CHAPTER FOUR

Hydrogels based on modified pectins capable of modulating neural cell behavior as prospective biomaterials in glioblastoma treatment

Andrei Belousov[a], Sergei Titov[a], Nikita Shved[a,b], Grigorii Malykin[b], Valeri Kovalev[b], Irina Suprunova[a], Yuri Khotimchenko[b,c], Vadim Kumeiko[a,b,*]

[a]School of Biomedicine, Far Eastern Federal University, Vladivostok, Russia
[b]A.V. Zhirmunsky National Scientific Center of Marine Biology, Far Eastern Branch of Russian Academy of Sciences, Vladivostok, Russia
[c]Department of Fundamental Medicine, School of Biomedicine, Far Eastern Federal University, Vladivostok, Russia
[*]Corresponding author: e-mail address: vkumeiko@yandex.ru

Contents

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Abstract

Glioblastoma is the most common malignant tumor of the brain, but its treatment outcomes can be improved by new therapeutic techniques using biocompatible materials. Utilizing controllable alkaline de-esterification we obtained pectin preparation with 27.4% esterification degree and used it for bio-artificial matrix production. We discovered optimal gelation conditions in the presence of Ca^{2+} by the analysis of viscoelastic properties of the gels and produced a series of biomaterials in hydrogel forms. Hydrogels based on low-esterified pectin significantly slow down the metabolism of C6 glioma cells and neural stem cells (NSCs) and slightly decrease the viability of the C6 glioma, but not of NSCs. This happens due to a decrease in cell proliferation rate, while apoptosis degrees remain stable or negligibly decrease. We created a set of pectin hydrogels supplemented with different ratios of two ECM proteins—collagens I and IV. We have shown that the formation of cell processes in glioma C6 can be regulated by varying the ratio of two ECM proteins in gels used for 3D cell cultivation. Thus, composite matrix materials obtained can be used for modeling brain tumor invasion. The results presented suggest that modified pectins supplemented with two collagen types may serve as prospective biomaterials for glioblastoma treatment due to their ability to regulate glioma cell dynamics.

1. Introduction

Brain tumors don't have the highest incidence rate among cancers, but they are definitely one of the deadliest. Each year, 296,000 new cases of nervous system cancers are being diagnosed worldwide, and these types of tumors cause 241,000 deaths annually, which makes up 2.5% of all cancer deaths. By mortality rate, tumors of the nervous system are surpassed only by pancreas cancer, liver cancer, and lung cancer (Bray et al., 2018). According to the latest reports, the ratio of deaths to newly diagnosed cases is 0.81 worldwide and varies by region (e.g., in the USA it was 0.74 as of 2019 estimates) (Bray et al., 2018; Siegel, Miller, & Jemal, 2019). According to reports based on statistics by Global Cancer Observatory, this ratio tends to increase over the last 10 years.

While lung, liver, and pancreas cancers are mostly diagnosed in the older age, it is brain cancers which are the scourge of younger patients, leading by mortality rate in men below the age of 40 and women below the age of 20 (Siegel et al., 2019).

When it comes to brain cancers, gliomas are the most notorious, due to their outstanding morbidity, mortality, and poor prognosis in most of the cases (Goodenberger & Jenkins, 2012; Ostrom, Gittleman, Stetson, Virk, & Barnholtz-Sloan, 2015). Median patient's life span, after a glioma is diagnosed, is usually estimated as 15 months; there are no viable preventive measures known or reliable conventional therapeutic strategies which can improve the outcome of the disease (Bastiancich et al., 2018; Pinel, Thomas, Boura, & Barberi-Heyob, 2019). Standard treatment of gliomas which consists of surgical resection of the tumor and concurrent radiotherapy and chemotherapy results in disappointingly low success, and relapse rate verges toward 100% in just 2 years (Bastiancich, Danhier, Preat, & Danhier, 2016; Pinel et al., 2019). Such a low efficiency of the traditional treatment strategy urges researchers to investigate novel therapeutic methods. Some of the most promising strategies focus not only on cell component of tumors, but on the extracellular matrix (ECM), which hasn't been taken into account in cancer treatment just until recently. However, tumor cells, which are usually considered the "main villains" of gliomas, are greatly affected by the ECM, which forms cancer stem cell niches and directs tumor development and progression (Kim et al., 2005; Mikhailova et al., 2018). Microenvironment of cancer stem cells (CSCs), formed by the ECM and surrounding cells is referred to as the niche. Cancer stem cell niche shares many similarities with the normal stem cell niche in terms of composition of the matrix, although there are some characteristics unique for each one of them. Cancer stem cell niche regulates the quiescence of CSCs, which is responsible for tumor relapse after its resection or chemotherapy. CSC niche delivers growth signals to the cells, shields them from the immune system, and increases their chemotherapy resistance (Gulaia et al., 2018). Therefore, the ECM is an important regulating and controlling factor for tumors and should always be taken into consideration when developing an anti-cancer therapeutic strategy. Promising techniques aiming to eliminate gliomas should rely on various matrix materials imitating normal ECM which will affect straggler glioma cells remaining after surgical resection of the tumor. Such materials can perform targeted drug delivery to greatly boost the effectiveness of chemotherapy; matrix materials also can mediate the behavior of surrounding cells (such as proliferation, migration, and

neurite growth), and not only by chemical composition or attached signaling factors but also just by mechanical properties of the matrix itself, such as stiffness and orientation of fibrils. Thus, proper matrix materials can greatly facilitate the treatment of a tumor per se, and promote the normal regeneration of surrounding tissues after resection (Shah & Kochar, 2018). Matrices supposed for glioma treatment should meet many important requirements though, such as low toxicity, biocompatibility, and biodegradability, and polysaccharides, or glycans, are one of those which can successfully achieve this. Hyaluronic acid (HA), glycosaminoglycan carrying ionogenic groups, is found in many human tissues, but it is of particular importance to the brain ECM, since the brain is especially rich in glycosaminoglycans (Mouw, Ou, & Weaver, 2014). HA forms the basic structural unit of the brain ECM, the perineuronal network. In gliomas, where its content appears to be even higher than in normal brain tissues, HA also increases the mobility of tumor cells facilitating their migration and tumor progression (Koochekpour, Pilkington, & Merzak, 1995), aside from being an essential component of the cancer stem cell niche (Reinhard, Brösicke, Theocharidis, & Faissner, 2016).

When used in hydrogels for anti-glioma treatment, various types of polysaccharides are applicable, and HA is not always the most desirable component, due to its high biological activity and dependency of glioma cells on its presence in their surroundings. It serves as the main stimulus for invasion of glioma cells, in which the HA receptor, CD44, is overexpressed (Akiyama et al., 2001).

Hyaluronic acid and its associated chondroitin sulfate proteoglycans and proteins of the lectican, tenascin families are abundantly present in the brain ECM (Song & Dityatev, 2018). The content of proteins is significantly reduced in the adult ECM of the central nervous system; though during the embryonic development of the nervous system extracellular matrix proteins such as laminin, fibronectin, and collagens play a crucial role.

