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Structural characteristics of thermosensitive chitosan glutamate hydrogels in variety of physiological environments

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HIGHLIGHTS

- Properties of thermosensitive chitosan glutamate are presented (FTIR, WAXS).
- Determined changes in hydrogel after conditioning in water, buffer at pH 7 and pH 2.
- On the basis of structure changes in water a mechanism formation was proposed.

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ABSTRACT

In this paper the properties of thermosensitive chitosan hydrogels prepared with the use of chitosan glutamate and β-glycerophosphate are presented. The study is focused on the determination of changes in the hydrogel structure in different environments: during conditioning in water and buffer at pH 7 and pH 2 respectively. The structure of gels was observed under the Scanning Electron Microscopy (SEM) and was investigated by infrared (IR) spectroscopy. The crystallinity of gel structure was determined by X-ray diffraction analysis (XRD). On the basis of structural changes during the conditioning in water a mechanism of their formation was proposed.

Introduction

Over the last few years, much attention has been paid to hydrogels prepared from both natural and synthetic or hybrid polymers [1]. Hydrogels from natural polymers are produced mainly from:

- proteins (collagen, gelatin, fibrin, silk, lysozyme, Matrigel™, and genetically engineered proteins such as calmodulin (a calcium-binding protein), elastin-like polypeptides and leucine zipper);
- polysaccharides such as hyaluronic acid (HA), agarose, dextran and chitosan;
- and hybrid systems: protein/polysaccharide hybrid polymers, such as collagen/HA, laminin/cellulose, gelatin/chitosan and fibrin/alginate;
- DNA X-, Y-, T-DNA, linear plasmid DNA.

Hydrogels from synthetic polymers are formed from nonbiodegradable and biodegradable polymers.

Nonbiodegradable synthetic hydrogels PHEMA, PHPMA, PNIPAm, can be prepared by copolymerization of various vinylated monomers or macromers such as 2-hydroxyethyl methacrylate (HEMA), 2-hydroxypropyl methacrylate (HPMA), acrylamide (AAm), acrylic acid (AAc), *N*-isopropylacrylamide (NIPAm) and methoxyl poly(ethylene glycol) (PEG) monoacrylate (mPEGMA or PEGMA), with crosslinkers such as *N*,*N*′-methylenebis(acrylamide) (MBA), ethylene glycol diacrylate (EGDA) and PEG diacrylate (PEGDA)®. Another method to form nonbiodegradable hydrogels is to use nonbiodegradable polymers such as self-assembly of Pluronic® polymers with a structure of poly(ethylene oxide) (PEO)–poly(propylene oxide) (PPO)–PEO, chemical cross-linking of modified poly(-vinyl alcohol) (PVA), and radiation cross-linking of linear or branched PEG.

Biodegradable synthetic hydrogels are mostly produced from polyesters. These are often hybrid systems containing poly(lactic

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acid) (PLA), poly(glycolic acid) (PGA), poly(ε-caprolactone) (PCL) and their copolymers PPF-PEG, PHEMA-PCL.

Synthetic/natural hybrid hydrogel PEG/dextran, heparin, HA, CS, protein, PNIPAm/proteins, chitosan, HA, alginate; synthetic peptides/proteins, polysaccharides, PVA/DNA, CS, pluronic/dextran and PHPMA/protein are also formed.

The main reason of this is a broad spectrum of their applications. Hydrogels can be used for the controlled release of bioactive molecules and as scaffolds in tissue engineering. However, introduction of drugs into the hydrogel structure requires the use of suitable cross-linking or coagulation agents which should not deactivate these active biomolecules. The use of hydrogels as scaffolds is beneficial because they provide a water environment for cell culture. The main disadvantage of these systems is the fact that they do not guarantee uniform spatial distribution of cells in the site of their application. Recent research of Drury and Mooney, Klouda and Mikos, and Barbucci [2–4] has been focused on hydrogels that are able to undergo transition from sol to gel at the physiological human body temperature.

One of the polymers with great potential is chitosan which is a natural polymer obtained in the process of chitin deacetylation. The use of chitosan seems to be very promising in tissue engineering. This is indicated by Alves and Mano, Muzzarelli, Ravi Kumar, Rinaudo and Shi et al. [5–10]. Chitosan scaffolds are formed most often by lyophilization, biomineralization, particle aggregation, electrospinning and gelation under the influence of cross-linking agents (UV or temperature) [11–14].

A new interesting form of scaffolds are gels whose sol–gel transition takes place at natural temperature of human body. They are usually formed from chitosan salt solutions of low viscosity with the use of β-glycerophosphate as a neutralizing agent [15–19] but also from polyvinyl alcohol and sodium bicarbonate [20–22] by the enzymatic method with the use of urease and urea [23] and by using β-tricalcium phosphate [24].

