CREATING A 3D NEURONAL-CULTURE USING ALGINATE AND COLLAGEN HYDROGELS OPTIMAL FOR NEURONAL SURVIVAL AND AXON GROWTH

by

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B.S., in Bioengineering, Istanbul Technical University & Montana State University, 2017

Submitted to the Institute of Biomedical Engineering
in partial fulfillment of the requirements
for the degree of
Master of Science
in
Biomedical Engineering

Boğaziçi University
2021

ACKNOWLEDGMENTS

Writing a thesis is not an individual work for me, and I am grateful to everyone that I mentioned here. First of all, I would like to thank my co-advisor Prof. Dr. Işıl KURNAZ due to her acceptance of me as a student during my challenging days. If her guidance and help did not exist, today I cannot write these words. The written part of the thesis and all experiments were done in favor of her and her lab, AXANLAB. With Prof. Dr. N. Hale SAYBAŞILI's guidance and collaboration, I learnt a lot about researching and different points of view of life.

During the thesis experiments, it was an honor to meet with Assoc. Prof. İsrafil KÜÇÜK and I am grateful to him for his knowledge that shared with me, collaboration in every part of the study, and all helps to improve the results.

I owe Prof. Dr. Bünyemin COŞUT, Asst. Prof. Doğuş ÖZKAN, Mustafa Alper Yılmaz a debt of gratitude for their collaborations. Also, I would like to record my gratitude to Assoc. Prof. Müge İŞLETEN HOŞOĞLU and Habibe KURT for alginate extraction which was as a part of the OBEK project. Also, I would like to thank my evaluation committee, Assoc. Prof. Bora GARİPCAN and Assoc. Prof. Duygu EGE.

One of the biggest my gratitude is to AXANLAB members; Ekin SÖNMEZ, M.Doğukan METİNER, Yiğit Koray Babal, Büşra Nur ÇİÇEK, and Betül ERTURAL. I learned what I know about wet lab from AXANLAB members, especially Ekin SÖNMEZ was in every corner when I needed help. Her guidance was important for me and I am grateful to her.

Being a master's student was backbreaking for me, and these two years were harder if I would not have my friends; Sevde Büşra BAYRAK, Zeynep GÜNER, and Alamira HAJJAR. They were always supportive of me to live stronger after all challenging times. Also, I would like to thank Merve YÜCE for her help, it was really

essential for me.

During my life, my fellows Nur POLAT and Bengisu KILIÇ were everywhere at every time that I need them. I feel lucky to have them in my life. If their motivational speeches did not exist, probably I would not be here now. Also, I would like to thank my dearest friend Cansu BUDAK for her support to make me a Bogazici student.

My last thanks were to my father Sedat DALBAYRAK and my mother Zülay ÖZKAN for their patients and supports in every moment in my life, also my brother Burak Ekin DALBAYRAK was like a motivational speaker in my life. I cannot finish my words without thanking to my sister-in-law Nilay ADAK DALBAYRAK for being a part of my family and my little sweet niece İpek DALBAYRAK for her sweetness. Who I am today is because of my family, I am lucky to have them and thank them for everything.

ACADEMIC ETHICS AND INTEGRITY STATEMENT

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ABSTRACT

CREATING A 3D NEURONAL-CULTURE USING ALGINATE AND COLLAGEN HYDROGELS OPTIMAL FOR NEURONAL SURVIVAL AND AXON GROWTH

Alginate is a natural linear polymer that found in brown algae; also, it is biocompatible, biodegradable, and non-toxic. These properties make alginate favorable for biotechnological applications. In this thesis, 3D cell culture properties of extracted alginate from Cystoseira barbata was compared with commercially available alginic acid, sodium salt. Alginate hydrogels were used to create a three-dimensional neural cell culture to achieve survival and axonal outgrowth of two cell lines, mouse motor neuron (NSC-34) and neuroblastoma (SH-SY5Y). The cells were embedded with 1% (w/v) alginate hydrogels. Due to the lack of alginate receptors in these cells, cells in alginate tended to become together as aggregate. To decrease the clusters and increase cellular survival, the alginate was also mixed with collagen type I at a ratio of 2:1 alginate/collagen. In both cell lines, the alginate/collagen hydrogels did not significantly alter cell proliferation or axonal outgrowth when compared to only alginate hydrogels. In order to enhance axonal growth and survival in these hydrogels, NSC-34 cells were differentiated with nerve growth factor (NGF, 50 ng/mL) and fibroblast growth factor (FGF, 10 ng/mL), while SH-SY5Y cells were differentiated with serum withdrawal and retinoic acid (RA, 10 µM) treatment when seeding them in hydrogels. Survival of NSC-34 cells in hydrogels was improved with FGF and NGF treatment, and cellular clusters were decreased upon differentiation with FGF, and survival was found to increase also with NGF treatment. On the other hand, SH-SY5Y cells were not successfully differentiated within hydrogels upon serum withdrawal and retinoic acid treatment; the cells were observed to undergo stress after the addition of RA.

Keywords: Alginate, Hydrogel, Collagen, NGF, FGF, RA.

ÖZET

ALJİNAT VE KOLAJEN HİDROJELİ İLE 3B HÜCRE KÜLTÜRÜNÜN NÖRONAL SAĞKALIM VE AKSON UZAMASI İÇİN OPTİMIZASYONU

Aljinat, kahverengi alglerde bulunan doğal, doğrusal bir polimerdir; ayrıca biyouyumlu, biyoçözünürdür ve toksik etkisi yoktur . Bu özellikler, aljinatı biyoteknolojik uygulamalar için uygun hale getirmektedir. Bu tezde, *Cystoseira barbata*'dan ekstrakte edilmiş aljinatın 3 boyutlu hücre kültürü özellikleri, ticari olarak temin edilebilen alginik asit, sodyum tuzu ile karşılaştırılmıştır. Aljinat hidrojelleri, iki hücre hattının, fare motor nöronunun (NSC-34) ve nöroblastomun (SH-SY5Y) sağkalımını ve aksonal uzamasını sağlamak, üç boyutlu nöral hücre kültürü oluşturmak için kullanılmıştır. Hücreler, %1'lik aljinat hidrojellerinin içerisine gömülmüştür. Bu hücrelerde aljinat reseptörlerinin bulunmamasından dolayı, aljinattaki hücreler kümelenme şeklinde bir araya gelme eğilimi göstermişlerdir. Kümelenmeyi azaltmak ve hücresel sağkalımını artırmak için, aljinat 2:1 aljinat/kolajen oranında kolajen tip I ile karıştırılmıştır. Her iki hücre hattında da, aljinat/kolajen hidrojelleri, sadece aljinat hidrojelleri ile karşılaştırıldığında hücre proliferasyonunu veya aksonal uzamayı önemli ölçüde değiştirmemiştir. Bu hidrojellerde aksonal uzamasını ve sağkalımı arttırmak için NSC-34 hücreleri sinir büyüme faktörü (NGF, 50 ng/mL) ve fibroblast büyüme faktörü (FGF, 10 ng/mL) ile farklılaştırılırken, SH-SY5Y hücreleri hidrojel içerisine ekilirken serumsuz ortam ve retinoik asit (RA, 10 µM) muamelesi ile başkalaştırılmıştır. FGF ve NGF ile farklılaşma sonrasında, hidrojellerde NSC-34 hücrelerinde sağkalımın arttığı ve FGF ile kümelenmelerin azaldığı görülmüştür. Öte yandan SH-SY5Y hücreleri, serumsuz ortamda retinoik asit muamelesi ile hidrojeller içinde başarılı bir şekilde başkalaştırılamamıştır.

Anahtar Sözcükler: Aljinat, Hidrojel, Kolajen, NGF, FGF, RA.

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LIST OF SYMBOLS

D_{cf}	Final concentration of collagen
D_{ci}	Initial concentration of collagen
V_c	Required collagen volume
V_f	Final volume of collagen
V_a	Volume of 0.02 M acetic acid
V_1	Required collagen volume
V_2	Total volume
V_3	Volume of NaOH
V_4	Volume of DPBS

LIST OF ABBREVIATIONS

AFM	Atomic Force Microscopy
AO	Acridine Orange
$CaCl_2$	Calcium Chloride
BDNF	Brain-Derived Neurotrophic Factor
DMEM	Dulbecco's Modified Eagle Medium
DPBS	Dulbecco's Phosphate Buffered Saline
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
FBS	Fetal Bovine Serum
FTIR	Fourier Transform Infrared
HCl	Hydrochloric Acid
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
Na_2CO_3	Sodium Carbonate
NSC-34	Mouse Motor Neuron Cell Line
PI	Propidium Iodide
SEM	Scanning Electron Microscopy
SH-SY5Y	Neuroblastoma Cell Line

1. INTRODUCTION

Cell culture techniques have been used since 1885; however, three-dimensional (3D) cell culture to mimic the natural environment of the cells has started at the beginning of the 20^{th} century [1]. Cells in 3D culture are presumed to behave as they would in their niche under appropriate culture conditions. With this technique, it is possible to mimic the cellular matrices found in the body; hence cell-cell interactions and cell-matrix interactions can be controlled by using these 3D environments. 3D cultures give cells different structural opportunities than two-dimensional cultures [2–4]. Cellular behavior is varied in 2D and 3D environments. Because there are too many parameters to control the cellular environment, in well-characterized and controlled 3D environments, in vitro results can represent physiological situation more accurately than 2D cultures. In order to ensure that 3D environments represent the extracellular environment for each cell type accurately, one needs first to understand the natural components of these environments [5] and then devise biomaterials that resemble or mimic these structures.

