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Alginate-based hydrogel promotes neuronal survival and axon outgrowth of neuron-like cells

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Abstract

Alginate is a natural polymer preferred for biotechnological applications due to its properties, such as biocompatibility, biodegradability, and low toxicity. However, neurons do not possess surface molecules interacting with alginate; therefore, alginate-based materials have limitations for neurodegenerative applications. Thus, increasing neuronal survival and promoting axonal outgrowth in the alginate-based hydrogels are the primary purposes of this study. We also aim to study the performance of alginate extracted from bioresources to that of commercial alginate. Cell-embedded alginate-based hydrogels were formed with $CaCl_2$ and either mixed with collagen type I or supplemented with differentiation protocols such as the addition of NGF or FGF and serum withdrawal and retinoic acid (RA). Cells were observed by fluorescence imaging with acridine orange and propidium iodide, and upon dissolving the hydrogel with EDTA, cells were counted with trypan blue staining. In this study, commercial alginate and alginate extracted from seaweed were compared for their performance and were found to be comparable. We determined that adding collagen to the alginate hydrogel increased neuronal survival but not axon outgrowth. NSC-34 cell differentiation with NGF and FGF was successful in both commercial and extracted alginate, with both growth factors increasing neural survival and axonal outgrowth, despite the clustering of cells immediately after treatment. However, the SH-SY5Y differentiation protocol using serum withdrawal and RA treatment did not yield good results. Both extracted and commercial alginates showed comparable performance in terms of neuronal survival in our study, which was further increased upon collagen addition. We also showed that NGF and FGF differentiation protocol in alginate hydrogels resulted in successful axon outgrowth in NSC-34 cells.

Keywords: Alginate, Nerve growth factor (NGF), Fibroblast growth factor (FGF), hydrogel, NSC-34 motor-like neuron cell line, SH-SY5Y human neuroblastoma cell line

1. Introduction

The cells' natural environment is three-dimensional (3D); thus, mimicking the cellular environment is vital to understanding cellular behavior in nature. For example, 3D biomaterials are commonly used to simulate the extracellular matrix (ECM) (Frantz et al., 2010; Kular et al., 2014; Ravi et al., 2015). Each cell has a different ECM requirement; therefore, mimicking the environment is vital for each cell type. Thus, common ECM properties, such as a meshwork fibrous structure, are preferred. Hydrogels are among the most preferred biomaterials to mimic the cellular microenvironment (1,2) and can be obtained from synthetic (poly-(acrylic acid) (PAA), poly (ethylene glycol) (PEG)), or natural polymers (collagen, chitosan, alginate, hyaluronic acid) (1,3,4). Instead of synthetic polymers, natural polymers are preferred for biomedical applications due to their biocompatibility, low toxicity, and biodegradability properties (1,3,4).

Alginate is a natural polymer found in the cytoplasm of brown seaweeds to provide mechanical strength or flexibility to the algae (5,6). Alginate is preferably used in biotechnological applications due to its biodegradability, biocompatibility, and nontoxicity properties. Mainly, alginate is composed of two monomers: (1-4)-linked β-D-mannuronate (mannuronic acid, M) and L-guluronate (guluronic acid, G); however, the specific composition and ratio of these monomers in alginate vary depending on the algae type and harvesting time. The ratio of M residues to G residues determines the characteristics of the hydrogel due to crosslinking capacity (7,8). Alginate forms hydrogel if the environment has divalent cations, such as Ca^{2+}, $Sr^{2+,}$ or Ba^{2+}; primarily, Ca^{2+} is used to obtain alginate hydrogels in biotechnological applications (1,6,9). If the M/G ratio exceeds 1, the alginate hydrogel becomes more flexible, and vice versa. Dissolving the alginate hydrogels is possible by breaking the crosslinking; therefore, a calcium chelator provides the dissolution (5,10).

