CYTOTOXICITY OF CHITOSAN BASED THERMO-SENSITIVE HYDROGELS INTENDED FOR NERVOUS TISSUE ENGINEERING

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Abstract

The damage to the central nervous system is one of the most difficult cases of trauma to treat. Over the last few years, increasing attention has been focused on the development of strategies based on biomaterials for regeneration and repair of the spinal cord injury. In particular, materials in the form of hydrogels based on chitosan are being actively investigated due to their intrinsic properties that are favorable in spinal cord tissue regeneration. The purpose of this study was to develop a thermo-gelling chitosan solution that will be prepared with the use of acids that naturally occur in the human nervous tissue. For this purpose, two types of chitosan gels were prepared based on chitosan glutamate and chitosan lactate. In order to reduce toxic action of the system obtained gels were conditioned in distilled water with pH 5.00. The changes in the structures of systems obtained were determined with the use of FTIR method. Biocompatibility was primarily evaluated through cytotoxicity testing by MTT assay with respect to mouse fibroblast cells.

Keywords: *Chitosan, hydrogel, biomaterial, nervous tissue regeneration*

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1. Introduction

Among materials for tissue engineering applications, major biomaterial classes considered to increase the *in vivo* integration of newly formed tissues with the surrounding cells are hydrogels. This interest is related to the fact that the human body is mainly composed of water. Hydrogels are created by hydrophilic homopolymer or copolymer networks that are able to swell in the presence of water or physiological fluids (90-99% w/w water). Hydrogel can be of either synthetic or natural origin [1-3]. Crosslinking of the hydrogel polymer chains can be achieved by wide spectrum of chemical or physical methods [4-6].

Nowadays, research is focused on development of an in situ gelling hydrogel system which is liquid at room temperature and turns into gel form at the temperature of human body (37°C). Among the natural polymers which have been extensively studied for preparation of such systems one of the most promising candidates is chitosan. Chitosan is derived from chitin, the most abundant polysaccharide found in nature after cellulose, and as a copolymer of D-glucosamine and N-acetyl-D-glucosamine is an aminopolysaccharide.

Chitosan is regarded as a biologically compatible polymer. It is allowed for use in dietary applications in Japan, Italy, and Finland. Chitosan was approved as a component of wound dressings in the USA by the Food and Drug Administration Agency [7]. Molecules of chitosan reveal biological and chemical properties that are favorable for biomedical applications. First of all, chitosan possesses low toxicity with excellent biological activities such as antimicrobial activity, low immunogenicity and antitumor activity. Moreover, this natural polymer indicates good biocompatibility and biodegradability which are crucial in biomedical applications.

Recently, it has been shown that chitosan possesses excellent mucoadhesive properties in its swollen state. It can easily adhere to hard and soft tissues. Clinical tests conducted with the use of materials containing chitosan and its derivatives revealed that they do not cause any inflammatory or allergic reactions in the human body. Chitosan is well tolerated by living tissues, including connective [8], muscle [9], epithelial [10], and nervous [11].

The cytocompatibility of chitosan and its derivatives has been studied *in vitro* with cardiomyocytes [12], keratinocytes, fibroblasts [13], endothelial cells [14], epithelial cell [15], hepatocytes [16], and chondrocytes [17]. Recently it has been shown that chitosan reveals excellent biomimetic properties that are favorable in promoting regeneration of nerve cells both in the central and in the peripheral system [18]. Chitosan has excellent neuroglail cell affinity and exhibits almost no cytotoxicity on Schwann cells growth [19]. Molecules of chitosan are hydrophilic what is beneficial when contacting them with water. Its molecular structure is very similar to the structure of glycosaminoglycans that create membranes and extracellular matrices of nervous tissue. Therefore, it can create poly-porous constructs that will mimic natural extracellular matrix and will be suitable for the growth of neural cells. Moreover, it has been shown that chitosan attracts extracellular molecules (laminin, fibronectin and collagen IV)

that are responsible for promoting nervous cells to adhere, migrate and differentiate [20, 21].

The use of chitosan involves its prior dissolution in certain organic or inorganic acids. Recently, it has been shown that there are two organic acids that naturally occur in the nervous tissue of the human body: L-glutamic acid and L-lactic acid. L-glutamic acid is one of the most common amino acids found in nature [22, 23]. It is present in many human proteins and peptides and most tissues, for example the spinal fluid contains 0.34-1.64 (mean 1.03 mg/l) of free glutamic acid. L-glutamic acid is one of a group of amino acid neurotransmitters in the brain and spinal cord. However, it has been reported that too high concentration of glutamic acid can be toxic for neuronal cells [24]. This specific type of excitotoxicity triggered by the amino acid glutamate is the key mechanism involved in the mediation of neuronal death in many disorders by excessive or prolonged activation of neuronal amino acid receptors. The excessive accumulation of glutamate in the synaptic space alters the normal cycling of it. Normal extracellular glutamate concentration is about 0.6 μmol/l. Substantial neuronal excitotoxic injury occurs with glutamate levels of 2 to 5 μmol/l.