1.1 Extracellular Matrix (ECM)

The extracellular matrix (ECM) is a non-cellular constituent in living organisms. It is one of the most critical parts of the body in terms of its cellular activities such as cell proliferation, migration, and differentiation [2, 6, 7]. It can be said that the ECM is like glue surrounding the cells and connects cells to connective tissues. In other words, the ECM is the environment that contains fibrous meshwork of ECM proteins such as elastic fibers and collagen. It regulates essential biomechanical and biochemical incidents vital for cellular morphogenesis, homeostasis, and differentiation [6, 7]. Basically, the ECM comprises of proteins and polysaccharides. Yet, every tissue has a unique composition, i.e., the ECM varies for each tissue [7]. It can be thought that the ECM is a gel-like structure, and provides interactions between cell-cell, cell-tissue, cell-protein, protein-proteoglycan [7, 8]. In addition to these, cell adhesion to

the ECM is mediated through integrins and the ECM receptors. Moreover, signal transduction from growth factors that bind cell-surface receptor interactions is, in some cases, arbitrated by the ECM [7].

The ECM is known to contain more than 300 proteins, but it is composed of mainly two macromolecules: fibrous proteins and proteoglycans [1, 7, 8]. The structural roles of proteoglycans are hydration, buffering, and filling the internal space to form a hydrogel. Fibrous proteins consist mainly of collagen, elastin, fibronectin, and laminin [6, 8]. The most abundant protein found in the ECM is collagen and accounts for 30% of all proteins [7]. Collagens are mainly responsible for providing tensile strength to support migration, chemotaxis, regulating cell adhesion, and directing tissue development. Collagen is mostly found in triple-stranded helix form. This structure provides collagen to form fibrils and networks [7, 8]. There are 28 different types of collagen found in vertebrates. Each type of collagen has its specific responsibilities. For instance, collagens type I,II,III are fibril-forming collagens, while collagen type IV is network-forming collagen [8]. Elastin is important for the elasticity of tissues, such as the lungs and dermis of the skin; moreover, it provides tendons with the property of stretch and recoil [8, 9]. Fibronectin is the other vital protein for the extracellular matrix, and its role is known to be the attachment and migration of the cells [8, 9]. Laminin has a property of self-polymerization that makes it important for basement membrane formation, also cellular interactions are based on cellular interactions with glycoproteins, integrins, and glycolipids. Laminins are essential for cellular development [10].

1.2 Biomaterials

The porous structure of the ECM contains fibrous meshwork, and this fibrous network structure gives the cell everything it needs; therefore, the ECM is the environment where the cells live, and it also supports their growth, proliferation, and differentiation. For cellular experiments, mimicking the ECM is one of the most important requirements to control physiological and cellular responses. Creating such an

artificial environment is possible by using specifically designed biomaterials. Biomaterials can be made from natural, synthetic, or a combination of these substances. These must be used in the living organisms without any side effects in any periods of life to augment, treat, or replace the unhealthy parts of the body [9]. In other words, biomaterials are the artificial extracellular matrix that surrounds and supports the cells mechanically, help their survival, proliferation, or differentiation, and even direct their tissue organization.

The history of biomaterials is dated back to 600 A.D. when Mayan people were interested in nacre teeth. They produced the nacre teeth by using seashells without any knowledge of biomaterials, medicine, and biology [11]. An iron dental implant was found in a corpse in 200 A.D in France; like Mayan people, they used osseointegration techniques [11].

Ancient people needed some materials to improve their quality of life or just for fashion, although they did not have the scientific information in the modern sense. With today's knowledge, biomaterials can be designed to be non-toxic, biocompatible, and structurally stable in the long term. They can restore or replace the missing or defective function, causing no or minimum inflammation to the body. The properties mentioned above are vital for an effective biomaterial.

Materials are categorized into four sub-types depending on host response: bioinert, bioactive, bioresorbable, and toxic. Bioinert materials cause less or no-host responses, and this type of biomaterials are biologically non-active; metallic implants are examples of bioinert materials. In bioactive biomaterials, the situation is different from bioinert ones since this type of biomaterials may cause the host response; bioactive glasses and calcium phosphate ceramics are bioactive materials. They are usually designed for specific and goal-oriented responses and are non-toxic. Host tissue and material usually interact and form bonds. Bioresorbable materials are non-toxic, and host tissue is replaced or reabsorbed by these materials; the materials made from biodegradable polymers are examples of bioresorbable materials. Lastly, toxic materials directly or indirectly cause the surrounding tissue's death. The toxicity of materials

should be minimized to reduce the cell response. Biomaterials should be tested before implanted to prevent failure caused by these materials [12].

Chemically, there are mainly four types of biomaterials: metals, ceramics, polymers, and hybrid [13]. Metallic materials are mostly used due to their high ductility, toughness, mechanical properties, and they are promising materials for joint and bone replacement. Ceramics are preferred in bone tissue engineering applications because they are bioresorbable, bioactive, and microbial attack resistant, but they are not suitable for fractures. Polymers are preferred in health care because they could be biodegradable or bioinert, flexible or rigid, depending on targeting function. Water interacts with polymers and is utilized as hydrogels. Because there is no or little interfacial tension with the environment, this non-tension decreases cell adhesion and protein adsorption. The last type of biomaterials is composite materials. These materials are a combination of the best two materials to increase mechanical strength or material properties. To mimic the mechanical properties of the tissues can be possible with hybrid materials, so hybrid materials are preferred in tissue engineering applications [14].

Hydrogels are hydrophilic, and they have a high percentage of water, which makes hydrogels favorable as biomaterials [15, 16]. Hydrogels can be components of both natural (e.g., collagen, chitosan, alginate, hyaluronic acid) and synthetic (poly-(acrylic acid) (PAA), poly (ethylene glycol) (PEG)) polymers [16–19]. Natural polymers are preferred for biomedical applications because of their biocompatibility, biodegradability, and low toxicity. Still, there are some limitations of natural hydrogel usage, like low mechanical properties [16–18].

Crosslinking can be done ionically, covalently, or physically. This variety of crosslinking ability makes hydrogels more controllable and applicable structures. The tissue-like hydrogel structure makes it possible for embedded cells to create a 3D cell culture both in vitro and in vivo [16]. The polymer choice is an essential factor for hydrogel usage area; for this purpose, collagen, chitosan, fibrin, hyaluronic acid, alginate, or agarose can be used [15].

In summary, to obtain 3D environments that mimic the ECM, hydrophilic gels are the most preferred biomaterials. Although to procure this complex structure in vitro seems challenging to build, artificial extracellular matrices can be generated with hydrogels for not only 3D cell cultures but also for cell transplantation, tissue engineering, or cell immobilization applications [20]. Among them, alginate has been widely used as a natural polysaccharide for synthetic ECMs as a hydrogel [20].

1.3 Alginate

E.C.C Stanford, a British chemist, first described alginate in 1881; 83 years later, it was understood that alginate is in the intercellular matrix. Alginate is a linear anionic polysaccharide found in the cell wall or the cytoplasm of brown seaweeds or obtained from bacteria [21, 22]. It is one of the most abundant polysaccharides that is industrially produced, amounting to about 38,000 tons annually [4, 21]. In the algae, the alginate's function is mostly structural, such as providing mechanical strength and flexibility, depending on the algae and with varying degrees based on compositional differences within the alginate. Alginate is used in different areas because of its water retention, viscosity, stabilization, and gelation properties. Food, technical applications, and pharmaceutical industries are the most profitable areas, but biotechnological applications continue to increase with recent research [19, 21].

Alginate hydrogels are preferred in dental applications as impressions. Also, alginate is used to prevent gastric diseases, cell carriers, transplantation, drug delivery system, and tissue engineering. Alginate should be pure for biotechnological and pharmaceutical applications because a natural source of alginate might give some impurities like heavy metals, polyphenolic compounds, proteins, and endotoxins. Because of these impurities, before cellular experiments, alginate purity tests should be done to avoid contamination [22–24].

Alginate is composed of (1→4) linked β-D-mannuronate (mannuronic acid, M), α-L-guluronate (guluronic acid, G), and these M and G residues can be found struc-

turally either homogeneously or heterogeneously (Figure 1.1, [23]). There is no single specific configuration of alginate; the alginate structures can differ based on the type of algae it is extracted from, the collection time and season, the environments of the algae, and many other parameters. In order to determine the specific structure of an alginate sample, a chemical analysis should be done to identify M and G composition [1]- [21].

