STRUCTURAL RESEARCH OF THERMOSENSITIVE CHITOSAN-COLLAGEN GELS CONTAINING ALP

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Abstract

Introducing collagen, which is basic ingredient of bone tissue, into the structure of chitosan gels which are formed at the physiological body temperature, is aimed at creating the so-called biomimetic structures, i.e. close in their composition to the natural composition of bone tissue. Within the research the influence of collagen on structural properties of thermosensitive chitosan gels and the influence of ALP on structural properties of chitosan and chitosan-collagen gels was determined.

Key words: *thermosensitive hydrogels, collagen, chitosan, alkaline phosphatase,*

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1. Introduction

Alkaline phosphatase (ALP) participates in bone mineralization processes, which is the reason for interest in it also in the case of scaffolds for osteoblast culture.

Hydrogels formed under the influence of temperature increase (thermosensitive gels) are one of the most interesting forms for used as scaffolds for cell culture, due to the possibility of injecting of the scaffold in the tissue which requires reconstruction [1-5].

Chitosan is well polymer for the use as scaffolds for cell culture. This polymer possesses a range of properties predisposing it to this type of use: it is biocompatible and biodegradable. It can be transformed into utility forms as membranes, sponges, micro-pellets or nanopellets, fibres and capillary fibres. These forms may be in the shape of hydrogels, dried forms (through lyophilization, by atomization method or in supercritical conditions), or matrixes woven from fibres created for instance by the method of electrospinning [6-11].

All the above forms could pose interesting material for scaffolds, especially hydrogels formed under the influence of temperature increase, are currently the subject of intensive research for use as scaffolds. They are interesting due to the presence of water in the structure, which makes them similar to live tissue; moreover, they are soft and flexible, which minimizes the risk of damage of the surrounding tissue during their implantation and secures the functional and morphological qualities of the regenerated tissue. They constitute a more efficient environment which ensures faster cell growth, because the water present in the structure allows permeability for oxygen and other metabolites soluble in water, and moreover their hydrophilicity is connected with the process of cell adhesion, which enables greater cell flattening [5].

The current research concerns the evaluation of the structure of chitosan-collagen gels containing alkaline phosphatase.

Introducing collagen, the basic ingredient of bone tissue, into the structure of chitosan gels which are formed at the physiological body temperature, is aimed at creating the so-called biomimetic structures, i.e. close in their composition to the natural composition of bone tissue. Chitosan-collagen systems, including those formed at the human body temperature are known in literature [12-14], whereas chitosan-collagen hydrogels containing alkaline phosphatase have not been published before.

Within the research were determined:

1. The influence of collagen on structural properties of thermosensitive chitosan gels,
2. The influence of ALP on structural properties of chitosan gels,
3. The influence of ALP on structural properties of chitosan-collagen gels.

2. Materials and methods

Thermosensitive chitosan gels were prepared according to the method described by Chenite [15]. To prepare hydrogels shrimp chitosan (Sigma Aldrich) of a degree of acetylation AD ~ 19,5% and molecular weight of 860 kD was used. 0.4 g of chitosan was dissolved in 16 ml of 0.1 M HCl (Sigma Aldrich). The chitosan solution was left on the mechanical shaker for approximately 12 h to ensure complete dissolution. At the same time 2 g of sodium β-glycerophosphate (Na-β-GP) (Sigma-Aldrich Product no. 50494) was dissolved in 2 ml of deionized water (18 mS). Subsequently at a low temperature, i.e. in the conditions of crushed ice and with vigorous stirring, a solution of Na-β-GP was added to the chitosan solution (sol) drop by drop. The resulting chitosan salt solution was cooled to 4°C.

Thermosensitive chitosan gels containing alkaline phosphatase ALP (SIGMA Aldrich Product no. P) were created by adding. 1 ml of alkaline phosphatase dissolved in deionized water at concentration of 2.5; 10; 25 mg/ml to the 16 ml chitosan chloride containing Na-β-GP.

Thermosensitive chitosan-collagen gels were created by adding 1 and 0.5 ml 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 alkaline phosphatase dissolved in deionized water at concentration of 2.5; 10; 25 mg/ml in to a chitosan salt chitosan chloride containing Na-β-GP and collagen. The resulting solutions were incubated at 37°C for 24 h in order to complete their gelation.

