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# MULTIFUNCTIONAL CHITOSAN-BASED HYDROGELS: CHARACTERIZATION AND EVALUATION OF BIOCOMPATIBILITY AND BIODEGRADABILITY *IN VITRO*

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*Creation of novel remedies efficient in supporting wound healing remains an actual task in pharmacology. Hydrogels showed high efficiency in wound healing and tissue regeneration due to viscosity, elasticity and fluidity that provide them with functional characteristics similar to that in extracellular matrix. The aim of the study was to create chitosan-based hydrogels functionalized with different components (chondroitin-6-sulfate, hyaluronic acid, N-stearoylethanolamine) and to estimate their biocompatibility and biodegradability in vitro. For the first time, a lipid substance N-stearoylethanolamine (NSE) known as suppressor of pro-inflammatory cytokines expression was used as hydrogel component (1.95 mg/g). FTIR analysis confirmed the complexation of chitosan molecule with hyaluronate, chondroitin-6-sulfate, NSE. MTT-test and Trypan blue exclusion test were used to study hydrogels cytotoxicity towards human cells of different tissue origin. Biodegradability of hydrogels was evaluated using direct hydrogel contact with cells and cell-independent degradation. It was shown that chondroitin-6-sulfate (<2 mg/ml), hyaluronic acid (<2 mg/ml) and NSE (26 µg/ml) did not demonstrate significant toxic effects towards pseudonormal human cells of the MCF10A, HaCat, HEK293 lines and mouse cells of the Balb/3T3 line. The studied hydrogels were stable in saline solution, while in a complete culture medium containing 10% fetal bovine blood serum they underwent degradation in >24 h. The identified biodegradability of the chitosan-based hydrogels is important for the release of noncovalently immobilized NSE into biological medium. Further studies on laboratory animals with experimental wounds are expected to explore the potential of created hydrogels as anti-inflammatory and wound-healing agents.*

K e y w o r d s: *chitosan hydrogels, chondroitin-6-sulfate, hyaluronic acid, N-stearoylethanolamine, FTIR analysis, human pseudonormal cells, toxicity, biodegradability.*

Various biological processes are activated under tissue injury, and their deterioration, for example, in the diabetic patients, alters normal wound healing causing severe complications that may lead even to amputation. Thus, a creation of novel remedies efficient in supporting wound healing remain an actual task in pharmacology [1]. Different types of cells are involved in the processes of inflammation, cell proliferation, re-epithelialization and tissue remodeling that take place during regeneration of damaged tissues [2].

The maintaining of moisture condition in the wound microenvironment contributes to faster and better healing. The moisture in the wound is important for autolysis, pain relief, induction of collagen synthesis, promotion of keratinocyte migration through wound surface, reduction of scar size, proper supply of nutrients, and for action of cytokines and other bio-regulators [3]. During principal stages of wound healing, such as hemostasis, antibacterial and anti-inflammatory action, the hydrogels showed the highest efficiency, especially at the regeneration of

the granulation tissues [4]. That might be due to the viscosity and elasticity of hydrogels, as well as their fluidity, that provide them with structural and functional characteristics similar to such characteristics in the extracellular matrices.

The hydrogels are effective wound dressing materials that is due to their biocompatibility, biodegradability, capability of water uptake and retention. Chitosan that is obtained during de-acetylation of chitin possesses physico-chemical properties necessary for preparation of hydrogel-like materials [4]. Chitosan is well known for its hemostatic and antibacterial activities, as well as an inducer of skin regeneration. Thus, its role in promoting wound repair may be easily predicted [5].

Chondroitin sulfate and hyaluronic acid are principal components of the extracellular matrix that is of critical importance for tissue remodeling taking place in wound healing [6]. Chondroitin sulfate–Zn complex demonstrated biocompatibility towards NIH3T3 fibroblasts, as well as a capability to induce the production of fibroblast growth factor, collagen III, and vascular endothelial growth factor, and to suppress an expression of the pro-inflammatory cytokines, such as IL-6, IL-1β and TNF-alpha. Besides, this biopolymer possessed an antibacterial activity towards *Escherichia coli* and *Staphylococcus aureus.*

Hyaluronic acid is present in human tissues and body fluids, participates in all stages of wound healing process, and is applied in wound dressings [7].

Earlier, we have shown that a lipid substance, N-stearoylethanolamine (NSE), suppressed the expression of pro-inflammatory cytokines (IL-1, IL-6 and TNF alpha) in rats with inflammation induced by thermal burn of skin [8]. The role of NSE in the modulation of biological processes in human body requires additional exploration. In general, lipids are known to participate in several signaling mechanisms that modulate various intercellular communications, and also in wound healing [9].

In the present work, we created chitosan-based hydrogels functionalized with chondroitin-6-sulfate, hyaluronic acid, or agarose, and NSE for stimulation of wound healing. In such hydrogels, NSE was used for the first time. The physico-chemical characteristics of the developed hydrogels, as well as the biocompatibility of their components *in vitro* towards human cells of different tissue origin were investigated. Besides, the biodegradability of the created hydrogels *in vitro* was explored depending on their composition and microenvironment. Altogether, these characteristics are important for the development of hydrogels to be applied for the suppression of inflammation and enhancement of wound healing in experiments conducted on laboratory animals.

## Materials and Methods

*Chitosans.* Shrimp chitosan samples, namely Chitopharm L, M, and S, were obtained from Chitinor AS (Norway). Besides, chitosan from shrimp shells was obtained in our laboratory using an earlier developed method with slight modifications [10]. To improve the solubility of chitosan preparations for obtaining a high concentration of the solution (>4%), the limited acid hydrolysis of the chitosan samples was carried out by a prolonged incubation (about 24 h with stirring) of 40 mg of chitosan in 6 ml of water mixed with 3 ml of 0.1 M HCI.

Agarose was purchased from Chemapol (Czechoslovakia).

Chitosan-based hydrogels were prepared using chondroitin-6-sulfate and hyaluronic acid purchased from YUNATIS (China). Before use, these compounds were converted into anionic forms.

Different methods of preparation of chitosan gels were applied, namely: slow increase of the pH of concentrated chitosan solutions, fixing the gel in order to obtain films by mild treatment with aldehyde vapors or epychlorohydrin, gel formation on supporting materials substrates (bandage meshes made of cotton or polyamide fibers). Anionic polysaccharides, such as heparin, hyaluronic acid, and chondroitin sulfate in free acid form, were used to form ion-type crosslinks between chitosan chains in the gel.

*Preparation of chitosan-based gel–films.* A solution of the hyaluronic acid (sodium salt) was poured evenly gently into Petri dish of the desired size. After pouring, the dish form was placed on a flat surface and cooled. To saturate the film with NSE, a solution of this cannabimimetic was mixed with a solution of the hyaluronic acid and heated for 1 h at 60°C in order to achieve uniform dissolution.

*Preparation of chitosan-based hydrogels that contain hyaluronic acid and agarose.* Two-layer chitosan-based gels were prepared. At first, hyaluronic acid was dissolved in water (18 h with periodic shaking) to 1% final concentration. Agarose was added to the hyaluronic acid solution and heated to boiling for complete dissolution of the agarose. This

mixture was solidified in a Petri dish for 3-5 min. In order to bring the pH in the gel to neutral, after making a two-layer gel, 50 ml of 0.2 M phosphate buffer, pH 8.5, was poured on the top of the gel. After 15 min, the chitosan layer of the gel became cloudy, and the pH of the buffer decreased to 7.5.

Preparation of chitosan-based hydrogels that contain chondroitin-6-sulfate and agarose. Chondroitin-6-sulfate (CS) has a lower molecular mass comparing to chitosan and it dissolves in water in 10 min. In order to conduct a complexation of the poly-cation (chitosan) with the poly-anion (chondroitin-6-sulfate), a conversion of sodium salt of the commercial preparation of this anionic polymer to its free acidic form was performed.

The bi-layer gels of chitosan with agarose and CS were prepared, as described above.