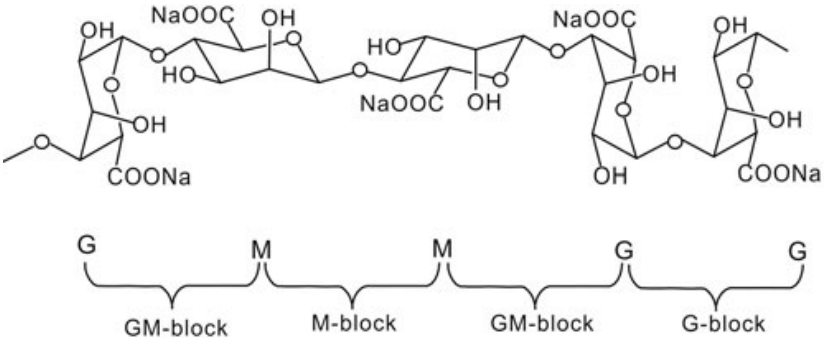


Figure 1.1 Typical sodium alginate chemical structure.

M/G ratio is an essential ratio for alginate gel's characteristics. Most alginate has 30% to 70% M residues, and alginate isolated from bacteria is composed of 100% mannuronic acid [4, 21].

The chemical structure of alginate is vital for forming ionic gelation. G-content has an essential role in alginate's gel property: increasing G-content causes increasing gel elasticity, porosity, and stability [4, 21]. G-blocks are cross-linked with divalent cations called egg-box, with different affinity as Ca^{2+}<Sr^{2+}<Ba^{2+} (Figure 2.1, [22]) [4, 16, 22]. Gel stiffness also depends on alginate chemical distribution, which is indirectly affected by the growth conditions of the algae source, i.e., when the algae were collected and how alginate was extracted [20]. In general, the hydrogel is stiffer if G residues are more than M blocks; if the M/G ratio is higher than one, then the hydrogel gets more flexible [22].

There are two main gelation methods for alginate, namely ionic and covalent gelation. Two main approaches can be used for ionic gelation. The first one is diffusion; it merely uses diffusion force from high concentration to low concentration to procure a gel. There is a gel maker solution tank in this type of gelation technique, and the gel

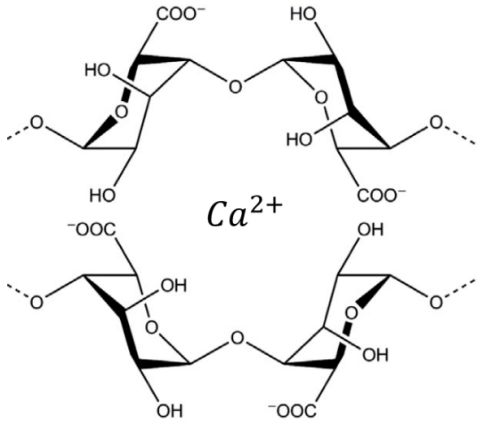


Figure 1.2 Crosslinking was done between guluronic acid and Ca^{2+} ions in egg-box shape.

solution is directly put in this reservoir, and simultaneously crosslinking is done. The other process is internal gelation, where gel-forming ions are used; for alginate, these ions can be carbonate solutions, calcium citrate, calcium EDTA. Controlled release is procured for gel-forming ions, and this release is dissolved with an alginate solution. On the other hand, covalent gelation can be achieved by chemical modification of the alginate. Chemically modified alginate hydrogels are generally more stable than ionic gelation [4, 21].

The alginate hydrogel's pore size can range from 5 to 200 µm; this scale is enough to embedded cells and allow access of cells to nutrients as well as easy removal of cellular wastes [4]. Even though alginate is a useful tool for embedding cells, the adhesion is not supported by cells because alginate polymer's receptors do not exist in mammalian cells. Chemically modified alginates are preferred for cellular embedding with laminin, fibronectin, or collagen [20]. Besides, cellular behavior can be directed by adding specific ligands because cell attachment in alginate is not supported [20].

1.4 Aim of the thesis

The aim of this thesis is to create a 3D neural culture using either extracted or commercial alginate and compare the quality of the extraction procedure. We further

wish to address the suitability and applicability of extracted alginate alone or in combination with collagen in supporting axonal outgrowth and cellular survival. In order to investigate the potential of alginate in neuronal differentiation 3D models, we have also supplemented the 3D hydrogels with nerve growth factor, and fibroblast growth factor for mouse motor neurons, NSC-34, and serum withdrawal and retinoic acid for SH-SY5Y, neuroblastoma cells.

2. MATERIALS AND METHODS

2.1 Materials

Materials, equipment, and devices used during experiments are shown in Table 2.1, Table 2.2, and Table 2.3. Most of these materials are provided by AXANLAB.

2.1.1 Alginate characterization

FTIR spectroscopy, fluorescence microscopy, density bottle, and viscometer were used for alginate characterization.

Table 2.1
Devices used during experiments.

Device	**Brand**
Centrifuge	Centurion
CO_2 incubator- Steri-cycle i60	Thermo Scientific
Density Bottle	ISOLAB
Fluorescence Spectroscopy	Varian Eclipse Spectrophotometer
Fluorescent Cell Imager	ZOE
Fourier Transform Infrared Spectroscopy (FTIR)	Perkinelmer Spectrum 100
Laminar Flow Cabinet- Safe 2020	Thermo Scientific
Light Microscope	Leica
MR Hei-tec Magnetic Stirrer with Heating	Heidolph
Steam Sterilizer (Autoclave)	NUVE OT-90L
Viscometer, Sv-10	Vibro
Water Bath	JeioTech

2.1.2 Alginate solution preparation

The seaweeds, *Cystoseira barbata*, were obtained as part of the OBEK project and extraction of alginate was done by Habibe Kurt, and commercial alginate for comparisons was a gift from Assoc. Prof. Israfil KUCUK. Besides alginates, $CaCl_2$ and NaCl were used to obtain alginate hydrogel (Figure 2.1) (extraction method, Appendix A).

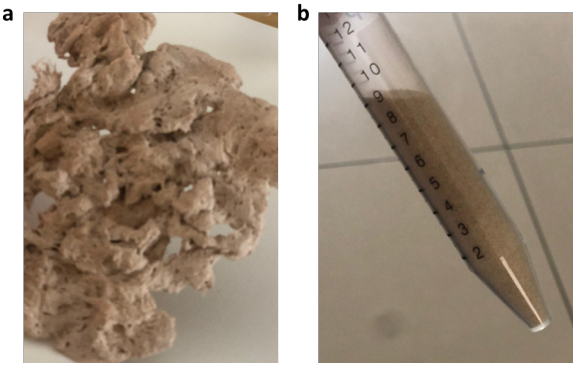


Figure 2.1 Extracted and commercial alginate. a. extracted alginate in lyophilized form, b. commercial alginate in powder form.

2.1.3 Cell culture

Two cell lines were used for cellular experiments, NSC-34, mouse motor-like neuron cells, CEDERLANE CLU400, and neuroblastoma neuron-like cells, SH-SY5Y, a gift from Acibadem University. These two cell lines were used from previous experiments done in AXANLAB. Nerve growth factor (NGF), fibroblast growth factor (FGF), and retinoic acid were used for cell differentiation, NGF and FGF for NSC-34, and RA for SH-SY5Y. For cell thawing, passaging, and freezing DMEM, DPBS, and trypsin were used.

Table 2.2
Equipment used for all experiments.

Equipment	**Brand**
6-,24-, 96-well plate	TRP
40 mm, 60 mm, 100 mm Tissue Culture Plate	TRP
15 mL, 50 mL Centrifuge Tube	CAPP
5 mL, 10 mL Serological Pipettes	CAPP
0.45 µm Syringe Filter	ISOLAB
T25, T75 Tissue Culture Flask	TRP
Haemocytometers	Marienfield
Micropipette Eppendorf Micro Centrifuge Tube 2 mL, 1.5 mL	CAPP

2.2 Methods

Before starting the experiments with cells, the extracted alginate was characterized, and the similarity between extracted and commercial alginate was investigated. The characterizations were done using FTIR spectroscopy to determine chemical properties, fluorescence spectroscopy for purity, viscometer for viscosity (FT-RAMAN, surface tension, and SEM were in Appendix B).

2.2.1 Chemical analysis with Fourier-transform infrared spectroscopy

Extracted sodium alginate from *Cystoseira barbata* was subjected to FTIR characterization to find the M/G ratio and percentages of mannuronic acid and guluronic acid residues. Approximately 5 mg of sodium alginate powder were placed in the spectroscopy chamber, and the wavenumber measurement was set between 450 cm^{-1} and 4000 cm^{-1} [25, 26]. The area under these wavenumbers gives the mannuronic acid and guluronic acid composition in the alginate, and this area was calculated using the OriginPro program, (https://www.originlab.com/) [27].

Table 2.3
Materials used for all experiments.