Besides several advantages of alginate, one disadvantage for neurobiology is that it is not recognized by neuronal cells

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due to a lack of receptors; thus, the hydrogel modifications are necessary for alginate to be used for neuronal model cells as a scaffold material (5,9). In this article, alginate extracted from *Cystoseira barbata* was mixed with collagen type I to increase the attachment of neuronal model cells to the hydrogel, and differentiation techniques were applied to create a 3D neuronal culture to increase cell survival and supportive cell axonal outgrowth. In addition, in the literature, two cell lines (NSC-34 and SH-SY5Y) were not studied with alginate material. Thus, this study investigated the comparison of extracted alginate to commercially available alginate in terms of alginate recognition improvement with neuron-like cells.

2. Materials and Methods

2.1. Alginate Solution Preparation

Alginate was extracted from *Cystoseira barbata* collected from Izmit Korfezi, Turkey, and lyophilized as described before (11). The lyophilized alginate extract and commercial alginate (Sigma, 9005-38-5) powders were dissolved in the distilled water at 500 rpm at 40°C for 30 min. The stock solutions were prepared using 0.9% NaCl (w/v) and 4.5% alginate (w/v) in distilled water. Also, 0.1 M $CaCl_2$ was prepared with distilled water to obtain alginate gel formation.

2.2. Sterilization Procedure

Alginate solutions and $CaCl_2$ were autoclaved at 121°C and 20 min to eliminate the contamination risk for cellular experiments. After autoclaving, the solutions were filtered under the Class II Laminar flow cabinet with a 0.45 µm syringe filter. After the sterilization procedure, the solutions were stored at 4°C for up to one week.

2.3. Hydrogel Preparation

Due to the alginate content, Ca^{2+} crosslinks with alginate and alginate solution turn the hydrogel form. For cellular and characterization experiments, the concentrations of alginate solutions were set to 1% with high glucose with 4.5 g/L D-Glucose, L-Glutamine, and sodium pyruvate Dulbecco's Modified Eagle Medium (DMEM) (Gibco) including 10% Fetal Bovine Serum (Gibco) and 1% penicillin-streptomycin (Gibco). The hydrogel was formed with the addition of $CaCl_2$, and the mixture was incubated for 10 minutes at room temperature. The alginate hydrogel formed from extracted alginate and the hydrogel formed from commercially available alginate (Sigma, 9005-38-5) are presented in Fig. 1a and 1b, respectively.

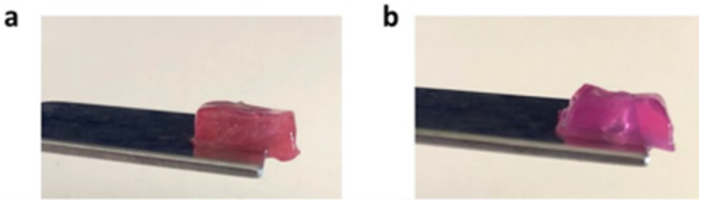

Fig. 1. Alginate hydrogels (1% w/w)prepared with DMEM and 1M $CaCl_2$. a. formed by using alginate extracted from *Cystoseira barbata*, b. formed by using commercial alginate

2.4. Cell Culture

NSC-34 (motor-like neuron cell line) and SH-SY5Y (human neuroblastoma cell line) were incubated for cell culture experiments at 5% CO_2 and 37°C. NSC-34 cells were grown in the DMEM high glucose without pyruvate with 10% FBS and 1% penicillin/streptomycin (Pen/Strep), and SH-SY5Y cells were seeded on DMEM high glucose with pyruvate, supplemented with 10% FBS and 1% Pen/Strep. When cells reached 80 % confluency, the existing medium was discarded, and the cells were collected with trypsin/EDTA (Gibco). Before centrifugation at 1500 rpm for 5 min, the cells were counted with Haemocytometer (Marienfield). The cell pellet was dissolved with the appropriate amount of DMEM to obtain $1x10^6$ cells/mL. The stock alginate solution (4.5% w/v) was diluted to 1% (w/v) with DMEM and cell solution (50,000 cell/well for NSC-34 and 75,000 cells/well for SH-SY5Y for 96-well plate). The prepared alginate/DMEM/cell solution was seeded onto the 96-well plate.