In addition to CS, hyaluronic acid, or agarose, the created chitosan-based hydrogels were supplemented with the NSE. When doing that, we took into account poor solubility of this agent in water, as well as its surface activity. The NSE was dissolved in water-alcohol (1 : 1) solution. The content of the created hydrogels was (during synthesis): chitosan–hyaluronate-NSE – 1 : 1 : 0.5 in the form of gel-film, chitosan–hyaluronate – 1 : 1 in the form of gel-film, chitosan-agarose-NSE – 1 : 1 : 0.8 in the form of gel-film, and chitosan–chondroitin-6-sulphate (1 : 0.7) in the form of gel-ointment.

The NSE was prepared by the authors of this study using the condensation of ethanolamine and stearic acid, as described in the Patent of Ukraine for Invention [11].

The potentiometric titration was applied for determination of values of the cationic or anionic equivalents in alkaline (chitosan) and acidic (chondroitin-6-sulfate and hyaluronic acid) biopolymers (measured with laboratory ion meter, model I-160 M (RF). A distinct quantity of analyzed substance was dissolved in 2 ml of distilled water and 0.1 M hydrochloric acid was added in order to achieve pH 2.0±0.2. The plots were constructed from the obtained data and the value of the reactive equivalents (acidic or alkaline) was calculated for the analyzed substance.

Fourier transform infrared spectroscopy (FTIR) was used to identify organic materials in the created chitosan-based hydrogels supplemented with chondroitin sulfate or hyaluronic acid and NSE. The samples for FTIR analysis were prepared as films, solutions, hydrogels. Obtained results standardized and analyzed by licensed program of device (IRSpirit Fourier Transform Infrared (FTIR) Spectrophotometer (Shimadzu) with QATR-S Single-Reflection ATR) [12].

Gas Chromatography-Mass Spectrometry (GC-MS) was used for measuring the NSE content in the chitosan-based hydrogels. NSE was extracted, as previously described [13]. To identify TMS derivatives of the NSE, the Agilent Technologies 7890A gas chromatograph (USA) with Agilent 8987 mass-detector and HP-5MS column (30 m × 250 µm × 0.25 µm, Germany) were used. The NSE was identified on the basis of its retention time compared to appropriate commercially available standard (Sigma-Aldrich, USA). The results of the analysis were expressed as µg of NSE per mg of gel. All the experiments were repeated three times.

The water content in gel samples was determined via weighing the gel before being placed in a thermostat (37°C) for 24 h, and then weighed again. The difference was counted as a content of the evaporated water.

Evaluation of biocompatibility *in vitro* (colorimetric MTT-test and Trypan blue exclusion test) of the components of created chitosan-based hydrogels towards pseudonormal cells of the epithelial human tissues and the connective murine tissue was performed, as described by us earlier [14]. Pseudonormal cells of HaCat (human keratinocytes), HEK-293 (human embryonic kidney), and Balb/3T3 (embryonic mouse fibroblasts) lines were obtained from Prof. Walter Berger (Institute for Cancer Research, Vienna Medical University, Austria) and cells of MCF-10A line (human mammary gland epitheliocytes) were obtained from Prof. Anna Bielawska (Medical University of Bialystok, Poland). HEK293, Balb/3T3 and HaCat cells were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM) (Merck, Burlington, MA, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Merck, Burlington, MA, USA) and 50 µg/ml gentamicin (Merck, Burlington, MA, USA) in a humidified atmosphere containing 5% $CO_2$ at 37°C. MCF10A cells were cultured in special medium based on DMEM/F12 medium (Merck, Burlington, MA, USA) supplemented with 5% horse blood serum, 1% of non-essential amino acids (NEAA), 20 ng/ml EGF, 0.5 mg/ml Hydrocortisone, and 10 µg/ml insulin. Cells were sub-cultured every 72 h at the rate of $5×10^4$ cells per 1 ml of culture medium. For morphologic assessment, cells were checked un-

der an inverted microscope IB-100 (Delta Optical, Gdansk, Poland).

Hyaluronic acid and chondroitin-6-sulfate were dissolved in sterile double distilled water to obtain 10 and 50 mg/ml stock solutions. The NSE was prepared in our lab, as mentioned above, and dissolved in 96% ethanol to obtain 20 mM stock solutions. Before addition to cultured cells, stock solutions of the NSE were dissolved in full culture medium in order to obtain appropriate concentrations.

For cytotoxicity studies, cells were seeded into 24-well tissue culture plates (Greiner Bio-one, Frickenhausen, Germany) at a concentration of $2\times10^5$ per well and allowed to attach for additional 24 h. Short-term (24 h) cytotoxic effects were studied under an Evolution 300 Trino microscope (Delta Optical, Gdansk, Poland) after cell staining with Trypan blue dye (0.1%). For evaluation of the anti-proliferative activity of studied compounds, cells were seeded into 96-well tissue culture plates (Greiner Bio-one, Frickenhausen, Germany) at a concentration of $3\times10^3$ per well, and allowed to grow for 24 h. Drugs were added in another 100 µl culture medium, and cells were exposed for 72 h. The proportion of viable cells was determined by the EZ4U assay according to the manufacturer's recommendations (EZ4U, Biomedica, Vienna, Austria) on BioTek ELx800 Absorbance Microplate Reader (Thermo Fisher Scientific, Waltham, MA, USA) at 450 nm wavelength with 620 nm as reference. Cytotoxicity was expressed as $IC_{50}$ values calculated from full dose-response curves (drug concentration including 50% reduction in cell survival comparing to the control cultured in parallel without drug).

*Biodegradability testing.* Incubation studies were carried out in order to determine a behavior of synthesized materials in selected liquids by checking the pH. Samples of hydrogels were incubated in physiological solution (0.9% sodium chloride) or DMEM supplemented with 10% FBS for a period of about two weeks. Incubation of cells was conducted at 37°C, 5% $CO_2$ and 100% humidity, and pH value was measured every two days [15, 16].

*Studies of sorption capacity of gels.* The chitosan-based hydrogels of known weight were immersed in the DMEM medium supplemented with 10% FBS and kept at 37°C for 1 or 8 h. The swollen hydrogels were removed and immediately weighed with a microbalance (Techniweights TBE, Ukraine) after an excess of water lying on the surfaces was absorbed with a filter paper. Sorption capacity was determined as a swelling ratio (Q) calculated using the equation: $Q = (w - w_0)/w_0$,
where $w$ – weight of hydrogel in a swollen state, $w_0$ – weight of hydrogel before swelling [17].

Statistical analysis of the results was performed using GraphPad Prisms (GraphPad Software, Inc, La Jolla, CA, USA) using Tukey's multiple comparison test (ordinary one-way ANOVA). The significance level of differences will be set below 0.05 ($P \leq 0.05$). All experiments were repeated at least 3 times.

## Results

Physico-chemical characteristics of chitosan-based hydrogels. Potentiometric titration. The ionic bonds play a crucial role in formation of complexes of the poly-cation, chitosan, with various poly-anions, such as chondroitin-6-sulfate or hyaluronic acid. Here, we used potentiometric titration for measuring the cationic equivalents of intact chitosan and chitosan fragmented during limited hydrolysis in acid medium. It was found that the cationic equivalent of chitosan equaled 4.5 ± 0.1 mEq/g, the anionic equivalents of chondroitin-6-sulfate equaled 1.5 mEq/g, and of hyaluronic acid – 3.06 mEq/g, correspondingly (Fig. 1).

At the titration of the commercial preparation of chondroitin-6-sulfate, the amount of the anionic equivalents was shown to be 0.86 mEq/g, while at the titration of the acidic form of this biopolymer, that indicator doubled its value and reached 1.65 mEq/g. Thus, in order to obtain reproducible results in preparing complexes of chitosan with other biologically active components, the interacting ingredients should be converted into their acidic forms before mixing.

Additionally, we calculated the pKa (pKa = -log10 Ka of the acid dissociation constant Ka) values of chitosan, chondroitin-6-sulfate and hyaluronate that equaled 6.2, 3.2 and 2.6, correspondingly. The lower pKa value means the stronger acid, thus, we found that the hyaluronic acid is a stronger acid comparing to chondroitin-6-sulfate.