The mechanisms of gel formation from thermosensitive hydrogel building blocks based on chitosan and β-glycerol phosphate were proposed previously by Chenite et al. [16,17] and Filion, Lavertu and Buschmann [25,26]. According to Chenite et al. [16,17], the following types of interactions are involved in the mechanism of gelation: (1) electrostatic attraction, (2) hydrogen bonding and (3) interchain chitosan–chitosan hydrophobic interactions. On the other hand, Filion, Lavertu and Buschmann [25,26] state that protons are bound by phosphate groups if the amount of GP exceeds 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.

In this paper, a new form of chitosan gel with thermosensitive properties is presented. The thermo-gelling chitosan solution was prepared with the use of L-glutamic acid. L-glutamic acid being one of the most common amino acids found in nature [27,28] is one of the group of amino acid neurotransmitters in the brain and spinal cord. Therefore, thermosensitive gels can be used in tissue engineering to regenerate nerves.

The study is focused on the determination of changes in the hydrogel structure and acid content due to water conditioning. The determination of structural changes in different environments is important when considering possible applications of hydrogels, e.g., as scaffolds for tissue regeneration or carriers for controlled drug release. Structural changes were investigated during conditioning of samples in different conditions simulating the physiological environment (demineralized water at pH 5 – mucosa and skin, pH 7 – blood, pH 2 – stomach). On the basis of structural changes during the conditioning in water a mechanism of their formation was proposed.

Materials and methods

Preparation of chitosan hydrogels

Chitosan glutamate solution (CH/GLU/GP system) was prepared by swelling 400 mg of chitosan in 18 ml of distilled water of specific conductivity 17 μS/m for 30 min. Parameters of chitosan from shrimp shells (CH) – weight average molecular weight Mw = 862 kDa; number average molecular weight Mn = 145 kDa and degree of deacetylation 80%, (SIGMA–ALDRICH®).

The molecular weight (MW) of chitosan preparations was determined by gel permeation chromatography (GPC/SEC) with the application of a high-performance liquid chromatography (HPLC) Knauer Smartline apparatus equipped with an analytical isocratic Pump 1000 and differential refractive index (DRI) detector (S-2300/2400, Knauer). The determination was made in the connected Tessek HEMA 1000 BIO 10 μm and HEMA BIO 40 μm columns at a temperature of 30 °C and eluent flow rate of 1 ml/min (0.1 M NaCl, 0.3 M CH3COOH). 20 μl of a standard dextran solution (PSS, Germany) or a solution of the sample at a concentration of 0.2% was fed to the column. The solutions were filtered beforehand through an injection filter with a membrane consisting of regenerated cellulose with a pore size of 0.2 μm. To obtain calibration curves, molecules of defined molecular weights ranging from 180 to 277,000 g/mol were used. Their retention times ranged from 10.5 to 16.8 min. On the basis of calibration curves, the MW of chitosan preparations was determined by applying a system for the calculation of GPC integrated into "Chromgate" software (Knauer) to verify and analyze the HPLC data.

The deacetylation degree (DD) (defined as the ratio of the number of $—NH_2$ groups formed in chitosan to the initial number of —NH—CO—CH_3 groups present in chitin) was determined by a titration method. Chitosan (0.13–0.3 g) was dissolved in 43% orthophosphoric acid (100 ml). Next, a distillation process was carried out until the solution reached a temperature of 160 °C. The distillate was titrated with a 0.1 M aqueous solution of sodium hydroxide against phenolphthalein. A blank test was performed by distilling a 43% solution of orthophosphoric acid that was titrated with 0.1 M sodium hydroxide solution. The acetylation degree (AD) (%) was calculated from the formula:

$$AD = 2.03 \times Vm$$

where V is the number of ml of 0.1 M sodium hydroxide used for distillate titration and m denotes the weighed portions of chitosan samples after taking into account moisture and ash content [g].

while the deacetylation degree (DD) was calculated from the formula:

$$DD = 100 - AD$$

Then, 200 mg of glutamic acid (GLU, Fluka®) was added to the solution. The solution was stirred at a flow rate of 120 rev/min. until complete dissolution. Next, the sample was cooled to 4 °C. To the cooled sample 2 g of β-glycerol phosphate disodium salt pentahydrate (GP, SIGMA–ALDRICH®) dissolved in 2.5 ml of distilled water of specific conductivity 17 μS/m was added dropwise under stirring in an ice bath. The final solution was mixed for another 20 min at a flow rate of 120 rev/min. and stored at 4 °C for 12 h.