The second solvent, lactic acid, has been suggested to be exported by astrocytes to neurons to power their mitochondria [25]. In the white matter of the brain, lactic acid can support axon function in conditions of energy deprivation following the spinal cord injury. Moreover, lactate can also support oligodendrocyte development and myelination that prevents axons from rapid damage in low energy conditions. The mean cerebrospinal fluid (CSF) lactate level is 2.00 mM/l [26] and a dramatic injury to the spinal cord is associated with about a 3-fold increase in CSF lactic acid [27].

The aim of this work is to develop a thermo-gelling chitosan solution that will be prepared with the use of acids that naturally occur in the human body and are able to enhance nervous tissue regeneration. For this purpose, two types of chitosan gels were prepared based on chitosan glutamate and chitosan lactate. It seems that the concentration of organic acid is crucial factor that must to be taken into account when developing systems for nervous tissue engineering applications. Therefore, the cytotoxicity was tested on hydrogel systems prepared in two ways. First test was conducted with the use of the system just after preparation. In order to reduce toxic action of the system, obtained gels were conditioned in distilled water with pH 5.00. The changes in the structures of these two systems were determined with the use of FTIR method. Biocompatibility was primarily evaluated through cytotoxicity testing by MTT assay with respect to mouse fibroblast cells.

2. Materials and methods

2.1. Preparation of thermo-gelling chitosan solutions

The samples were prepared with the use of two acid solvents: glutamic acid (the CH/GLU/GP system) and lactic acid (the CH/LA/GP system).

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A thermo-gelling chitosan glutaminate 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.

A thermo-gelling chitosan lactate solution (the CH/LA/GP system) was prepared by dissolving 400 mg of chitosan (SIGMA-ALDRICH®) in 18 ml of 1% or 0.75% lactic acid (SIGMA-ALDRICH®). The obtained solution was stirred (under slow rotations) until complete dissolution. Then the sample was placed in the ice bath and chilled to 4°C. Then 2 g of β-glycerophosphate disodium salt hydrate (SIGMA-ALDRICH®) dissolved in 2.5 ml of distilled water was added dropwise under stirring to the chilled sample. The obtained solution was stirred (under slow rotations) for 20 min. Prepared sample was placed at 4°C. Then sample was kept at 37°C for 12 h. In these conditions it undergoes phase transition from sol to gel.

The *in vitro* cytotoxicity test was conducted on samples just after preparation and after 72 h of the *in vitro* conditioning in pH 5 distilled water.

The samples obtained were sterilized by autoclaving (SterilClave 24B, COMINOX®) using the cycle special destined to sterilize liquids.

2.2. *In vitro* release studies

Before the *in vitro* release studies, the samples were stored at 37 °C for 12 h in order to obtain a gel phase system with well-defined 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 (pH = 5.0 ± 0.5) with a capacity of 900 dm^3. The temperature of the release medium was kept constant at 37°C during the whole release process.

2.3. FTIR studies

The FTIR spectra were obtained using a Perkin-Elmer apparatus in the range 4000-500 cm^{-1}.

2.4. *In vitro* cytotoxicity studies

The samples for *in vitro* cytotoxicity tests were prepared according to the ISO standard 10993-12 (2010): Biological evaluation of medical devices – Sample preparation and reference material, and the tests were conducted in accordance with ISO 10993-5 (2009) Tests for *in vitro* cytotoxicity. Mouse fibroblast cells L-929 (NCTC clone 929: CCL 1, American Type Culture Collection ATCC®), were cultured in culture medium composed of Eagle's minimum essential medium (EMEM) with L-glutamine (ATCC®) and

supplemented with 10% fetal bovine serum (FBS, Lonza®). The tests were performed using extracts prepared using culture medium with serum. Extracts were prepared in the following proportions: (1) negative control – high density polyethylene (HDPE, U.S. Pharmacopeia - Rockville, MD, USA) with the surface area of 6 cm^2 /1 ml, (2) positive control - sodium lauryl sulfate (SLS, SIGMA-ALDRICH®) with the following concentrations: 0.2, 0.15, and 0.1 mg/ml, and (3) chitosan gels with the sample mass of 0.2 g/ 1 ml of culture medium. In addition, a blank test was used - medium with serum containing no sample.In order to conduct the *in vitro* cytotoxicity assessment, the samples of chitosan gels extracts were prepared with the following concentrations: 100, 50, 25 and 12.5%.