The structural properties of the gels were determined by infrared spectroscopy with Fourier transformation (FTIR) using a Nicolet 6700 spectrometer (Thermo Nicolet) equipped with a snap Photoacoustics MTEC model 300.

3. Results and Discussion

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

Figure- 1 present the FTIR spectra of thermosensitive chitosan gels with the addition of 0.5ml and 1.0ml of collagen in the scope of 4000-500cm^{-1} and 2000-500cm^{-1} .

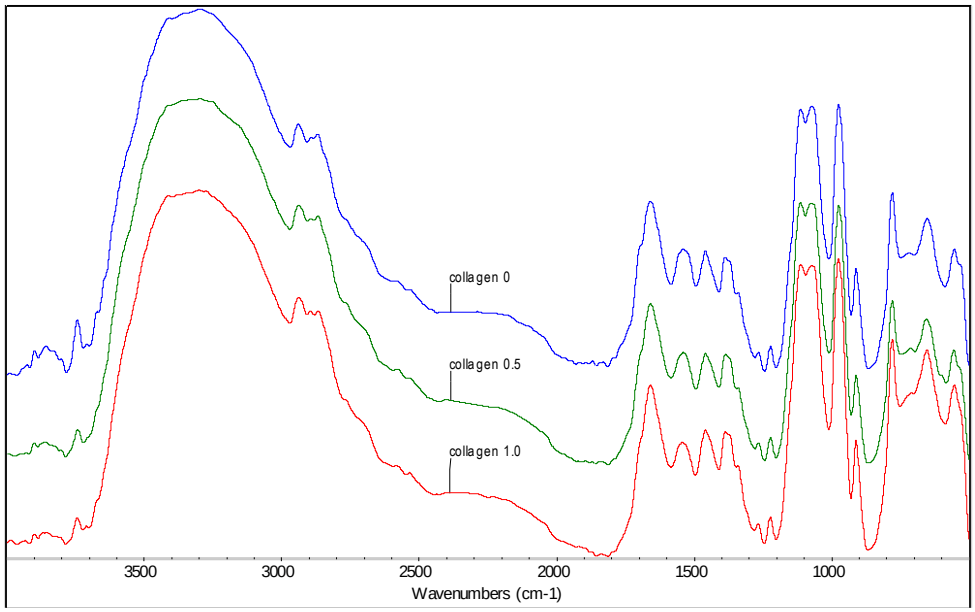

Figure 1. FTIR spectra for chitosan gel without collagen and containing collagen in the amount 0.5 and 1.0ml in the scope of 4000-500cm^{-1}.

The presence of collagen in these amounts does not cause any significant changes to the oscillations of chemical groupings observed by means of FTIR spectra.

After the introduction of collagen to the structure chitosan gels can observe a lower intensity of the arm of amide I band occurring for wave number 1700cm^{-1} and the amide II band of secondary amides for wave number 1560cm^{-1} [16].

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

The FTIR phantoms of samples of chitosan and chitosan-collagen hydrogels containing ALP in the amount of 0.0ml; 2.5ml; 10ml and 25ml are presented on the figures (Fig.2 – 4).