Material	**Brand**	**Cat/Lot No**
Acetic Acid	Sigma	27225
Acridine Orange (AO)	Sigma	A6014
Alginic Acid Sodium Salt	Sigma	9005-38-5
Calcium Chloride, 96% Extra Pure, Powder, On Hydrous, $CaCl_2$	Acros Organics	10043-52-4
Collagen I, Rat Tail 3mg/mL	Gibco	A10483-01
Dimethyl Sulfoxide for Molecular Biology, DMSO	Biofroxx	67-68-5
Dulbecco's Modified Eagle Medium, DMEM, 4.5 g/L D-Glucose, L-Glutamine, (+) Pyruvate	Gibco	2183101
Dulbecco's Modified Eagle Medium, DMEM, 4.5 g/L D-Glucose, L-Glutamine, (-) Pyruvate	Gibco	2007829
Dulbecco's Phosphate Buffered Saline, DPBS, (-) $CaCl_2$, (-) $MgCl_2$	Gibco	2062129
Ethylenediaminetetraacetic Acid Disodium Salt Dihydrate, EDTA	Sigma	E5134
Fetal Bovine Serum, FBS, Qualified	Gibco	41A0006K
Fibroblast Growth Factor 2 (FGF-2)	ThermoFisher	13256-029
Nerve Growth Factor, 2.5S (NGF)	Sigma	N6009
Pen Strep, Penicillin Streptomycin,(+)10,000 units/mL Penicillin, (+) 10,000 µg/mL Streptomycin	Gibco	1953096
Poly-L-Lysine Solution 0.1 % (w/v) in H_2O	Sigma	25988-63-0
Propidium Iodide (PI)	Sigma	P4170
Retinoic Acid (RA)	Sigma	R2625
Sodium Chloride (NaCl)	Merck	7647-14-5
Sodium Hydroxide	Sigma	06203
Trypan Blue Stain (0.4 %)	Gibco	1674980

2.2.2 Purity with Fluorescence Spectroscopy

0.1 g of extracted and commercial alginate each was separately weighed and dissolved in 10 mL distilled water. The 1% (w/v) alginate solutions were filtered (0.45 µm syringe filter). 3 mL of the solution was used to examine under fluorescence

spectroscopy (excitation wavenumber was 366 nm) [28–30]. The excitation/scanning was between 370-600 nm.

2.2.3 Viscosity

50 mL of 1% (w/v) alginate solution was solved in distilled water at 500 rpm. The sample volume was 40 mL.

After measuring the viscosity, the density measurement was done with the same alginate solutions. 25 mL of solutions were used for this purpose.

2.2.4 Degradation

To check the weight loss of alginate hydrogel, alginate solution was prepared with the same procedure but without cells as in 2.2.6. The alginate was diluted with DMEM (pyruvate free) to 1% (w/v) and adding 1 mL alginate into the 40 mm dish, 1.5 mL $CaCl_2$ was used for gelation. After 10 minutes of incubation, the hydrogel was washed with DPBS twice and once with DMEM, and 1 mL DMEM was added to the hydrogel. The alginate hydrogels were prepared for alginate solution prepared one week ago (old alginate), alginate solution that was not autoclaved just filtered with 0.45 µm syringe filter (filtered alginate), and fresh alginate for both extracted and commercial. The hydrogels were measured at 0, 30 minutes, and 1, 2, 6, 12, 24 hours. Also, the measurement was repeated every 24 hours, 7 days. The last measurement was performed on the 10^{th} day [29].

2.2.5 Cell culture

For the entire thesis, the words ‘medium’ or ‘DMEM’ was used for a complete medium which contains 10% FBS and 1% penicillin-streptomycin (pen/strep), if it

is not mentioned differently. For the NSC-34 cell line, DMEM, 4.5 g/L D-Glucose, L-Glutamine, (-) Pyruvate was used while DMEM, 4.5 g/L D-Glucose, L-Glutamine, (+) Pyruvate was used for SH-SY5Y (cell thawing and cell freezing were described in Appendix C).

2.2.5.1 Cell passaging. Before embedded the cells, cells were passaged at least one time to recover. The passaging procedure for both cell lines was the same.

When cells reached confluency, they were either passaged or set for experiments. Cells were received from the incubator, the existing medium was removed, and the flask was washed with 1x sterile DPBS. Trypsin was added to detach the cells. When cells were all detached, the medium was inset 1:5 ratio to dilute trypsin. Then cell solution was placed in the centrifuge tube. Centrifugation was done at 1500 rpm for 5 minutes. The supernatant was discarded, and the cell pellet was dissolved with 1 mL medium. The cells were counted by using a haemocytometer. The last step of passaging cells was seeding cells on the planned area and in the required density.

2.2.6 Alginate solution preparation

The alginate solutions were prepared for 0.9% NaCl, 4.5% alginate (w/v) dissolved in distilled water [20, 31, 32]. Alginate was dissolved at 500 rpm 40°C on the magnetic stir for approximately 30 minutes [33, 34]. Prepared alginate was placed to autoclave at 121°C for 20 minutes to remove any impurities and contamination risks for cellular use [35, 36]. This procedure was the same for both extracted and commercial alginate (Figure 2.2). After sterilization, alginate solutions were stored at 4°C overnight. Before using solutions, they were filtered with 0.45 µm syringe filters under a laminar flow hood [33, 34]. Solutions were prepared fresh and diluted to the desired concentration and were done with the appropriate medium for which the cell line was embedded.

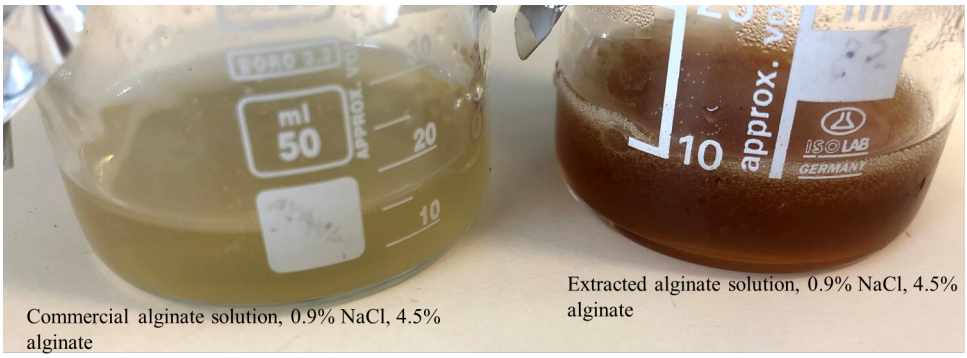


Figure 2.2 Alginate solutions; the left one is the commercial, and the right one is the extracted alginate.

2.2.7 3D cell cultures with alginate hydrogel

Cells were detached with the same method as the passaging procedure, and then cells counted with a haemocytometer. After centrifugation, cells were seeded to alginate hydrogels at 2×10^6 cells/mL; to that end, the required amount of cell solution, medium, and alginate was mixed to achieve a 1% (w/v) or other concentrations alginate solution. The alginate and medium were mixed and left for interpenetration before adding the cells into the medium/alginate mix. During the hydrogel preparation, the tip of the pipette could affect the fluidity of alginate solution; therefore, the tip of the pipette was cut by a sharp knife under the hood to remove viscosity effect on the solution. Alginate/cell mixture was gently pipetted several times to make the solution homogeneous. For 24-well plate 300 µL or for 96-well plate 100 µL, alginate/cell solution was placed in the well, and 450 µL or 150 µL 0.1M $CaCl_2$ was added on the alginate/cell solution. The crosslinking between the guluronic acid and Ca^{2+} form almost instantaneously; hence, it has to be quickly applied; otherwise, cells tend to escape from alginate before gelation takes place. The solution was incubated for 10 minutes at room temperature and washed with an equal amount of DPBS twice and medium once [31]- [33, 37]. The medium was added to the hydrogel and placed in the incubator with 37°C and 5% CO_2. The whole procedure of 3D culture with alginate hydrogel is illustrated in Figure 2.3 (illustration with BioRender.com). The medium on the hydrogel was changed every two days. The time interval for imaging is set to 24h, and images are taken under light microscopy.

For most of the experiments, a 96-well plate was used for gelation observations. For example, if it is 50,000 cells/well, then the required cell solution is prepared 25 µL/well. The alginate/cell solution was placed 100 µL/well to well, and the amount of $CaCl_2$ was 150 µL. To illustrate, after giving these parameters, to have 3-well of alginate/cell hydrogel, the calculations were done for 4 wells because of pipetting error. 89 µL of 4.5% alginate, 211 µL of a medium, and 100 µL cell solution were placed in 2 mL centrifuge tube and pipetting, then 100 µL of the solution was taken and put into the well, after that the procedure was same with explained.

