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Characterization of a 3D Neuronal-Culture Using Alginate Hydrogels and Optimize For Neuronal Survival and Axon Growth

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Abstract

Alginate is a polysaccharide that found in the brown algae, the main function of the alginate in the algae is structure-forming like strength and flexibility. Alginate has wide range of uses in textile, food, pharmaceutical, and biomedical industries. It is preferred because of its properties such as biocompatibility, nontoxicity, biodegradability. Alginate chemical properties can be varied by collection time, weather, and the type of algae.

Alginate can be used as a hydrogel because of cross-linking capability with divalent cations. There are two main residues of alginate, mannuronic acid (M) and guluronic acid (G). The ratio of M/G is one of the most important features, if M/G is bigger than 1, hydrogel would be more elastic and if G residues are more than M residues, the hydrogel is become stiffer.

The algae in this study are collected from Tuzla seaside, Istanbul, and alginate extraction is performed. The extracted alginate is used for 3D cell culture. Characterization of the alginate is essential before making it hydrogel by using divalent cations. Before cellular experiments the chemical analysis,

purity test, and viscosity of alginate are checked by using Fourier-transform infrared spectroscopy (FTIR), fluorescence spectroscopy, and viscometer. Extracted alginates M/G ratio is found 0.6, there is no fluorescence is observed and the viscosity is 29 mPas. All these results are compared with alginic acid, sodium salt (SigmaAldrich, 180947), and the results are similar with each other. For cellular experiments, the alginic acid, sodium salt (SigmaAldrich, 180947), and extracted alginate are used.

Alginate can be used as a 3D environment for cells, but the neuronal model cells used in this study did not exhibit differentiation, which may be due to these cells not having integrins that recognize and bind the alginate. To improve cellular growth and differentiation in the alginate hydrogel, extracellular matrix (ECM) proteins can be used.

Motor neuron cells (NSC-34) are used for cell encapsulation, the alginate concentration is optimized 1% after trying the concentrations of 0.5%, 1%, 2.2%, 2.5%. Cells are imaged every 24h and after 72nd hour the cells undergo necrosis, and there is observed cellular growth, barely. Non-growth is one of the expected results. To extend the lifetime and the growth of neurons, the collagen is added but the optimization step continues.

The expectations for these experiments are to create an environment for neurons and optimize the alginate hydrogel and collagen. These alginate and alginate-collagen platform can be used for implantation after optimizations and the physical properties are improved.

Keywords: Alginate, Hydrogel, 3D cell culture

1. INTRODUCTION

Alginate is a polysaccharide found in the brown algae, and the function in the algae is structure-forming. Alginate is located in the intracellular space and the cell wall.[1] Alginate can be used in different sectors, such as pharmaceutical, textile, food, etc. Alginate is biocompatible and biodegradable, these properties make alginate favorable material in biomedicine and biotechnology like wound dressing, gastric reflux, cell carrier, cell therapy, drug delivery.[2]

Alginate is composed of 2 residues, 1-4 linked β-D-mannuronic acid (M), and α-L-guluronic acid (G), also these residues can be located homogenously or heterogeneously. Alginate composition is directly affected by seasonal and growth conditions of the algae.[3] Alginate that collected from bacteria has 100% mannuronic acid blocks.[3] If alginate has more G-residue, the gelation property becomes stiffer. When M-residue is more than G-residue, the hydrogel is more elastic.[4]

[1] Inger-Lill Andresen et al., "Some Biological Functions of Matrix Components in Benthic Algae in Relation to Their Chemistry and the Composition of Seawater," *ACS Symposium Series Cellulose Chemistry and Technology*, (1977), p. 361.
[2] Jerome P. Paques, "Alginate Nanospheres Prepared by Internal or External Gelation with Nanoparticles," *Microencapsulation and Microspheres for Food Applications*, 2015, p. 39. Biopolymers Online, (2002), p. 811.
[3] Kurt Ingar Draget, Olav Smidsrød, and Gudmund Skjåk-Bræk, "Alginates from Algae," *Biopolymers Online*, (2002), p. 811.
[4] Paques, Alginate Nanospheres, p. 43