Hydrogels formed by various glycans can effectively imitate the normal ECM structure; they generate a highly-hygroscopic 3D polymer network which can be used as a drug-delivery system in chemotherapy, due to the capability of such gels to gradually release loaded drugs (Bastiancich et al., 2016). We have demonstrated that pectin-based hydrogels can successfully mimic the natural brain ECM and replace the native matrix polysaccharide component in composite hydrogels. To create an optimal microenvironment for anti-cancer therapy, it is necessary to thoroughly adjust the ratio of matrix components which are capable of remodeling native ECM in

the remaining cancer cells microenvironment, affect cell migration, proliferation, processes outgrowth and determine cell fate. We showed that two ECM proteins—collagens I and IV, supplementing pectin-based hydrogels, can modulate glioma cells behavior depending on their certain ratio.

2. Material and method

2.1 Materials

High esterified pure citrus pectin was obtained from Copenhagen Pectin A/S, Lille Skensved, Denmark. The stated degree of esterification of this preparation was 60.0%. The pectin contained no acetyl or amide groups.

2.2 Preparation of low-esterified pectin

Preparation of low-esterified pectin was executed using the method of alkali de-esterification of high esterified pectin in a water–ethanol solution as was previously described by Khotimchenko, Kolenchenko, Khotimchenko, Khozhaenko, and Kovalev (2010). Briefly: highly esterified citrus pectin was washed with ethanol and dried at 70 °C. After drying, the pectin sample was ground and fractionated according to the particle size. In the study, the fine fraction was sieved through a sieve with a cell size of 74 μm. The process of alkaline de-esterification of pectin included the initial neutralization of free carboxyl groups of anhydrogalacturonic acid, and then, with an increase of pH above 8.5, the necessary de-esterification of pectin. When the desired degree of esterification was achieved, the reaction mixture was acidified with 1 M HCl solution in 50% ethanol, reaching a pH of 5–6 with vigorous stirring. The obtained pectin preparation was separated from the water-ethanol solution by filtration and washed with 300 mL of a 50% ethanol solution and 150 mL of a 95% ethanol in succession. Washed pectin was dried at 70 °C. This method allowed to obtain a set of products that differ by the content of methoxylated groups.

Pectin sample characterization was performed according to previously described technique (Khotimchenko et al., 2010).

2.3 Rheological properties of pectin hydrogels

Rheological properties of pectin hydrogels were evaluated using a HAAKE Mars-III rheometer equipped with a 20 mm serrated parallel plate geometry with a gap of 1000 μm (upper plate P20/Ti/SE, lower plate Meβplate TMP 20, Thermo Fisher Scientific Inc., USA).

Hydrogel samples for selecting of the optimal Ca^{2+} concentration were obtained using a cylinder made from a disposable syringe with a diameter of 20 mm. One end of the cylinder was sealed with parafilm and placed on a heater set to 40 °C. 250 μL of the 3% pectin solution and 250 μL of 300 mM NaCl solution with 100 mM HEPES-NaOH pH 7.4, and Ca^{2+} in concentrations of 12, 14, 16, 18 or 20 mM were poured into the cylinder. The solutions were thoroughly mixed using the syringe plunger and incubated for 30 min at +4 °C till hydrogels formation. After incubation, the parafilm was carefully removed from the cylinder and hydrogels were transferred to the lower plate using gel releaser (Bio Rad).

All samples were covered to prevent moisture loss during measurements. Viscoelastic characteristics were measured, and optimal concentration of Ca^{2+} ions was selected to form hydrogel from modified pectin. All measurements were carried out on samples of hydrogels 20 mm in diameter and 1.0 mm thick, at a controlled shear stress of 1 Pa and a variable frequency of 1–0.01 Hz. At all investigated concentrations, the storage modulus (G') and loss (G'') modulus were recorded.

2.4 Cell cultures

2.4.1 Glioma C6

Rat C6 glioma cells were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% Fetal Bovine Serum (FBS; Gibco) and 1% penicillin/streptomycin (Gibco), at 37 °C in a humidified atmosphere of 5% CO_2. After cells reached 70% confluency, the cells were harvested, washed with DPBS buffer (Gibco), resuspended (1×10^6 cells/mL) and used for experiments.

To study cell proliferation and viability, the cells were grown in 24-well plates at a density of 1.5×10^5 cells/well. The culture medium was replaced by fresh one on the third day.

2.4.2 Neural stem cells (NSCs)

Ventral midbrain regions of 15-day-old Wistar rat embryos were used to obtain culture of neural stem cells (Ahlenius & Kokaia, 2010). The animal experiment was approved by Animal Care and Use Committee the School of Biomedicine of Far Eastern Federal University. Pregnant rats were euthanized with ether anesthesia and decapitation. The uterus was prepared, the embryos were removed, washed in sterile Phosphate-Buffered Saline (PBS; Gibco) and placed in Petri dishes with Neurobasal medium. In sterile conditions, the embryos were dissected, the cranium was opened, and the

ventral regions of the midbrain were isolated. The isolated parts of the brain were placed in 6-well Eppendorf plates with Neurobasal medium (Gibco) containing 1% B-27 supplement without vitamin A (Gibco), 20 ng/mL EGF and 20 ng/mL bFGF, 1% amphotericin B, 100 U/mL penicillin, 100 μg/mL streptomycin. The resulting cell suspension was seeded with a density of 50,000 cells/cm^2 in culture flasks for further incubation at 37 °C, 5% CO_2, in a humid chamber. The medium was changed every 3 days. Neurospheres were ready for reseeding after 4–5 days of cultivation. Neurospheres were carefully collected for reseeding, disaggregated with a sterile Pasteur pipette, and plated with a density of 12,500 cells/cm^2. Passage was carried out every 3 days. When cells were plated on polysaccharide substrate, Neurobasal medium supplemented with B-27 Supplement with Vitamin A (Gibco) was used to stimulate neuronal differentiation.

2.5 Proteins preparation

Type I collagen was isolated from rat tails as described by Price (1975).

Tendon fibers were isolated from rat tails, washed with several portions of distilled water, and dried. The fibers were placed in a solution of 30 mM acetic acid with penicillin-streptomycin (Gibco, 10,000 units/mL penicillin, 10,000 μg/mL streptomycin) at the proportion of 100 mL of solution per 1 g of fibers and incubated for 7 days at 4 °C with constant stirring. The mixture was centrifuged for 15 min at 3000 *g*, 4 °C. The supernatant was collected and centrifuged for 40 min at 10,000 *g*, 4 °C.

Type I collagen was salted out by adding 2 M sodium chloride. Then, the solution was centrifuged (10,000 *g*, 45 min, 4 °C), the supernatant was decanted, and the precipitate was resuspended in 30 mM acetic acid. The resulting protein sample was freeze dried and stored at −80 °C.

To prepare the matrices, the protein was sterilized by dialysis against 30 mM acetic acid with 0.5% chloroform, followed by a three-fold change of the acid solution to remove chloroform.

Type IV collagen in the form of NC1-hexamers was isolated from chicken gizzards as described by Perris, Syfrig, Paulsson, and Bronner-Fraser (1993). The gizzards purified from connective tissue (100 g) were homogenized in 500 mL of buffer containing 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM dithiothreitol (DTT). The suspension was centrifuged at 10,000 *g*, 30 min, 4 °C. The purification procedure was repeated. The precipitate was resuspended with the addition of 250 mL of buffer containing 50 mM

Tris-HCl pH7.4, 0.15 M NaCl, 2 mM PMSF, 2 mM DTT, 10 mM EDTA, incubated for 1 h with constant stirring, 4 °C. The mixture was precipitated by centrifugation at 3000 *g*, 30 min, 4 °C, washing was repeated, the suspension was centrifuged again.

