealed through an increase of resonant frequencies of C-O groupings occurring both in the heterocyclic ring and in the oxygen bridge. They all shift towards higher vibration energies after the mineralization process.

Mineralization of chitosan-collagen gels containing ALP indicates the influence of ALP on aliphatic phosphatase and it is probably connected with a strong interaction on the N-H oscillator in the amide grouping and the creation of strong ionic bonds. ~~An increase of ALP concentrations causes the separation of macromolecules, thus releasing them from the strong mutual deformations.~~ The increasing ALP concentrations cause a relaxation of the polymer and shifting of the oscillation band C-N in the amide group towards lower wavenumbers, which is revealed as an appearance of this band in the spectra. The addition of ALP does not reduce the tensions in the oxygen bridges, which is proved by the tension remaining along the chain.

The XPS analysis suggests that collagen and chitosan in a solution of hydrochloric acid is connected through C1 carbon, and also amine and hydroxide groups; ALP in the chitosan-collagen gel reacts with NH-(C=O)-C groups and during the mineralization process CaGP was decomposed by ALP to Ca and P and disconnected from NH-(C=O)-C groups, ~~hence indicated formation of amorphous calcium phosphate Conjugates of the phosphate residue interacting with amine groups, the glycosidic bonds break; compounds of calcium and phosphorus are created in the hydrogel pores.~~

1.7 % of calcium was obtained after the mineralization chitosan hydrogels containing ALP and 8.3 % in the structure of chitosan-collagen gels containing ALP, thus it is five times more Ca, and the Ca/P ratio is close to that of hydroxyapatite and such chitosan hydrogel containing collagen and alkaline phosphatase may pretend for use for biomedical applications.

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HIGHLIGHTS

- Chitosan hydrogels containing ALP and collagen have been prepared
- Results before and after mineralization are compared, showing clear differences
- Apatite-like mineral within chitosan hydrogels after mineralization were discussed