When alginate is obtained from nature, some impurities can be found in the alginate like endotoxins, phenolic compounds, proteins.[1,2,3] These impurities can be problem for pharmaceutical or biotechnological application, so it is important to removed impurities from alginate.[5]

Alginate can cross-link easily when divalent cations are found in the environment, depending on the divalent cation and density of ion the gelation quality may be changeable.[6,4]

Cross-linking is occurred between guluronic acid and the divalent ions, this structure is called egg-box. Neurons are lack of attachment part to alginate, to improve attachment extracellular matrix (ECM) proteins can be used to modify the alginate hydrogels.[5] Alginate hydrogel is a good tool to mimic brain environment even though there is no integrin to recognize.

The aim of this project was to characterize the alginate extracted from *Cytoseira barbata* that was collected from Tuzla, Istanbul seaside and compare extracted alginate with commercial alginate for cellular experiments by using neuronal cell line.

2. MATERIALS AND METHODS

Alginate Characterization

Alginate's chemical analysis is vital before starting the cellular experiments since different chemical properties can vary the alginate hydrogel characterization. The extraction method and the type of algae that extracted alginate can be affected by alginate properties. Characterizing the alginate and finding the appropriate commercial alginate is one of the essential parts of this study. Besides the chemical analysis, purity is necessary to ensure the non-toxic effect of the alginate. The alginate's viscosity can affect the hydrogel properties, so the alginate's viscosity was controlled.

Alginate chemical characterization was done using Fourier Transform Raman Spectroscopy; for a purity test, fluorescence spectrometer was used.

The aim was to find the commercial alginate with similar properties with the extracted alginate and compare them in terms of the cellular experiment (Figure 1a). The commercial alginate was procured from the Institute of Nanotechnology at Gebze Technical University, alginic acid, sodium salt SigmaAldrich, 180947 (Figure 1b).

[1] Anu Shilpa, S. S. Agrawal, and Alok R. Ray, "Controlled Delivery of Drugs from Alginate Matrix," *Journal of Macromolecular Science, Part C: Polymer Reviews* 43/2 (2003), p. 189.
[2] Paques, Alginate Nanospheres, p. 41
[3] M.l Hans and A.m Lowman, "Biodegradable Nanoparticles for Drug Delivery and Targeting," *Current Opinion in Solid State and Materials Science* 6/4 (2002), p.319.
[4] Draget, Smidsrød, Skjåk-Bræk, Alginates from, p.818
[1] Marina Matyash et al., "Novel Soft Alginate Hydrogel Strongly Supports Neurite Growth and Protects Neurons Against Oxidative Stress," *Tissue Engineering Part A* 18/1-2 (2012), p. 56.

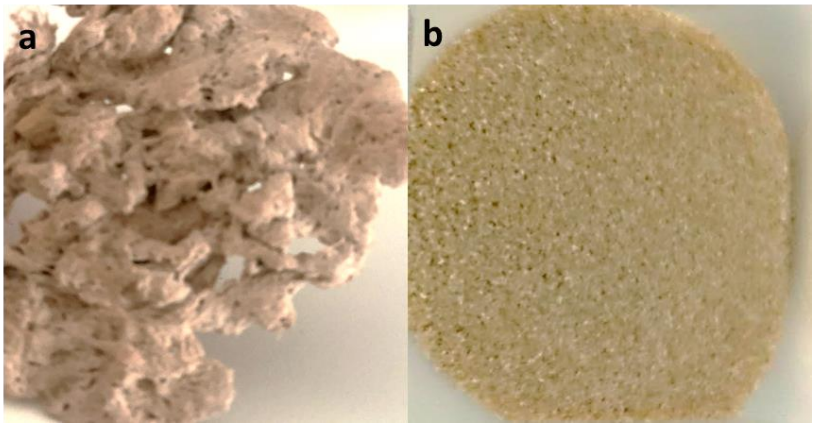

Figure 1. Sodium alginate a. extracted and b. commercial, SigmaAldirich 180947.