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 form of cylinders with a diameter of 3 cm and height of 3 cm.

The *in vitro* release studies were performed using an ERWEKA apparatus which conforms to the requirements of the Pharmaco-

peia. The frequency of fluid mixing was fixed at 20 rev/min. The release study was performed in distilled water at pH 5, buffer at pH 2 and pH 7 with a capacity of 900 dm^3 and pH = 5 ± 0.5.

Water at pH 5 of specific conductivity17 μS/m.

Buffer at pH 7 (0.2 M NaH_2PO_4−39 ml and 0.2 M Na_2HPO_4−61 ml) supplemented with distilled water to 200 ml.

Buffer at pH 2 (50 ml 0.2 M KCl + 13 ml of 0.2 M HCl).

The temperature of the release medium was kept constant at 37 °C during the whole release process.

Structural studies

The structural properties of hydrogels after preparation (CH/GLU/GP BR system) and after conditioning in water at pH 5, buffer at pH 7 and pH 2 were studied.

Samples for analysis (SEM, FTIR, XRD) were taken after sufficient time of conditioning in a given medium (water, buffer at pH 2 and pH 7). Next, they were frozen to temperature −25 °C and lyophilized at temperature −6 °C for 5 h on Christ Alpha 2–4 aparature.

The structure of gels was observed using the FEI QUANTA 200 F scanning electron microscope. The elemental composition was analyzed using the FEI QUANTA 200 F scanning electron microscope.

The structural characteristics were based on the analysis of XRD and IR spectra. The XRD analysis was used to identify structural changes in the analyzed samples. Room temperature powder X-ray diffraction patterns were collected using a PANalytical X'Pert Pro MPD diffractometer in Bragg–Brentano reflecting geometry. Copper Cu Kα radiation from a sealed tube was applied.

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.

Results and discussion

Structural characteristics of the CH/GLU/GP system

The SEM image of chitosan hydrogels before and after conditioning in water, buffer at pH 7 and pH 2 is shown in Fig. 1.

Fig. 2 shows the FTIR spectrum of the lyophilized chitosan hydrogels during conditioning in water at pH 5. For comparison Figs. 3–4 show the FTIR spectra for samples of hydrogels conditioned in a buffer at pH 7 and pH 2, respectively. The analysis of the elemental composition is given in Table 1.

The X-ray diffraction (XRD) patterns of CH/GLU/GP hydrogel samples during conditioning in water at pH 5 are shown in Fig. 5. For comparison Figs. 6 and 7 show XRD patterns for CH/GLU/GP conditioned in the buffer at pH 7 and pH 2, respectively.

Infrared spectra of gels before (BR) and after conditioning in water (1 h, 7 h, 10 h, 24 h, 72 h) indicate changes in the structure. The broad spectrum in the range of wave number of 3600–3100 cm^{-1} (corresponding to the oscillations of O—H) is observed in all cases. The asymmetric shape of a peak that is visible in the range of lower wave numbers indicates the presence of strong hydroxyl bounds and amine N—H groups in the structure. In hydrogel the band is shifted toward lower frequencies compared to the spectrum of chitosan. After conditioning in water this band moves again to higher frequencies. Band shift to higher frequencies may be associated with the presence of additional amine groups derived from glutamic acid. After conditioning in water the glutamic acid is probably eluted and the band becomes more symmetrical.

In the range of 2850–2950 cm^{-1} the spectrum of chitosan has one asymmetric band at 2871 cm^{-1}. This band probably consists of two overlapping bands, one less intensive at 2910 and the other one more intensive at 2870, which represents stretching vibrations of aliphatic groups (—CH_2 and —CH_3) characteristic of the pyranose *ring of chitosan*. The spectrum of BR is split into two distinct bands at 2926 and 2858 cm^{-1}. For the system after conditioning in water this band appears at 2870 cm^{-1} and has a minor shoulder like in the spectrum of pure chitosan.

The spectrum of chitosan shows a band at 1648 cm^{-1} which is assigned to the C=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 BR and of the system after conditioning in water for less than 10 h shows one distinct band at 1571 cm^{-1} with a minor shoulder. The system conditioned in water for above 10 h 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 of 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 —CH_2—OH. For the BR system, the bands at 1419, 1375 and 1315 cm^{-1} are shifted to 1448, 1404 and 1346 cm^{-1}, respectively. For the system conditioned in water for more than 24 h these bands appear at 1419, 1375, 1315 cm^{-1} as for chitosan.