The precipitate was resuspended in 250 mL of 0.5 M acetic acid and incubated at 4 °C for 3 days with constant stirring for extraction. The suspension was centrifuged at 10000 *g*, 40 min, 4 °C, and the precipitate was lyophilized.

The lyophilized precipitate was resuspended in 40 mL of 0.2 M ammonium bicarbonate pH7.9, chymotrypsin inhibitor tosylphenylalanyl-chloromethylketone (TPCK) was added to a final concentration of 100 μM in solution, and treated with trypsin (160 mg, MP Biomedicals, with activity 284.5 BAEE units/mg) at room temperature for 4 h with constant stirring. Trypsinization was stopped by the addition of a trypsin inhibitor (30 mg, Sigma; 1 mg inhibits 1 mg of trypsin with an activity of 10,000 BAEE units/mg). The mixture was centrifuged at 10000 g, 40 min, 4 °C. Since a flotation precipitate was observed, they were centrifuged again at 15000 *g*, 30 min, 4 °C. Sodium chloride was added to the supernatant with stirring to a final concentration of 2 M. The mixture was incubated for 2 h at 4 °C without stirring. After centrifugation 15,000 *g*, 30 min, 4 °C, the precipitate was resuspended in 10 mL of 0.2 M ammonium bicarbonate pH7.9, 2 mm DTT, and dialyzed overnight against the same buffer. The resulting preparation was packaged, freeze-dried and stored at −80 °C. Type IV collagen preparation was sterilized by filtration (pore diameter 0.45 μm).

2.6 Preparation of pectin-based hydrogel samples

Stock 3% solution of pectin was prepared in deionized water by dissolving dry preparation of polysaccharide in a + 60 °C water bath for 15 min.

Pectin-based hydrogel was obtained by mixing in sterile conditions in equal proportions the stock 3% solution of pectin and a gelation initiator consisting of 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH7.4, 300 mM NaCl, 16 mM $CaCl_2$. Afterward, hydrogel was incubated for 30 min for complete gelation.

Thin films of hydrogel were prepared in 24 well plates (Eppendorf, Germany) under sterile conditions. 0.5 mL of a 1.5% solution of pectin was applied to each well, incubated for 1 h at room temperature.

After incubation, the solution was taken of the wells. After that, the plate was left open to dry and to form a thin film of polysaccharide. 0.5 mL of previously sterilized solutions of gelation initiator containing 50 mM HEPES pH 7.4, 150 mM NaCl, 8 mM $CaCl_2$ were added to the dried films after their formation, then incubated for 1 h to gelatinate. Solutions of gelation initiators were collected from the wells, after washing the wells with a similar volume of PBS for washing out unbound calcium ions.

2.7 Development of three-dimensional cell cultures based on composite hydrogels

Three-dimensional composite hydrogels consisting of modified pectin and extracellular matrix proteins in different concentrations were obtained: type I collagen and type IV collagen NC1-hexamers. Samples were prepared in chambered coverslips (μ-Slide, ibidi).

To obtain 1.5% of hydrogel samples with a volume of 100 μL, gelation initiator based on 37 mM sodium hydroxide, HEPES pH 7.4, sodium chloride and calcium chloride was quickly mixed with cell suspension, proteins or their solvents and 50 μL stock 3% solution of pectin. After 30 min of incubation at 37 °C, DMEM culture medium was added. The gelation initiator contains NaOH in an amount sufficient to neutralize acetic acid (since collagen I was solubilized in 0.03 M acetic acid), sodium chloride (up to physiological concentration in gel), calcium chloride (up to 16 mM in gel) and HEPES (up to 30 mM in gel).

The concentrations of Ca^{2+} were selected experimentally; we picked those concentrations which brought the interval of hydrogel storage and loss modulus (G′, G″) to the plateau.

2.8 Cell viability assay

Cultures were plated at a density of 7500 cells per cm^2 and cultured in DMEM with 10% FBS and 1% penicillin-streptomycin (Thermo Fisher Scientific) for 10 days. Media was changed every 3 day through the experiment.

After 3 and 10 days of cultivation, cells were detached from the substrate with a 0.25% trypsin solution and labeled with the Calcein AM and propidium iodide (PI) (Thermo Fisher Scientific). Viability assessment was performed using a MoFlo Astrios EQ flow cytometer (Beckman Coulter) at excitation wavelengths of 488 and 561 nm emission.

2.9 Apoptosis analysis

One of the earliest features of apoptosis is the translocation of phosphatidylserine from the inner to outer leaflet of the plasma membrane, which can be detected by the binding of annexin V. Apoptosis was analyzed with an annexin V—FITC conjugate (Thermo Fisher Scientific). Cells were trypsinised and centrifuged at 100*g* for 5 min. Cells were resuspended in PBS, and then stained with 5% annexin V conjugate and PI reagent for 30 min at room temperature (RT) in the dark. The cells were immediately analyzed by MoFlo Astrios EQ flow cytometer (Beckman Coulter).

2.10 Cell proliferation assay

Cell proliferation was determined by the thiazolyl blue tetrazolium bromide (MTT, Sigma) assay. Viable cells convert MTT to insoluble formazan crystals. Cells were seeded at a density of 5000 cells per well in 96-well plates. Cells were treated with MTT solution for 4 h at 37 °C. The absorbance at 595 nm was measured using a microplate reader (iMark™ Microplate Absorbance Reader, Bio-Rad).

2.11 Visualization of C6 glioma cells in 3D composite hydrogels

Cells were stained intravitally with a bioconvertible tracer succinimidyl ester of carboxyfluorescein diacetate (CFDA SE), which can penetrate cell membranes and turn into a fluorescent product with cytoplasmic localization after interaction with enzymes of a living cell. Cell marking was performed according to the method recommended by the manufacturer (Invitrogen, USA). 3D composite cell culture system was incubated in the resulting tracer solution for 15 min at 37 °C. The tracer was drained, then fresh DMEM medium with 10% fetal bovine serum was added to a well and incubated for 30 min for fluorescence enhancement.

2.12 Multiphoton laser microscopy and methods of morphometric analysis

Samples were analyzed using a FluoView FL1200MPE optical imaging system based on an IX83 inverted microscope workstation (Olympus). A two-photon observation mode with reflected light detectors was used. A femtosecond infrared laser with a tunable Chameleon wavelength (Coherent) was used.

To visualize three-dimensional patterns of cell distribution in composite matrix materials, samples were scanned using $20^{\times}$ lenses with a 0.45 numerical aperture (LUCPlanFLN), in a unidirectional scan mode. In each sample,

a material region 200 μm thick was examined. For this purpose, a series of optical sections with a step of 1.5 μm was obtained (in the *Z*-stack mode). Using the Imaris software module (version 7.6.5), we obtained three-dimensional reconstructions of the images. For each sample, at least 200 cells were analyzed, among which cells close to a spherical shape and cells forming processes were identified.

The compactness criterion was calculated as $C = P^2/S$, where C—the value of the criterion of compactness of the cell, P—the perimeter of the cell, S—the area of the cell.

We considered cells with processes as objects which compactness criterion was at least 20 units. Parameters were evaluated using Sigma Scan Pro 5.0 software (Systat Software, Inc).

2.13 Statistical processing

At least 15,000 events were collected in three independent repetitions for apoptosis and vitality assays. Nine independent retries were made for MTT. Compactness was measured for 200 cells in five Z-stacks for each sample. Statistical data processing was performed using GraphPad Prism 6 Software.