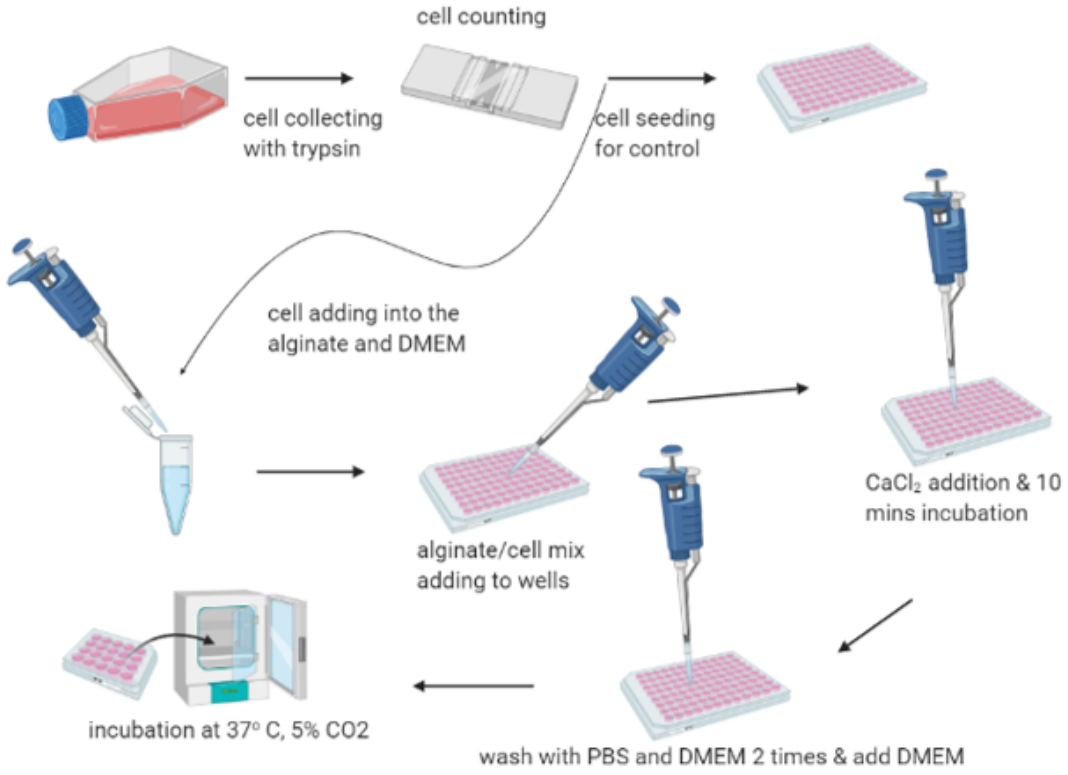


Figure 2.3 Illustration of cell encapsulation in alginate hydrogel preparation.

2.2.8 Alginate hydrogel staining

Propidium iodide (PI) and acridine orange (AO) were procured from BIOSCUBUS LAB, Bogazici University, and the staining procedure of BIOSCUBUS LAB was used with some modifications. For staining, propidium iodide (3 mg/mL in ethanol) and AO (5mg/mL in ethanol) solutions were prepared. 1 µL of AO and 2 µL of PI were dissolved in 1 mL DPBS. The existing medium above the hydrogel was removed, 100 µL

of staining solution was added onto the hydrogel and incubated for 15 minutes. After that, the hydrogel was washed twice with the same amount of DPBS, adding the same amount of DPBS, and incubated 10 minutes more to diffuse stains every level of alginate. The incubations were done at 37°C and 5% CO2. The fluorescence images were taken under a fluorescent cell imager, ZOE with 480/517 nm (excitation/emission) (green fluorescence) for AO to see live cells and 556/615 (excitation/emission) (red fluorescence) to observe dead or dying cells with PI.

2.2.9 Cell counting with EDTA

0.5 M ethylenediaminetetraacetic acid (EDTA) was prepared with distilled water and filtered with 0.22 µm syringe filters under laminar flow. Medium on hydrogel was removed, and 100 µL EDTA solution was added. After addition, the alginate hydrogel turned into a liquid form. This solution was collected, and trypan blue was adding in a 1:9 ratio (trypan blue:cell/alginate/EDTA solution); after approximately 5 minutes of incubation, 10 µL of the solution was taken, and cells were counted with a haemocytometer.

2.2.10 Collagen coating & Alginate/collagen/cell hydrogel preparation

The surface coating was done by following the collagen protocol was provided by GIBCO; the recommended concentration for coating was 5 µg/cm^2. The surface area of one well of a 96-well plate is 0.32 cm^2; therefore, the coating was set for 1.6 µg/mL. The calculations were done using Eq. 2.1 and Eq. 2.2. The initial concentration was 3 mg/mL, and collagen was diluted with 0.02 M acetic acid.

$$V_c = \frac{D_{cf} \cdot V_f}{D_{ci}} \tag{2.1}$$

$$V_a = V_f - V_c \tag{2.2}$$

where V_c: required collagen volume, D_{cf}: final concentration of collagen, V_f: final volume of collagen, D_{ci}: initial concentration of collagen, V_a: volume of 0.02 M acetic acid.

25 µL collagen coating solution was added per well and incubated in the incubator at 37°C and 5% CO_2. The coating solution was then removed and washed three times with DPBS, and it was ready for cell seeding. Required collagen concentration was set for alginate/collagen mixture, and the calculations were performed for collagen, Eq. 2.3.

$$V_1 = \frac{D_{cf} \cdot V_2}{D_{ci}} \tag{2.3}$$

where V_1 is the required collagen volume.

Initial concentration was 3 mg/mL, desired concentration was set to 1 mg/mL after trying 1.5 and 2 mg/mL. The final volume of collagen changeable depending on the collagen/alginate ratio. The concentration of alginate was set using 1x DPBS, and pH was adjusted to 7.4 with 1N NaOH, Eq. 2.4 and Eq. 2.5.

$$V_3 = V_1 \cdot 0.025 \tag{2.4}$$

$$V_4 = \frac{V_2}{10} + V_2 - (V_1 + V_3) \tag{2.5}$$

where V_3: volume of NaOH, V_4: volume of DPBS.

Collagen was stored in a dark and cold during the experiment; after collagen was diluted with DPBS and pH was set, collagen was mixed with alginate. Also, alginate concentration was adjusted according to the collagen amount. After the addition of cell solution, the blend was gently pipetted to acquire a homogeneous structure. 100

µL solution was put into each well and incubated at 37°C for 1 hour to form a collagen fibril structure. After incubation, 150 µL, 0.1 M $CaCl_2$ was added and incubated for 10 minutes at room temperature. The formed hydrogel was washed with 150 µL DPBS twice and with the medium for once. The last step was adding the medium and placed into the incubator. Hydrogels were imaged every 24h, and the medium was changed every two days.

2.2.11 Differentiation in 3D cultures

Differentiation of NSC-34 was ensured using two different growth factors, but the differentiation procedures were the same. Nerve growth factor (NGF) and fibroblast growth factor (FGF) were used with concentrations of 50 ng/mL and 10 ng/mL in DMEM, respectively, to differentiate NSC-34 cells [38].

After cells were detached with trypsin and precipitated, the cell pellet was dissolved with a medium with FGF or NGF; also, alginate was diluted with this medium. Then the gelation procedures were the same as described above. The medium was changed every two days with medium including FGF or NGF.

To differentiate SH-SY5Y, retinoic acid and serum withdrawal were used. After cells reached confluency, they were passaged and seeded; after 36-hour to 48-hour incubation, the growth medium was changed with serum-free medium (DMEM with 1% FBS, 1% pen/strep). Cells were incubated one day with this medium, and for differentiation, it was replaced with differentiation medium (DMEM with 1% FBS, 1% pen/strep, 10 µM retinoic acid). The differentiation medium was refreshed every two days. For 3D cultures, the cells were detached one-day after serum-free medium addition, and the cell pellet was dissolved with a differentiation medium. The cell solution was added to the alginate solution that was diluted with a differentiation medium. The medium was changed every two days with a differentiation medium.

3. RESULTS

3.1 Alginate characterization

3.1.1 Chemical analysis with Fourier-transform infrared spectroscopy

In the literature, FTIR wavenumbers for alginate are reported to be 950 cm^{-1} O-H, 1400 cm^{-1} CH_2, 1200-1290 cm^{-1} C-O-C, and 1000-1025 cm^{-1} C-OH [26,27]. Mannuronic acid and guluronic acid have specific wavenumbers to identify the percentage of transmission. For guluronic acid, the number is 1025 cm^{-1}, and for mannuronic acid, the wavenumber is 1100 cm^{-1} [25, 26]. Alginate was reported to be rich in guluronic acid when extracted from Cystoseira barbata, with M/G ratio values between 0.59 and 0.64 [24, 39, 40].

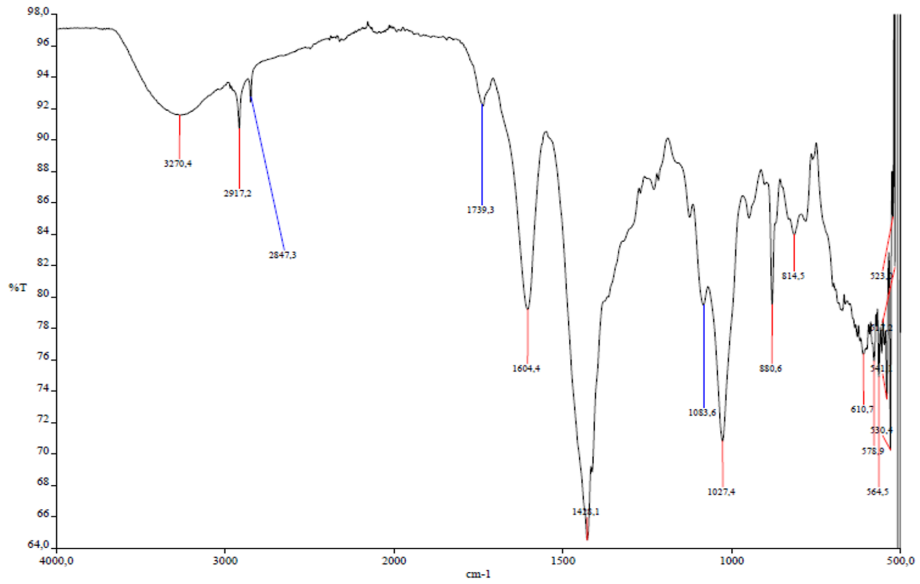


Figure 3.1 FTIR results of extracted alginate, peak points were tagged.