After the accomplishment of the 1st stage of our study (potentiometric titration), various types of chitosan-based hydrogels, were prepared for further studies.

*FTIR was used for analysis of chemical bonds in different chitosan-based hydrogels.* We detected an overlapping of FTIR-signals of the components of gels, in particular, chitosan and NSE, which caused the broadening of the peaks in the range of 3,600-

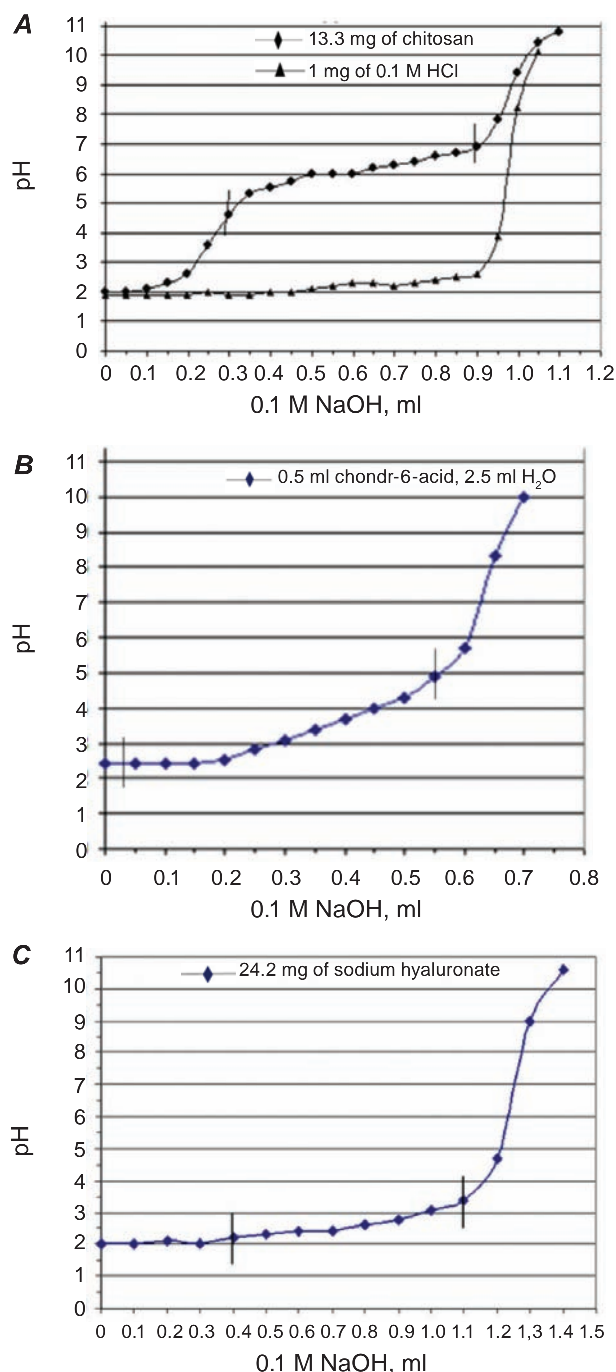


*Fig. 1. Potentiometric titration curves of chitosan (**A**), chondroitin-6-sulfate (**B**), and hyaluronic acid (**C**). | – Points of dissociation of functional groups. **A** – 13.3 mg of chitosan in 2 ml of water was mixed with 1.0 ml of 0.1 M HCl and after dissolution, it was titrated with 0.1 M NaOH. **B** – 50.0 mg of chondroitin-6-sulfate in 2 ml of water was mixed with 1.0 ml of 0.1 M HCl and titrated with 0.1 M NaOH. **C** – 24.2 mg of sodium hyaluronate was dissolved in 3 ml of water, 1.3 ml of 0.1 M HCl was added. The solution was titrated with 0.1 N NaOH*

2,800 $cm^{-1}$ (Fig. 2). Two separate peaks appeared at 2,800 $cm^{-1}$ that correlates with the presence of hyaluronate. At the analysis of chitosan-hyaluronate gel film, the intensity of the peak at 1,600 $cm^{-1}$ decreased. In our opinion, that was caused by binding of the hyaluronic acid to chitosan molecule. After including NSE in chitosan-based gels, a high peak appears at 1,640 $cm^{-1}$, which might be a marker of the presence of the NSE. It may be caused by C=O bond in the NSE that is lacking in both chitosan and hyaluronate.

The overlapping of FTIR-signals of gels composed of chitosan and chondroitin-6-sulfate caused the broadening of the peaks in 3,600-3,200 $cm^{-1}$ range (Fig. 3). Besides, in this case, the peak in $1{,}400^{-1}$-1,200 $cm^{-1}$ range disappeared. These changes (Fig. 2 and Fig. 3) may indicate a complexation of positively charged amino groups of chitosan with negatively charged sulfate groups of the chondroitin-6-sulfate that is in accordance with the results of the potentiometric titration (Fig. 1).

GC-MS was applied to measure NSE content in the chitosan-based hydrogels. High purity of the prepared NSE was detected.The obtained results also demonstrated that the NSE content in chitosan-hyaluronate gel-films equaled 1.95 mg/g (or 0.062 mg/$cm^2$ of hydrogel).

The water content in the chitosan-based gel films was calculated to be approximately 6% of the dry mass of the gel.

Swelling study was conducted in order to define the sorption capacity of the hydrogels. DMEM, which has a physiological composition of electrolytes, was used as a simulator of fluids of human body in order to test the ability of hydrogels to absorb these fluids from tissue microenvironment. This property is important in the biomedical and pharmaceutical applications, especially for characterization of materials used in wound dressings. Two prepared hydrogels were shown to possess relatively high swelling capability. The weight of hydrogel Chitosan - hyaluronate was doubled during 1 h incubation period (Q = 2). It should be noted that the NSE reduced twice the sorption efficiency of that hydrogel (Q = 1). However, while the weight of the gel Chitosan – hyaluronate continued to increase at 8 h incubation (Q = 2.7), the Chitosan – hyaluronate - NSE gel did not change its weight in dependence on the duration of incubation. We assumed that these swelling peculiarities of gels under study are caused by the hydrophobic nature of the NSE that could hinder the absorption of water and other hydrophilic