3. Results

3.1 Pectin samples

Low-esterified pectin preparation was obtained, and its physicochemical properties were analyzed. The sample was characterized by 27.4% degree of esterification. The total content of anhydrogalacturonic acid was 67.8% by weight; the content of unesterified anhydrogalacturonic acid was 49.2% by weight. The intrinsic viscosity was 452 mL/g anhydrogalacturonic acid.

3.2 Viscoelastic properties of the hydrogels and optimization of calcium concentration

To evaluate the dependence of viscoelastic properties of low-esterified pectin hydrogels on various Ca^{2+} concentration, the storage modulus (G′) and loss (G″) modulus were recorded performing frequency sweeps (1–0.01 Hz, 1 Pa) (Fig. 1). It was found that the gelation of pectin was observed for all Ca^{2+} concentrations studied, as evidenced by the values of $G' > G''$, which is typical for hydrogels. Rheological analysis of the hydrogels revealed an increasing storage modulus in response to a 1 mM incremental increase in the concentration of Ca^{2+}. For 1.5% pectin hydrogels, a significant rise

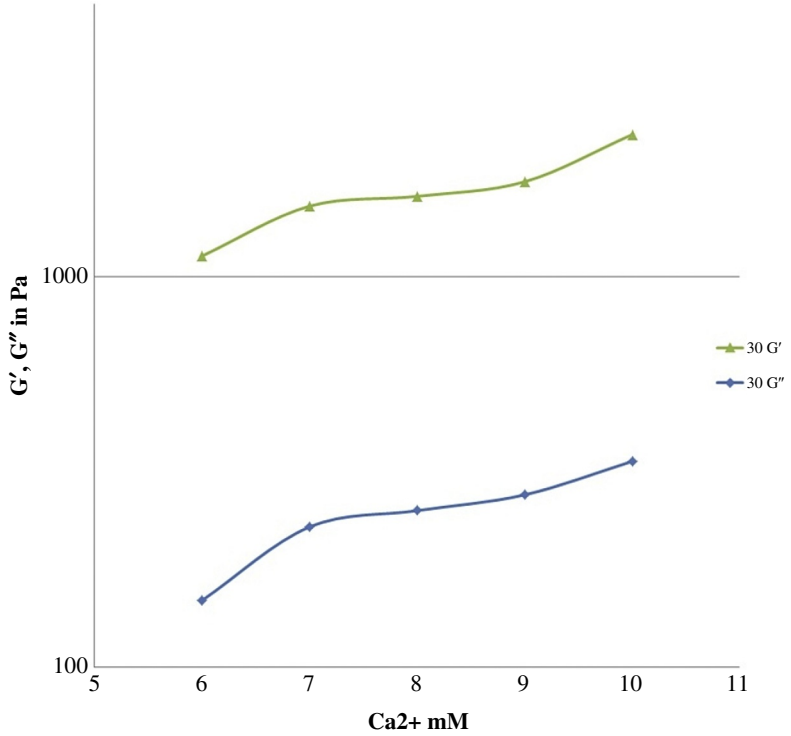

Fig. 1 Dependence of the storage modulus (G′) of the hydrogel on a concentration of Ca^{2+} ions at 1 Hz.

in G′ was observed when Ca^{2+} concentration increases from 6 to 7 mM (6 mM: 1129 Pa, 7 mM: 1516 Pa), then on the range from 7 to 9 mM intermediate plateau was identified (7 mM: 1516 Pa, 8 mM: 1607 Pa, 9 mM: 1752 Pa), then the slope changes dramatically (9 mM: 1752 Pa, 10 mM: 2314 Pa) and hydrogel viscoelastic properties moved out of the range suitable for neural cell microenvironment.

For the formation of hydrogels based on low-esterified pectin, 8 mM concentration of Ca^{2+} was chosen, because this concentration corresponds to the intermediate plateau of the graph with viscoelastic properties suitable for neural cells.

3.3 Effects of materials on cell viability and apoptosis in cell cultures

The viability of C6 rat glioma cells during their cultivation on the surface of hydrogel remained high throughout the experiment. The viability of

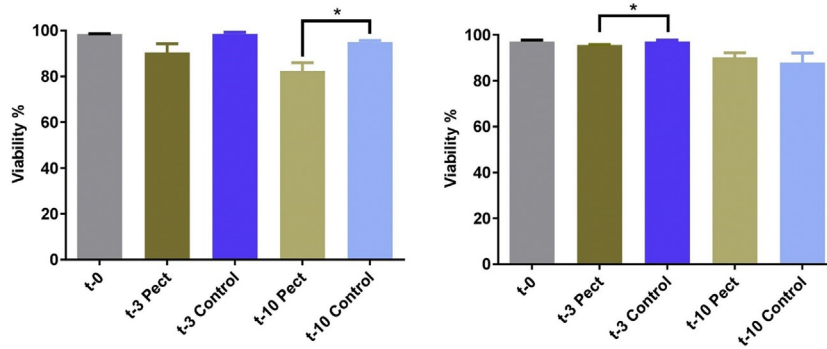

Fig. 2 Cell viability on pectin hydrogel: (A) Glioma C6, (B) NSCs. *P value <0.05. t-0—cell viability before culturing; t-3 Pect—cell viability after 3 days culturing on pectin hydrogel; t-3 Control—cell viability after 3 days culturing on plastic control; t-10 Pect—cell viability after 10 days culturing on pectin hydrogel; t-10 Control—cell viability after 10 days culturing on plastic control.

C6 cells after 3 days cultivation was 90 4 ±2.2% for pectin hydrogel and 98.5 ±0.5% in the control. The viability for C6 cells after 10 days cultivation was 82.4 ±2.1% for pectin hydrogel and 94.9 ±0.4% for control, respectively (Fig. 2A). NSCs showed a similar trend. The level of NSCs viability after 3 days cultivation was 95.7±0.1% on pectin hydrogel and 97.1±0.4% for the control. After 10 days, the viability was 90.2 ±1.1% for pectin hydrogel and 87.9 ±2.4% for the control (Fig. 2B).

MTT analysis showed a decrease in proliferative activity on the hydrogel in comparison with the control after 3 days and after 10 days in both cell lines (Fig. 3).

The degree of apoptosis in the C6 culture was low and amounted to about 0.74 ±0.02% on the hydrogel and about 1.75 ±0.09% in the control (Fig. 4A). Apoptosis of NSCs was about 4.75±0.04% on pectin hydrogel and 4.24±0.02% for the control (Fig. 4B).

This data allowed to conclude that the change in the number of viable cells during their cultivation on pectin hydrogel was due to inhibition of proliferation, and not a high degree of apoptosis.

3.4 Macroscopic examination of the material

We developed 16 types of hydrogels that differ in protein content. All preparations, upon visual assessment, were optically transparent with visually uniform structure after gelation. No syneresis was observed.

