

STRUCTURAL CHARACTERISTICS OF THERMOSENSITIVE CHITOSAN GLUTAMINATE HYDROGELS

**Zofia Modrzejewska, Katarzyna Nawrotek,
Roman Zarzycki, Timothy Douglas¹**

*Faculty of Process and Environmental Engineering,
Lodz University of Technology
ul. Wólczańska 175, 90-924 Łódź, Poland
E-mail: zofia.modrzejewska@p.lodz.pl*

*¹Polymer Chemistry and Biomaterials (PBM) group,
Ghent University,
Krijgslaan 281 S4, 9000 Gent, Belgium*

Abstract

Hydrogels that possess the ability of gelling in response to changes in the local environment, such as pH or temperature, have been examined extensively recently. In this paper the properties of thermosensitive chitosan hydrogels prepared with the use of chitosan glutamate and β -glycerophosphate are presented. The sol/gel transition point was determined based on the rheological properties. The structure of gels was observed under the Scanning Electron Microscopy (SEM) and was investigated by thermogravimetric (TG) and differential thermogravimetric (DTG) analysis and infrared (IR) spectroscopy. The crystallinity of gel structure was determined by X-ray Diffraction analysis (XRD).

Key words: *hydrogel; chitosan; natural polymer; structural properties.*

1. Introduction

Over the last few years, the great interest has been put in hydrogels prepared from both natural and synthetic polymers [1 - 4]. The main reason of this great attention is the broad spectrum of their applications. Hydrogels can be used for the controlled release of bioactive molecules and as tissue engineering scaffolds. Introduction of drugs into the structure of hydrogel, however, requires the use of suitable crosslinking, or coagulation agents which should not deactivate these active biomolecules. The use of hydrogels as scaffolds, is beneficial because it provides a water environment for cell culture. The main disadvantage of these systems is the fact that it does not guarantee uniform spatial distribution of cells within the site of their application.

Recent research has been focused on hydrogels that are able to undergo transition from sol to gel at the physiological temperature of human body [5 - 13]. The main advantage of this structures is the possibility to introduce cells to water environment of sol *in situ*. The structure of the hydrogel can additionally contain nutrients (proteins) or growth-supporting factors. Inclusion of cells in the entire gel structure of the matrix under the influence of normal human body temperature does not result in their destruction and enables a uniform distribution over the whole scaffold space. Moreover, pharmacological agents introduced into the gel formed under the influence of temperature are distributed throughout the volume, and are not inactivated by cross-linking agents or coagulation. Thermosensitive hydrogels are obtained on the basis of

- poly(N-isopropylacrylamide) (PNIPAm),
- poly(ethylene oxide-co-propylene oxide-co-polyethylene oxide)
- polyethylene glycol, a copolymer of lactic acid with glycolic acid and poly(ethylene glycol)
- polysaccharides: xyloglucan, ethylcellulose, hydroxyethylcellulose and chitosan.

One of the polymers with great potential is chitosan [14 - 21]. Chitosan is a natural polymer obtained in the process of chitin deacetylation.

Advantageous properties of chitosan include its ability to form chelate compounds, non-toxicity, biocompatibility with the human organism, biodegradability and ability to form gels in an acidic environment. Its therapeutic action is also known; as a polycation it combines with bile acids and decreases the level of LDL cholesterol and level of lipids. It is used as substance supporting weight loss diets. According to some researchers, by controlling acid and base management chitosan reveals anticarcinogenic properties. Others claim that it hampers excessive glycolysis in neoplastic cells by slowing down lactate production and decreasing the ATP level. It also decreases elasticity of neoplastic cells. Chitosan is also used as a dressing material. Its static and cidal action towards bacteria and yeast-like fungi has been proved.

The use of chitosan seems to be very promising in tissue engineering. Chitosan scaffolds are formed most often by lyophilization, biomimetic mineralization, particle aggregation, electrospinning and gelation under the influence of crosslinking agents (UV or temperature).

In this paper, a new form of chitosan gel with thermosensitive properties is presented. Hydrogels formed at the physiological temperature of the human body (37 °C). Hydrogels are elastic, soft and their properties are similar to properties of living tissues. The study focused on determining the change in the structure due to water conditioning.

Determination of structural changes of hydrogels in different environments is important when considering their envisaged application, e.g. as scaffolds for tissue regeneration. Structural changes during conditioning of the sample in demineralized water also allow elucidation of the mechanism of their formation.

2. Materials and methods

2.1. Preparation of chitosan hydrogels

Chitosan glutamate solution (the CH/GLU/GP system) was prepared by swelling 400 mg of chitosan (CH, chitosan from shrimp shells with low viscosity and degree of deacetylation ~79.5%, SIGMA-ALDRICH®) in 18 ml of distilled water for 30 min. Then, 300 mg of glutamic acid (GLU, Fluka®) was added to the solution. The solution was stirred (under slow rotation) until complete dissolution. Next, the sample was cooled to 4 °C. To this cooled sample, 2 g of β -glycerol phosphate disodium salt pentahydrate (GP, SIGMA-ALDRICH®) dissolved in 2.5 ml of distilled water was added dropwise under stirring in an ice bath. The final solution was mixed for another 20 min and stored. at 4 °C for 12 h.