L-929 cells were seeded in 96-well flat-bottomed cell culture plates (at 1×10^4 cells in 100 µl of medium per well), incubated at 37 °C for 24 h to allow attachment and then the medium was replaced with 100 µl of test extract or control group extract. Cytotoxicity was assessed after a further 24 h of incubation. The changes from normal morphology were evaluated with the use of an inverted microscope *(*CKX 41, Olympus®). The viability of cells was assessed with the use of the MTT assay (1 mg /1ml MEM without phenol red, without glutamine and without $NaHCO_3$, SIGMA-ALDRICH®).

To perform MTT 50 µL of MTT reagent was added to each well and plates were incubated at 37°C in 5% CO2 for 3 h. Then, the MTT solution was discanted and 100 µl of analytical grade isopropanol (STANLAB®) was added to each well. After 30 min, the absorbance values were recorded at 570 nm using an Epoch Microplate Spectrophotometer (BioTek®).

Cell viability was calculated according to the following formula:

$$V = \frac{100\ x\ \ OD_{570A}}{OD_{570B}}\%$$

where: V - cell viability; [%]; $OD_{570\ A}$ - mean value of the measured optical density of the test sample [-]; $OD_{570\ B}$ - mean value of the measured optical density of the blank sample [-].

3. Results and Discussion

3.1. Structural characterization

FTIR studies were conducted in order to determine the structural changes in the glucosamine units of the chitosan molecule upon gel formation. Figure 1 shows the FTIR spectrum of the CH/GLU/GP BR system and the CH/LA/GP BR system lyophilized immediately after gelation, before subjecting the samples to the *in vitro* release studies. In Figure 2, the FTIR spectra of both systems are shown after 72 h of the *in vitro* conditioning in distilled water (denoted as the CH/GLU/GP AR and the CH/LA/GP AR system). As a comparison, the FTIR spectra of native chitosan (red curve) and β-glycerol phosphate disodium salt (violet curve) are also depicted.

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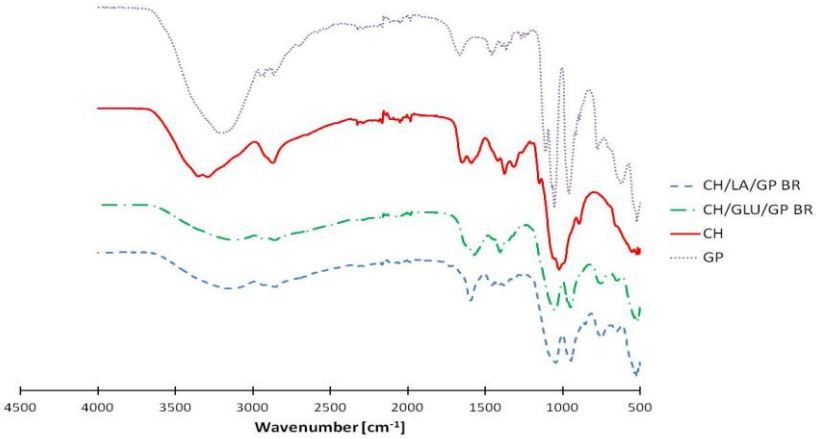

Figure 1. The FTIR spectra of the CH/GLU/GP BR system and the CH/LA/GP BR system just after preparation. As comparison, the spectra of native CH (red curve) and GP (blue curve) are shown.

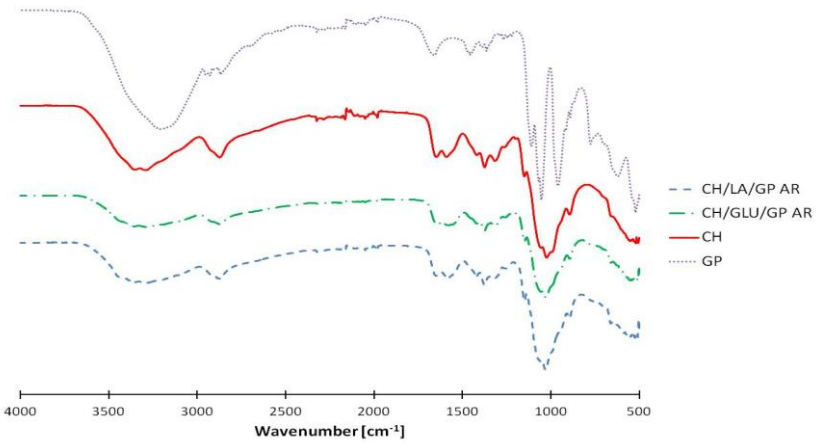

Figure 2. The FTIR spectra of the CH/GLU/GP AR system and the CH/LA/GP AR system after 72 h of conditioning in distilled water. As comparison, the spectra of native CH (red curve) and GP (blue curve) are shown.

