BIOLOGICAL PROPERTIES OF THERMOSENSITIVE CHITOSAN GELS CONDITIONING IN WATER

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Abstract

In this paper the properties of thermosensitive chitosan hydrogels prepared with the use of chitosan salz (chloride and lactate) and β-glycerophosphate are presented. Biological studies were carried out on thermogels without conditioning in water and on thermogels after 24 h conditioning in water. Experiments were conducted on a reference cell line L 929 (NCTC clone 929) American Type Culture Collection according to Annex C PN-EN ISO 10993-5

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1. Introduction

Thermosensitive structures are made from polymers which in aqueous solutions show temperature-dependent sol-gel transition. The transition from a viscous liquid to hydrogel is a result of a quick increase of viscosity at a temperature called the lower critical solution temperature (LCST) [1-3]. For biomedical applications, thermo-gelling injectable systems with an LCST around or below 37 °C would be ideal, as they would transform from a solution to a gel upon injection into a body cavity [4,5].

Currently, there is much interest in hydrogel materials of natural origin, in particular chitosan which is a product of chitin deacetylation [6]. Chitosan hydrogels, whose sol-gel transition takes place at natural temperature of human body, are formed mainly from chitosan salt solutions of low viscosity with the use of β-glycerophosphate as a neutralizing agent [7-13] but also from polyvinyl alcohol and sodium bicarbonate [14-16] by the enzymatic method with the use of urease and urea [17] and by using β-tricalcium phosphate [18].

2 Materials and Methods

2.1 Preparation of chitosan hydrogels

Chitosan chloride and lactate solutions was prepared by dissolution 400 mg of chitosan (CH, chitosan from shrimp shells with low viscosity and degree of deacetylation ~79.5% SIGMA-ALDRICH®) in 16 ml of 0.1 M HCl or $C_3H_6O_3$; The solutions was stirred (at slow rotations) 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 drop-wise under stirring in an ice bath. The final solution was mixed for another 20 min and stored at 4°C for 12 h. Samples were stored at 37°C for 24 h, to obtain a gel structure.

2.2. Methods

2.2.1. Material tested

Biological studies were carried out on thermogels without conditioning in water and on thermogels after 24 h conditioning in water. The formed samples were subjected to thermal sterilization (in a sterilizer using the fluid sterilization program – 126^{o}C for 20 min.).

Studies on the cytotoxic activity were carried out by the direct method using extracts in accordance with PN-EN ISO 10993-5:2009 standard "Biological assessment of medical products. Cytotoxicity tests", and PN-EN ISO 10993-12:2009 standard "Biological assessment of medical products" – part 12: "Preparation of samples and reference material".

Experiments were conducted on a reference cell line L 929 (NCTC clone 929) American Type Culture Collection according to Annex C PN-EN ISO 10993-5. Cells for the experiments were stored in liquid nitrogen in the culture broth EMEM with L-glutamine (ATCC - 30001265 series) with 10% FBS (FBS Brazilian Origin, Lonza Sales Ltd. - 1SB010 series), 7% DMSO (Sigma-Aldrich - RNBC1951 series).

After thawing the cells were passaged twice using trypsin (0.25% Trypsin - EDTA Solution, Sigma-Aldrich - 11C508 series). The cell culture medium had the following composition: EMEM with L glutamine (ATCC - 30001265 series) with 10% FBS (Lonza Sales Ltd - 1SB010 series).

Cells were grown in the conditions recommended for tissue culture in a Steri Cycle 381 incubator (Thermo Scientific) with the required parameters: 5% CO_2, 37°C, constant wetting of the chamber.

2.2.2. Preparation of extracts

Extracts from the tested samples were prepared according to the recommendations given in PN-EN ISO 10993-12:2009 "Biological assessment of medical products" – Part 12: "Preparation of samples and reference material" in the ratio: 2 g sample/1 cm^3 culture broth EMEM with L glutamine (ATCC - 30001265 series) with 10% serum (Lonza Sales Ltd - 1SB006 series). As negative control high density polyethylene was used (HDPE U.S. Pharmacopeia - Rockville, MD, USA), and as positive control the solution of sodium lauryl sulfate (SLS) at concentrations 0.1 mg/ml and 0.2 mg/ml was applied. The samples were incubated at a temperature of 37°C for 24 h.