The indicated amount of the growth factors or the DMEM without FBS was applied during the dilution of the alginate solution and cell pellet dissolving as a differentiation medium for neuronal differentiation. The differentiation medium for NSC-34 contained 50 ng/mL nerve growth factor (NGF) or 10 ng/mL fibroblast growth factor (FGF). For SH-SY5Y, the differentiation medium contained retinoic acid (RA, Sigma R2625, final concentration at 10 µM) in DMEM with 1 % FBS. After cells reached 80% confluency, the cells were collected and seeded onto the flask and treated with DMEM with 1% FBS and 1% Pen/Strep for 24 h. Embedding the cells with alginate was achieved after 24h serum withdrawal, the cells were collected, and the cell pellet was dissolved with DMEM, including 1% FBS, 1% pen/strep, and 10 µM RA. The cell solution was blended with alginate, and this alginate/cell/differentiation medium solution was seeded onto the 96-well plates. The hydrogel form was obtained with the same procedure as described previously. Cell-embedded hydrogels were incubated at 37°C with 5% CO_2, and the differentiation medium was refreshed every two days for a 7-day treatment. The schematic representation of the cell embedding into the alginate hydrogel is shown in Fig. 2.

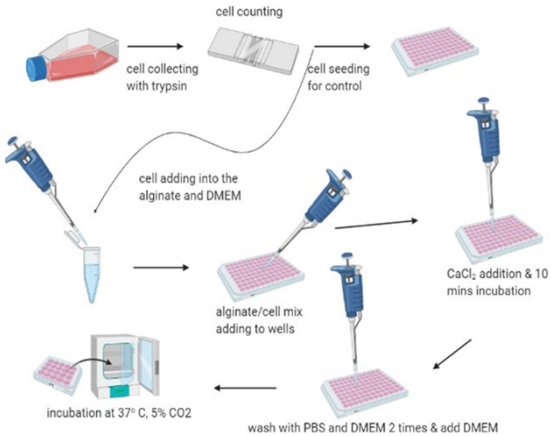

Fig. 2. The schematic representation of cell embedding into the alginate hydrogels

2.5. Fluorescence Staining

5 mg/mL Acridine Orange (AO) (Sigma, A6014) and 3 mg/mL propidium iodide (PI) (Sigma, P4170) were used to identify living cells and dead cells, respectively. The staining solution was prepared with 1x DPBS, and cells were treated with this solution for 15 min; after incubation, the existing solution was discarded, and the hydrogels were washed with 1x DPBS twice. Hydrogels were imaged under ZOE fluorescent cell imager with appropriate excitation/emission wavelength.

2.6. Dissolving Alginate and Cell Counting

Due to the Ca^{2+} crosslinking, the alginate hydrogel was dissolved with 0.5 M ethylenediaminetetraacetic acid (EDTA). After adding EDTA into the hydrogels, the solution was stained with trypan blue when the hydrogels dissolved completely. Trypan blue staining was applied to count the viable and dead cells via Haemocytometer after 5 min incubation at room temperature. While counting the cells, colored cells indicated dead cells, and the others represented living cells. The calculations were achieved with the percentage of the ratio of dead cell numbers over the number of live cells. A two-way ANOVA test was applied for both cell lines, with Sidak multiple comparison methods for comparing commercial and extracted alginate samples with a confidence interval of 95%.

2.7. Collagen/alginate Hydrogel Preparation

The initial concentration of collagen I, Rat Tail (Gibco), was 3mg/mL, and the working concentration of the collagen solution was diluted with 0.02 M acetic acid, 1x DPBS, with controlled pH of 1 mg/mL. Then, the collagen and alginate concentrations and ratios were adjusted using an appropriate amount of DMEM and cell solution.