The FTIR spectra of the CH/GLU/GP and the CH/LA/GP systems after gelation indicate the characteristic peaks for chitosan and glycerol phosphate disodium salt.

The spectrum of chitosan shows a peak at 1648 cm^{-1} that is assigned to the C=O stretch of the amide bound and a peak at 1589 cm^{-1} that is assigned to the NH_2 group of chitosan. These peaks indicate the partially deacetylated form of chitin being applied in the present work. The spectrum of the CH/GLU/GP BR system shows an asymmetric peak, one pronounced at 1571 cm^{-1} (from amino groups) with a minor shoulder at 1620 cm^{-1} (from acetyl groups). The distinct peak characteristic of amino groups, that partially absorbs peak characteristic of acetylamino groups, probably results from higher concentration of amino groups in the CH/GLU/GP BR system structure due to the presence of glutamic acid. The CH/LA/GP BR system shows a peak at 1595 cm^{-1}. This peak can indicate the presence of protonated amino groups or can result from the higher concentration of carboxylic groups.

In the range of 1200-1500 cm^{-1}, chitosan molecule shows four peaks at 1419, 1375, 1315 and 1264 cm^{-1}. The peaks at 1419 and 1314 cm^{-1} are associated to oscillations characteristic for OH and C-H bending of CH_2 groups. The peak at 1375 cm^{-1} represents the C-O stretching of the primary alcoholic group -CH_2-OH. For the CH/GLU/GP BR system, the peaks at 1419, 1375 and 1315 cm^{-1} are shifted to respectively 1448, 1404 and 1346 cm^{-1} and for the CH/LA/GP system these peaks are shifted to respectively 1454, 1416 and 1371 cm^{-1}.

In the wavenumber range of 800-1200 cm^{-1}, the FTIR spectrum of chitosan shows three peaks at 1159, 1024 and 894 cm^{-1}. The wide band at 1155-1037cm^{-1} represents the bridge O stretch of the glucosamine residues. Both systems, CH/GLU/GP BR and CH/LA/GP BR do not reveal peaks characteristic for chitosan in this wavenumber range. However, two new peaks characteristic for GP appear in this region. The peak at 1050 cm^{-1} is characteristic for GP and indicates the aliphatic P-O-C stretching. The peak at 960 cm^{-1} is present with a minor shoulder at 980 cm^{-1}. The peak at 980 cm^{-1} is characteristic for the nonprotonated phosphate group (-PO_4^{2-}) whereas the peak at 960 cm^{-1} may indicate the presence of the -HPO_4^- group. Both systems developed reveal these peaks being shifted to 975 and 948 cm^{-1}, respectively. For the CH/GLU/GP AR and the CH/LA/GP AR system, the peaks characteristic for chitosan (at 1150, 1030 and 895 cm^{-1}) can be observed.

The FTIR spectra obtained for the systems conditioned in distilled water show only peaks characteristic for chitosan molecule. The peaks connected with the presence of phosphorus in the system structures, P-O-C and -PO_4^{2-}, disappear.

These results are in accordance with our previous work suggesting that in the case of chitosan gels based chitosan and β-glycerophosphate during the process of the system gelation charged groups of chitosan are being deprotonated what makes the groups of acid and glycerophosphate free to diffuse out of the system structure [28]. The washing out of these groups can be observed with the use of the FTIR spectra. The FTIR spectrum of system just after gelation shows peaks characteristic for all components. After the *in vitro* conditioning process only peaks characteristic for native chitosan are visible. This observation suggests that the system is composed mainly from polymer chains. Moreover,

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the *in vitro* conditioning leads only to structural changes of the system without its macroscopic dimensional changes.

3.2. Cytotoxicity evaluation

The results of the *in vitro* cytotoxicity assessment of the CH/GLU/GP system and the CH/LA/GP system just after preparation and after 72 h of the conditioning in pH 5 are presented in Table 1 and Table 2, respectively. The photos of cell cultures are shown in Figure 3 and 4.

Table 1. Results of the *in vitro cytotoxicity assessment* of the CH/GLU/GP system just after preparation and after 72 h of the conditioning in pH 5 distilled water. Data represent the mean of three independent measurements.

sample	extract	cell viability	description	grade
CH/GLU/ GP	100%	14.43 %	complete destruction of the cell layers, wide lysis of cells (Fig. 3)	4
	50%	16.91 %	complete destruction of the cell layers, wide lysis of cells (Fig. 3)	4
	25%	94.58 %	discrete intracytoplasmatic granules, no cell lysis, no reduction of cell growth, cell cover all cell culture plates, a lot of cells in fission (Fig. 3)	0
	12.5%	104.79 %	discrete intracytoplasmatic granules, no cell lysis, density of culture is comparable to density of negative control culture	0
CH/GLU/ GP 72 h	100%	94.0 %	discrete intracytoplasmatic granules, no cell lysis, a lot of cells in fission (Fig. 3)	0
	50%	101.6 %	discrete intracytoplasmatic granules, no cell lysis, a lot of cells in fission, density of culture is comparable to density of negative control culture (Fig. 3)	0
	25%	107.56 %	discrete intracytoplasmatic granules, no cell lysis, a lot of cells in fission (Fig. 3)	0
	12.5%	108.72 %	discrete intracytoplasmatic granules, no cell lysis, a lot of cells in fission	0