To evaluate the cytotoxic activity the extracts from chitosan materials were used at the following concentrations: 100%, 50% and 25%. In the tests the extract from negative control and blank sample was used in 100% concentration.

2.2.3. Experimental conditions

Mouse fibroblast cells were placed on NUNC 96-well plates. To each well 100 µl cell suspension of density 1×10^5/ml (1×10^4/well) was added. After 24 hours the cells got stuck to the bottom and divided, covering around 80% of the plate surface and forming a monolayer. After that time the culture broth was removed and to each well extracts from the tested samples or control extracts in the amount of 100 µl were added and incubated for 24 h at a temperature of 37±1°C in the atmosphere of 5% CO_2.

2.2.4. Evaluation of the cytotoxic activity

The cytotoxic activity was estimated after 24 h incubation of cells in the extracts. Morphological changes after the contact with the tested material were estimated in an Olympus CKX 41 phase-contrast microscope and cell viability was assessed with MTT assay (1 mg /1ml MEM without phenol red and supplements – General Chemistry Laboratory II and TD PAN; 15.05.2013 series) according to Annex C PN-EN ISO 10993-5:2009.

After 24 h since adding the extracts, cell morphological evaluation was performed according to the criteria given in PN-EN ISO 10993-5:2009.

After evaluation of the culture under the phase-contract microscope, the tested and control extracts were removed from the plates and to each well 50 µl of MTT solution was added (10 mg Thiazolyl Blue Tetrazolium Bromide, Sigma-Aldrich, MKBH9792V series suspended in 10 ml medium MEM without phenol red and supplements) and incubated for 3 h at 37°C in the atmosphere of 5% CO_2.

After that period the MTT solution was decanted and to each well 100 µl isopropyl alcohol was added (P.P.H. STANLAB – 02/07/11 series). After 30

minutes (until complete dissolution of formazan) the plate was placed in an Epoch spectrophotometer (Biotek) and reading of absorbance was performed at the wavelength of 570 nm, for tested and control samples at n=6 and for blank sample at n=12. Cell viability was calculated from the formula:

$$V\% = \frac{AB \times 100}{As}$$

where: *Ab* – average absorbance of the tested samples; *As* – average absorbance of the blank sample.

Changes in the cultures exceeding 2 grades and decrease of cell viability by more than 30% are considered to form a cytotoxic effect.

3. Results and Discussion

Results of studies on cytotoxic activity are presented in Tables 1-3 and Figures 1-5.

Table 1. Estimation of cytotoxic activity of chitosan chloride

Sample	Extract	Cell viability [%]	Changes in the culture	Estimation of changes in the culture
Negative sample	HDPE 100%	103.3	Single intraplasmatic grains. No cell lysis is observed. Cells cover the whole well. Many cells in divisions (Fig.1)	**0**
Positive sample	SLS 0.1 mg/ml	57.7	More than 30% rounded, shrunken cells separating from the base. Single cells disrupted. Empty space between cells (Fig. 2a)	**2**
	SLS 0.2 mg/ml	11.14	Completely destroyed cell culture (Fig. 2b)	**4**
Thermogel conditioned in H_2O for 24h	Extract 100%	96.6	Single intraplasmatic grains. No cell lysis is observed. Cells cover the whole well (Fig. 3a)	**0**
	Extract 50%	103.3	Single intraplasmatic grains. No cell lysis is observed. Cells cover the whole well. Culture density is comparable to the negative control (Fig. 3b)	**0**
	Extract 25%	103.7	Single intraplasmatic grains. No cell lysis is observed. Cells cover the whole well. Culture density is comparable to the negative control (Fig. 3c)	**0**
Thermogel immediately after formation	Extract 100%	101.1	Single intraplasmatic grains. No cell lysis is observed. Cells cover the whole well. Culture density is comparable to the negative control (Fig. 3d)	**0**
	Extract 50%	101.1	Single intraplasmatic grains. No cell lysis is observed. Cells cover the whole well. Culture density is comparable to the negative control (Fig. 3e)	**0**
	Extract 25%	103.3	Single intraplasmatic grains. No cell lysis is observed. Cells cover the whole well. Culture density is comparable to the negative control (Fig. 3f)	**0**