2.2. Rheological measurements

The rheological properties of the solutions obtained were examined using a Rheoplus (Anton Paar GmbH) rheometer equipped with a parallel-plate sensor PP25-SN7371 in oscillatory mode. The storage modulus G' and loss modulus G'' were measured as a function of time using a gap of 0.500 mm and a frequency of 1.0 Hz. The sol/gel transition point was determined as the time at which viscous and elastic behavior of the solution exactly match (i.e. $G' = G''$). The research was carried out at 37 °C

The curves of the obtained storage modulus G' and loss modulus G'' are the mean of 3 independent measurements.

2.3. In vitro conditioning

Before the *in vitro* release studies, the samples were stored at 37 °C for 12 h, to obtain a gel structure. Gel samples were prepared in the shape of cylinders with a diameter of 3 cm and a height of 3 cm.

The *in vitro* release studies were performed using an ERWEKA apparatus which conforms to the requirements of the Pharmacopeia. The frequency of fluid mixing was fixed at 20 reversions/min. The release study was performed in distilled water with a capacity of 900 dm³ and a pH = 5 ± 0.5. The temperature of the release medium was kept constant at 37 °C during the whole release process.

2.4. Structural studies

The structural properties of hydrogels after preparation (the CH/GLU/GP BR system) and after conditioning in the water for 24 h (the CH/GLU/GP AR system) were studied.

The structure of gels was observed under the scanning electron microscopy FEI QUANTA 200 F and atomic force microscopy Nanoscope IIIa, Veeco.

Thermogravimetric (TG) and differential thermogravimetric (DTG) analysis were carried out with a Perkin-Elmer calorimeter V3 1E TA. All measurements were performed with 5 mg hydrogel samples under a dynamic nitrogen atmosphere in the temperature range from room temperature to 600 °C at a scanning rate of 10 °C/min and gas flow of 100 mL/min.

The structural characteristics were based on the analysis of XRD and IR spectra.

The X-ray diffraction patterns were determined using a wide-angle X-ray Siemens D5000 diffractometer and $K\alpha$,Cu radiation.

The FTIR spectra were obtained using a Bio-Rad apparatus in ATR-FTIR mode, in the range 4000 - 500 cm^{-1} with a resolution of 4 cm^{-1} and at 100 scans.

3. Results and discussion

3.1. Auto-gelling of the CH/GLU/GP system

Addition of β -glycerol phosphate disodium salt pentahydrate to the solution of chitosan dissolved in glutamic acid enables quick gelation at 37 °C.

The relationship between the storage modulus (G') and the loss modulus (G'') as a function of time is shown in *Figure 1*.

The phase transition occurs at the intersection of the storage and the loss modulus curves. The damping factor at this point is equal to 1 after 196 s. After this time, the systems exist in the gel state. Upon heating, the initial part of the storage modulus curve obtained is steeper. The sharp rise of this modulus upon heating indicates that the liquid solution passes into solid-like gel.

3.2. Structural characteristic of the CH/GLU/GP system

The SEM image of CH/GLU/GP BR system after lyophilization is shown on *Figure 2*. *Figure 3* shows the AFM images of CH/GLU/GP BR before conditioning in water and *Figure 4* AFM images of CH/GLU/GP AR after conditioning in water at 37 °C.

TGA and DTG for CH/GLU/GP BR and CH/GLU/GP AR hydrogels are shown in *Figure 5*.

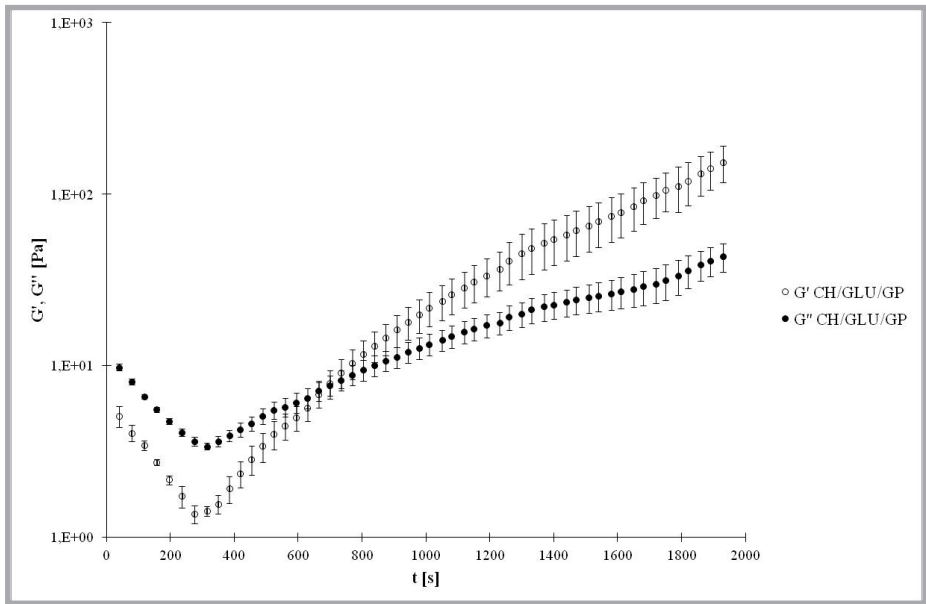


Figure 1. The relationship between the storage modulus (G') and loss modulus (G'') as a function of time for the CH/GLU/GP system.