The cell solution was added to the collagen/alginate mixture, and 100 µL of 50,000 cells (NSC-34) or 75,000 cells (SH-SY5Y) was seeded onto the 96-well plate for each well. To obtain collagen gelation, the seeded cell solutions were incubated at 37°C with 5% CO_2 for 1 hour to obtain collagen gelation. After incubation, 0.1 M $CaCl_2$ was added to the mixture, and 10 min incubation at room temperature was applied for gelation, and the hydrogels were washed with DPBS twice to reduce $CaCl_2$ existence. Finally, the plate was incubated under the same conditions with cell growth conditions after adding DMEM to the hydrogels.

3. Results

Fluorescence images were taken from cell-embedded alginate (extracted and commercial) hydrogels with acridine orange and propidium iodide stains. The staining results for NSC-34 motor neuron cells showed that cell viability decreased on Day 4 of embedding in hydrogels with both extracted and commercial alginate (Fig. 3a). In the case of SH-SY5Y cells, the number of the clustered cells was lower than that of NSC-34 cells, while cell survival was supported until Day 4 of embedding in both extracted and commercial alginate hydrogels, similar to the case in NSC-34 cells (Fig. 3b).

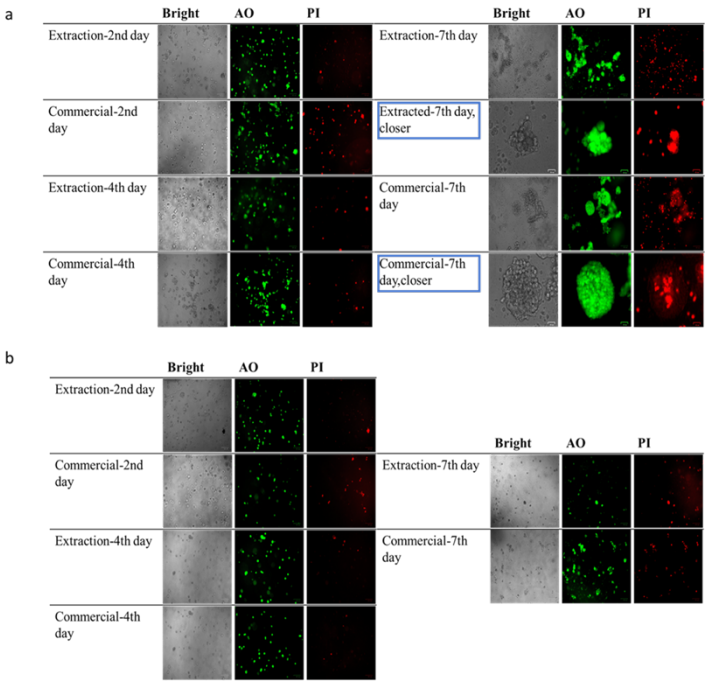

Fig. 3. Fluorescence imaging of a. NSC-34 and b. SY-SY5Y cells taken on the 2nd, 4th, and 7th days after embedding of either extracted (extraction) or commercial alginate hydrogels. Scale: 100 µm, for closer to 25 µm. Bright: brightfield; Green: Acridine orange (AO); Red: Propidium iodide (PI)