The relevant wavenumbers were observed for extracted alginate from the FTIR spectroscopy were 1027.4 and 1083.6 cm^{-1} for guluronic acid and mannuronic acid, respectively (Figure 3.1). After calculated the areas under these wavenumbers, the

M/G ratio was calculated 0.6. In other words, the percentage of mannuronic acid composition was 37.5%, and guluronic acid composition in the alginate extraction was estimated at 62.5%. When compared to literature, the extracted alginate was found to exhibit comparable chemical composition. The next step was to analyze whether the chemical composition of our extracted alginate was similar to commercially available counterparts.

Alginic acid, sodium salt (complements of Assoc. Prof. Israfil Kucuk) was used in a controlled manner and examined under FTIR under the same conditions and calculated as before. The results showed that the M/G ratio of commercial alginate was 0.71, and the percentage of M residues was 41.5, and the rate of G residues was 58.5. Both extracted and commercial alginates were found to be similar and guluronic acid-rich (Figure 3.2).

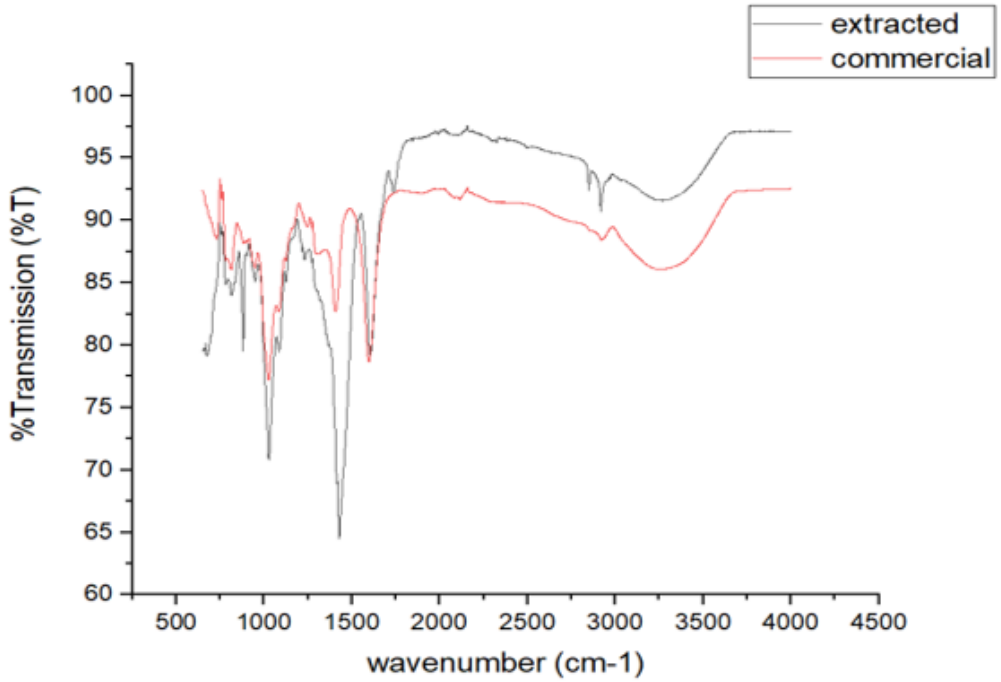


Figure 3.2 FTIR results of extracted and commercial alginate; black for extracted and red for commercial.

3.1.2 Purity with fluorescence spectroscopy

The phenolic compounds in the alginate extracts are the biggest problem of impurity [41]. The impurities are significant problems, particularly for cellular applications, so to detect any such impurities and purify the alginate from them is vital for cells and cellular experiments [4, 39, 42, 43]. The phenolic compounds are fluorescent, the excitation wavelength being at 366 nm, and the peak at 455 nm; therefore, the impurity caused by phenolic compounds can be detected using fluorescence spectroscopy [28, 29].

To understand and if it was necessary to purify extracted alginate any further or whether this extraction procedure was efficient for tissue culture purposes, the phenolic compounds in alginate were determined with fluorescence spectroscopy. Since the excitation wavelength of phenolic compounds was 366 nm, the alginate samples were checked at 366 nm for excitation wavelength. The results of the spectroscopy were in Figure 3.3, and there were no significant emissions for extracted alginate, indicating minimum or negligible impurities, at even less emission intensities than commercial alginate [28, 44]. This indicated that alginate extracted by the OBEK platform was suitable for tissue culture purposes.

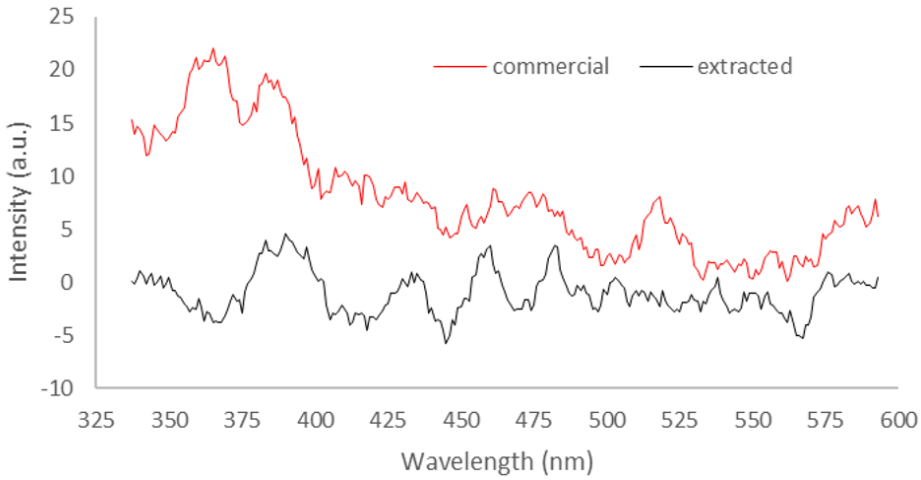


Figure 3.3 Fluorescence spectroscopy results, red for commercial and black for extract. The excitation wavelength was at 366 nm, and emission was at 450 nm.

3.1.3 Viscosity & Density

The viscosity of the sodium alginate solution is essential for the gelation property [29]. The viscosity of extracted and commercial sodium alginate was compared using viscometer; to that end, 1% (w/v) alginate solutions were filtered with a 0.45 µm syringe filter and applied to viscometer (courtesy of Assoc. Prof. Israfil Kucuk). The results were 29 mPas and 29.5 mPas, respectively. The density calculations in the same setup were 1.01 g/mL and 1.009 g/mL for extracted and reference sodium alginate, respectively, indicating both viscosity and density of extracted alginate was comparable to commercially available counterpart.

3.1.4 Degradation

Degradation was tested with six samples, three extracted, and three commercial alginate samples, for 10 days. Figure 3.4 shows that the percentage of weight loss of extracted and commercial alginates; these alginates were used as regular alginate hydrogel. Samples were weighed three times for each measurement time; all samples' standard deviation was negligible.

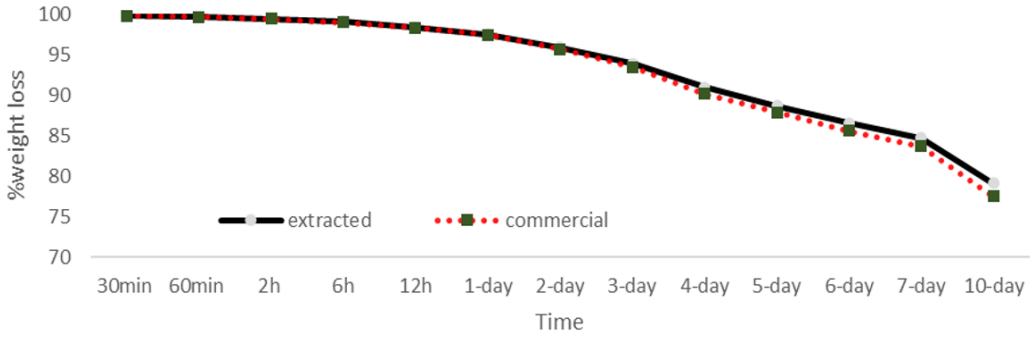


Figure 3.4 Percentage of weight loss of hydrogels for extracted and commercial alginate, hydrogels were weighted for 10 days, red dots represent commercial alginate weight loss while the black line is for extracting.

The results of other samples (only filtered alginates or one-week-old alginates, as indicated in the figure) were not significantly different from standard alginates (Figure 3.5).