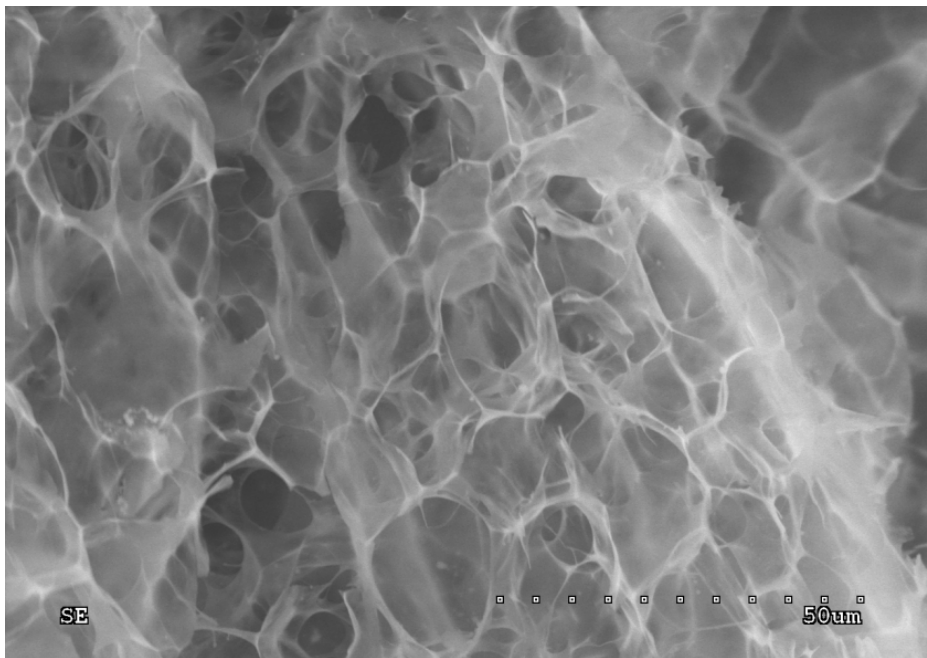


Figure 2. SEM image of hydrogel after lyophilization.

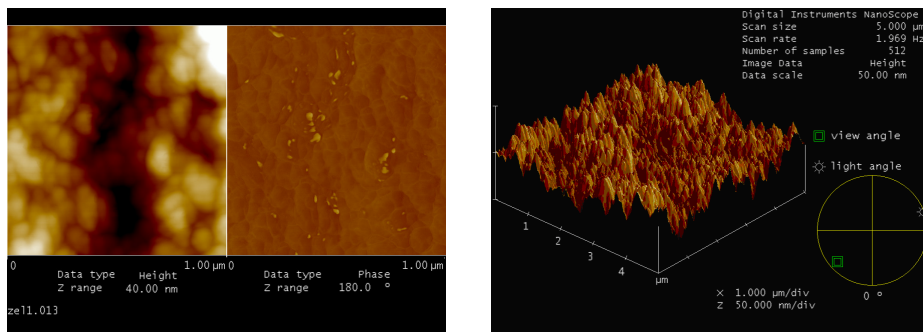


Figure 3. The AFM image of hydrogel surface before conditioning in water (the CH/GLU/GP BR system).

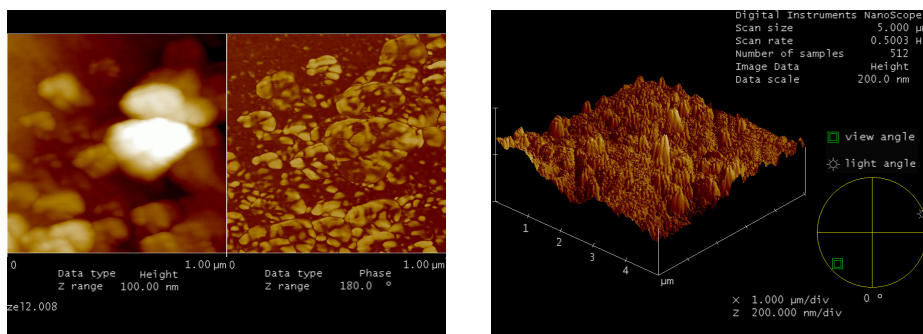


Figure 4. The AFM image after conditioning in water at 37°C (the CH/GLU/GP AR system).