Table 2. Results of the *in vitro* cytotoxicity assessment of the CH/LA/GP system just after preparation and after 72 h of the conditioning in pH 5 distilled water. Data represent the mean of three independent measurements.

sample	extract	cell viability	description	grade
CH/LA$_{1.0}$ /GP	100%	95.9 %	discrete intracytoplasmatic granules, no cell lysis (Fig. 4)	0
	50%	96.7 %	discrete intracytoplasmatic granules, no cell lysis (Fig. 4)	0
	25%	98.4 %	discrete intracytoplasmatic granules, no cell lysis, no reduction of cell growth, cell cover all cell culture plates, a lot of cells in fission (Fig. 4)	0
	12.5%	100.8 %	discrete intracytoplasmatic granules, no cell lysis, density of culture is comparable to density of negative control culture (Fig. 4)	0
CH/LA$_{1.0}$ /GP 72 h	100%	91.9 %	discrete intracytoplasmatic granules, no cell lysis (Fig. 4)	0
	50%	94.3 %	discrete intracytoplasmatic granules, no cell lysis (Fig. 4)	0
	25%	94.3 %	discrete intracytoplasmatic granules, no cell lysis (Fig. 4)	0
	12.5%	99.2 %	discrete intracytoplasmatic granules, no cell lysis, density of culture is comparable to density of negative control culture (Fig. 4)	0

Based on the results obtained, it seems that there is a critical concentration of L-glutamic acid above which chitosan extracts are toxic for L-929 cells. It seems that during the *in vitro* conditioning in distilled water L-glutamic acid is being washed out of the system and the system becomes non-toxic for living cells. The foreseen application of the system obtained is nervous system tissue engineering. Recently, it has been reported that too high concentration of glutamic acid can cause toxic effect on neuronal cells [24]. This specific type of excitotoxicity triggered by the amino acid glutamate is the key mechanism involved in the mediation of neuronal death in many disorders by excessive or prolonged activation of neuronal amino acid receptors. The excessive accumulation of glutamate in the synaptic space alters the normal cycling of it. Normal extracellular glutamate concentration is about 0.6 μmol/l.

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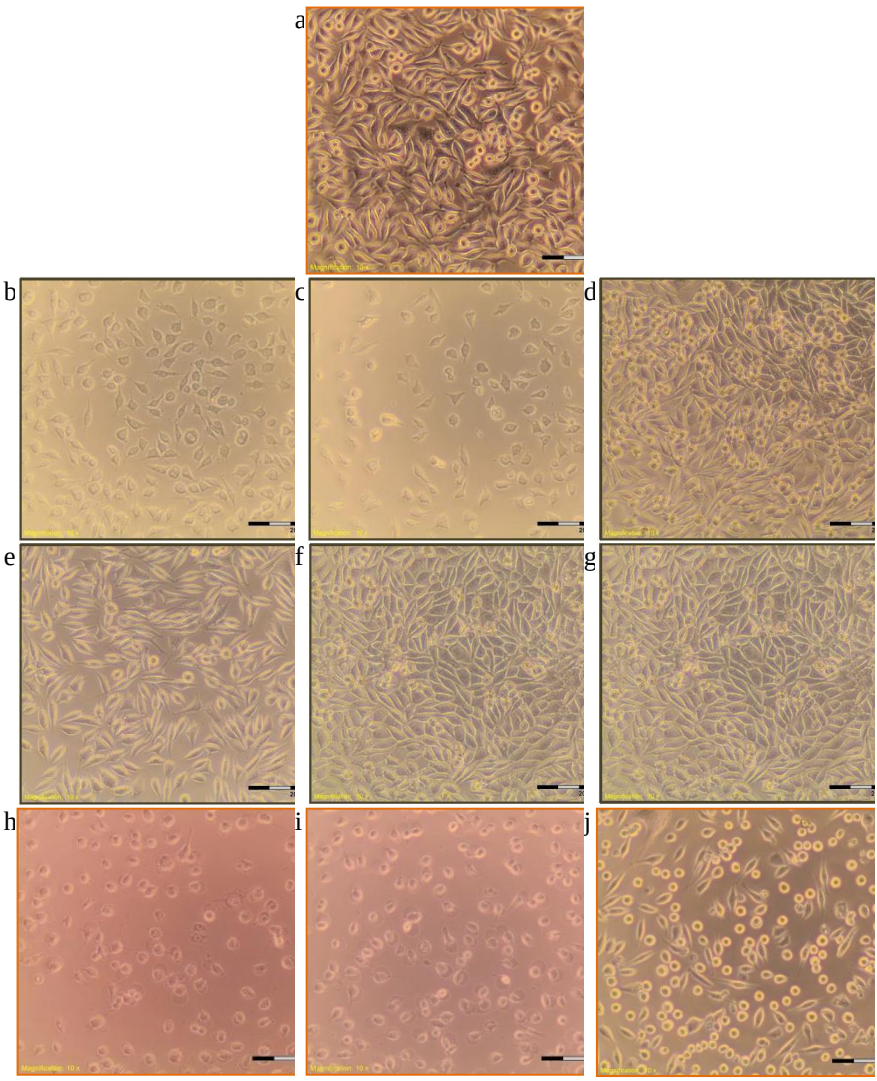