The cell experiments were done with mouse motor neuron cell line NSC-34. The cells were maintained in the pyruvate free medium, Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine and 4.5g/L Glucose, without Pyruvate, Gibco 2007829, with 10% FBS, Fetal Bovine Serum, Gibco 10270, and 1% Penicillin, Streptomycin, PAN Biotech P06-07100. The medium in the flask where the cells are located was removed, and the flask was washed with DPBS, Dulbecco's Phosphate Buffered Saline 1X Gibco 14190-94 to remove all medium. After PBS, cells were detached by 0.05% Trypsin/EDTA, Gibco 25300-54. When the detachment was done trypsin, the cells were diluted with DMEM by the ratio of 1:5 and centrifuged at 1,500 rpm for 5 minutes. The supernatant was discarded, and the medium was adding to dissolve the pellet. The medium amount is depending on the cell number and cell seeding density. After cells were ready to encapsulate, they were mixed with alginate.

$CaCl_2$, calcium chloride, 96%, extra pure, powder, anhydrous, Acros Organics 10043-52-4, was prepared for 0.1 M, and alginate solutions were designed with 0.9% NaCl, sodium chloride, Merck 7647-14-5, and 4.5% alginate, on the magnetic stir (Figure 2). The extracted alginate was dissolved at 40^0C, and commercial one was dissolved at room temperature for 30 minutes. The stock solution of alginates and $CaCl_2$ were autoclaved for 20 minutes at 121^0C. After autoclave, these solutions were stored at 4^0C. Before using solutions, 0.45 µm syringe filters were used to filter. Every experimental set up was set with new alginate solutions to prevent pigmentation. Alginate solutions were diluted by using the appropriate medium and the cell amount. The critical part for dilution is to calculate the correct amount considering the cell solution.

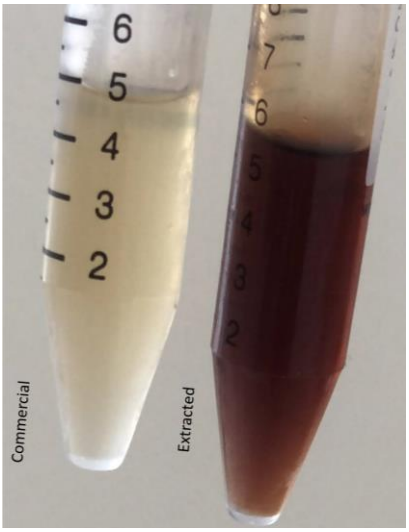

Figure 2. Extracted and commercial alginate solutions, the left falcon has a commercial alginate solution, and the left one is extracted alginate solution. The concentrations of the solutions are the same, and 4.5% alginate in 0.9% NaCl solution.

Cellular Experimental Setup

After cells are collected and mixed with an alginate solution to the desired concentration, for this study, the alginate concentrations were 1%, 2.2%, and 2.5%. The cell-alginate solution is added into the $CaCl_2$ as quickly as possible. This step should be quick because when the alginate contacts with the Ca^{2+} ion, the cross-linking starts. After 10 minutes of incubation at room temperature, the $CaCl_2$ is removed, and the gel is washed two times with PBS, then adding DMEM onto the alginate hydrogel. Removing the $CaCl_2$ is essential for this step. The hydrogels are incubated at 37^0C with 5% CO_2 (Figure 3). The medium was changed every two days.