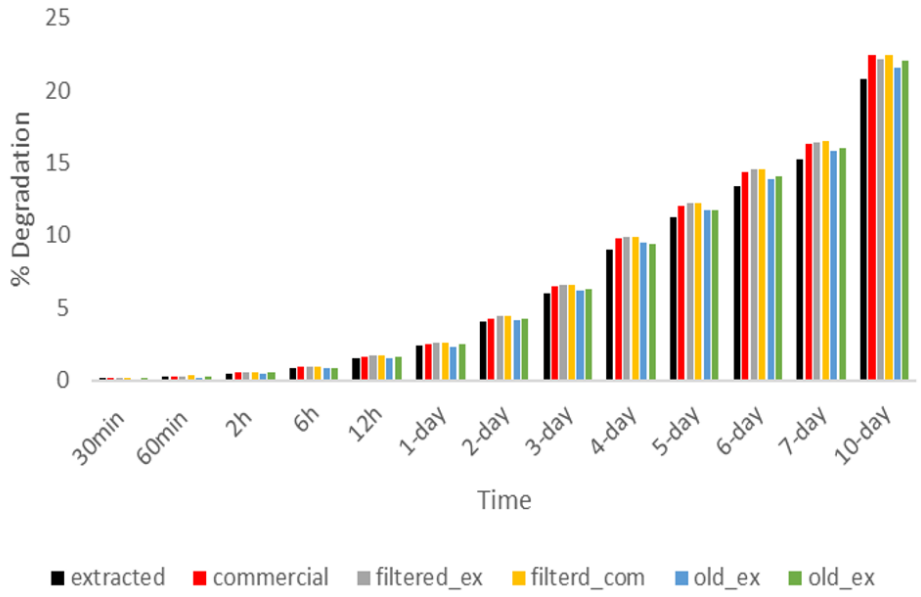


Figure 3.5 Percentage of degradation of extracted, black, commercial, red, filtered extracted, grey, filtered commercial, yellow, old extracted, blue, and old commercial, green.

The degradation test showed no significant difference between extracted and commercial alginate samples ($p= 0.0051$, $p<0.05$, student t-test), and the purification method (autoclaved or non-autoclaved) or shelf-life of solution did not seem to affect the degradation profile of alginate hydrogel. After the 10^{th} day, the sample weights were not measured because the existent medium was almost gone, and the hydrogel started to dry.

During the degradation test, the medium color changed on the 3^{rd} day; it turned pink to yellow, primarily commercial hydrogels (Figure 3.6).

3.2 Optimization

3.2.1 Alginate concentration & Cell density

Alginate concentration optimizations were done using the NSC-34 cell line, and a 24-well plate, 300 µL alginate solution/well were used. In the literature, the most favorable alginate concentration for cell survival was 2.2%; it was estimated that al-

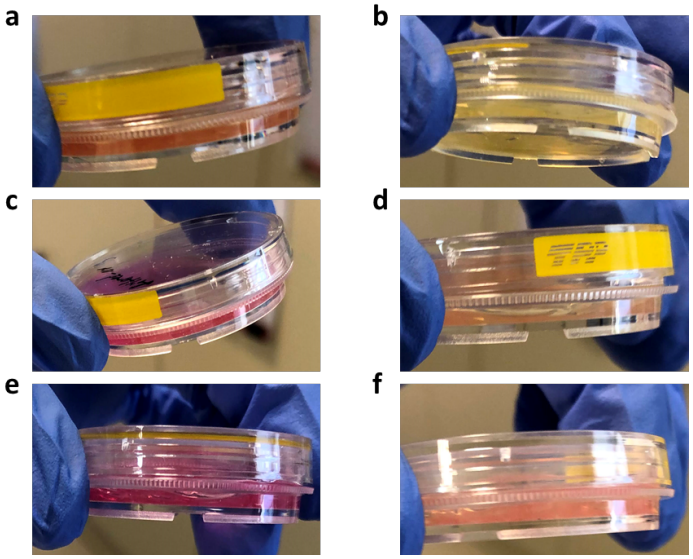


Figure 3.6 3^{rd} day of the degradation test, the color of the medium started to change pink to yellow. a.extracted, b.commercial, c.filtered extracted, d. filtered commercial, e. old extracted, f. old commercial.

ginate concentrations could be around 2% [33, 34]. Also, 1% alginate has a similar Young's modulus with brain grey matter [45]; therefore, the 1% alginate concentration was chosen. Increasing alginate concentration was reported to affect the hydrogel stiffness; 2.5% alginate concentration was selected to obtain solid hydrogel than other concentrations (Figure 3.8) [11].

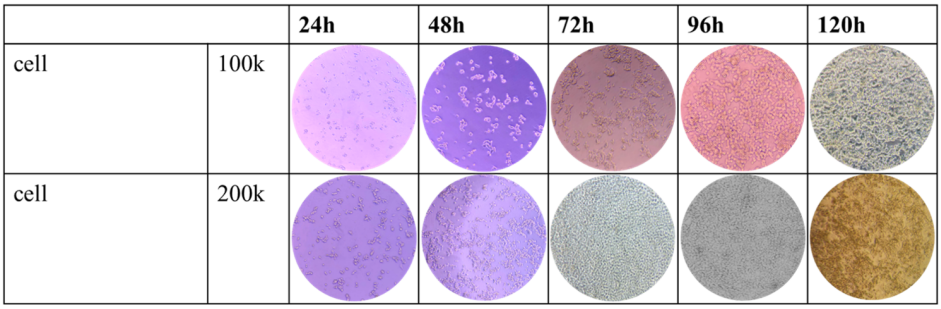


Figure 3.7 NSC-34 cell control, cells were seeded on a 24-well plate; the numbers represent cell seeding per well.

In addition to alginate concentration, the initial cell density was also optimized. Due to the depth and porosity of different percentages of the hydrogel, the cell density was found to be a crucial issue. The first sets carried out with 10^5 cells, and $2{\times}10^5$ cells/well were found to be successfully embedded in alginate hydrogel. In every step of this thesis, the cell controls were used to compare cell behavior out and in of the hydrogel (Figure 3.7). Alginate concentration best suited for cell survival in these

a

Extacted		24h	48h	72h	96h	120h
1% alginate	100k					
2.2% alginate	100k					
2.5% alginate	100k					
commercial		**24h**	**48h**	**72h**	**96h**	**120h**
1% alginate	100k					
2.2% alginate	100k					
2.5% alginate	100k					

b

Extacted		24h	48h	72h	96h	120h
1% alginate	200k					
2.2% alginate	200k					
2.5% alginate	200k					
commercial		**24h**	**48h**	**72h**	**96h**	**120h**
1% alginate	200k					
2.2% alginate	200k					
2.5% alginate	200k					

Figure 3.8 Cell embedded in alginate hydrogels with 1%, 2.2%, and 2.5% for both extracted and commercial. a. 100k cells/well were embedded into hydrogels, b. 200k cells/well were embedded into extracted and commercial alginate hydrogels.

initial cell amounts was found to be 1%, after trying concentrations of 0.5%, 1%, 2.2%, and 2.5% (Figures 3.8 and 3.10).

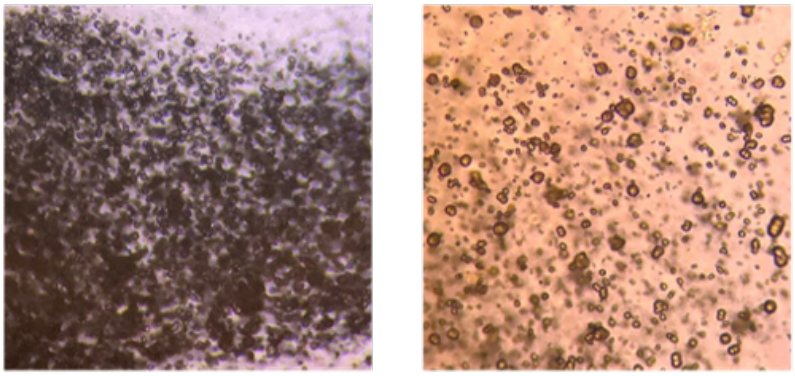


Figure 3.9 Extracted alginate had some pigmentation on 2.2% and 2.5% alginate hydrogels. The left image was from 2.2% extracted alginate hydrogel and taken on the 2nd day; the right one was from 2.5% extracted alginate hydrogel on the 3^{rd} day.

Cells were found to reach confluency after 3^{rd} day in the controls, and cellular proliferation and cellular growth were observed during imaging time. On the 3^{rd} day, cells in 1% alginate hydrogels extracted and commercial were migrated; the images were taken this migration through levels. However, cells exhibited signs of poor survival after the 4^{th} day and started to die after day 5. Cellular behaviors were not different in hydrogels prepared from either extracted or commercial alginate. During taking the images, finding the cells and capturing them were difficult in 10^5 cells/well, and it was decided to increase cell density to $3{\times}10^5$ cells/well (Figure 3.10).

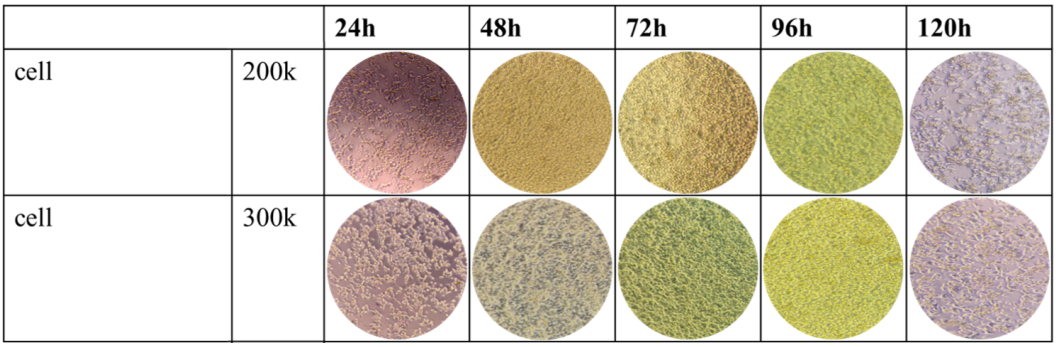


Figure 3.10 NSC-34, $2{\times}10^5$ cells, and $3{\times}10^5$ cells/well were seeded in a controlled manner.