Figure 3. Exemplary photographs of mouse fibroblasts cells (L-929), with (a) negative control, (b),(c),(d) the CH/GLU/GP system (100, 50, and 25% of extract concentration); (e), (f), (g) the CH/GLU/GP 72 h system (100, 50, and 25% of extract concentration) and (h), (i), (j) positive control (0.2, 0.15, and 0.1 mg/ml of SLS).

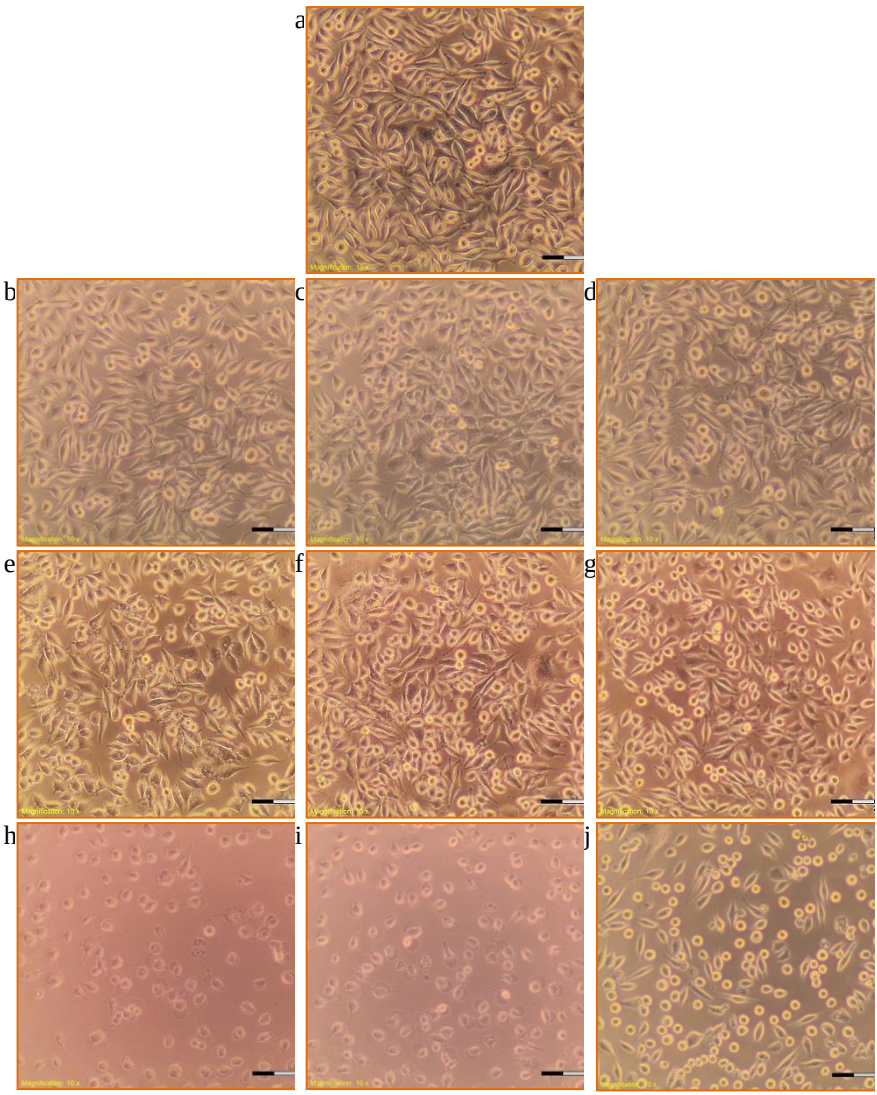

Figure 4. Exemplary photographs of mouse fibroblasts cells (L-929), with (a) negative control, (b),(c),(d) the CH/LA/GP system (100, 50, and 25% of extract concentration); (e), (f), (g) the CH/LA/GP 72 h system (100, 50, and 25% of extract concentration) and (h), (i), (j) positive control (0.2, 0.15, and 0.1 mg/ml of SLS).