In the wave number 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 —O— stretch of the glucosamine residues. The FTIR spectrum of BR indicates characteristic bands for chitosan and glycerol phosphate disodium salt – two new bands characteristic of GP appear in this region. The band at 1050 cm^{-1} is characteristic of 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 of the —PO_4^{2-} group, whereas the band at 960 cm^{-1} may indicate the presence of the —HPO_4^- group. The FTIR spectra obtained for the systems conditioned in distilled water after conditioning in water for more than 24 h show only peaks characteristic of 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 —PO_4^{2-}, disappear. The spectrum ranging from 1200 to 900 cm^{-1}, that is assigned to the saccharide structure, is similar to the spectrum of a chitosan molecule.

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 O=C—N group. The BR displays two bands in this range at 752 and 648 cm^{-1}. The band at 750 cm^{-1} is characteristic of GP (aliphatic stretching of P—O—C). For the system conditioned in water for longer than 24 h only bands characteristic of the chitosan molecule are observed.

The spectrum of hydrogel conditioned in the buffer at pH 7 varies with time like the spectrum of a sample conditioned in water at pH 5. However, changes occurring on the sample (cylinder) wall are different than these observed inside it which was not reported in the case of the sample conditioned in water at pH 5. Heterogeneity of the structure is also confirmed by SEM photographs.

Changes in FTIR spectra are observed in the broad band range 3000–3500 cm^{-1} but primarily in the band range from 1100 to 700 cm^{-1} corresponding to saccharide structure and the bands connected with the presence of phosphate ions.

After conditioning in the buffer at pH 7 the band in the frequency range of 3600–3100 cm^{-1} moves toward higher frequencies. In the spectrum of pure chitosan two maxima 3350 and 3290 cm^{-1} can be observed, in hydrogel the O—H and N—H bands overlap (the presence of the two bands is confirmed by the asymmetric shape). Asymmetry observed in the spectra of gels conditioned in the buffer at pH 7 is higher than in the spectra