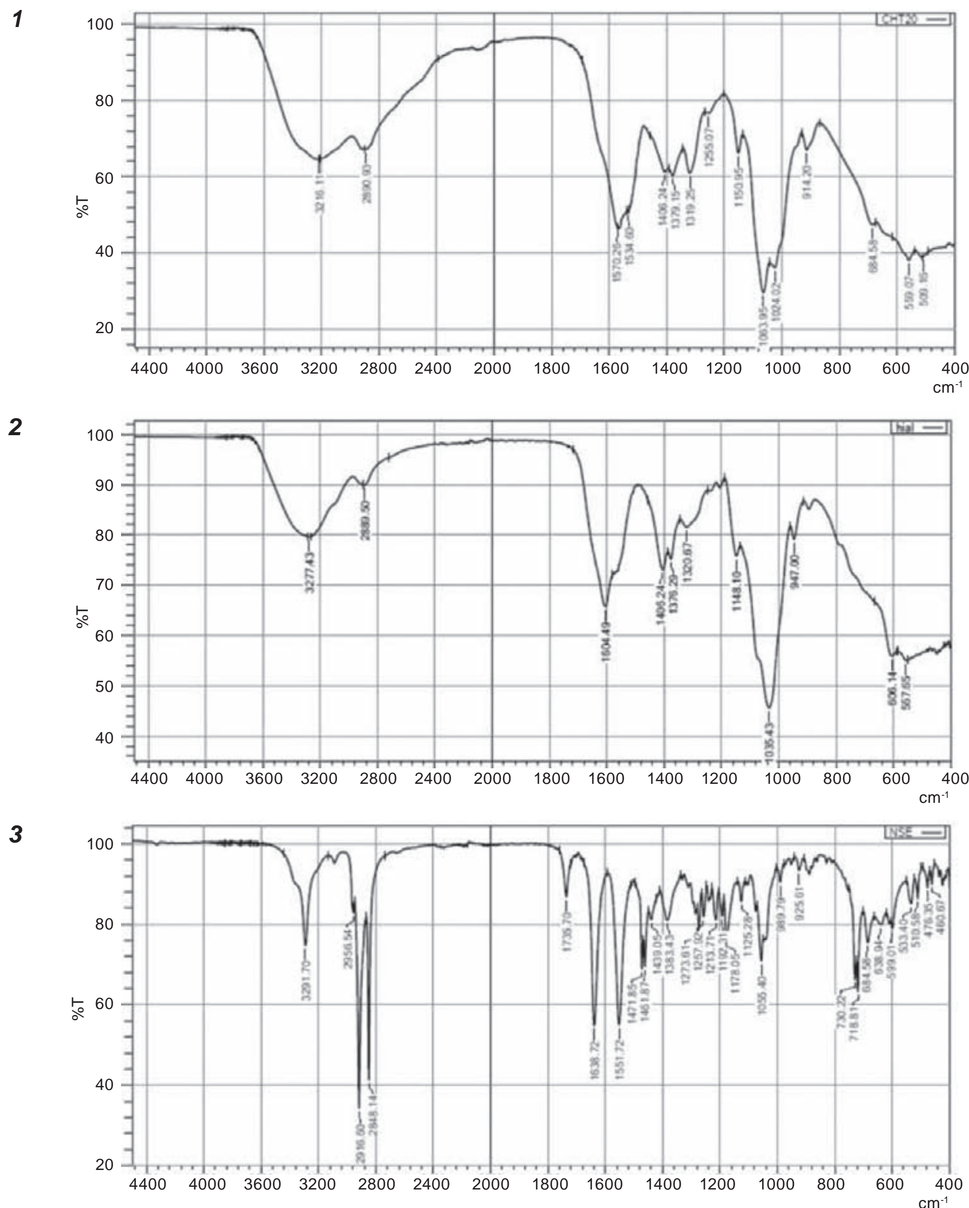


*Fig. 2. The results of FTIR analysis of components of chitosan-based hydrogels:* ***1*** *– chitosan;* ***2*** *– sodium hyaluronate;* ***3*** *– NSE*

compounds into the gel matrix. The sorption capacity of chitosan-agarose hydrogel that contained the NSE - Chitosan – Agarose - NSE – was relatively low (Q = 0.5).

Evaluation of biocompatibility *in vitro* of components of the created chitosan-based hydrogels was performed towards pseudonormal cells of epithelial and connective tissues of the mammalians. In order to check the biocompatibility *in vitro* of components of the created chitosan-NSE compositions, their impact on the viability (72 h) and survival (24 h) of human and murine pseudonormal cells of the epithelial and connective tissues was carried out. The results of the evaluation of short-term and long-term cytotoxic effects of wide concentration range of the NSE towards different cell lines are presented in Fig. 4. One can see that even in a high (80 μM) dose that is equivalent to ~26 μg/ml, the NSE did not kill the aforementioned cells either at 24 h, or at 72 h time points, thus, demonstrating its biocompatibility towards these pseudonormal cells. The maximum decrease in cellular viability was less than 25% at 100 μM concentration of the NSE at 24 h treatment of murine Balb/3T3 cells (see Fig. 4).

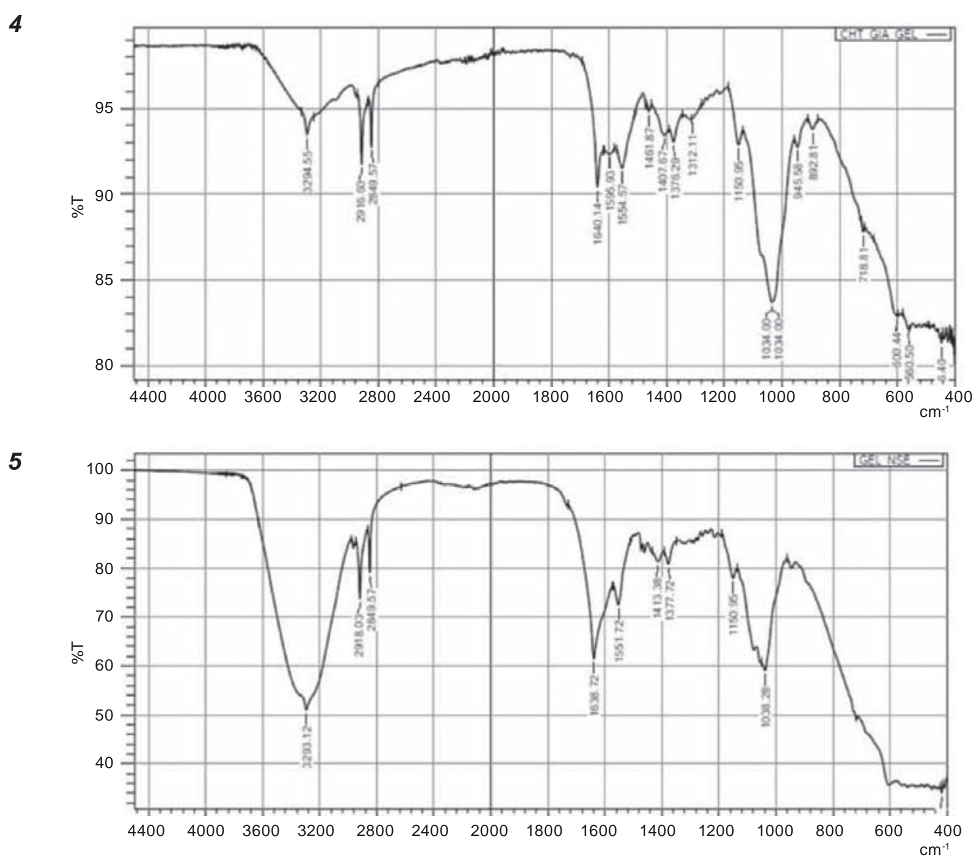


*Fig. 2 (continuation). The results of FTIR analysis of components of chitosan-based hydrogels:* ***4*** *– chitosan-hyaluronate gel-film;* ***5*** *– chitosan-hyaluronate gel-film containing NSE*

At the next stage of our studies, the biocompatibility *in vitro* of the hyaluronic acid (HA) and chondroitin-6-sulfate was estimated using the same panel of cell lines in a wide concentration range (up to 4 mg/ml). Hyaluronic acid was found to be non-toxic towards HEK293 and HaCat cells (100% viability at the highest dose) both at 24 and 72 h incubation time, while the epithelial cells of MCF-10A line demonstrated slight decrease in viability (~15%) under treatment with 4 mg/mg dose of the HA (Fig. 4). Murine embryonic fibroblasts of Balb 3T3 line were also sensitive to the action of the HA, with average decrease by 15% in their viability at 4 mg/ml dose. The same tendencies were observed for chondroitin sulfate, which did not impact the viability (100% survival) of human MCF-10A, HaCat, HEK293 cells at 2 mg/ml dose at 72 h, and only weakly (~15%) affected a survival of murine Balb/3T3 fibroblasts.

In order to check if the mean of the molecular weight of chitosan might affect the biocompatibility of this biopolymer *in vitr*o, three different samples of chitosan – low molecular weight (150 kDa), prepared from lobster carapaces (#1), medium (500 kDa, #2) and high molecular weight (740 kDa, #3) were used to treat the pseudonormal cells under study. We did not detect any significant differences in the biocompatibility of these chitosan samples towards MCF-10A, HaCat, HEK293 and Balb/3T3 cell lines checked at 24 h and 72 h time points (Fig. 5), thus, pointing out that molecular weight of chitosan does not affect its bio-compatibility at least *in vitro*. Weak toxicity of chitosan was observed only at its high concentration (250 μg/ml) causing a decrease in cell viability by 15-25%, depending on the cell line. There was no significant difference in toxicity of chitosan samples of different molecular weights at 24 and 72 h time points, thus, excluding the risk of adverse side effects of application of this biopolymer in animal studies.