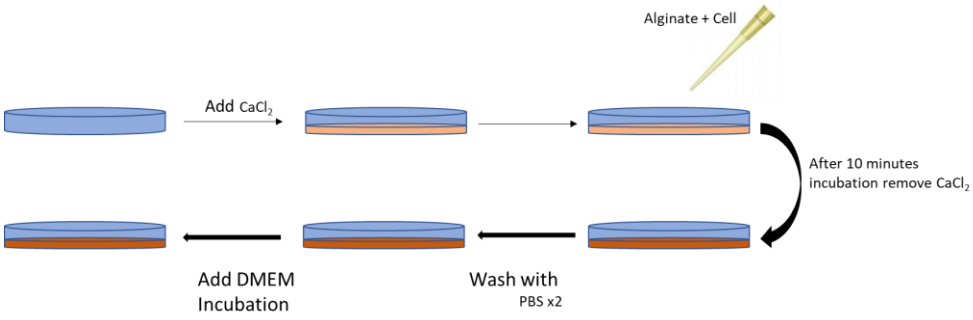

Figure 3. Experimental setup.

3. RESULTS

Chemical analysis/FT-RAMAN Spectroscopy

To analyze and calculate the ration between mannuronic acid and guluronic acid is the priority for this study, for that the FT-RAMAN was used. There are some binding specific wavenumbers such as 950 cm-1, 1000-1025 cm-1, 1200-1290 cm-1, and 1400 cm-1 for O-H to understand the FT-RAMAN results C-OH, C-O-C, and CH_2, respectively.[1] In the literature, around 1025 cm^{-1} and 1100 cm^{-1} are specified for guluronic acid and mannuronic acid, respectively.

The samples were put into the spectrometer chamber, and the results were analyzed using EXCEL, and the graph was plotted by ORIGIN PRO (Figure 4). After plotting the extracted and commercial alginates wavenumbers versus %Transmission, the areas under the curve for 1025 and 1100 cm^{-1} were calculated, and the M/G ratio for extracted alginate was found to 0.6. This ratio for commercial alginate was 0.71. These numbers are coherent with not only each other but also literature value.[2] Also, the extracted and commercial alginates have guluronic acid-rich properties.

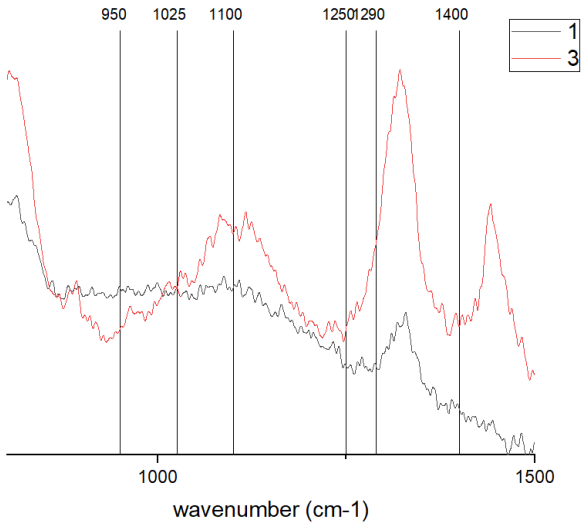

Figure 4. FT-Raman spectroscopy results, 3 on the graph (red line) represents commercial, and 1 on graph (black line) is for extracted alginate.

[1] Leonel Pereira et al., "Use of FTIR, FT-Raman and 13C-NMR Spectroscopy for Identification of Some Seaweed Phycocolloids," *Biomolecular Engineering* 20/4-6 (2003), p.226.
[2] Trica et al., "Extraction and Characterization of Alginate from an Edible Brown Seaweed (*Cystoseira Barbata*) Harvested in the Romanian Black Sea," *Marine Drugs* 17/7 (2019), p.5.

Purity Control-Fluorescence Spectroscopy

Alginate has phenolic compounds due to its growth area.[1,2] These phenolic compounds can have toxic effects, understand how toxic extracted alginate is, and ensure its purity before experiments fluorescence spectroscopy was used. The excitation wavelength was set to 320 nm; due to extracted alginate pigmentation, the solution's concentration was decreased by ten-fold. The results show that extracted alginate and commercial alginate have ignorable emission at 320 nm excitation wavenumber (Figure 5).