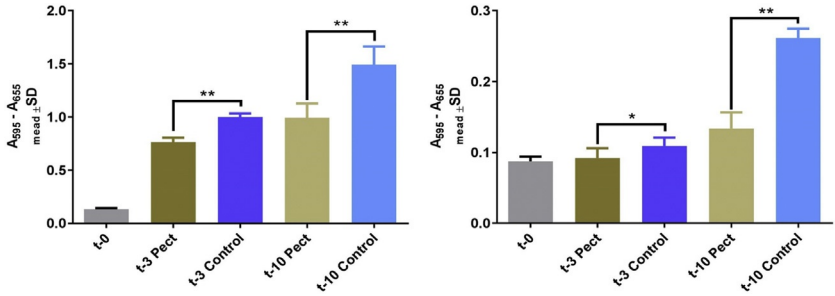

Fig. 3 MTT assay of the cell cultures on pectin hydrogel: (A) Glioma C6, (B) NSCs. *P value <0.05. **P value <0.0001. t-0—MTT test before culturing; t-3 Pect—MTT test after 3 days culturing on pectin hydrogel; t-3 Control—MTT test after 3 days culturing on plastic control; t-10 Pect—MTT test after 10 days culturing on pectin hydrogel; t-10 Control—MTT test after 10 days culturing on plastic control.

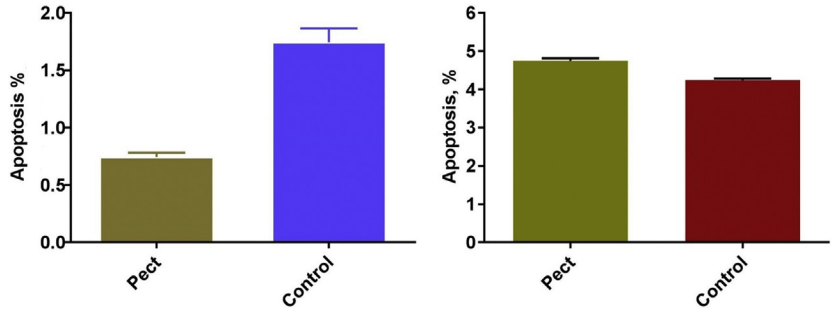

Fig. 4 Apoptosis levels for the cell cultures on pectin hydrogel: (A) Glioma C6, (B) NSCs. **P value <0.001. Pect—cell apoptosis degree after culturing on pectin hydrogel; Control—cell apoptosis degree after culturing on plastic control.

3.5 Cell morphology analysis after cultivation of three-dimensional composite cell system

After 3 days of cultivation, differences in cell morphology were noted. Cells with compactness of more than 20 units were considered elongated, fusiform and branched.

In the material, consisting of 1.5% pectin and protein solvents (Fig. 5A), all cells retained a spherical shape and did not have processes; groups of cells were observed in material.

In the material containing 1.5% pectin and type I collagen at a concentration of 250 μg/mL (Fig. 5B), the proportion of cells with processes was insignificant and numbered $1.5 \pm 0.9\%$. Cells also formed clusters in material.

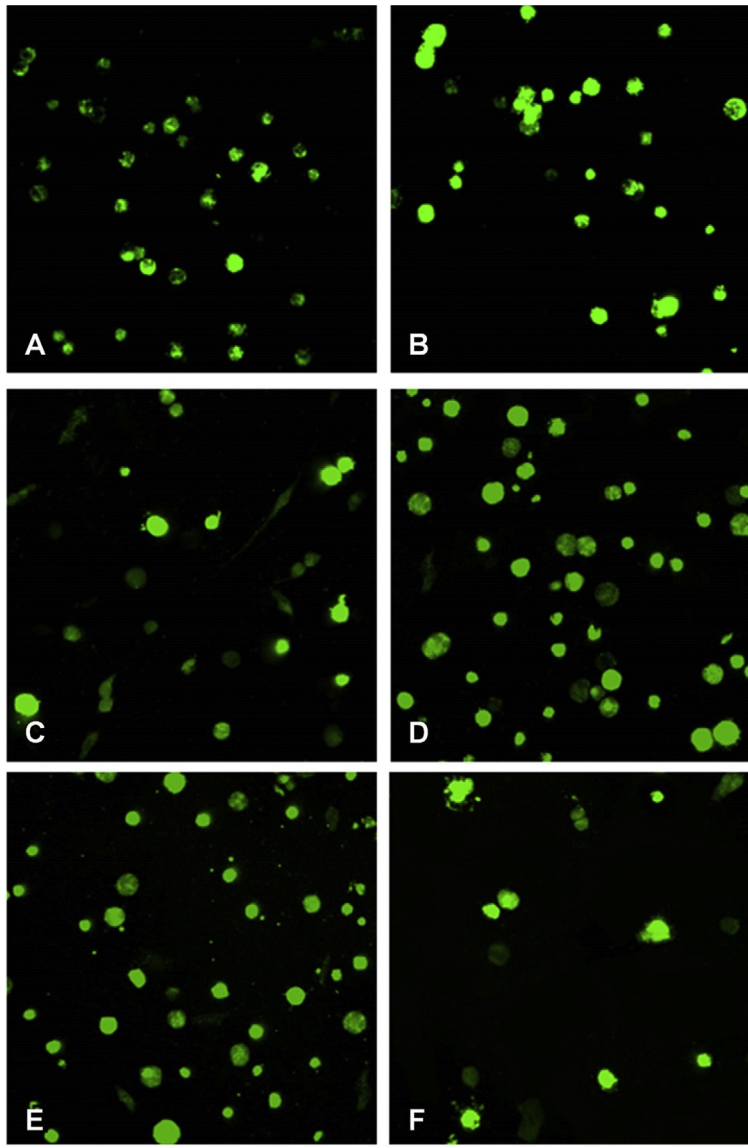

Fig. 5 Morphology of C6 glioma cells in matrix materials of various compositions after 3 days of cultivation (part 1). Magnification 200×. (A) Hydrogel based on pectin. (B) Hydrogel based on pectin and collagen type I (250 μg/mL). (C) Hydrogel based on pectin and collagen type I (500 μg/mL). (D) Hydrogel based on pectin and collagen type I (1000 μg/mL). (E) Hydrogel based on pectin and collagen type IV (50 μg/mL). (F) Hydrogel based on pectin and collagen type IV (150 μg/mL).

In the composite matrix material of a similar composition, but with type I collagen concentration of 500 μg/mL, 13.0 ± 9.3% of the cells formed processes; the degree of flattening in the substrate and the length of the processes was different (Fig. 5C). Cells were distributed diffusely in the material.

In the hydrogel with the doubled concentration of type I collagen (up to 1000 μg/mL), shown in Fig. 5D, the processes on cells were not detected.

In the composite hydrogel containing 1.5% pectin and type IV collagen at a concentration of 50 μg/mL, the processes were formed in 1.5 ± 1.0% of the cell population. The result of cultivation is shown in Fig. 5E.

The behavior of C6 glioma cells in the hydrogel of 1.5% pectin and type IV collagen at a concentration of 150 μg/mL was similar to that in a pectin gel. Cells were arranged in groups; however, 7.8 ± 0.1% of them formed processes (Fig. 5F). Most of the processed cells had small outgrowths on their surface, while the shape of the cells was close to round. Elongated cells had small processes.

In the composite hydrogel containing 1.5% pectin and type IV collagen at a concentration of 600 μg/mL, shown in Fig. 6A, C6 glioma cells did not form processes.

Fig. 6B shows the result of culturing C6 glioma cells in the hydrogel containing 1.5% pectin, type I collagen at a concentration of 250 μg/mL and type IV collagen at a concentration of 50 μg/mL. Most of the cells did not form processes. The proportion of glioma cells with processes was 16.1 ± 6.9%.

In the hydrogel containing 1.5% pectin, type I collagen at a concentration of 500 μg/mL and type IV collagen at a concentration of 50 μg/mL (Fig. 6C), a significant part of the cells formed 1–2 long processes. Amount of cells with processes was 79.1 ± 2.6%.