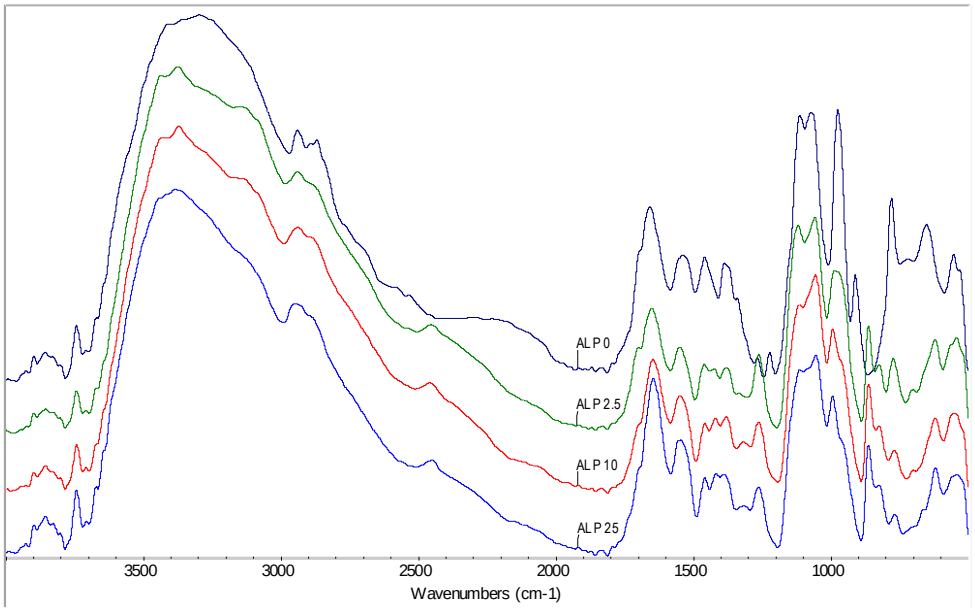

Figure 2. FTIR spectra for chitosan hydrogel with ALP; ALP 0; 2.5%; 10%; 25% in the scope of 4000-500cm^{-1}.

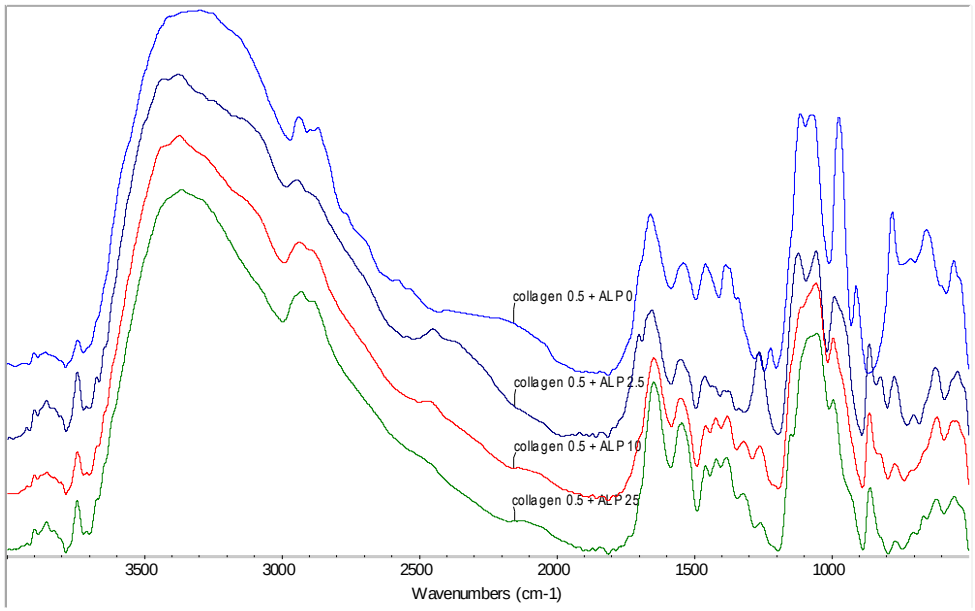

Figure 3. FTIR spectra for chitosan-collagen hydrogel containing 0.5 ml collagen and ALP 2.5%; 10%; 25%in the scope of 4000-500cm^{-1}.