Table 2. Estimation of cytotoxic activity of chitosan lactate

Sample	Extract	Cell viability [%]	Changes in the culture	Estimation of changes in the culture
Thermogel conditioned in H_2O for 24h	Extract 100%	95.9	Single intraplasmatic grains. No cell lysis is observed. Cells cover the whole well (Fig. 4a)	**0**
	Extract 50%	96.7	Single intraplasmatic grains. No cell lysis is observed. Cells cover the whole well. Culture density is comparable to the negative control (Fig. 4b)	**0**
	Extract 25%	98.4	Single intraplasmatic grains. No cell lysis is observed. Cells cover the whole well. Culture density is comparable to the negative control (Fig. 4c)	**0**
Thermogel immediately after formation	Extract 100%	91.9	Single intraplasmatic grains. No cell lysis is observed. Cells cover the whole well. Culture density is comparable to the negative control (Fig. 4d)	**0**
	Extract 50%	94.3	Single intraplasmatic grains. No cell lysis is observed. Cells cover the whole well. Culture density is comparable to the negative control (Fig. 4e)	**0**
	Extract 25%	94.3	Single intraplasmatic grains. No cell lysis is observed. Cells cover the whole well. Culture density is comparable to the negative control (Fig. 4f)	**0**

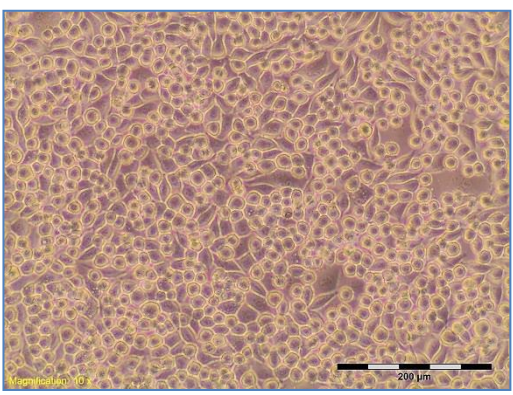

Figure 1. Negative control sample

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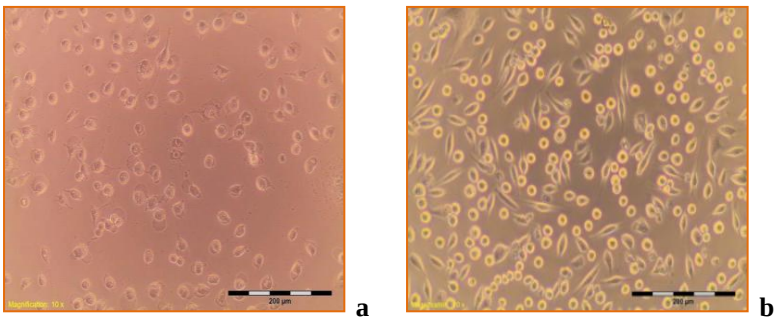

Figure 2. Positive control sample (**a** - 0.2 mg/ml of SLS; **b** - 0.1 mg/ml of SLS).