In the composite matrix material of a similar composition, but with a type I collagen concentration increased to 1000 μg/mL (Fig. 6D), the processes formed 8.9 ± 2.8% cells. The vast majority of spread cells forms only a single long process.

Fig. 6E shows the result of cell cultivation in the bulk of hydrogel containing 1.5% pectin and type I and IV collagen at a concentration of 250 μL/mL and 150 μg/mL, respectively. One part of the cells in the material was arranged in groups, the other was distributed diffusely. In both cases, some cells formed 1–2 processes. The proportion of cells with processes in the gel sample was 16.8 ± 5.2%.

In the hydrogel containing 1.5% pectin and type I collagen at a concentration of 500 μg/mL and type IV collagen at a concentration of 150 μg/mL

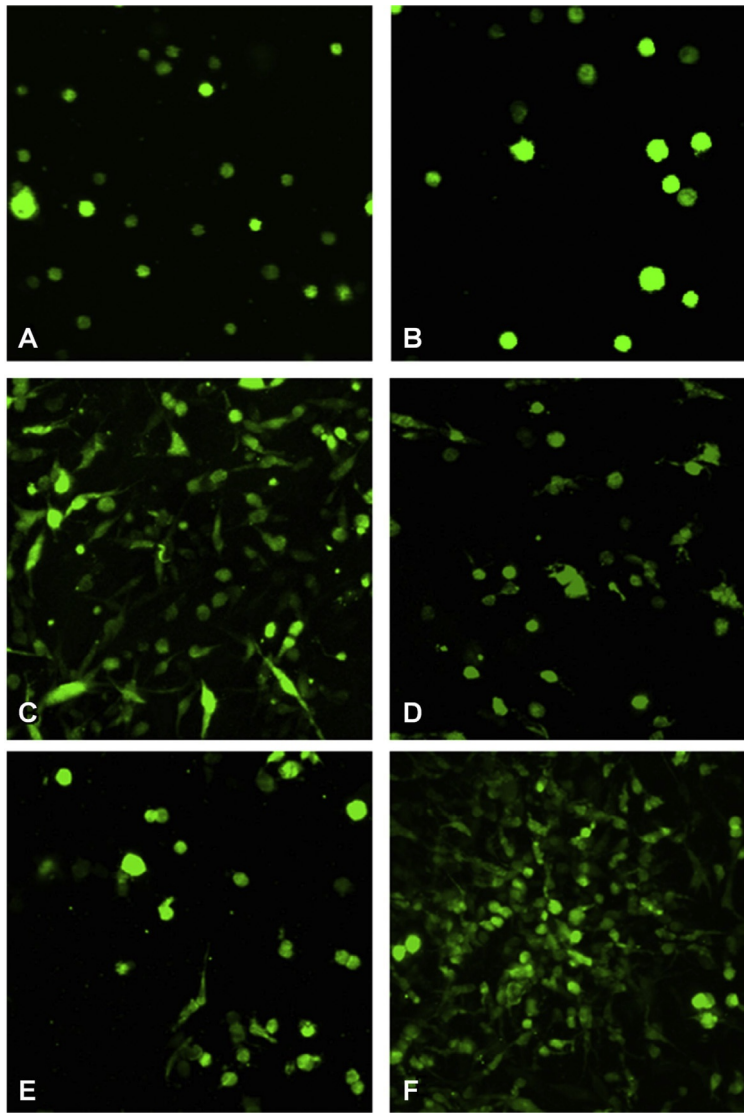

Fig. 6 Morphology of C6 glioma cells in matrix materials of various compositions after 3 days of cultivation (part 2). Magnification 200×. (A) Hydrogel based on pectin and collagen type IV (600 μg/mL). (B) Hydrogel based on pectin, collagen type I (250 μg/mL) and collagen type IV (50 μg/mL). (C) Hydrogel based on pectin, collagen type I (500 μg/mL) and collagen type IV (50 μg/mL). (D) Hydrogel based on pectin, collagen type I (1000 μg/mL) and collagen type IV (50 μg/mL). (E) Hydrogel based on pectin, collagen type I (250 μg/mL) and collagen type IV (150 μg/mL). (F) Hydrogel based on pectin, collagen type I (500 μg/mL) and collagen type IV (150 μg/mL).

(Fig. 6F), 67.8 ± 8.4% of cells formed processes; the degree of protrusive cell growth in the substrate and the length of processes was different. In some cases, cells formed processes in an organized manner, probably along collagen fibers, which turned out to be linearly located in the gel sample.

In the hydrogel containing 1.5% pectin, type I collagen at a concentration of 1000 μg/mL and type IV collagen at a concentration of 150 μg/mL (Fig. 7A), many cells formed 1–2 long processes. The parallel arrangement of processes in many cells may indicate elongation of cells along collagen fibers. Cells with processes numbered 58.2 ± 2.3%.

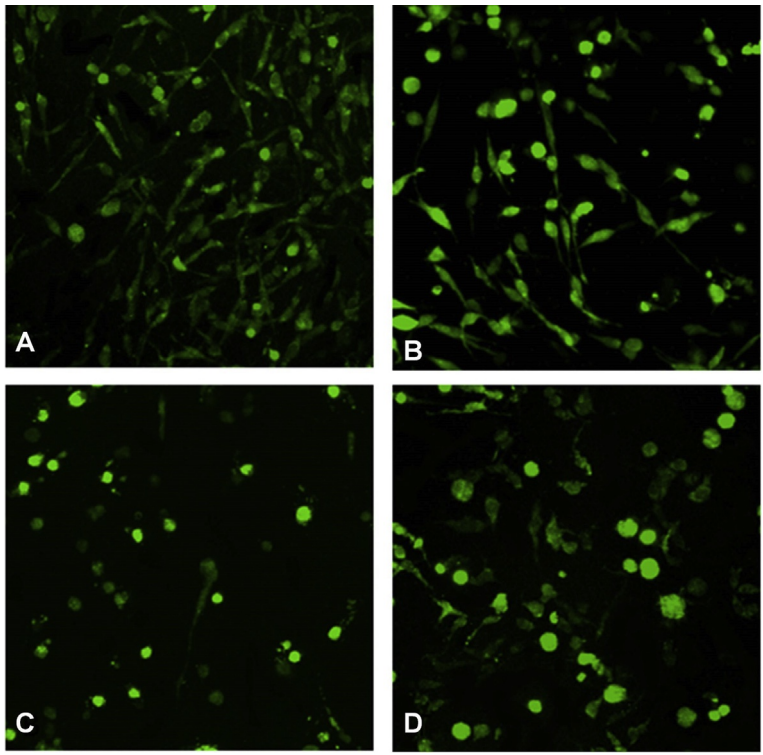

Fig. 7 Morphology of C6 glioma cells in matrix materials of various compositions after 3 days of cultivation (part 3). Magnification 200 ×. (A) Hydrogel based on pectin, collagen type I (1000 μg/mL) and collagen type IV (150 μg/mL). (B) Hydrogel based on pectin, collagen type I (250 μg/mL) and collagen type IV (600 μg/mL). (C) Hydrogel based on pectin, collagen type I (500 μg/mL) and collagen type IV (600 μg/mL). (D) Hydrogel based on pectin, collagen type I (1000 μg/mL) and collagen type IV (600 μg/mL). The data obtained are presented in the diagram (Figs. 8 and 9).