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Substantial neuronal excitotoxic injury occurs with glutamate levels of 2 to 5 μmol/l. These values are significantly lower than the amount of L-glutamic acid used to dissolve chitosan molecules and prepare the CH/GLU/GP system. The *in vitro* conditioning in distilled water significantly reduced its toxicity with respect to mouse fibroblast cells; however it seems reasonable to replace it with different acid when neural cells are considered. In order to reduce the cytotoxicity of the system obtained L-glutamic acid was replaced by L-lactic acid. The mean cerebrospinal fluid (CSF) lactate level is 2.00 mM/l [26] and a dramatic injury to the spinal cord is associated with about a 3-fold increase in CSF lactic acid [27]. Therefore, the samples were prepared initially with the concentration equal to 1% in order to test their *in vitro* toxicity to mouse fibroblast. The data obtained indicate that neither the CH/LA/GP system nor the CH/LA/GP 72 h system reveals toxic action on mouse fibroblasts.

4. Conclusions

The chitosan systems obtained with the use of glutamic acid as well as lactic acid reveal satisfactory biocompatibility with respect to mouse fibroblast cells. However, the application of the system based on glutamic acid needs its prior *in vitro* conditioning in distilled water. The conditioning in distilled water causes the washing out of excessive amount of glutamic acid from the system structure. According to this process the proportion of all original ingredients is changed and the resulting system structure is composed mainly of native chitosan. In conclusions, both system obtained are excellent candidates for skin as well as spinal cord tissue regeneration.

5. Acknowledgements

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6. References

1. Hoffman AS; (2002) Hydrogels for biomedical applications. Adv Drug Deliv Rev 54, 3-12. **DOI**: 10.1016/S0169-409X(01)00239-3
2. Qiu Y, Park K; (2001) Environment-sensitive hydrogels for drug delivery. Adv Drug Deliv Rev 53, 321-39. **DOI**: 10.1016/S0169-409X(01)00203-4
3. Censi R, Di Martino P, Vermonden T, Hennink WE; (2012) Hydrogels for protein delivery in tissue engineering. J Control Release 161, 680-92. **DOI**: 10.1016/j.jconrel.2012.03.002
4. Wang L, Stegemann JP; (2010) Thermogelling chitosan and collagen composite hydrogels initiated with beta-glycerophosphate for bone tissue engineering. Biomaterials 31, 3976-85. **DOI**: 10.1016/j.biomaterials.2010.01.131
5. Grolik M, Szczubiałka K, Wowra B, Dobrowolski D, Orzechowska-Wylęgała B, Wylęgała E, Nowakowska M; (2012) Hydrogel membranes based on genipin-cross-linked chitosan blends for corneal epithelium tissue

engineering. J Mater Sci Mater Med 23, 1991-2000. **DOI**: 10.1007/s10856-012-4666-7