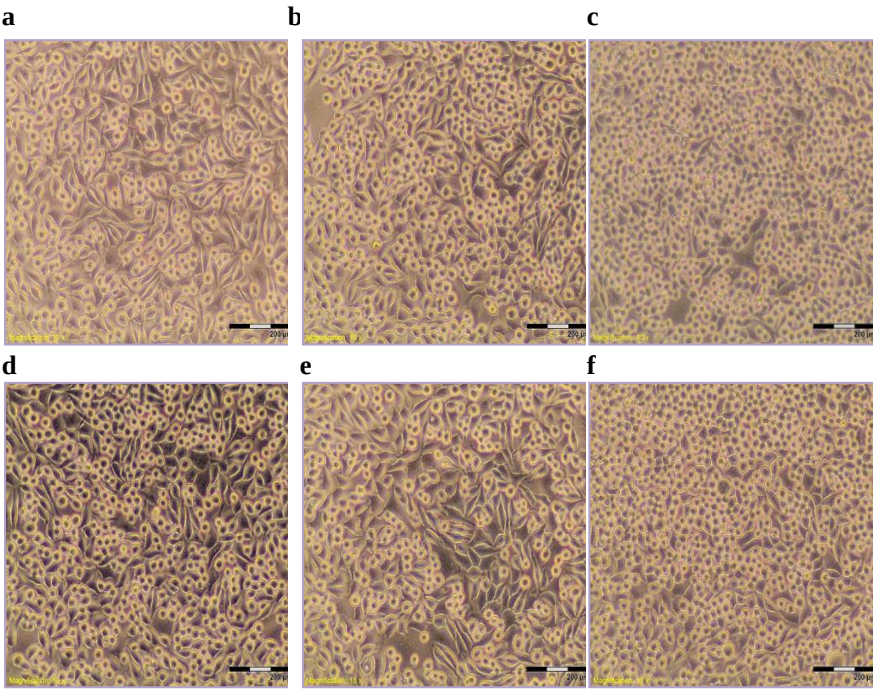

Figure 3. Samples of chitosan chloride gel directly after formation. **a**. Extract 100%, **b**. Extract 50%, **c**. Extract 25%, Samples of chitosan chloride gel after conditioning in H_2O, 37^oC, **d**. Extract 100%, **e**. Extract 50%, **f**. Extract 25%

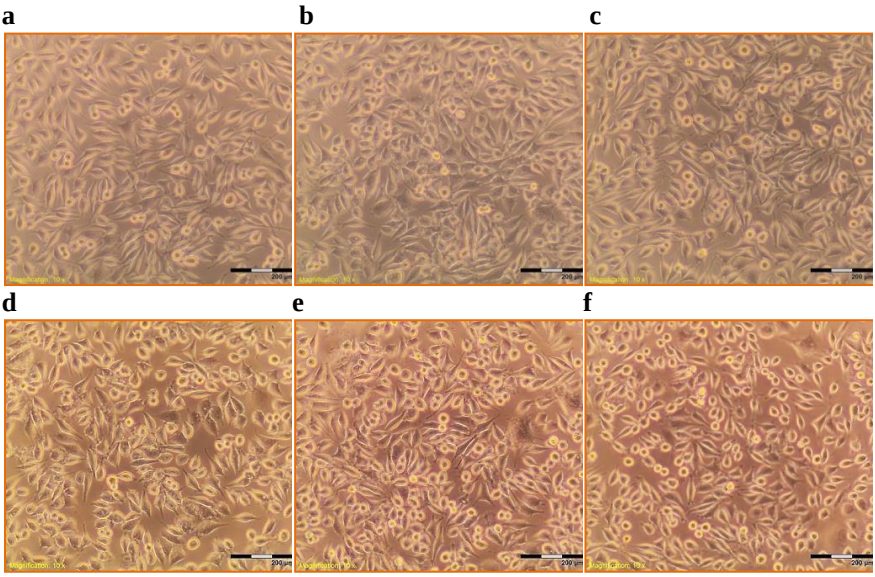

Figure 4. Samples of chitosan lactate gel directly after formation. **a**. Extract 100%, **b**. Extract 50%, **c**. Extract 25%, Samples of chitosan chloride gel after conditioning in H_2O, 37°C, **d**. Extract 100%, **e**. Extract 50%, **f**. Extract 25%

On the basis of studies on the cytotoxic effect of chitosan material it was found that extracts from chitosan chloride and lactate hydrogels immediately after formation and conditioning in water at 37°C at any concentration do not induce changes in the culture. Cell viability is comparable to that in the negative sample and so chitosan hydrogels have no cytotoxic effect.

4. Conclusions

Thermosensitive chitosan gels do not show any toxic activity. The systems obtained are candidates for scaffolds for tissue regeneration.

5. Acknowledgment

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