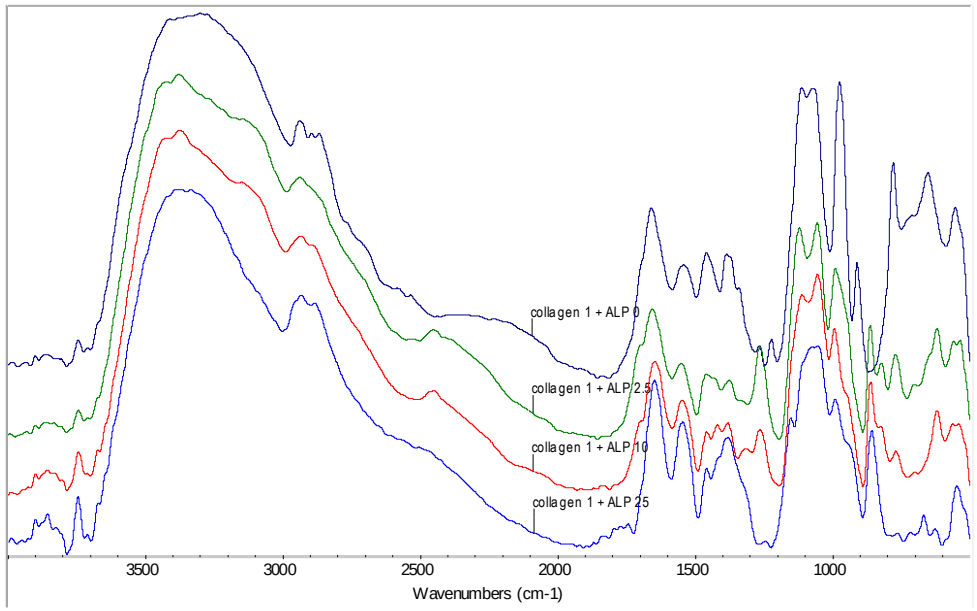

Figure 4. FTIR spectra for chitosan-collagen hydrogel containing 1 ml collagen and ALP 2.5%; 10%; 25%in the scope of 4000-500cm^{-1}.

Changes in the FTIR phantoms of chitosan hydrogels containing ALP can be observed in the scope of wave numbers 3000-3500cm^{-1} (Fig.3), but the main differences occur in the scope of wave numbers 1100-700cm^{-1} (Fig.4), which correspond with the saccharide structure, and in the scope of wave numbers connected with the presence of phosphate ions.

Spectrum of chitosan hydrogels containing ALP in the scope of wave numbers 3600-3100cm^{-1}, similarly to hydrogels without ALP, have a wide, asymmetrical band corresponding to O-H vibrations. The asymmetry is visible while moving towards lower frequencies, and is higher than in the case of spectrum of hydrogels without ALP, which indicates the presence of strong hydrogen-oxygen bonds and amine groups N-H in the structure.

In the scope of wave numbers 2800-2970cm^{-1} the spectrum of chitosan hydrogel is divided into three maximum bands at the wave numbers 2870cm^{-1}, 2890cm^{-1} and 2940cm^{-1}. These bands derive from stretching vibrations of aliphatic groups ($-CH_2$ and $-CH_3$) and are characteristic of a chitosan ring. For hydrogels containing ALP on the obtained bands in this scope the two maximum bands can be observed: at the wave number 2940cm^{-1} and 2890cm^{-1}

Moving towards lower wave numbers on the obtained bands containing ALP, a new band can be observed at wave number 2450cm^{-1}.

In the spectrum of chitosan hydrogel (Fig.4) amide I band for wave number 1658cm^{-1} is distinguishable; these are vibrations corresponding to C=O (N-acetylamine group) and amide II band of secondary amides for wave number 1540cm^{-1} NH_2. These peaks indicate that chitosan is directly the product of chitin deacetylation. The presence of ALP in the structure makes the intensity of bands connected with amine and acetyl-amine groups decrease, which would indicate their participation in the reaction with ALP.

The major changes can be observed in the scope of wave numbers 1200-900cm^{-1}; in the case of chitosan hydrogel spectrum one can observe bands for wave numbers 1110, 1070,

975, 910, 780, 650 and 554cm^{-1} – these are bands derived both from the saccharide structure and resulting from the presence of glycerophosphate GP:

1. bands for wave numbers 1070cm^{-1} and 780cm^{-1} are characteristic for GP and correspond to stretching vibrations P-O-C;
2. a band for wave number 975cm^{-1} characteristic for (-PO_4^{3-})
3. a band for wave number 910cm^{-1} indicating the presence of groups (-HPO_4^{2-}).

For pure chitosan one can distinguish several overlapping bands: for wave numbers 1110; 1070, 975cm^{-1} and a clear maximum for wave number 910cm^{-1}. These are bands characteristic for a saccharide structure. A connection of oxygen bridges (C-O-C) similar to that of ether is probably connected with the peaks for wave numbers 1115–1070cm^{-1}, whereas the peak for wave number 910cm^{-1} – with groups CH_3COH.
A wide band for wave number 650cm^{-1} can also be attributed to amide structures and it is caused by the vibrations of bonding systems O=C-N.

After the introduction of ALP, the spectrum in this scope of wave numbers does not change: the intensity of band 1110cm^{-1} decreases, band at 1070cm^{-1} moves towards lower wave numbers 1055cm^{-1}, the intensity of band for wave number 975cm^{-1} decreased and for the larges addition of ALP moves towards to higher wave numbers 994 cm^{-1}; the bands for wave number 910 and 780cm^{-1} decrease, and the band for wave number 650 cm^{-1} moves towards lower wave numbers 610cm^{-1}. While, in spectra obtained after adding ALP, bands appear for wave numbers at 860cm^{-1} and 820cm^{-1}.