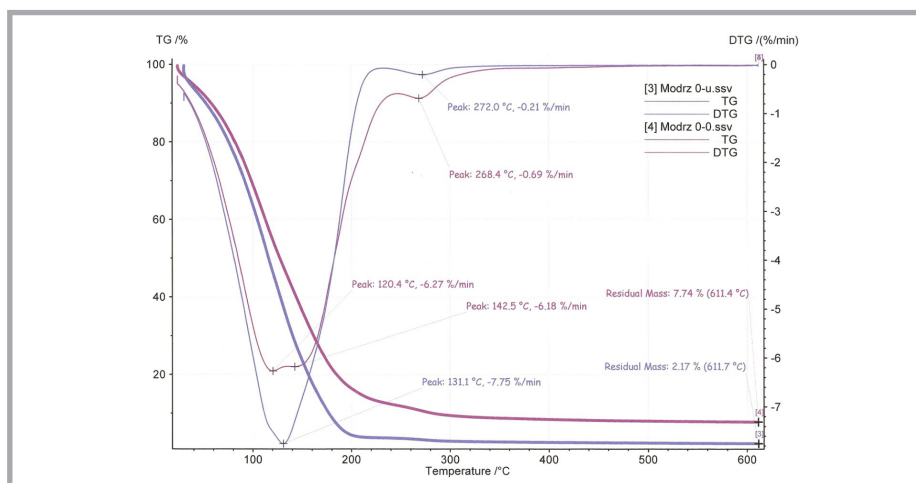


Figure 5. TGA and DTG curves of hydrogels CH/GLU/GP BR - claret and CH/GLU/GP AR - dark blue.

The TG curves indicate that the decrease of hydrogel weight residual mass for CH/GLU/GP BR amounts to 7.74%, for CH/GLU/GP AR to 2.17%. This proves the partial release of components during conditioning of the hydrogel in water. The DTG analysis for the CH/GLU/GP BR and CH/GLU/GP AR samples indicates a multi-step decomposition process. For CH/GLU/GP BR samples, the first stage occurs in the range 120 - 140 °C and corresponds to water in materials. The shape of the curve (two bands overlapping) suggest that the water in the hydrogel exists in two forms (free water in the pores of the gel and partially bound water) or that there are two systems containing water. For CH/GLU/GP AR samples, the first stage occurs at approximately 130 °C. Its shape is different than that of the CH/GLU/GP BR samples. Specifically, the band at 120 °C is reduced. This suggests that free water in the pores of gel has been removed from the structure during conditioning in water or that compounds with which water is associated (Phosphorous components) are removed. For both systems, the decomposition of chitosan is observed, for CH/GLU/GP BR at 268 °C, for CH/GLU/GP AR at 272 °C. The decomposition process is completed at ca. 320 °C.

The X-ray Diffraction (XRD) pattern of CH/GLU/GP BR hydrogel samples before and during conditioning in water is shown in **Figure 6**. For comparison, in **Figure 7** XRD patterns for CH/GLU/GP BR, CH/GLU/GP AR and chitosan samples after drying are shown.

Diffraction patterns prove that the structures of CH/GLU/GP BR and CH/GLU/GP AR samples differ significantly. A maximum at an angle of $2\theta \approx 5$ is visible on the diffraction

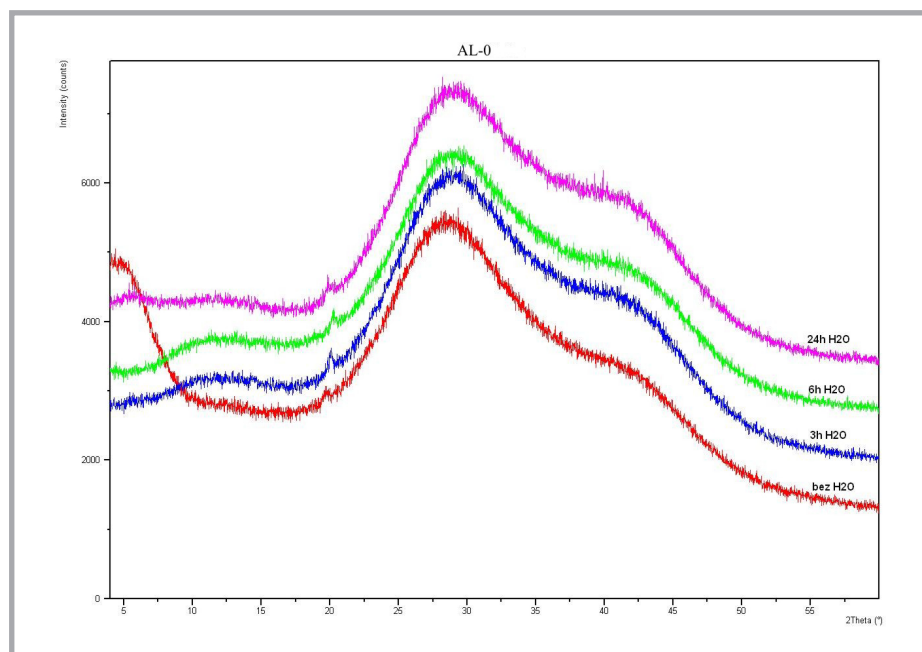


Figure 6. The diffraction pattern (XRD) – hydrogel.