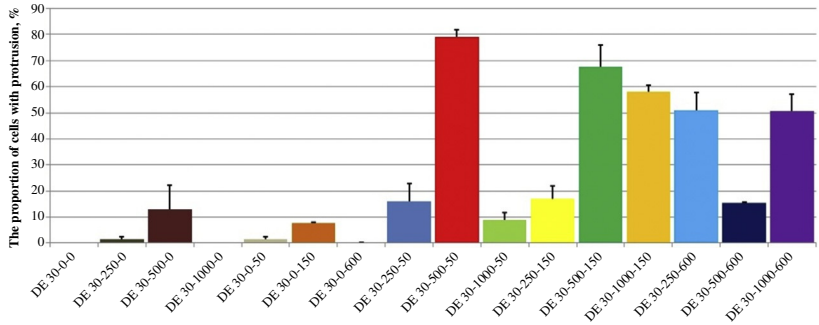

Fig. 8 Cells outgrowth in matrix materials of different composition (means ± SD). Materials entitled as "pectin degree of esterification—collagen type I concentration (μg/mL)-collagen type IV concentration (μg/mL)."

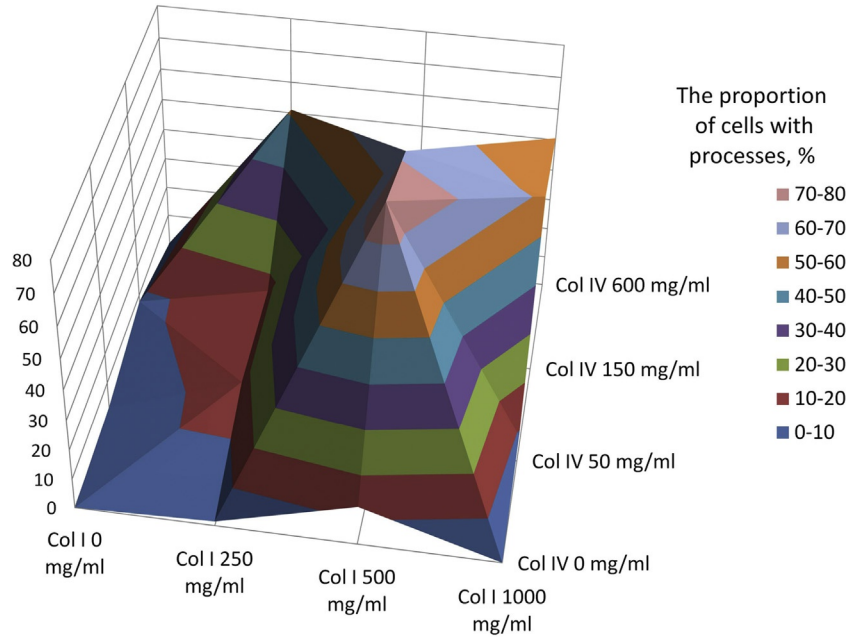

Fig. 9 The dependence of the formation of processes on concentration of collagen type I and IV.

On the fourth day of cell cultivation in the material consisting of 1.5% pectin, type I collagen at a concentration of 250 μg/mL, and type IV collagen at a concentration of 600 μg/mL (Fig. 7B), the length of processes was different. The proportion of spread cells was 51.1 ± 6.7%.

Fig. 7C shows the result of cell cultivation in the material consisting of 1.5% pectin, type I collagen at a concentration of 500 μg/mL and type IV collagen at a concentration of 600 μg/mL. Most cells retained spherical morphology, cells with processes were located individually; 15.6 ± 0.1% of cells formed processes. Some cells formed a single long process, some formed 5–6 small and short processes.

In the composite hydrogel containing 1.5% pectin and type I collagen at a concentration of 1000 μg/mL and type IV collagen at a concentration of 600 μg/mL (Fig. 7D), 50.6 ± 6.5% of cells formed processes. The material contained both rounded cells and cells with long processes.

4. Discussion

Polysaccharides have weak cell-adhesion properties, therefore, to stimulate adhesion, polysaccharide-based matrix materials are either chemically modified or enriched with components that stimulate cell adhesion. A number of studies have shown that polysaccharides have the ability to support the formation of processes of cells of the nervous tissue (Bombaldi de Souza, Bombaldi de Souza, Drouin, Mantovani, & Moraes, 2019; Sadeghi, Moztarzadeh, & Aghazadeh Mohandesi, 2019; Yao et al., 2018). In this case, effects can be opposite and depend not only on the type, but also on visco-elastic properties of materials and the concentration of the polymer. Thus, culturing rat cortical neurons in agarose hydrogels with concentration of up to 1.5% supported the formation of neurites; gels with an agarose concentration of more than 2% retained cells in spherical morphology. Cell viability in this case is higher the higher the concentration of agarose in the gel surrounding the cell in 1.5% gel, cells retained normal viability (about 80%) and actively formed processes on day 8, (Cullen, Lessing, & LaPlaca, 2007). The viability of C6 glioma cells cultured on films of 2% chitosan is higher than that on 1% films (Martín-López, Nieto-Díaz, & Nieto-Sampedro, 2013).

The effect of hyaluronic acid, alginic acid, and pectins on the microenvironment of cells is largely similar, since all these glycopolymers have a similar structure—they are polymers of uronic acids.

A composite material based on agarose and chitosan promotes cell attachment and survival much more efficiently than pure agarose (Cao, Gilbert, & He, 2009). It was also shown that the concentration of chitosan affects the morphology of the processes of neurons. The increase in

concentration leads to the fact that the processes become more branched. In a medium with a minimal concentration of chitosan, long, unbranched processes are formed. A composite material of chitosan and alginate with longitudinally oriented channels coated with laminin contributes to the formation of longer neurites compared to a material without a protein coating (Francis et al., 2013). Hydrogel based on HA increases the number of neurons, and also significantly increases the viability of NeuN-positive neurons compared with collagen hydrogel, when cultivating organotypic sections of the spinal cord in gels. It is interesting to note that the HA storage modulus (G′) based hydrogel is significantly higher (1200 Pa) than the collagen hydrogel (≈25 Pa) (Schizas et al., 2014).

Changes in viscoelastic properties of the tissue, which coincide with the development of the tumor, can contribute to the progression of glioma growth. Human glial tumors have storage modulus of about 300–1000 Pa (Niu et al., 2015; Pogoda et al., 2014), but glioma cell lines in vitro respond strongly to substrate stiffness. Thus, the glioma cell line LN229 responds to substrate stiffness in the range from 100 to 2000 Pa. When culturing glioma cells on polyacrylamide gels with an elastic modulus similar to or exceeding the normal brain, they adhere and survive on both substrates coated with either collagen type I or laminin, but their morphology and area strongly depend on the stiffness of the substrate. On softer substrates, LN229 cells have a round morphology without processes (Pogoda et al., 2014).

Since changes in viscoelastic properties affect the fate of tumor cells, this can be used in anticancer therapy. Our material has stiffness similar to that of a tumor, but has weak adhesive properties and is able to slow down the metabolism and proliferation of cells. Thus, material variants containing type I collagen and type IV collagen hexamers of low type IV collagen that do not stimulate the formation of processes can be introduced into the tumor site after its removal together with the anticancer drug, and will slow down the proliferation of residual tumor cells. After completing a course of chemotherapy and destroying tumor cells, the initial material can be replaced by material enriched with collagen of both types to stimulate cell migration into the resection cavity and neuroregeneration.