Summarizing the results of the biocompatibility evaluation *in vitro* of components of the created chitosan-based hydrogels, one may conclude that all these components were non-toxic towards the mammalian cells of the epithelial and connective tissues that take part in the healing of surface wounds.

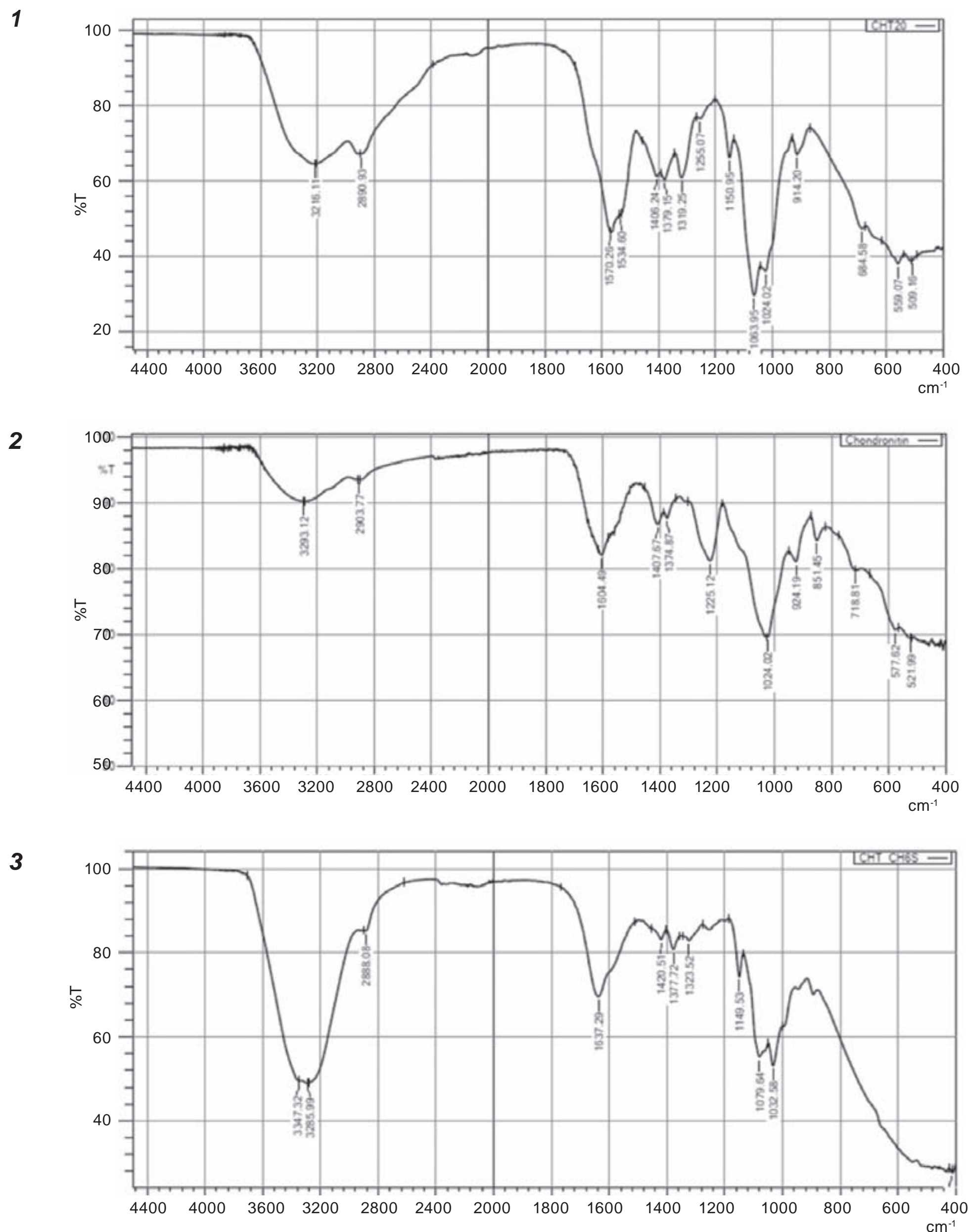


*Fig. 3. The results of FTIR analysis of components of chitosan-based hydrogels: **1** – Chitosan; **2** – Chondroitin-6-sulfate; **3** – Chitosan - Chondroitin-6-sulfate complex*

Biodegradability of chitosan-based hydrogels was evaluated using two approaches: 1) direct gel contact with cells; 2) cell-independent degradation of gels.

Sample Chitosan - hyaluronate (Fig. 6, *A*, *B*) did not cause significant changes in cell morphology and only a small number of cells with condensed nucleus was observed in control (untreated) cells (Fig. 6, *G*, *H*). In the presence of Chitosan – hyaluronate - NSE sample (Fig. 6, *C*, *D*), a large number of cells with condensed chromatin appeared. Besides, these cells were heavily covered with fragments of the destroyed hydrogel. The fibroblasts that were in a direct contact with Chitosan – Agarose - NSE gel (Fig. 6, *E*, *F*), showed a morphology similar to control (untreated) cells, although the sticking of gel fragments to these cells was also present.

Cell-independent degradation of gels was measured as an indicator of stability of hydrogels in the DMEM/10% FBS via monitoring swelling of gels in such medium. We found signs of degradation of samples Chitosan - hyaluronate and Chitosan – hyaluronate - NSE after 24 h incubation, and a complete dissolution of the gel Chitosan - hyaluronate in