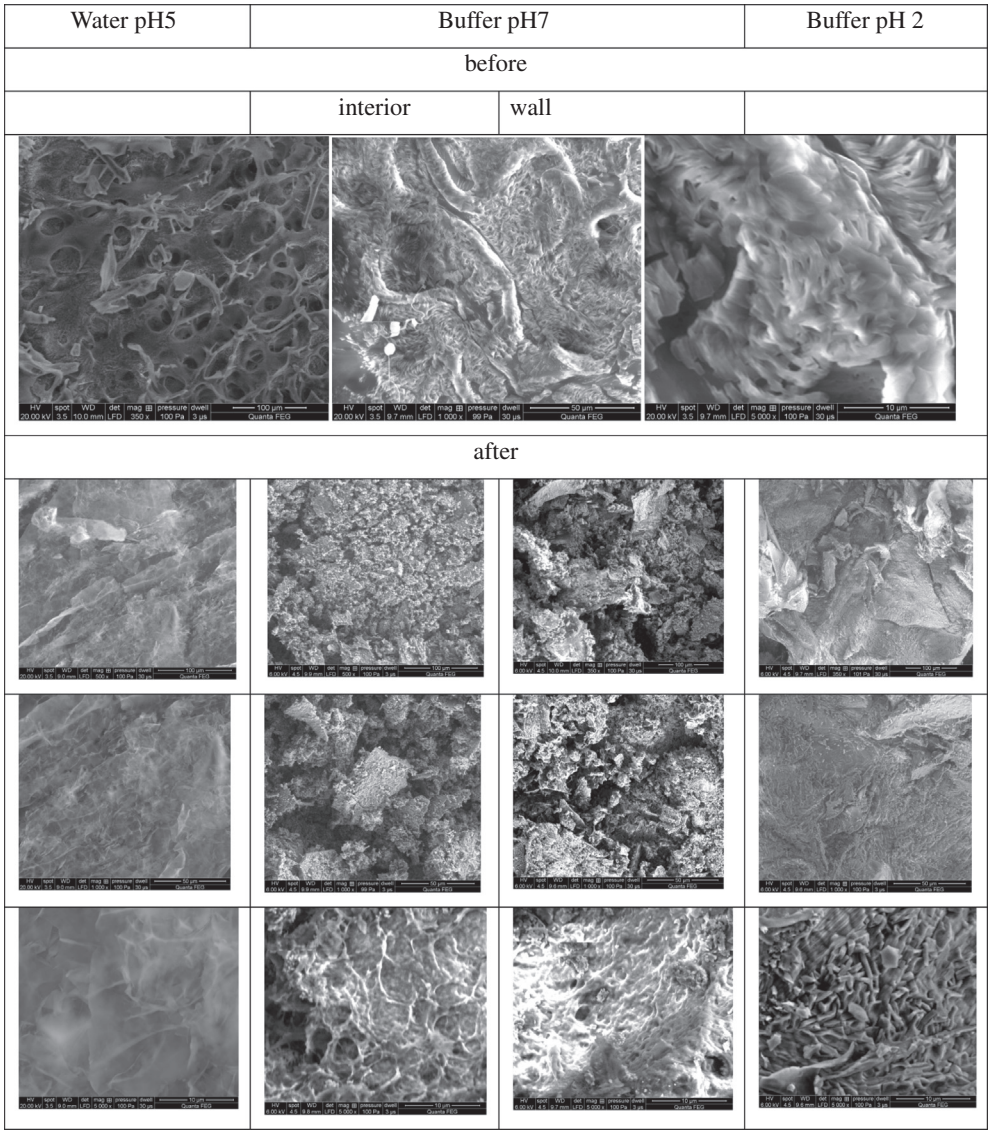

Fig. 1. SEM image of hydrogel conditioning in different environments.

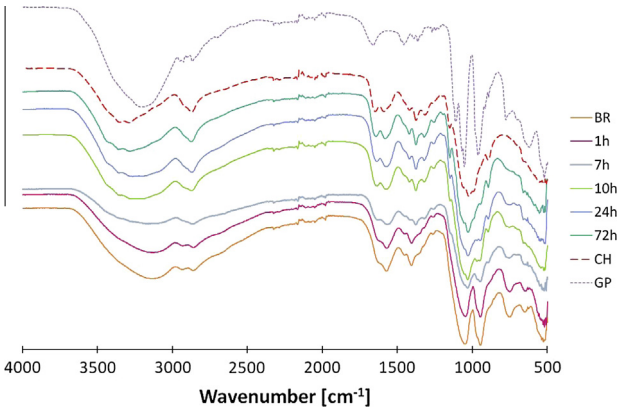

Fig. 2. The comparison of infrared spectra of CH/GLU/GP system during conditioning in water at pH 5.

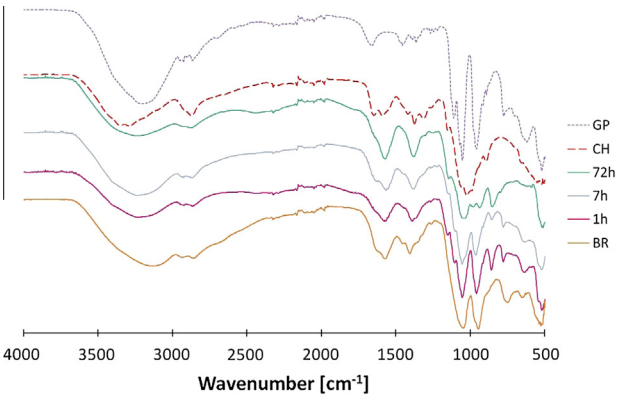

Fig. 3a. A comparison of infrared spectra of chitosan hydrogels during conditioning at pH 7 – the wall.

obtained in the interior of the gel. This may indicate a change in the gel wall structure caused by conditioning in the buffer containing phosphate ions at pH 7 which makes it difficult to elute GP and GL acid from inside the sample.

Analyzing the frequency range of 1200–900 cm^{-1} where the biggest changes are observed, it can be stated that the bands coming from GP, i.e., 1050 cm^{-1} and 950 with a shoulder at 980 cm^{-1}, are clearly visible up to defined time (7 h) and then they disappear

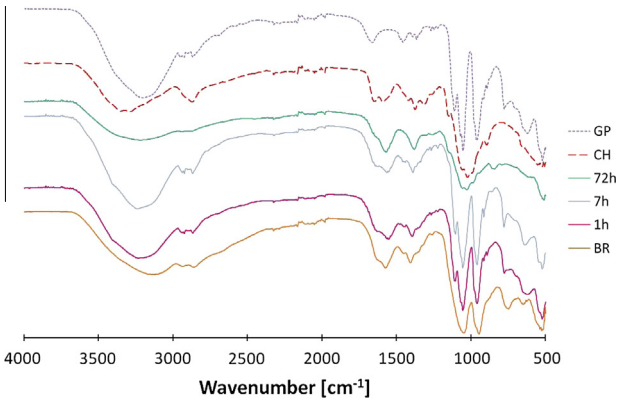

Fig. 3b. A comparison of infrared spectra of chitosan hydrogels during conditioning at pH 7 – interior.