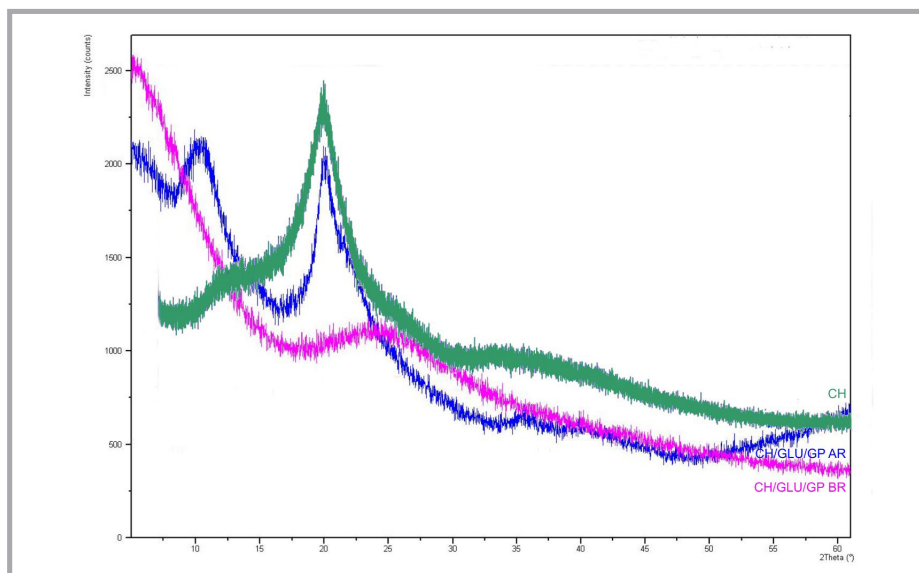


Figure 7. The diffraction pattern (XRD) hydrogel after drying.

pattern of the gel without conditioning in water. This peak is hardly visible in the spectra for the gels after short conditioning times (3 - 6 h). It is clear after sample conditioning in the water for 24 h. Broad halo at an angle of $2\theta \approx 11^\circ$ is apparent on all of the diffraction patterns. A significant difference is observed at the characteristic maximum for chitosan at an angle of $2\theta \approx 20$. This maximum is more pronounced for gels conditioned in water. The two remaining overlapping broad peaks at angles of $2\theta \approx 30^\circ$ and 40° do not change.

A vast change in the crystal structure is observed for the dried form. The structure of the CH/GLU/GP BR gels are amorphous in character. The structure of the CH/GLU/GP AR gels is more crystalline and similar to the structure of the polymer, as shown by the characteristic maximum for chitosan at an angle of $2\theta \approx 20^\circ$.

Figure 8 shows the FTIR spectrum of the lyophilized CH/GLU/GP system immediately after gelation (CH/GLU/GP BR blue curve) and after subjecting the samples to 24 h of conditioning in distilled water (CH/GLU/GP AR green curve). As a comparison, the FTIR spectra of native chitosan (red curve) and β -glycerol phosphate disodium salt (violet curve) are also depicted.

Infrared spectra of gels before (BR) and after conditioning (AR) in the water indicate changes in the structure. The broad spectrum corresponding to the oscillation of O-H is observed in the range of wave number of $3600 - 3100 \text{ cm}^{-1}$ in both cases (BR and AR). However, it is far less intensive than for chitosan. It proves the presence of hydroxyl groups in the structure. The asymmetric shape of this peak that is visible in the range of lower wave numbers indicates the presence of strong hydroxyl bounds and amine groups N-H in the structure.

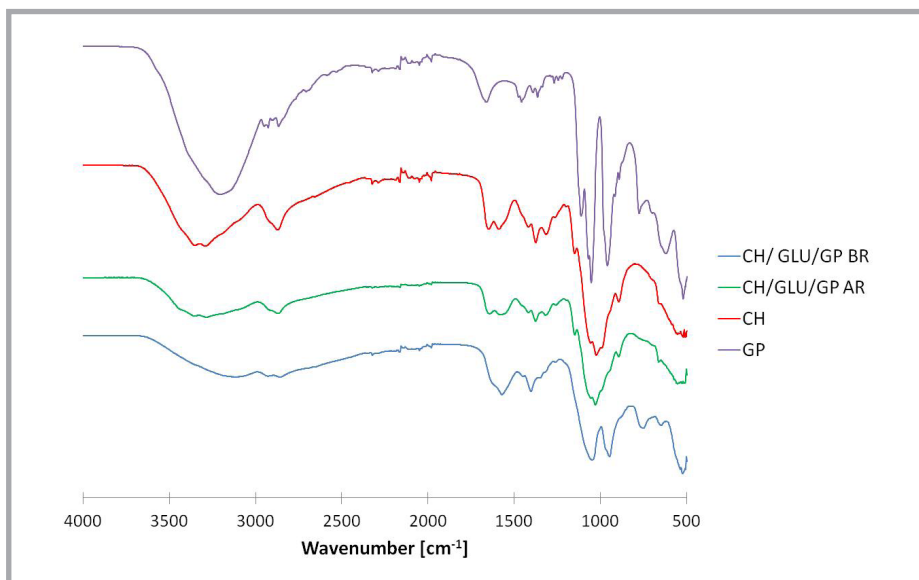


Figure 8a. The comparison of infrared spectra of CH/GLU/GP system before (CH/GLU/GP BR) and after conditioning (CH/GLU/GP AR) in water.