6. Fatimi A, Chabrot P, Berrahmoune S, Coutu JM, Soulez G, Lerouge S; (2012) A new injectable radiopaque chitosan-based sclerosing embolizing hydrogel for endovascular therapies. Acta Biomater 8, 2712-21. **DOI**: 10.1016/j.actbio.2012.04.006
7. Dash M, Chiellini F, Ottenbrite RM, Chiellini E; (2011) Chitosan - A versatile semi-synthetic polymer in biomedical applications. Prog Polym Sci 36, 981–1014. **DOI**: 10.1016/j.progpolymsci.2011.02.001
8. Tsai WB, Chen YR, Liu HL, Lai JY; Fabrication of UV-crosslinked chitosan scaffolds with conjugation of RGD peptides for bone tissue engineering. Carbohyd Polym 85, 129–37. **DOI**: 10.1016/j.carbpol.2011.02.003
9. Zakhem E, Raghavan S, Gilmont RR, Bitar KN; (2012) Chitosan-based scaffolds for the support of smooth muscle constructs in intestinal tissue engineering. Biomaterials 33, 4810-7. **DOI**: 10.1016/j.biomaterials.2012.03.051
10. Hsu SH, Kuo WC, Chen YT, Yen CT, Chen YF, Chen KS, Huang WC, Cheng H; (2013) New nerve regeneration strategy combining laminin-coated chitosan conduits and stem cell therapy. Acta Biomater 9, 6606-15. **DOI**: 10.1016/j.actbio.2013.01.025
11. Xu H, Yan Y, Li S; (2011) PDLLA/chondroitin sulfate/chitosan/NGF conduits for peripheral nerve regeneration. Biomaterials 32, 4506-16. **DOI**: 10.1016/j.biomaterials.2011.02.023
12. Chiu LL, Janic K, Radisic M; (2012) Engineering of oriented myocardium on three-dimensional micropatterned collagen-chitosan hydrogel. Int J Artif Organs 35, 237-50. **DOI:** 10.5301/ijao.5000084
13. Chatelet C, Damour O, Domard A; (2001) Influence of the degree of acetylation on some biological properties of chitosan films. Biomaterials 22, 261-8. **DOI**: 10.1016/S0142-9612(00)00183-6
14. Deng C, Zhang P, Vulesevic B, Kuraitis D, Li F, Yang AF, Griffith M, Ruel M, Suuronen EJ; (2010) A collagen–chitosan hydrogel for endothelial differentiation and angiogenesis. Tissue Eng Part A 16, 3099-109.
15. Teijeiro-Osorio D, Remuñán-López C, Alonso MJ; (2009) Chitosan/cyclodextrin nanoparticles can efficiently transfect the airway epithelium in vitro. Eur J Pharm Biopharm 71, 257-63. **DOI**: 10.1016/j.ejpb.2008.09.020
16. Loh JW, Yeoh G, Saunders M, Lim LY; (2010) Uptake and cytotoxicity of chitosan nanoparticles in human liver cells. Toxicol Appl Pharmacol 249, 148-57. **DOI**: 10.1016/j.taap.2010.08.029
17. Fehrenbacher A, Steck E, Roth W, Pahmeier A, Richter W; (2006) Long-term mechanical loading of chondrocyte-chitosan biocomposites in vitro enhanced their proteoglycan and collagen content. Biorheology 43, 709-20.

18. Gnavi S, Barwig C, Freier T, Haastert-Talini K, Grothe C, Geuna S; (2013) The use of chitosan-based scaffolds to enhance regeneration in the nervous system. Int Rev Neurobiol 109, 1-62. **DOI**: 10.1016/B978-0-12-420045-6.00001-8
19. Yuan Y, Zhang P, Yang Y, Wang X, Gu X; (2004) The interaction of Schwann cells with chitosan membranes and fibers in vitro. Biomaterials 25, 4273-8. **DOI**: 10.1016/j.biomaterials.2003.11.029
20. Nomura H, Zahir T, Kim H, Katayama Y, Kulbatski I, Morshead CM, Shoichet MS, Tator CH; (2008) Extramedullary chitosan channels promote survival of transplanted neural stem and progenitor cells and create a tissue bridge after complete spinal cord transaction. Tissue Eng Part A 14; 649-65.
21. Li X, Yang Z, Zhang A, Wang T, Chen W; (2009) Repair of thoracic spinal cord injury by chitosan tube implantation in adult rats. Biomaterials 30, 1121-32. **DOI**: 10.1016/j.biomaterials.2008.10.063
22. Young VR, Borgonha S; (2000) Nitrogen and amino acid requirements: the Massachusetts Institute of Technology amino acid requirement pattern. J Nutr 130, 1841S-1849S.
23. Armstrong M, Jonscher K, Reisdorph NA; (2007) Analysis of 25 underivatized amino acids in human plasma using ion-pairing reversed-phase liquid chromatography/time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 21, 2717-26. **DOI**: 10.1002/rcm.3124
24. Mark LP, Prost RW, Ulmer JL, Smith MM, Daniels DL, Strottmann JM, Brown WD, Hacein-Bey L; (2001) Pictorial review of glutamate excitotoxicity: fundamental concepts for neuroimaging. AJNR Am J Neuroradiol 22; 813-24.
25. Rinholm JE, Hamilton NB, Kessaris N, Richardson WD, Bergersen LH, Attwell D; (2011) Regulation of oligodendrocyte development and myelination by glucose and lactate. J Neurosci 31, 538-48. **DOI**: 10.1523/JNEUROSCI.3516-10.2011
26. Regenold WT, Phatak P, Makley MJ, Stone RD, Kling MA; (2008) Cerebrospinal fluid evidence of increased extra-mitochondrial glucose metabolism implicates mitochondrial dysfunction in multiple sclerosis disease progression. J Neurol Sci 275, 106-12. **DOI**: http://dx.doi.org/10.1016/j.jns.2008.07.032
27. Nyberg F, Wiesenfeld-Hallin Z, Sharma HS, Neuropeptides in the Spinal Cord, 1995, Elsevier, ISBN: 978-0-444-81719-8
28. Modrzejewska Z, Nawrotek K, Zarzycki R, Douglas T; (2013) Structural Characteristics of Thermosensitive Chitosan Glutaminate Hydrogels. Progress in the Chemistry and Application of Chitin and its Derivatives 8, 93-105.