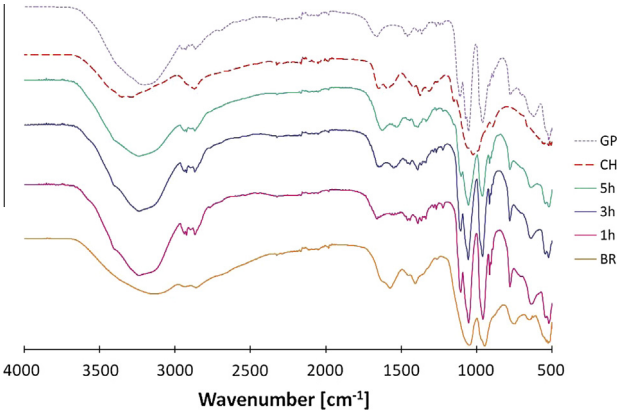

Fig. 4. A comparison of infrared spectra of chitosan hydrogels during conditioning in the buffer at pH 2.

leaving trace amounts. The broad band at the frequency 750 becomes sharp and moves toward higher frequencies, however, after a sufficiently long time (72 h) it disappears. In this frequency range no difference is observed between the spectra of hydrogel wall and its interior. A significant difference between the wall

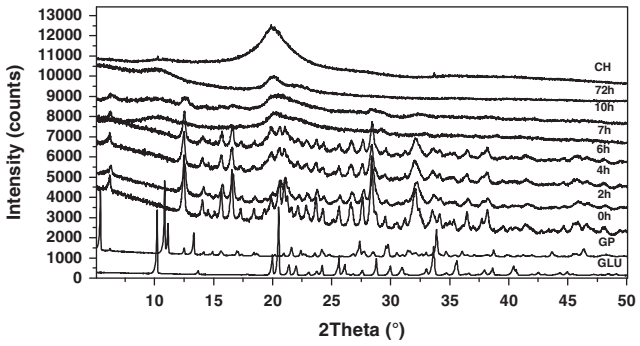

Fig. 5. The diffraction patterns (XRD) – hydrogel conditioned in water at pH 5.

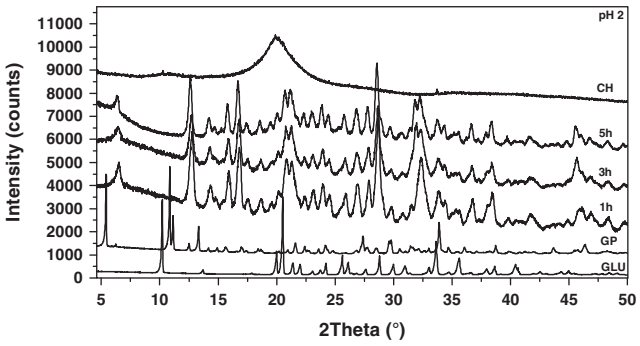

Fig. 6. The diffraction patterns (XRD) – hydrogel conditioned in the buffer at pH 2.

and interior spectra is reported at a frequency of 850 cm^{-1}. It appears in the wall spectrum after 1 h conditioning of the gel, and after 72 h of conditioning this band seems to be present also inside the hydrogel sample. This is probably the band from HPO_4^{2-} (C_{3v}) and $H_2PO_4^-$ (C_{2v}) from the buffer solution. In the hydrogel wall spectrum besides the band at 850 cm^{-1} there are also bands at the frequencies 940 and 990, while in the spectra of hydrogel interior they do not occur.

The differences in the wall and interior spectra confirm the cross-linking effect of phosphate buffer.

Table 1
Elemental composition of thermogel during conditioning in different environments.

		C	N	O	Na	P	P/Na
Elemental composition of thermogel during conditioning in water at pH 5 (at%)							
Interior and wall	CH/GLU/GP	34.65	5.17	47.67	7.91	4.60	0.58
	CH/GLU/GP 1 h	36.09	5.43	47.38	7.16	3.93	0.55
	CH/GLU/GP 7 h	47.02	8.45	38.63	3.42	2.46	0.72
	CH/GLU/GP 24 h	52.01	8.75	37.95	0.43	0.50	1.16
	CH/GLU/GP 72 h	51.73	11.01	36.51	0.35	0.30	0.85
Elemental composition of thermogel during conditioning in the buffer at pH 7 (at%)							
Wall	CH/GLU/GP	34.65	5.173	47.67	7.91	4.6	0.58
	CH/GLU/GP 1 h	41.54	5.17	47.67	7.91	4.60	0.58
	CH/GLU/GP 7 h	40.01	6.08	41.66	7.8	4.46	0.57
	CH/GLU/GP 24 h	32.74	4.72	44.48	11.67	6.39	0.55
Interior	CH/GLU/GP	34.65	5.173	47.67	7.91	4.6	0.58
	CH/GLU/GP 1 h	38.55	5.486	44.17	7.49	4.13	0.55
	CH/GLU/GP 7 h	41.15	5.70	41.40	7.33	4.39	0.6
	CH/GLU/GP 24 h	41.46	6.41	43.58	5.59	2.9	0.51
Elemental composition of thermogel during conditioning in the buffer at pH 2 (at%)							
Interior and wall	CH/GLU/GP	34.645	5.173	47.672	7.91	4.597	0.58
	CH/GLU/GP 1 h	38.825	5.1	44.59	7.32	3.835	0.52
	CH/GLU/GP 3 h	39.181	6.08	44.183	6.27	3.525	0.56
	CH/GLU/GP 5 h	42.342	7.01	40.263	5.69	3.394	0.6