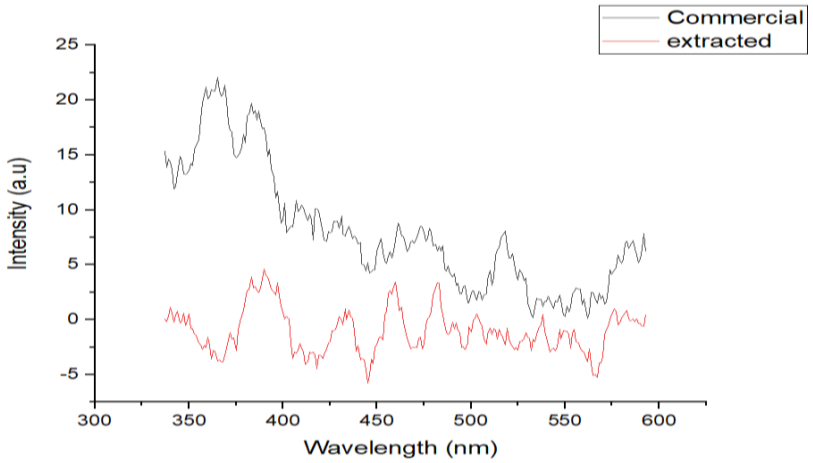

Figure 5. Fluorescence spectroscopy, excitation wavelength set to 320 nm. The red line represents extracted alginate, and the black line is for commercial.

Cellular Experiments

Cells were imaged for five days; after the fourth day, the cells started to undergo cellular debris in the alginate hydrogel. These results were the same as extracted and commercial alginate (Figure 6). Depending on alginate concentration, cellular behavior showed differences. On the third day, some cellular movements were observed in both 1% extracted and alginate hydrogels. After observations, it can be said that increasing the alginate concentration causes cellular debris earlier. Due to cellular behaviors, 1% alginate concentration was optimized for both extracted and commercial alginate.

[1] Sabrine Sellimi et al., "Structural, Physicochemical and Antioxidant Properties of Sodium Alginate Isolated from a Tunisian Brown Seaweed," *International Journal of Biological Macromolecules* 72, (2015), p. 1359
[2] Shilpa, Agrawal, Ray, Controlled Delivery, p. 189.