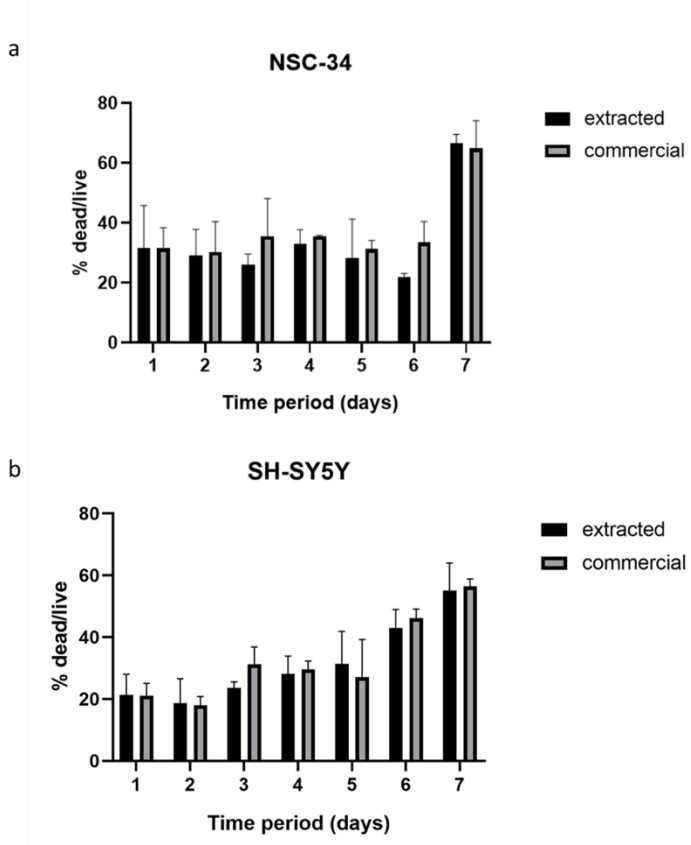

Fig. 4. The percentages of the ratio of dead cells to the living cells a) NSC-34 cells and b) SH-SY5Y for 7 days. Error bars indicate the standard deviation of 3 biological replicates

The live and dead cells were counted as described in Materials and Methods, and reported as a percentage of cell death for both NSC-34 and SH-SY5Y cells embedded in alginate hydrogels (Fig. 4). We observed that both hydrogels

exhibited similar performance in terms of supporting cell survival: While cell death appears to sharply increase after Day 6 for NSC-34 cells, SH-SY5Y cell death appeared to show a gradual increase (Fig. 4). Statistical analysis of the quantitative results indicated no significant differences between extracted alginate and commercial alginate results for both cell lines, ensuring the tissue culture applicability of the alginate extraction procedure.

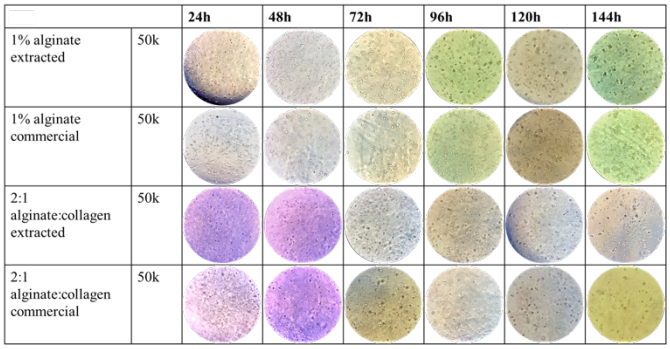

Fig. 5. The appearance of cell-embedded alginate hydrogels (%1 extracted alginate, %1 commercial alginate, extracted alginate:collagen (2:1), commercial alginate:collagen (2:1) under light microscopy during 6-days to identify the effect of collagen on cell survival and axonal outgrowth for NSC-34 cell lines. k: 1000 cells / well.

Cell-embedded alginate hydrogels were further observed under light microscopy during a 6-day period to identify the effect of collagen on cell survival and axonal outgrowth for both NSC-34 (Fig. 5) and SH-SY5Y (Fig. 6) cell lines. Alginate/collagen mixture at the ratio of 2:1 was used to embed the cells with the cell density of 50,000 cells per well in the 96-well plate. The cellular survival period was found to extend with the addition of collagen, decreasing cellular migration compared with only alginate hydrogels. Under these conditions, no significant axon outgrowth of the NSC-34 cells was observed either in alginate or alginate/collagen hydrogels (Fig. 5).