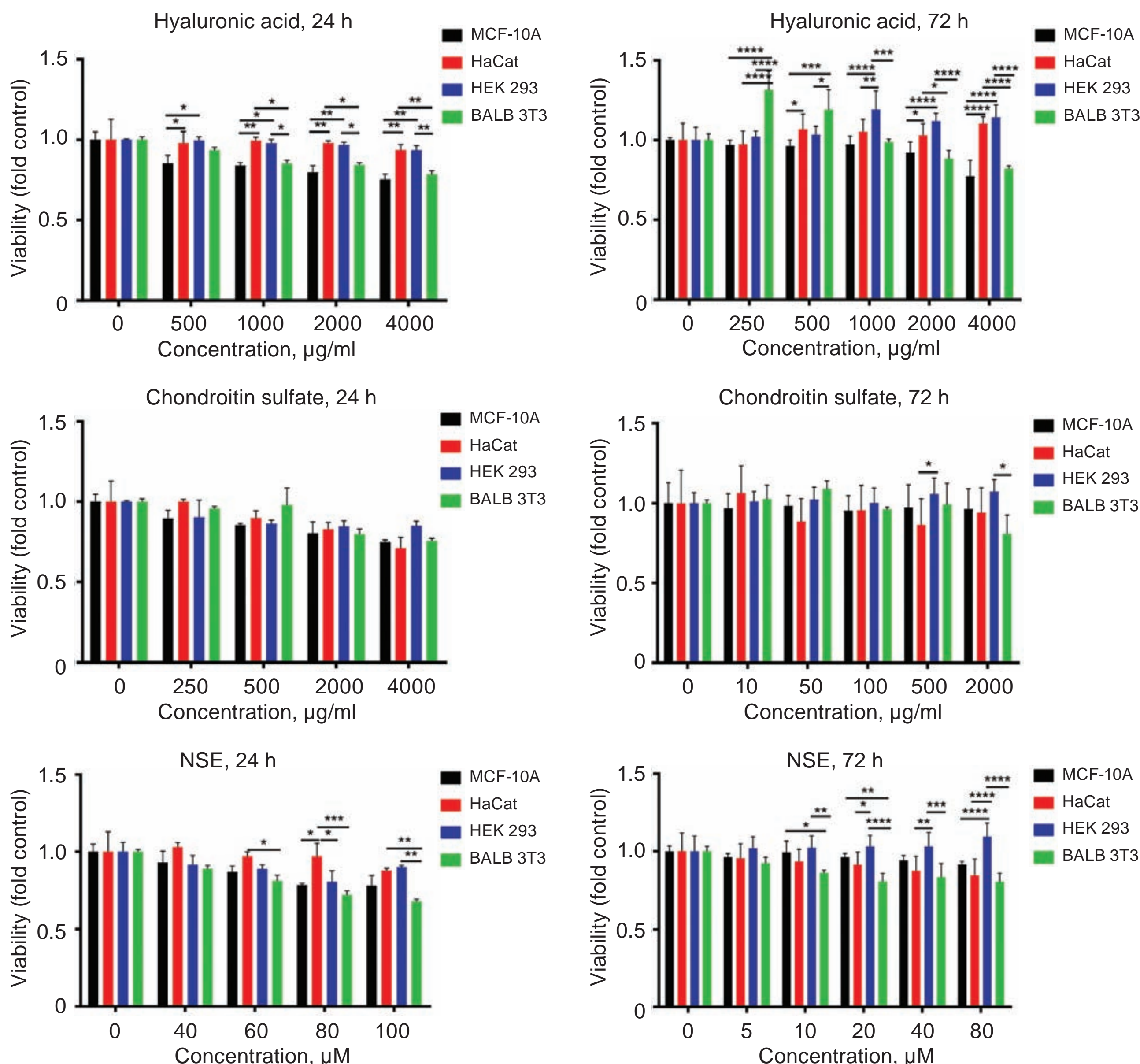


*Fig. 4. Comparison of short-term (24 h) and long-term (72 h) effect of the NSE, hyaluronic acid and chondroitin-6-sulfate towards human breast epithelial cells of MCF-10A line, human keratinocytes of HaCat line, human embryonic kidney cells of HEK 293 line, and murine embryonic cells of Balb/3T3 line. Trypan blue exclusion assay for counting dead cells was used with 24 h treatment, and MTT assay of cell survival was used with 72 h treatment. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001*

that medium was observed on the 4th day of gel incubation. The Chitosan – Agarose - NSE sample that contained agarose, remained undamaged throughout the experiment. Changes in the pH of the DME medium were monitored during gel incubation lasting for 14 days. It was maintaining at about the value of pH equal of 7.4, however, at the end of the incubation term, the pH value increased to approximately 7.8 (Fig. 7, *A*).

Stability of hydrogels in the physiological solution (0.9% sodium chloride) was detected during their incubation for up to 14 days. All hydrogels kept their appearance and shape, and at the end of the incubation, the pH of the medium equaled 6.2 (Fig. 7, *B*).

Summarizing the results of the MTT-testing of the biocompatibility of various components of chitosan-based gels and the results of studying gel

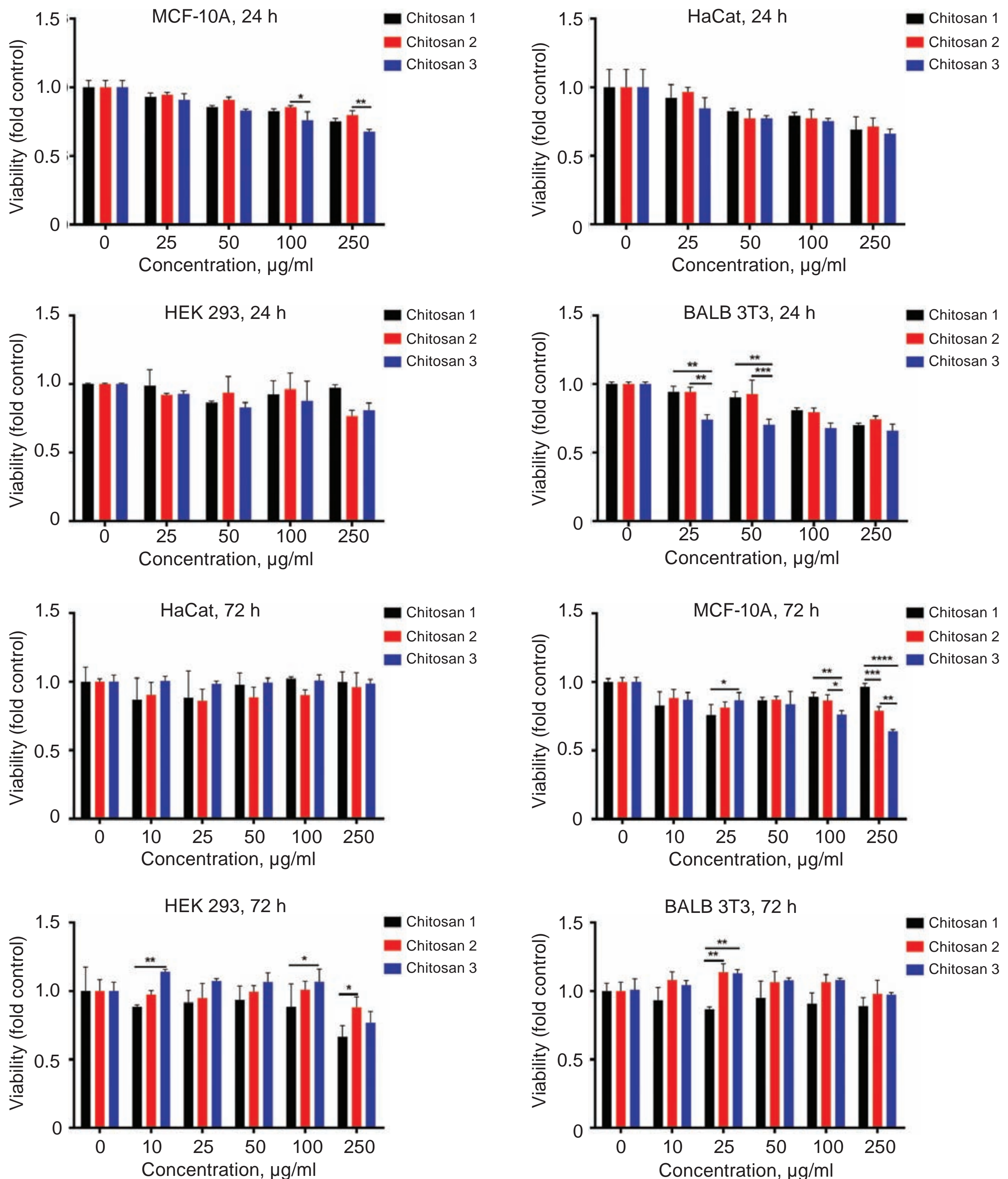


*Fig. 5. Comparison of short-term (24 h) and long-term (72 h) effects of chitosan preparations of various molecular weights towards human breast epithelial cells of MCF-10A line, human keratinocytes of HaCat line, human embryonic kidney cells of HEK 293 line, and murine embryonic cells of Balb/3T3 line. Trypan blue exclusion assay for counting dead cells was used with 24 h treatment and MTT assay of cell survival was used with 72 h treatment.*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Chitosan 1 – extracted from lobster carapaces, Mw = 150 kDa, pH 6.3, 0.5% solution. Chitosan 2 – Mw = 500 kDa, pH 6.3, 0.5% solution. Chitosan 3 – Mw = 740 kDa, pH 6.3, 0.5% solution*

degradation at a direct interaction of treated cells with these gels, we suggest that Chitosan - hyaluronate gel is better comparing to other gels. Besides, this gel possesses good adsorption capacity and does not form small fragments during biodegradation that could affect growing cells.

## Discussion

Chitosan is characterized by a biodegradability (digestion with the lysosomal enzyme in mucosa), biocompatibility and non-toxicity. Since chitosan decomposition is not accompanied by an appearance of any toxins and other adverse effects in human body, this biopolymer was approved by the FDA (USA) [18]. The cationic nature of chitosan is important for its self-structuring around the anionic groups and for bio-adhesion [19]. It is well seen during formation of complexes of chitosan with the hyaluronate (Fig. 2).