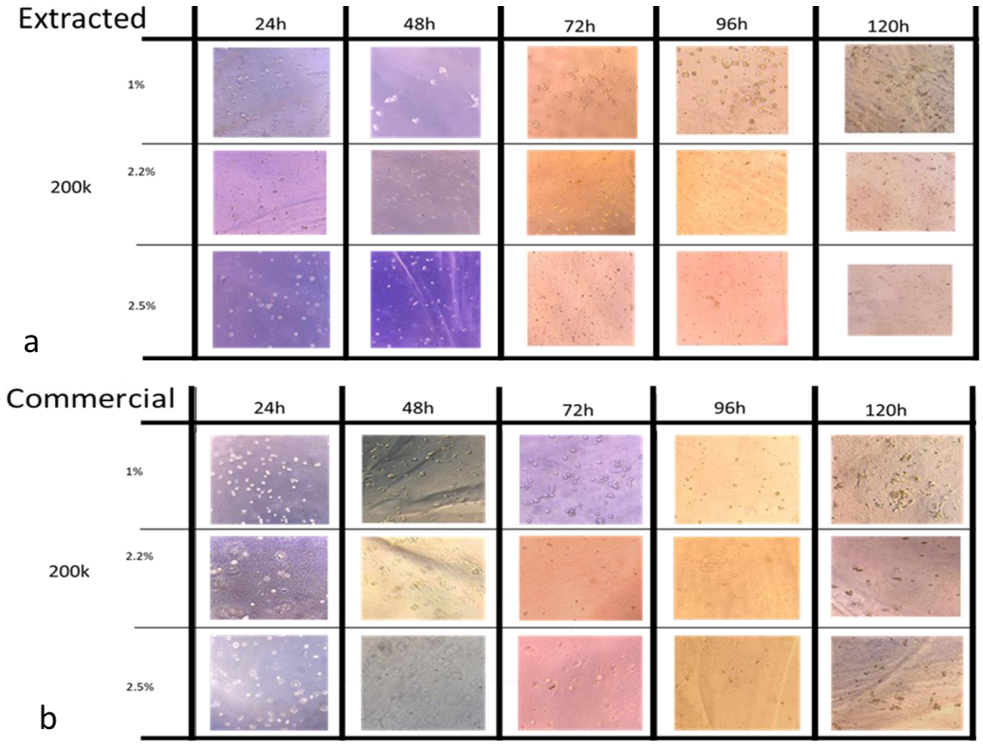

Figure 6. Cellular experiment results. a. extracted alginate b. commercial alginate.

During the experiments, it was observed some pigmentation problems with extracted alginate (Figure 7). The pigmentation increased with alginate concentration and with time.

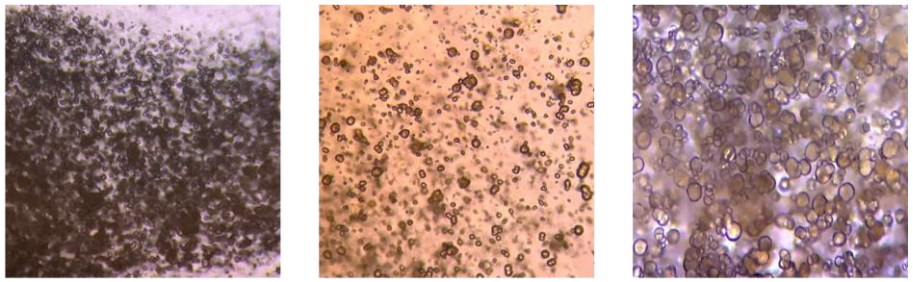

Figure 7. Extracted alginates pigmentation problems in 2.2% second day, 2.5% fourth day, and 2.2% forth day.

4. DISCUSSION

F-RAMAN spectroscopy results showed that extracted and alginate have the same properties considering the M/G ratio. Both are guluronic acid-rich, which means extracted and commercial alginate are stiffer hydrogel when they are cross-linked with divalent cations. The fluorescence emissions for extracted and commercial alginate were ignorable, so there are no significant impurities for these samples.

Cellular experiments showed that the alginate concentration is important for cellular behavior and increasing alginate concentration caused decreasing with cellular migration. Before starting the experiments, it was expected that 2.2% alginate concentration should provide better growth and differentiation for neuronal cell models than the other concentrations. But in the 2.2% alginate hydrogel especially with extracted alginate exhibited some pigmentation problems. To prevent the pigmentations, alginate can be further filtered by using 0.45 or 0.22 µm syringe filters; also, extraction method can be modified to decrease the pigmentation. $CaCl_2$ amount and the adding method can affect the hydrogels homogeneity, after trying different techniques and ratio, it can be said that 1:1.5 ratio for alginate solution: $CaCl_2$ ratio is better than other ratios. For bigger surface areas, adding the alginate solution onto $CaCl_2$ is better than reversed method.

Cell seeding density is a different essential part of this study because the layer structure of the hydrogel can cause some imaged difficulties. To find the cell and image it is harder when the cell density is less. Furthermore, when cells and seeded too sparsely, they tend to form clusters or migrate, most likely in an attempt to benefit from growth factors secreted by other cells in a paracrine manner. This can also benefit these model cells since they do not have the integrin subtypes to attach the alginate, further forcing them to cluster together.

Modifying alginate hydrogels with ECM proteins could improve cellular growth using extracted alginate hydrogel. Alginate-collagen mixtures were not found to improve neuronal growth and survival; however, addition of growth factors could mimic paracrine stimulation thus decrease the clustering of the cells, allowing for differentiation.

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