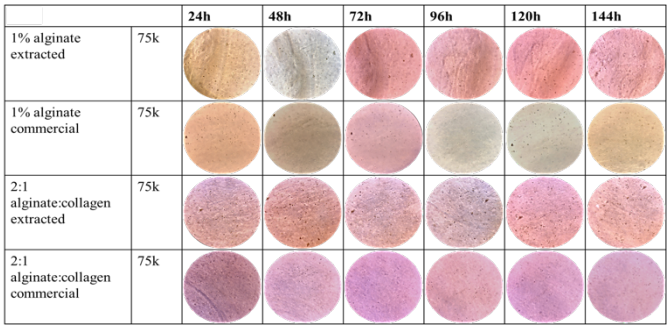

Fig. 6. Collagen mix with 2:1 ratio (alginate/collagen), SH-SY5Y cells embedded with alginate and alginate/collagen hydrogels. k: 1000 cells / well

Similarly, SH-SY5Y cells were embedded in the alginate (extracted and commercial) hydrogels with alginate/collagen hydrogels with a 75,000-cell density per well. Cell survival did not appear to be significantly enhanced in SH-SY5Y cells upon collagen addition. Therefore, no cellular proliferation or axonal outgrowth was observed during the 6-day period (Fig. 6).

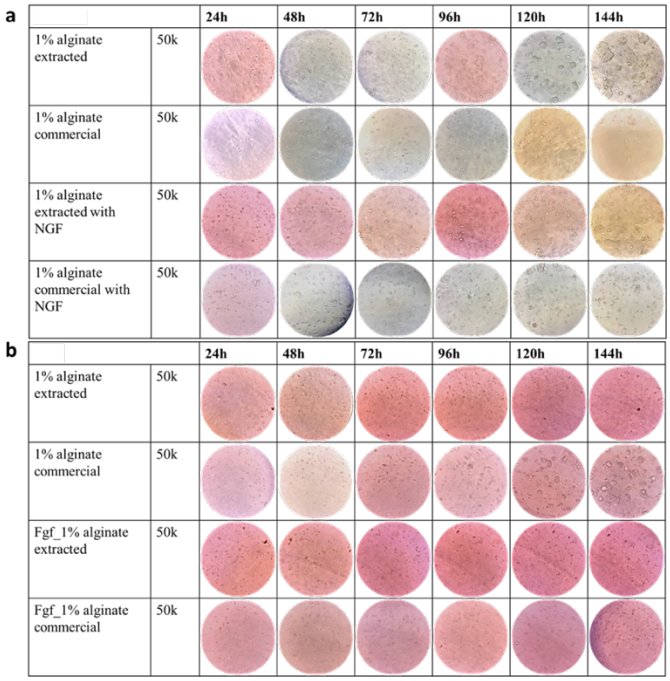

Fig. 7. NSC-34 cells embedded with & without included a. NGF b. FGF included medium. k: 1000 cells / well

Next, the effect of differentiation protocols on axon outgrowth in these hydrogels was studied. We initially treated NSC-34 cells with 10 ng/mL FGF or 50 ng/mL NGF to induce differentiation. Neuronal differentiation, as measured by axon outgrowth, was observed after Day 4 of embedding in FGF-treated cells (Fig. 7a), while NGF-treated cells in the hydrogel started to differentiate after the 3rd day (Fig. 7b). In addition, cells were observed to form cellular clusters earlier (about the 2nd or 3rd day) compared to non-NGF-treated hydrogels; moreover, cellular survival increased with NGF treatment.