Changes in the FTIR spectrum of chitosan-collagen hydrogels (for both concentrations of collagen) containing ALP can be observed similarly to hydrogels without collagen in the scope of wave numbers 3000-3500cm^{-1} and 1100-700cm^{-1}.

In the spectra of chitosan-collagen hydrogels (Fig.3-4) containing ALP a wide, asymmetrical band corresponding to O-H vibrations in the scope of wave numbers 3600-3100cm^{-1} can be observed. The asymmetry is visible while moving to the lower frequencies, and is higher than in the case of spectra of hydrogels without ALP, which indicates the presence of strong hydrogen-oxygen bindings and amine groups N-H in the structure.

In the scope of wave numbers 2800-2970cm^{-1}, the spectrum of chitosan-collagen hydrogel without the addition of ALP is divided into three bands whose maximum are located at wave numbers 2870cm^{-1}, 2890cm^{-1} and 2940cm^{-1}. For chitosan-collagen hydrogels containing ALP can observed two bands at maximums 2940cm^{-1} and 2880cm^{-1} .

Moving towards lower wave numbers in spectra of chitosan-collagen hydrogels one can observe a very wide band 2500-2050 cm^{-1}; after introducing ALP this band disappears and similarly to spectra of chitosan hydrogels, the band appears at wave number 2450cm^{-1}, its intensity decreases together with an increase in ALP concentration in the gel. It is definitely lower than in the case of chitosan gels containing ALP.

In the scope of amide I band and amide II band of secondary amides in spectra of chitosan-collagen gels one can observe bands for wave numbers 1660 cm^{-1} with a slight asymmetry towards higher wave numbers (1700 cm^{-1}) and a number of bands for wave numbers 1540, 1460, 1380, 1340, 1270, 1220cm^{-1}. For low concentrations of ALP (2.5 mg/ml) the band 1700cm^{-1} is more visible; however for concentration of ALP 25 mg/ml the band disappears and the intensity of band 1540cm^{-1} increases In the scope of low ALP concentrations, similarly to spectra of chitosan gels, a very intensive band 1260 cm^{-1} , is present; however the intensity of this band decreases together with the increase of ALP concentration (at high ALP and collagen concentrations the band disappears).

In the scope of wave numbers 1200-900 cm^{-1}, spectra of chitosan-collagen and chitosan hydrogels are similarly; one can observe bands for wave numbers 1115 cm^{-1}, 1070cm^{-1}, 975cm^{-1}, 910cm^{-1}, 860cm^{-1}, 650cm^{-1} and 555cm^{-1}.

After introducing ALP the changes of spectra of chitosan-collagen and chitosan hydrogels are different and depend on the concentration of ALP. For low concentrations of

ALP the bands 1115cm^{-1} and 1055cm^{-1} are separated and at high concentrations of ALP they combine to; at high concentrations of ALP one can see a new band for wave number 1150cm^{-1}; the intensity of band for wave number 975cm^{-1} definitely decreases, and together with the increase of ALP concentration it becomes more asymmetrical. A band appears for wave number 860cm^{-1} with arm 820cm^{-1}, the arm disappears at high ALP concentrations. The band for wave number 910cm^{-1} disappears, the band for wave number 780cm^{-1} decreases intensity, and the band for wave number 650cm^{-1} moves towards lower wave numbers 610cm^{-1}.

Table 1. The analysis of changes in the structure of hydrogels.