Chitosan was also proposed for a use as a wound dressing remedy. The positive electric charge of chitosan is important for the interaction with the negatively charged components of plasma membrane of the red blood cells and the wall of bacteria that reduces scar formation in damaged tissue via blocking fibrin formation [20].

Combining chitosan with alginate having negatively charged carboxylic groups creates a strong electrostatic force with amino groups of chitosan that prevents a premature release of the hydrophobic drug(s) [21, 22]. We suggested an existence of such a preventing effect at a release of the lipophilic NSE from the chitosan gels that contain the poly-anions.

The creation of the non-covalent (ionic) complexes of chitosan with chondroitin-6-sulfate or hyaluronate and various biologically active hydrophilic components might ensure a faster and easier release of these components from chitosan's complexes, comparing to their release when they are immobilized through the covalent bonds.

Our team has a long term experience in isolation of various forms of chitosan from different sources, such as crustaceans, honey bees and fungi [23]. A high molecular weight form (up to 1 million Da) of chitosan demonstrated strong hemostatic activity [24], and chitosan-melanin complex possessed a potent anti-microbial activity [25].

The hyaluronic acid is a natural polysaccharide that consists of two monomers of glucuronic acid and N-acetylglucosamine. High viscosity and elasticity [26], hydrophilicity, biodegradability, and biocompatibility of the hyaluronic acid provide a great potential in its biomedical application, namely for drug delivery and wound dressings [27, 28]. HA is found in higher animals' intercellular space (such as vitreous humor of the eye and synovial fluid of articular joints) [29]. Hyaluronic acid penetration into the cell was prevented by a rapid enzymatic degradation with the hyaluronidase and metabolic absorbance [30].

NSE belongs to minor lipids known as N-acylethanolamines. It possesses the effect of an endogenous agonist of the cannabinoid receptors. This and other cannabinoid-like compounds demonstrated various biological activities. NSE exerted the membrane-protective, anti-oxidative, hypoglycemic, adaptogenic, and anti-inflammatory action in various experimental models under several pathological conditions [31-33].

Recently, we have shown that an anti-inflammatory effect of the NSE was realized through inhibition of NF-kB translocation into the nucleus of the LPS-activated rat peritoneal macrophages. That effect was followed by a decrease in content of the pro-inflammatory cytokine Il-1β and transcription factor NF-κB [34].

Recently, we reported that PPARγ (peroxisome proliferator-activated receptor type gamma) was involved in an anti-inflammatory action of NSE. The treatment of insulin-resistant rats with NSE influenced the expression of the mRNA of PPARγ targeting genes in their liver, such as SLC27A1 and IL1RN [35].

In rats with burn trauma, the application of NSE on damaged skin led to acceleration of wound healing and a reduction in content of pro-inflammatory cytokines and in level of glucocorticoids [36].

In the present work, we immobilized the NSE on a platform of the chitosan-based hydrogels. We assumed that such a form of the NSE application would improve the biological action of this cannabimimetic. The results of conducted FTIR analysis suggest an interaction between chemical groups of chitosan and chemical groups of the ingredients of chitosan-based hydrogels such as chondroitin sulfate, hyaluronic acid, or agarose, and the NSE. The created chitosan-based hydrogels contained 1.95 mg of the NSE per gram of gel, and in such a concentration, the NSE is practically non-toxic for the mammalian cells *in vitro* (Fig. 4-5).

The conducted FTIR analysis permitted detecting a complexation of chitosan molecule that is rich in amino groups with the poly-anionic molecules, such as hyaluronate or chondroitin-6-sulfate.

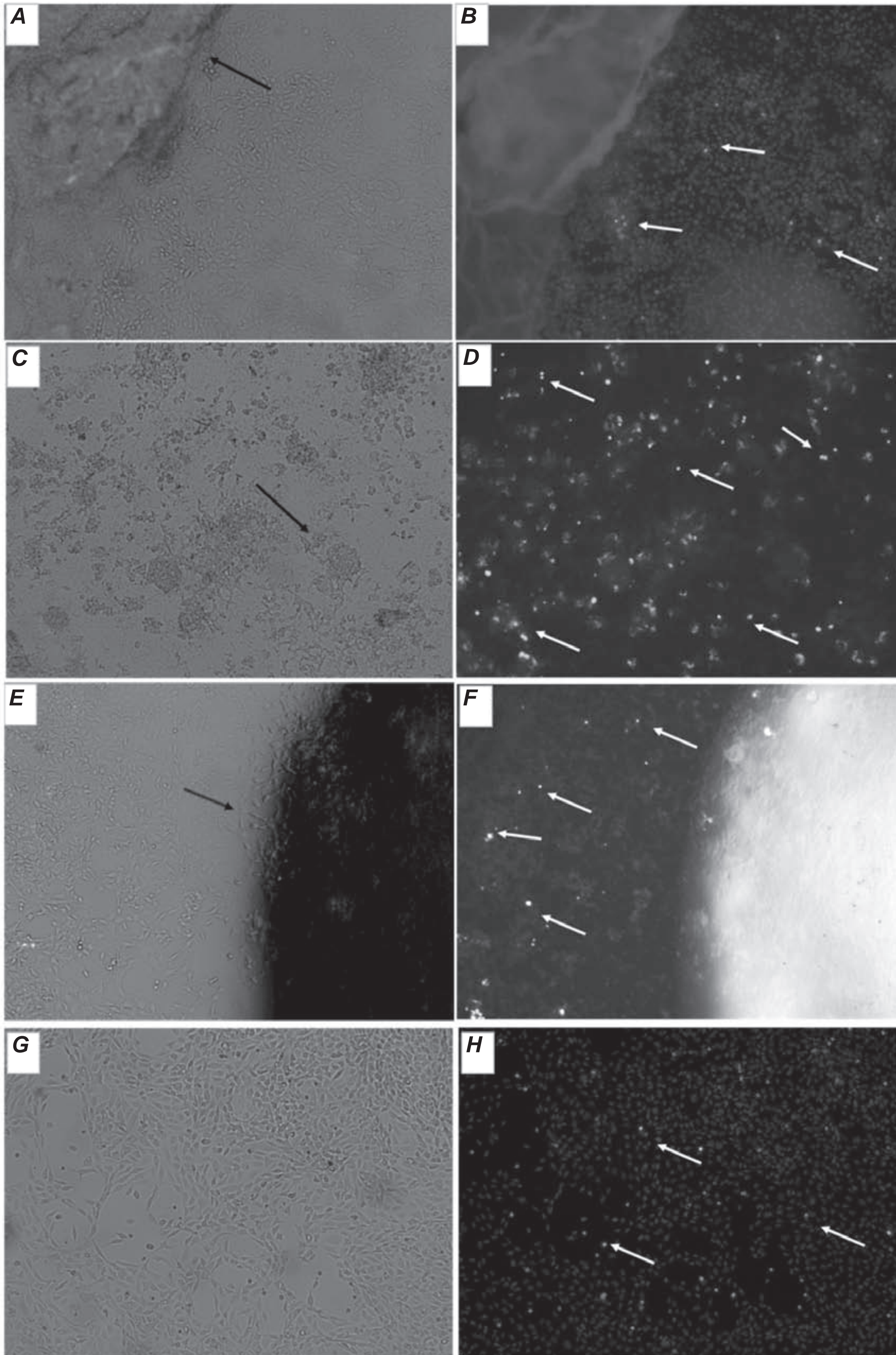


*Fig. 6. The results of direct contact assay of tested hydrogels with Balb/3T3 cells showing changes in the morphology of treated cells. Black arrows – hydrogel fragments; white arrows – cells with condensed chromatin.* ***A***, ***B*** *– Chitosan – hyaluronate;* ***C***, ***D*** *– Chitosan – hyaluronate – NSE;* ***E***, ***F*** *– Chitosan – Agarose – NSE;* ***G***, ***H*** *– Untreated cells*