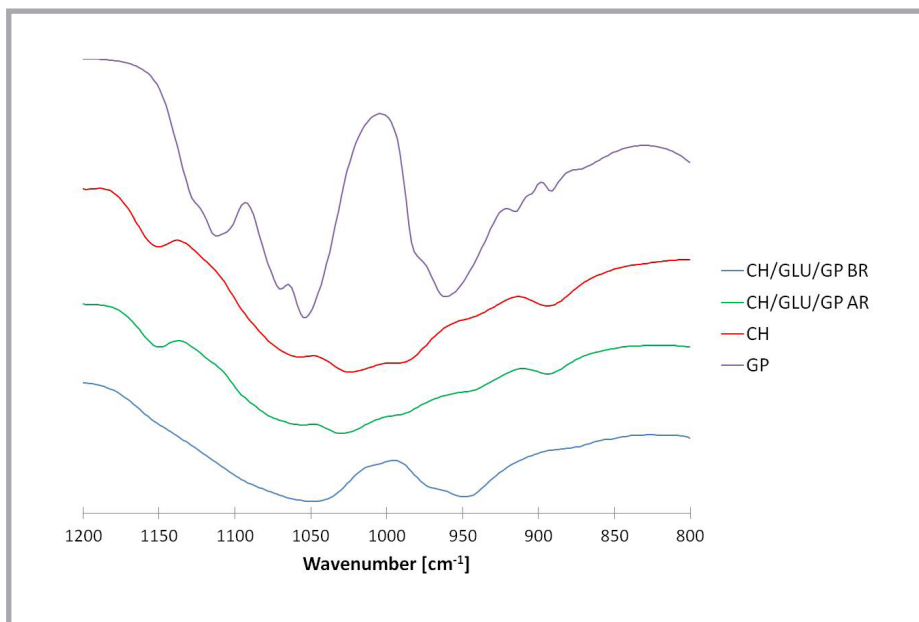


Figure 8b. The FTIR spectra of the CH/GLU/GP BR and CH/GLU/GP AR system. As comparison, the spectra of native CH (red curve) and GP (blue curve) are shown in the range of 1200-1700 cm⁻¹.

In the range of 2850 - 2950 cm^{-1} the spectrum of chitosan possesses one asymmetric band at 2871 cm^{-1} . This band probably consists of two overlapping bands, one less intensive at 2908 and one more intensive at 2871, which represents the stretching vibrations of aliphatic groups ($-\text{CH}_2$ and $-\text{CH}_3$) characteristic for the pyranose ring of chitosan. Both systems CH/GLU/GP BR and CH/GLU/GP AR have bands at this wavenumber. The spectrum of CH/GLU/GP BR is split into two distinct bands at 2926 and 2858 cm^{-1} . For the CH/GLU/GP AR system this band appears at 2870 cm^{-1} and has a minor shoulder.

The spectrum of chitosan shows a band at 1648 cm^{-1} that is assigned to the $\text{C}=\text{O}$ stretch of the amide bond and at 1589 cm^{-1} that is assigned to the NH_2 group of chitosan. These bands indicate that chitosan is a partially deacetylated product of chitin. The spectrum of CH/GLU/GP BR shows one distinct band at 1571 cm^{-1} with a minor shoulder. The CH/GLU/GP AR has two bands at 1642 and 1582 cm^{-1} .

In the range of 1200 - 1500 cm^{-1} , the chitosan molecule shows four peaks at 1419, 1375, 1315 and 1264 cm^{-1} . The bands at 1419 and 1314 cm^{-1} are associated with oscillations characteristic for OH and C-H bending of CH_2 groups. The band at 1375 cm^{-1} represents the C-O stretching of the primary alcoholic group $-\text{CH}_2\text{-OH}$. For the CH/GLU/GP BR system, the bands at 1419, 1375 and 1315 cm^{-1} are shifted to 1448, 1404 and 1346 cm^{-1} , respectively. For the CH/GLU/GP AR system these bands appear at 1419, 1375, 1315 cm^{-1} as for chitosan.

In the wavenumber range 800 - 1200 cm^{-1} , the FTIR spectrum of chitosan shows three bands at 1159, 1024 and 894 cm^{-1} . The wide band at 1155 - 1037 cm^{-1} represents the bridge $-\text{O}-$ stretch of the glucosamine residues.