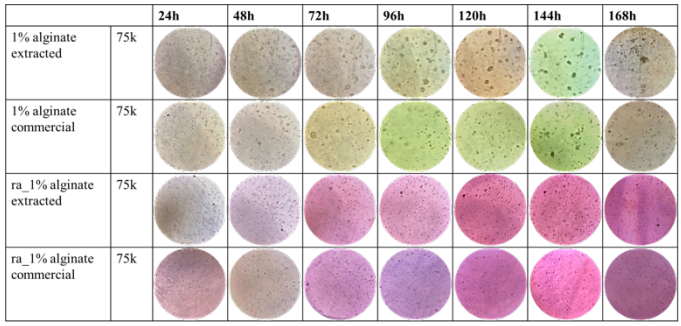

Fig. 8. SH-SY5Y cells were embedded in hydrogels, including RA, for 7 days. The medium exchanges were similar to usual embedding methods with medium, including RA. The scale bar of images is 450 µm. k: 1000 cells / well

The differentiation protocol used for SH-SY5Y cells included overnight serum withdrawal followed by RA treatment (12); however, neither in 3D nor in 2D conditions, the differentiation did not yield reliable axon outgrowth in SH-SY5Y cells (Fig. 8).

4. Discussion

The previously reported chemical and physical characterization (11), as well as the cellular results presented in this study, showed that the extracted alginate from *Cystoseria barbata* collected from Tuzla, Istanbul, Turkey, had

similar properties to commercially available alginate as a relatively cost-effective and locally produced alginate source.

The cell studies on the alginate hydrogels using two different neuronal model cells showed the applicability of alginate hydrogels and hydrogels of alginate/collagen mixtures, which were suitable for neuronal cell survival. It should be noted that fluorescence images of NSC-34 cells in this study also indicated that the cells migrate to each other to form a spheroid-like structure; this could be due to paracrine signaling to enhance survival (13). The cellular morphology of both NSC-34 and SH-SY5Y cells remained spherical after embedding in the hydrogels, which could be due to the lack of surface molecules that recognize and bind alginate (5), in turn lowering the cellular attachment to alginate hydrogel. Cellular proliferation or axonal outgrowth was not observed for either cell line in the alginate hydrogels alone.

Due to the low attachment of cells to alginate, we next mixed alginate with collagen in order to provide an extracellular matrix-like microenvironment to the cells. The results indicated that cellular survival was increased with the addition of collagen. More detailed work is necessary to determine optimum alginate mixtures suitable for different types of neuronal models.

We also studied the effect of differentiation protocols on axon outgrowth in hydrogels. NGF or FGF treatment of NSC-34 cells showed axon outgrowth in cells, indicating the hydrogels prepared in this study supported neuronal differentiation; however, the SH-S5Y5 differentiation protocol did not yield any axon outgrowth. Growth factor supplementation in neuronal differentiation protocol may be better suited in alginate-based hydrogels. The cellular clusters observed in the early periods of differentiation protocol can be due to the cells' need to maximize cell-to-cell communication and signaling as a means for cellular survival and differentiation (13,14).

This study aimed to create a 3D alginate-based environment that provides support for both neuronal survival and axonal outgrowth, using two different model cells, namely NSC-34 and SH-SY5Y cell lines. Our study showed that the collagen addition to the alginate hydrogel increased the cellular survival for both cell lines. Different ECM mimic molecules can be mixed with alginates in order to obtain optimum hydrogels for different neuronal cell types. We have also shown that neuronal differentiation of NSC-34 cells was achieved in alginate hydrogels supplemented with FGF and NGF, indicating alginate-based hydrogels can support neuronal differentiation, although optimum differentiation protocols need to be determined for different neuronal model cells and different alginate-hydrogel mixtures. In this study, we have also shown the biocompatibility of alginate extracted from algae in the Tuzla province of Türkiye as a local and cost-effective alternative bioresource.

Ethical Statement

Ethic commitee approval is not required for this study.

Conflict of interest

The authors declared no conflict of interest.

Funding

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Authors' contributions

Concept: B.D., I.K, M.I.H., Design: B.D., E.S., I.K, H.K., H.S., Data Collection or Processing: B.D., Analysis or Interpretation: B. D., Literature Search: B.D., Writing: B.D., I.K.

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