		Chitosan hydrogel	**Chitosan hydrogel + ALP**	**Chitosan-collagen hydrogel**	**Chitosan-collagen hydrogel +ALP**
3600-3100	γ(O-H) overlapped to γ_s(N- H)	wide, asymmetrical	higher asymmetry	wide, asymmetrical	higher asymmetry
2870; 2890; 2920	γ_{as}(C-H) γ_a(C-H)	three bands present	lower intensity of band 2890 cm^{-1}.	two bands present	lower intensity of band 2890 cm^{-1}
2500-2050	ν_{OH}	lack	lack	present	in decline
2450		lack	present	lack	present intensity decreases with the increase of ALP concentration
1660	γ (C=O) amide I	slight asymmetry towards higher wave numbers	intensity is decreasing	slight asymmetry towards higher wave numbers 1700 asymmetry is decreasing in comparison to chitosan gel	clear band 1770
1540	$\delta s(NH_2)$	present	intensity is decreasing	present	intensity is slightly decreasing
1460	ν_{CO} (CO_3^{2-})	present	present	present	band 1450 is divided into two: 1465 1430-1420
1420		lack	present	lack	present
1380	δs(C-H) amide group	present	present	present	lower intensity
1340		present	in decline together with the increase of ALP concentration	present	in decline

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1310	δ(O- H)	not present	present	present	not present
1260		present	increase of intensity	present low intensity	clear at low ALP concentrations together with the increase of concentration lower intensity
1110	γ_{as}(C-O-C) γs(C-O- C)	present	intensity decreases	present	in the scope of low ALP concentrations bands 1110 and 1050 cm^{-1} are separated, at high ALP concentrations they are joined; for high ALP concentrations you can see a new band for the wave number 1150 cm^{-1}
1070 - 1050	γ_{as}(C-O-C) γs(C-O- C) γ_3 (PO_4^{3-})	present	present	present	
975	γ_1 (PO_4^{3-})	present	intensity decreases	present, high intensity	distinctly decreasing intensity together with the increase of ALP concentration is becoming more asymmetrical, which indicates the presence of a band for wave number 950 cm^{-1}
910	γ_1 (PO_4^{3-})	present	in decline	present	in decline
860	ν_{CO} (CO_3^{2-})	not present	present		a band for wave number 870 cm^{-1} appears with arm 830 cm^{-1}, the arm disappears at high ALP concentrations
780	γ_{PO} P-O-C		intensity decreases		intensity decreases
650			moving towards lower wave numbers		650 cm^{-1} is moving towards lower wave numbers

The reactivity of alkaline phosphate (ALP) depend on the pH. In FTIR spectra of alkaline phosphatase (in pH =7 which corresponding to the conditions in hydrogel) can observed bands in the scope of amide I band and amide II band of secondary amides and bands for wave numbers. 1340cm^{-1}, 1260cm^{-1}, 1110cm^{-1} and 980cm^{-1} [17-18]. In spectra of chitosan hydrogels containing ALP in their structure changes can also be observed in this scope of wave numbers; mainly in the scope of amide I band and amide II band of secondary amides; moreover a band 1260 cm^{-1} appears, and changes take place in the scope of band of saccharide structure and those resulting from the presence of glycerophosphate GP (1110 – 830)cm^{-1}.

As a result of adding small amounts of ALP in proportion to the weight of chitosan gel one obtains a specific effect of a significant change of spectra. In the scope of oscillation of stretching groupings -OH and N-H at circa 3400cm^{-1} a change is visible - sharp tips are formed. This phenomenon can be explained by the occurrence of groupings -OH and -NH taking part in intermolecular bonds in various places of the polymer chain in the same or similar impacts of energy. This means that the supermolecular structure of chitosan is more organised.

A juxtaposition of spectra in Figure 4 on which you can observe deformation oscillations of groups containing bondings C-O and C-O-C in a wide scope of wave numbers depending on the presence in a given grouping. Spectra of chitosan gel after adding ALP show very significant changes in the supermolecular construction regardless of the amount of the addition.

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 enabling an unconstrained migration of molecules during binding. The result of this phenomenon is for instance a bond moving circa from 910cm^{-1} to 864cm^{-1}. Increasing the amount of ALP has influence on the changes in conformation, probably due to earlier saturation of groupings forming intermolecular bonds.

Juxtapositions of FTIR spectra of chitosan-collagen gel samples and with the addition of ALP show a strong influence of additions on the conformation and the intermolecular form of gels probably triggered by blocking polar groupings by ALP; this influence is stimulated by collagen.

4. Conclusions

The presence of collagen in the structure of chitosan hydrogel causes changes in FTIR spectra in the scope of wave numbers corresponding to amide I band and amide II band of secondary amides. Introducing ALP into the structure of chitosan-collagen gels causes changes in FTIR spectra close to the changes observed in chitosan gels. The differences are visible at high ALP and collagen concentrations.

5. Acknowledgements

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6. References

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