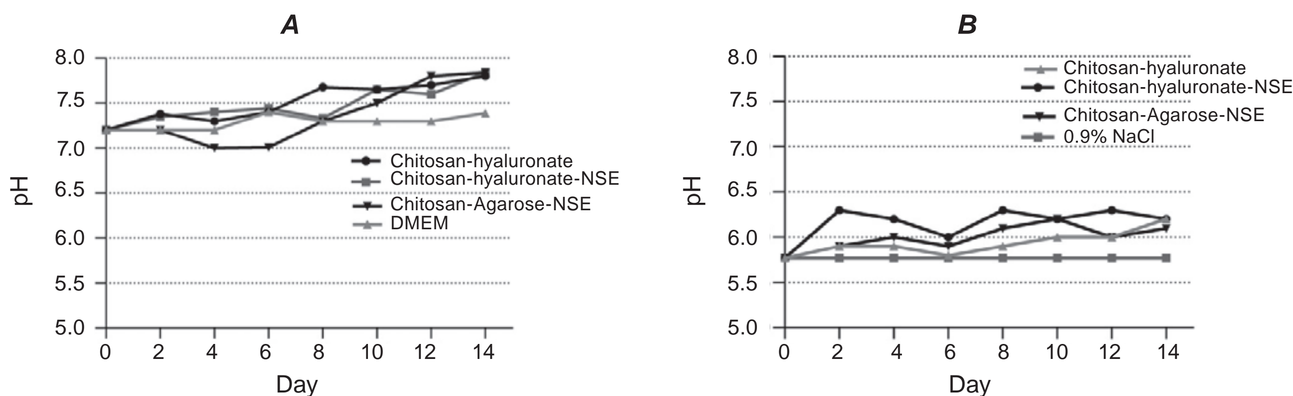


*Fig. 7. Results of pH measurements in DMEM/10% FBS medium (**A**) and physiological solution (0.9% sodium chloride) (**B**)*

While the NSE molecule that possesses lipid-like properties due to its aliphatic chain creates a new modality to such complexes whose properties should be studied additionally.

The developed chitosan-based hydrogels contained approximately 6% of water, however, they also demonstrated a significant sorption capacity with the highest swelling capability in chitosan-hyaluronate hydrogels. An additional functionalization of the created hydrogels with the lipophilic NSE decreased their sorption capacity, probably due to the hydrophobic properties of the NSE (this phenomenon needs more precise investigation).

We used various approaches to explore the biodegradability of the created multifunctional chitosan-based hydrogels, namely, study of their stability in saline solution and in a complete cell culture medium supplemented with 10% fetal calf serum, as well as a direct interaction of cultured cells with the hydrogels of different composition. It was found that most of these gels were destroyed in culture medium supplemented with 10% fetal calf serum, that means in the conditions close to those available in vivo. However, these gels were resistant to degradation in saline solution lacking any additives (Fig. 7, *A*). The chitosan hydrogel that contained agarose instead of the hyaluronate demonstrated resistance to degradation in both saline solution medium and culture medium supplemented with 10% fetal calf serum (Fig. 7, *B*).

Taking into account the results of experiments presented in Fig. 7, we suggest that an absence of pH fluctuations in the medium suggests that the degradation of the chitosan-based gels is not attributed to their chemical interactions with components of the micro-environment. It might be speculated that the degradation of samples Chitosan - hyaluronate and Chitosan – hyaluronate - NSE observed in the DMEM/10% FBS medium is probably linked to the enzymes and other biological components of blood serum that caused gel degradation. Since the above-mentioned medium is similar in its content to body fluids, one may predict that such a degradation of created gels would also take place in the organism. Rapid biodegradation of these gels in a simulated body fluid (DMEM supplemented with 10% FBS) without pH fluctuations is in accordance with the results of study of the biocompatibility of the tested chitosan-based gels.

We consider that the biodegradability of the chitosan-based hydrogels is a crucial advantage of these biomaterials. Besides, the non-ionic type of immobilization of the bioactive component (NSE) in these hydrogels facilitates easy release of this component during wound healing action of the hydrogels.

We evaluated the biocompatibility towards various mammalian pseudonormal cells *in vitro* of separate components (chitosan, hyaluronate, chondroitin-6-sulfate, and the NSE) present in the created chitosan-based hydrogels. None of these components (chitosan was applied in three molecular weight forms – 150, 500 and 740 kDa) demonstrated a significant toxicity for treated cells.

In future, we plan on verifying the activity of the developed multi-functional chitosan-based hydrogels in the experiments on wound healing in laboratory animals. Novel efficient wound healing remedies are particularly necessary in Ukraine that faced to the aggression of Russian Federation. Although we used in our work commercially produced chitosan, chondroitin-6-sulfate and hyaluronic acid,

we also have in our hands the biotechnologies of production of these substances that would make our future investigations cheaper.

*Conclusions.* New chitosan-based hydrogels supplemented with chondroitin-6-sulfate, hyaluronic acid, and N-stearoylethanolamine (NSE) were created. Their physico-chemical characteristics were studied using the Fourier transform infrared spectroscopy (FTIR analysis) and gas chromatography - mass spectroscopy (GC-MS). It was shown that the NSE content in chitosan-hyaluronate gel equaled 1.95 mg/g. The biocompatibility of components (chitosan, chondroitin-6-sulfate, hyaluronic acid, and the NSE) of the created chitosan-based hydrogels *in vitro* was confirmed via treatment of pseudonormal cells of epithelial tissues (MCF10A line of human mammary gland, HaCat line of human keratinocytes, HEK293 line of human embryonic kidney) and connective tissue (mouse fibroblasts of Balb/3T3 line). The bio-degradability of the created chitosan-based hydrogels *in vitro* depended on their composition and testing conditions. Most of the studied hydrogels were stable in saline solution, while in the presence of a complete culture medium (DMEM) containing 10% fetal bovine blood serum, these hydrogels underwent a degradation in >24 h.

In future, we will explore the created chitosan-based hydrogels for their anti-inflammatory and wound-healing effects in laboratory animals with experimental wounds.

*Conflict of interest.* The authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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## БАГАТОФУНКЦІОНАЛЬНІ ГІДРОГЕЛІ НА ОСНОВІ ХІТОЗАНУ: ХАРАКТЕРИСТИКА ТА ОЦІНКА БІОСУМІСНОСТІ ТА БІОДЕГРАДАЦІЇ *IN VITRO*

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Описані нові гідрогелі хітозану, доповнені хондроїтин-6-сульфатом, або гіалуроновою кислотою чи агарозою, а також N-стеароїлетаноламіном (NSE). При формуванні цих гелів враховували катіонні властивості хітозану та аніонні властивості трьох інших біополімерів. Результати FTIR-аналізу створених гідрогелів свідчать про утворення композицій, які містили вищевказані біополімери та NSE (1,95 мг/г або 0,062 мг/см$^2$ хітозан-гіалуронатного гідрогелю). Хітозан різної молекулярної маси (150, 500, 740 кДа), а також хондроїтин-6-сульфат (<2 мг/мл) і гіалуронова кислота (<2 мг/мл) не продемонстрували значної цитотоксичної дії *in vitro* (тести з МТТ та із виключення трипанового синього) щодо псевдонормальних клітин епітеліальних (лінія MCF10A людської молочної залози, лінія HaCat людських кератиноцитів і лінія HEK293 клітин ембріональної нирки людини) і сполучної (мишачі фібробласти лінії Balb/3T3) тканин. Менше ніж 20% інгібування виживання клітин спостерігали за дії 80 мкМ (26 мг/мл) NSE. Більшість гелів були стійкими до деградації у сольовому розчині, однак вони

руйнувалися протягом >24 год культивування в середовищі, що містило 10% ембріональної телячої сироватки крові. Обговорено перспективи застосування створених гідрогелів на основі хітозану як супресорів запалення та стимуляторів загоєння ран у лабораторних тварин.

Ключові слова: гідрогелі хітозану, хондроїтин-6-сульфат, гіалуронова кислота, N-стеароїлетаноламін, аналіз FTIR, псевдонормальні клітини людини, токсичність, біодеградація.

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