The system CH/GLU/GP BR does not reveal bands characteristic for chitosan in this wavenumber range. However, two new bands characteristic for GP appear in this region. The band at 1050 cm^{-1} is characteristic for GP and indicates aliphatic P-O-C stretching. The band at 960 cm^{-1} is present with a minor shoulder at 980 cm^{-1} . The band at 980 cm^{-1} is characteristic for the $-\text{PO}_4^{2-}$ group whereas the band at 960 cm^{-1} may indicate the presence of the $-\text{HPO}_4^-$ group. The FTIR spectra obtained for the systems conditioned in distilled water CH/GLU/GP AR show only peaks characteristic for chitosan molecule (at 1150, 1030 and 895 cm^{-1}). The peaks connected with the presence of phosphorus in the system structures, P-O-C and $-\text{PO}_4^{2-}$, disappear.

The FTIR spectrum of chitosan has one band at 656 cm^{-1} in the range of 500 - 800 cm^{-1} which is connected with vibrations of the $\text{O}=\text{C}-\text{N}$ group. The CH/GLU/GP BR displays two bands in this range at 752 and 648 cm^{-1} . The band at 750 cm^{-1} is the band characteristic for GP (aliphatic stretching of P-O-C).

The FTIR spectrum of the CH/GLU/GP system after gelation indicates characteristic bands for chitosan and glycerol phosphate disodium salt. For the CH/GLU/GP AR system only bands characteristic for the chitosan molecule are observed. The spectrum in the range of 1200 - 900 cm^{-1} , that is assigned to the saccharide structure, is similar to the spectrum of chitosan molecule. There are no additional bands and shifts observed.

Mechanisms of the gel formation of thermo-sensitive hydrogel building blocks based on chitosan and β -glycerol phosphate were proposed previously by Chenite [12] and Lavert [13]. According to Chenite, the following types of interactions are involved in the gelation mechanism: (1) electrostatic attraction (2) hydrogen bonding and (3) interchain chitosan–chitosan hydrophobic interactions. On the other hand, Lavertu stated that protons are bound by phosphate groups if the amount of GP passes a threshold concentration. The transfer of protons leads to chitosan precipitation and induces the sol-gel transition. After protonation, the phosphate molecules are free to diffuse out of the system.

Based on our results obtained for the CH/GLU/GP system, it seems that both theories hold correct assumptions. The theory of Lavertu can be supported by the fact that phosphate molecules diffuse out of the system structure (**Figure 9**) as the infrared spectra obtained for both systems after 72 h of conditioning in distilled water do not display characteristic phosphate bands. The theory proposed by Chenite can be supported by the fact that the diffusion of phosphate molecules occurs some time after conditioning in distilled water (further tests are currently ongoing). These data suggest the existence of electrostatic interactions.

The results presented are both very important and relevant, but merely represent an initial step in complete elucidation of the gel formation mechanism. The data obtained so far should be complemented with phosphate release kinetic studies.

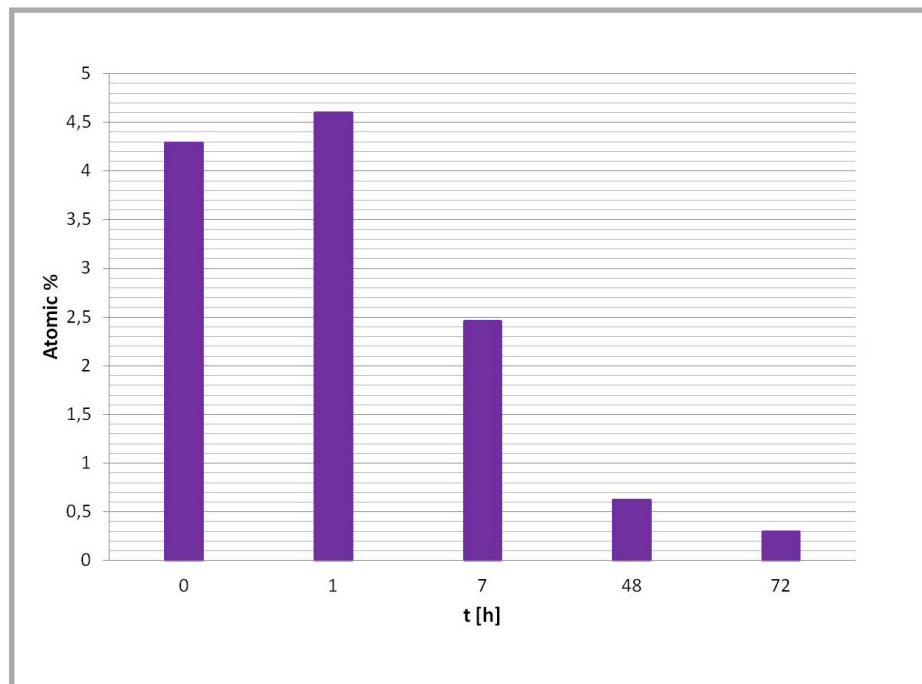


Figure 9. The phosphorus content in the structure of the hydrogel during the water conditioning.