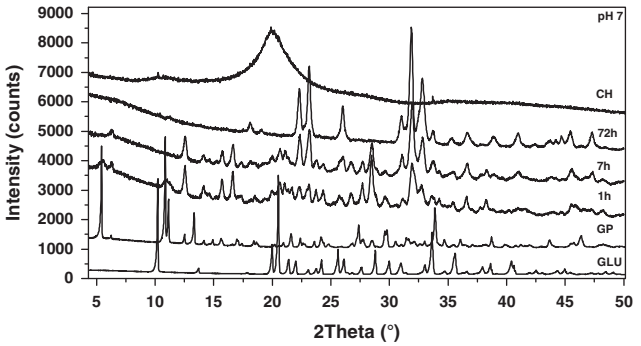

Fig. 7. The diffraction patterns (XRD) – hydrogel conditioned in the buffer at pH 7.

The spectrum of hydrogel conditioned in the buffer at pH 2 does not practically change with time in the range of either amino or saccharide bonds. In these samples phosphorus remains in the structure. In the band range of 1200–500 cm^{-1} there are bands coming from GP. After 6 h the sample is dissolved.

From the elemental composition given in Table 1 it follows that the elemental atomic composition of the gel changes due to conditioning in the investigated environments. The results for conditioning in water show that phosphorus remains in the structure up to ca. 10 h which coincides with the analysis of FTIR spectra. Before the conditioning in water, the P/Na atomic ratio for hydrogel is about 0.5 which is consistent with the ratio resulting from the structural formula. Up to about 7–10 h of conditioning in water, potassium and sodium occur in the hydrogel structure. However, with an increase of the time of conditioning in water, they are washed out from the structure. After 24 h only around 10% of the initial phosphorus remains in the structure. Between 1 and 7 h sodium disappears from the structure much faster than phosphorus: 37 at% of phosphorus and 52 at% of sodium. The P/Na ratio increases reaching the value close to unity after 24 h.

From the elemental composition of the gel conditioned in the buffer at pH 7 it appears that the P/Na ratio remains practically constant. Like for the FTIR spectra differences are observed in the elemental atomic percent of hydrogel on the wall and in the interior. Up to 7 h of conditioning, in the wall structure the content of potassium and sodium is constant. The atomic P/Na ratio is around 0.58 which is close to the stoichiometric ratio of sodium glycerophosphate. This might suggest that GP is not washed out from the structure. However, after sufficiently long time of conditioning (24 h), in the wall structure there is much more sodium and potassium, ca. 50% and 40%, respectively, and their ratio remains practically constant. This is probably a result of the cross-linking effect of the phosphate buffer (pH 7) which might be suggested by the FTIR spectra (the band of frequency 850 cm^{-1} appearing in the wall spectrum after the long time of 72 h is observed also in the spectrum of the sample interior). It should be noted that at the same time about 30% sodium and 37% potassium disappears from the internal structure. It is therefore probable that the increase of both sodium and potassium content in the wall structure is a synergistic effect of GP diffusion on the one hand and a cross-linking effect of the buffer on the other hand.

From the elemental composition of the gel conditioned in the buffer at pH 2 it appears that the elemental atomic percent in the gel structure changes only slightly. Sodium and potassium decrease (after 5 h a decrease of about 25%) but their atomic ratio is constant. This is probably a result of dissolution of subsequent layers of the hydrogel sample during conditioning in the buffer at pH 2.