The gelation of many materials occurs under conditions that are not completely suitable for cells placed in a material, so maintaining cells' viability can be a challenge. For example, the formation of gels from agarose occurs at a temperature of 60–90 °C. For more physiological polymerization conditions, agarose is chemically modified to produce "low melting" agarose. For example, agarose covalently bound to hydroxyethylene

polymerizes at 17 °C (Jain, Kim, McKeon, & Bellamkonda, 2006). Alginate is soluble in dilute aqueous solutions of acids at pH <6.5. Optimal gel formation occurs in an acidic environment, and even small changes in the concentration of hydrogen ions in the pH range of 4–5.5 lead to the formation of hydrogels with different properties (Delmar & Bianco-Peled, 2015). The formation of alginate gels also occurs in the presence of divalent metal salts.

Fibroblasts were cultured in 2% hydrogel from pectin, retain a high level of viability if gelation occurs at near neutral pH (Gentilini et al., 2018).

The gelation of our material occurs at physiological temperatures, pH and ionic composition, which simplifies its use for medical purposes. In this regard, alginate preparations can also be used for producing gels by adding cross-linking calcium ions in physiological concentration. However, commercially available materials from alginates have a rather variable composition and are characterized by a lower swelling rate, which makes their use in cell technologies not very convenient. In addition, it was reported that they significantly reduce the rate of cell metabolism (Novikova et al., 2006).

Unlike polymers based on alginic acid and commercially available pectins, modified pectins we used are standardized preparations, while alginates differ by the composition of glycopolymer chains depending on the batch, type of raw material and the method of their isolation.

Type I collagen acts as a fibrillar component of a composite material. It is known that cells on a two-dimensional substrate predominantly migrate along collagen fibrils (Stopak & Harris, 1982). Type I collagen in composite materials stimulates the formation of processes, however, the optimal protein concentration is variable, and varies from material to material. For example, when culturing the mouse dorsal spinal ganglion in a gel based on hyaluronic acid, the concentration of collagen type I 2 mg/mL turned out to be the most promising. At higher concentrations, the length of neurites was lower (Jin et al., 2013).

In our material based on modified pectin, but without the addition of type IV collagen, the largest proportion of cells with processes is observed at a concentration of type I collagen 500 μg/mL. However, on the fourth day of cultivation, cells with processes made up only 13 ± 9.3% of the cell population.

Type IV collagen is the main component of the Schwann cell basement membrane and is believed to modulate the activity of Schwann cells and neurons (Chen, Cescon, & Bonaldo, 2015). It was established that type IV collagen promotes adhesion and migration of glial cells through α1β1

and α2β1 integrins, and also enhances the proliferation of Schwann cells (Detrait et al., 1999). In addition, type IV collagen indirectly promotes axon growth through the binding of its NC1 domain to α1β1 integrin (Lein, Higgins, Turner, Flier, & Terranova, 1991).

Type IV collagen is known to enhance sciatic nerve regeneration after injury (Politis, 1989). A study of the influence of type IV collagen on the formation of neurites showed that this effect depends on protein concentration. When culturing nerve stem cells obtained from 17-day-old rat embryos in three-dimensional systems of composite hydrogels from agarose and type IV collagen, it was shown that the formation of the greatest number of long neurites occurs in materials with a collagen concentration of type IV from 150 to 300 μg/mL. In materials with a collagen content of type IV 600 μg/mL, significantly fewer neurites were found. Viability in all studied variants of the material remained at a high level, which indicates the absence of toxicity of any of the components.

Collagen in the nervous system of an adult organism is less common than in other tissues, it is concentrated mainly in connective tissues and basement membranes. As structural components of the meninges, collagens contribute to brain development, as well as axon growth and synapse formation (Heikkinen, Pihlajaniemi, Faissner, & Yuzaki, 2014).

Thus, by adjusting the ratio of extracellular matrix proteins to the carbohydrate base in the material, it is possible to change the properties of the matrix and control cell migration and proliferation.

As is known, the central nervous system ECM abundantly contains hyaluronic acid and its associated glycoproteins and proteoglycans of the lectan, tenascin and binding protein families (Burnside & Bradbury, 2014).

Normal brain cells produce extracellular matrix components, such as laminin, type IV collagen and fibronectin, and also express specific integrins and other receptors on their surface that interact with these extracellular matrix components that are not present in the corresponding normal cells when they collide with invasive cells gliomas, which promotes the migration and invasion of tumor cells (Knott et al., 1998).

In addition, glioma cells can secrete their own ECM components, which normally are actively expressed only in the ECM of the developing nervous system along cell migration pathways (Bilozur & Hay, 1988). Fibronectin and HA increase the mobility of glioma cells and increase their invasiveness. High-grade glioma cells synthesize hyaluronic acid at the level of its synthesis in the central nervous system in the embryonic period (Delpech et al., 1993).

Researchers offer a variety of materials to support the proliferation and differentiation of neural cells. These materials can also serve as a temporary extracellular matrix after transplantation. For example, silk fibroin coated with laminin promotes neural progenitor cell proliferation, differentiation, and survival (Li et al., 2019).

The incorporation of hyaluronic gel into the damaged area of the rat cerebral cortex inhibits the formation of the glial scar, but does not support axonal growth. The same gels, but modified with immobilized RGD peptides, lack this disadvantage (Cui, Tian, Hou, Xu, & Lee, 2006). Hyaluronic acid and PEG composite hydrogel functionalized with RGD support the viability and physiology of primary GBM cell lines (Xiao et al., 2018).

Polysaccharides inhibit the formation of glial scar in the post-traumatic period and interfere with the adhesion of connective tissue cells, but do not stimulate effective neurite growth by themselves. In this regard, carbohydrate hydrogels modified by the addition of collagen, fibronectin (Prang et al., 2006), laminin (Dhoot, Tobias, Fischer, & Wheatley, 2004), as well as specific amino acid signal sequences such as RGD tripeptide and PHSRN (proline histidine-serine-arginine-asparagine) (Nakaoka, Hirano, Mooney, Tsuchiya, & Matsuoka, 2013). Another modification option is the addition of poly-L-lysine and antibodies to the nogo-66 receptor to the material (Wei et al., 2010).

5. Conclusions

In this paper, we showed that modified pectins are able to support cell viability, but slow down cell proliferation, which looks very promising for local tumor treatment. Recently a new concept was proposed based on the use of hydrogels with various properties in the sequential stages of postoperative glioblastoma therapy. In the first stages of postoperative therapy, carbohydrate hydrogels are required to inhibit the proliferation and migration of remaining cancer cells. At subsequent stages composite hydrogels based on proteins and carbohydrates can be used to stimulate differentiation and regeneration of neural tissue. However, the exact composition and ratio of components requires further investigation (Belousov et al., 2019). We determined the optimal ratio of the components in composite hydrogels based on modified pectins and extracellular matrix proteins for creating three-dimensional cell cultivation systems. We showed that neither collagen I nor collagen IV alone, when individually introduced in hydrogels based on modified pectins, can effectively stimulate the formation of

processes by glioma C6 cells. It was first established that the ratio of collagen type I and IV in bio-artificial matrices based on composite hydrogels greatly influences the formation of processes of neural cells. These results are useful for creating materials applicable in postoperative anti-cancer therapy for gliomas, as well as for creating three-dimensional models of tumor growth.

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