4. Conclusions

The structure of thermosensitive chitosan gels prepared with the use of chitosan glutamate is high porous. This structure undergoes changes due to conditioning of gels in the water.

The changes in diffraction patterns and IR spectra in the range of amide spectrum and saccharide structure spectrum suggest that the arrangement of particles in the structure is changing.

Thermosensitive gels prepared with the use of chitosan glutamate can be an interesting material both as drug carrier and as high porous material, scaffold in tissue engineering.

5. Acknowledgement

The research was financed by the National Science Center of Poland-Grant N UMO-2011/01/B/ST8/06686. Timothy E.L. Douglas acknowledges the Research Foundation Flanders (FWO) for financial support in the framework of a postdoctoral research fellowship.

6. References

1. Barbucci R; (2009) *Hydrogels: Biological properties and applications*, Springer, Milan.
2. Berger J, Reist M, Mayer J M, Felt O, Peppas N A, Gurny R; (2004) Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications, *Eur. J. Pharm. Biopharm.*, 57, 19-34.
3. Bhattari N, Matesen F A, Zhang M; (2005) PEG-grafted chitosan as injectable thermoreversible hydrogel, *Macromol. Biosci.*, 5, 107-111.
4. Chenite A, Chaput C, Wang D, Combes C, Buschmann M D, Hoemann C D; (2000) Novel injectable neutral solutions of chitosan from biodegradable gels in situ, *Biomaterials*, 21, 2155-2161.
5. Drury J L, Mooney D J; (2003) Hydrogels for tissue engineering: scaffold design variables and applications, *Biomaterials*, 24, 4337-4351.
6. Klouda L, Mikos A G; (2008) Thermoresponsive hydrogels in biomedical applications, *Eur. J. Pharm. Biopharm.*, 68, 34-45.
7. Lee Y M, Kim S S, Kim S H; (1997) Synthesis and properties of poly(ethylene glycol) macromer/ β -chitosan hydrogel, *J. Mater. Sci.: Mater. Med.*, 8, 537-541.
8. Ravi Kumar M N V; (2000) A review of chitin and chitosan applications, *Reactive & Functional Polymers*, 46, 1-27.
9. Schuetz Y B, Gurny R, Jordan O; (2008) A novel thermoresponsive hydrogel based on chitosan, *Eur. J. Pharm. Biopharm.*, 68, 19-25.
10. Van Tomme S R, Storm G, Hennink W E; (2008) In situ gelling hydrogels for pharmaceutical and biomedical applications, *Int. J. Pharm.*, 355, 1-18.
11. Yao K D, Peng T, Goosen M F A, Min J M, He Y Y; (1993) pH sensitivity of hydrogels based on complex forming chitosan: polyether interpenetrating polymer network, *J. Appl. Polym. Sci.*, 48, 343-354.
12. Yao K D, Peng T, Xu M X, Yuan C, Goosen M F A, Zhang Q, Ren L; (1994) pH dependent hydrolysis and drug release of chitosan/polyether interpenetrating polymer network hydrogel, *Polym. Int.*, 34, 213-219.

13. Zhou H Y, Chen X G, Kong M, Liu C S, Cha D S, Kennedy J F; (2008) Effect of molecular weight and degree of chitosan deacetylation on the preparation and characteristics of chitosan thermosensitive hydrogel as a delivery system, *Carbohydr. Polym.*, 73, 265-273.
14. Alves N M, Mano J F; (2008) Chitosan derivatives obtained by chemical modifications for biomedical and environmental applications, *Int. J. Biol. Macromol.*, 43, 401-414.
15. Kurita K; (2006) Chitin and Chitosan: Functional Biopolymers from Marine Crustaceans, *Marine Biotechnology*, 8, 203-226.
16. Muzzarelli R A A; (2009) Chitins and chitosans for the repair of wounded skin, nerve, cartilage and bone, *Carbohydr. Polym.*, 76, 167-182.
17. Rinaudo M; (2006) Chitin and chitosan: Properties and applications, *Progr. Polymer Sci.*, 31, 603-632.
18. Shepherd R, Reader S, Falshaw A; (1997) Chitosan functional properties, *Glycoconj. J.*, 14, 535-542.
19. Shi C, Zhu Y, Ran X, Wang M, Su Y, Cheng T; (2006) Therapeutic Potential of Chitosan and Its Derivatives in Regenerative Medicine, *J. of Surg. Res.*, 133, 185-192 .
20. Sinha V R, Singla A K, Wadhawan S, Kaushik R, Kumria R, Bansal K, Dhawan S; (2004) Chitosan microspheres as potential carrier for drugs, *Int. J. Pharm.*, 274, 1-33.
21. Tharanathan R N, Kittur F S; (2003) Chitin – the undisputed biomolecule of great potential, *Crit. Rev. Food Sci. Nutr.*, 43, 61-87.