The high crystalline structure of the thermogel produced from chitosan glutamate directly after formation is confirmed by the observed intense and strong peaks in the diffraction pattern (Fig. 5). It is probably due to the formation of a complex between glutamic acid and glycerophosphate, since the observed diffractogram differs from the diffractograms of pure glutamic acid and glycerophosphate. In the case of hydrogel conditioned in water at pH 5 for up to 6 h the crystalline structure does not change although intensity of the observed peaks is decreasing. After 7 h the diffraction pattern starts to resemble the diffractogram of pure polymer. After further conditioning in water the diffractograms of thermogel and initial polymer are the same. Changing the way of conditioning, especially in the buffer of pH 7, we observed (Fig. 6) that with an increase of the conditioning time, the resulting complex between glutamic acid and glycerophosphate, becomes increasingly crystalline. This would confirm the synergistic effect of diffusion from the buffer and from inside of the sample. The presented XRD results confirm the general conclusions following from the FTIR spectra.

After conditioning in the buffer of pH 2 the diffractograms practically do not change which is in agreement with the results revealed by the FTIR spectra and with the elemental analysis and suggests that subsequent layers of the external gel structure are dissolved.

Conclusions on the mechanism of gel formation have been proposed on the basis of the above presented structural studies and analysis of the elemental composition shown in Table 1.

Based on our results obtained for the chitosan hydrogel, it seems that both theories hold correct assumptions. According to Chenite et al. [23] the formation of chitosan salt solutions in the presence of glycerophosphate in the gel is due to electrostatic interactions between positively charged amino groups of a chitosan molecule and negatively charged phosphate groups. Data in the presented investigations confirm partly these theories and suggest the existence of electrostatic interactions. Phosphorus is observed in the structure for 10 h.

On the other hand, Lavertu, Filion and Buschmann [25,26] state that protons are bound by phosphate groups if the amount of GP exceeds 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. The theory can be supported by the fact that the diffusion of phosphate molecules occurs some time after conditioning in distilled water – the phosphate molecules diffuse out of the system structure as the infrared spectra obtained for both systems after 24 h of conditioning in distilled water do not display characteristic phosphate bands.

By analyzing the change in elemental composition, FTIR spectra and XRD patterns the following model of hydrogel formation was proposed.

When glutamic acid solution is added to chitosan powder the polymer is dissolved. First, amino groups of chitosan are protonated. The protonated amino groups from the chitosan molecule are bound with COO^- groups from glutamic acid. From the stoichiometric analysis of hydrogel composition it follows that per 1 mol of amino groups there are 0.67 mol of glutamic acid, i.e., only about 35% amino groups from chitosan can be protonated, and the remaining 65% can react theoretically with the rest of glutamic acid. After the addition of β-glycerophosphate through O^- from glycerophosphate NH_3^+ groups from glutamic acid are bound. The acid is bound with amino groups of chitosan molecule and with protonated amino groups of chitosan. The COO^- group from glutamic acid and O^- group from glycerophosphate remain free. Most probably, sodium Na^+ ions are bound with these groups. In this way the system is neutralized which is evidenced by the fact that during the first hour the P/Na ratio is about 0.5 and remains constant.

In the first hours of conditioning the loss of GP is probably caused by a release of its excess which is in the pores, or of GP produced during neutralization of amino groups of chitosan. A faster release of sodium is a result of the release of its free ions present in the structure. Probably, after disconnection of glycerophosphate from amino groups, in the next stage the complex of glutamic acid bound with the protonated NH_3 groups is disconnected.

Conclusions

The structure of thermosensitive chitosan gels prepared with the use of chitosan glutamate is highly porous. This structure undergoes changes due to conditioning of the gels in water and buffer at pH 7 and pH 2.

On the basis of both structural studies on thermosensitive chitosan gels after conditioning in water and elemental analysis, the mechanism of gel formation was proposed.

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 depending on the conditioned medium.

Conditioning of hydrogel in water leads to production of a form from pure chitosan. Up to 6 h phosphorus is present in the structure, after 7 h it is released from the structure. The release of GP and glutamic acid from the structure during conditioning in water makes it possible to obtain structures with a specified amount of GP and glutamic acid which can be used when these structures are used as scaffolds for nerve regeneration (at glutamic acid concentration up to 1.03 mg/ml its effect on the culture of nerve cells is advantageous [27,28]).

Conditioning in the buffer of pH 2 does not change the hydrogel structure, phosphorus remains in the structure up to 5 h and then the hydrogel is dissolved. Probably, as long as phosphorus is in the structure (phosphorus is bound by electrostatic forces with protonated amino groups of glutamic acid), the structure does not dissolve. Dissolution of subsequent layers during conditioning in water can be used in controlled release of pharmacological agents that can be incorporated into the structure.

Conditioning in the buffer of pH 7 changes the hydrogel structure in a non-homogeneous way. There is also an additional cross-linking through phosphorus ions from the buffer.

Thermosensitive gels prepared with the use of chitosan glutamate can be an interesting material both as a drug carrier and as a highly porous material for scaffolds in tissue engineering.

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