It was observed that there were some pigmentation taints in the extracted alginate hydrogels, and these pigmentation taints were increased with time and increased

alginate concentrations (Figure 3.9).

a

Extracted		24h	48h	72h	96h	120h
0.5% alginate	200k					
1% alginate	200k					
Commercial		**24h**	**48h**	**72h**	**96h**	**120h**
0.5% alginate	200k					
1% alginate	200k					

b

Extracted		24h	48h	72h	96h	120h
0.5% alginate	300k					
1% alginate	300k					
Commercial		**24h**	**48h**	**72h**	**96h**	**120h**
0.5% alginate	300k					
1% alginate	300k					

Figure 3.11 NSC-34 cell embedding in alginate hydrogels. a. extracted and commercial alginate for the concentration of 0.5% and 1% for 200k cells/well, b. extracted and commercial alginate for the concentration of 0.5% and 1% for 300k cells/well.

In order to address whether this pigmentation could be toxic to the cells, lower alginate concentrations of 0.5% and 1% were also used for hydrogels (Figure 3.11).

Pigmentations were not prominent under the microscope for 1% and 0.5% algi-

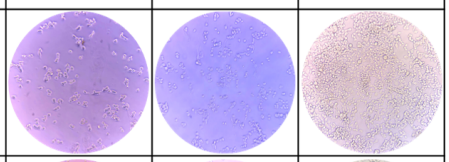
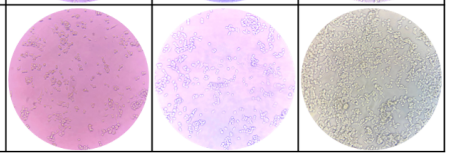
		24h	48h	72h
cell	100k			
cell	150k			

Figure 3.12 NSC-34 cell seeding with 10^5 and 1.5×10^5 cells/well to control cellular behavior with and without hydrogel.

nate hydrogels, yet cells exhibited poor survival to the same degree as previous hydrogel concentrations, indicating this long-term survival challenge is not necessarily related to the potential toxicity of pigmentations. Cell density in this set was too high, and overcrowding could be one reason for the loss of survival after day 5, due to waste removal problems in hydrogels. For that reason, 10^5 cell/well and 1.5×10^5 cells/well were used in 1% and 0.5% alginate hydrogels (Figure 3.12 and Figure 3.13).

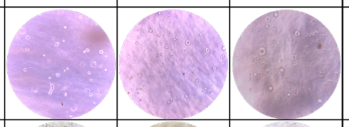
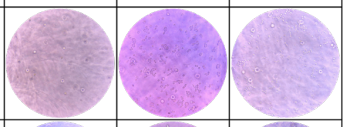
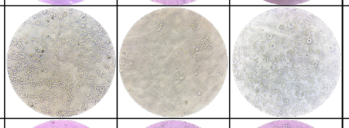
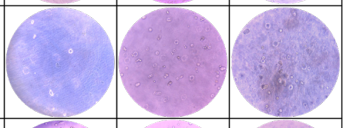
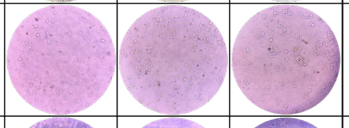
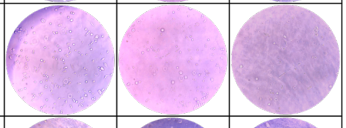
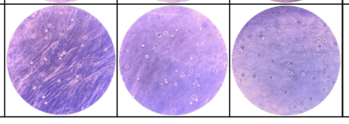
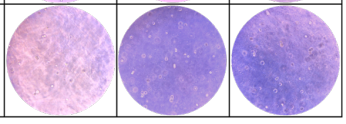
Extracted		24h	48h	72h	Commercial		24h	48h	72h
0.5% alginate	100k				0.5% alginate	100k			
0.5% alginate	150k				0.5% alginate	150k			
1% alginate	100k				1% alginate	100k			
1% alginate	150k				1% alginate	150k			

Figure 3.13 10^5 cells and 1.5×10^5 cells were embedded in 0.5% and 1% alginate hydrogels for both extracted and commercial alginate.

Alginate hydrogel with 0.5% exhibited more fluidic characteristics than previous hydrogels; nonetheless, in these four conditions (Figure 3.13), the cells did not grow or proliferate as good as in previous cases. However, they migrated away from the hydrogel, most probably to find more suitable attachment conditions outside the hydrogel. Cell density was optimized to 10^5 cells/well for a 24-well plate, and alginate

concentration was optimized to 1% for optimum cell survival for NSC-34. Cell density for a 96-well plate was calculated as 5×10^4 cells/well.

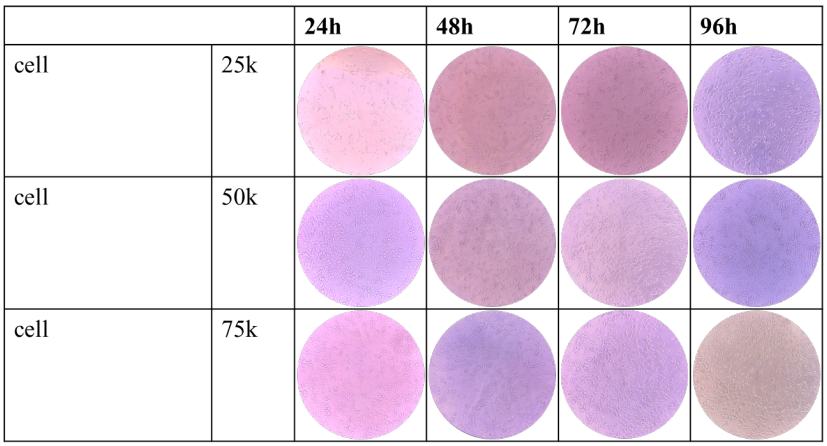


Figure 3.14 SH-SY5Y cell seeding in different densities, 2.5×10^4, 5×10^4, and 7.5×10^4 in a controlled manner.

After alginate hydrogel was optimized, the cell density for SH-SY5Y was optimized using a 96-well plate. The initial cell densities were assumed to be similar to NSC-34 results and determined as 2.5x 10^4, 5×10^4, and 7.5×10^4 cells/well (Figure 3.14, 3.15).

In the control part, there were not any problems with cellular behaviors (Figure 3.14).

Cell density for optimum survival was decided as 7.5×10^4 cells/well for the SH-SY5Y cell line. The hydrogel with a concentration of 1% looked as in Figure 3.16. These hydrogels were prepared with 8-well chamber slide.

3.3 Survival

The optimization results showed that both cell lines in alginate hydrogels did not tend to grow, and after the 6^{th} day, they were not looked healthy.

The porous structure of alginate hydrogel gives opportunities for using stains

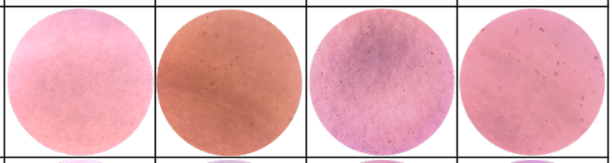
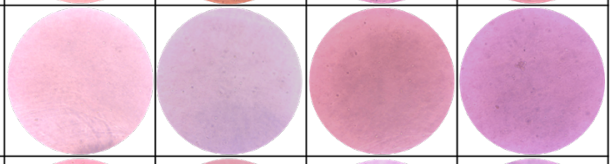
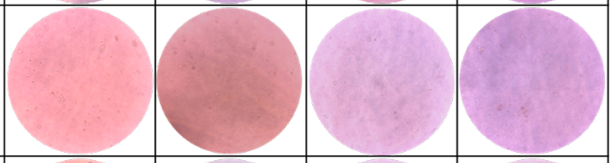
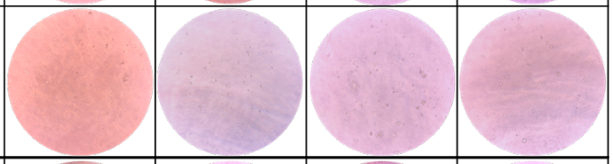
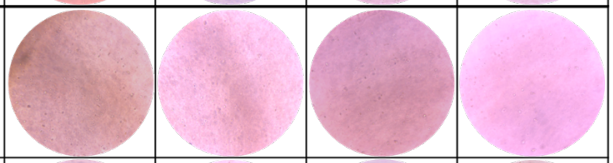
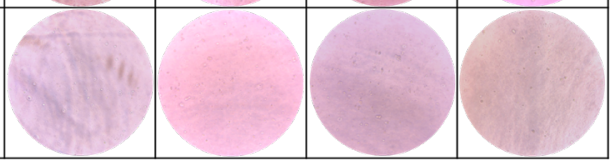
		24h	48h	72h	96h
1% alginate extracted	25k				
1% alginate commercial	25k				
1% alginate extracted	50k				
1% alginate commercial	50k				
1% alginate extracted	75k				
1% alginate commercial	75k				

Figure 3.15 25k, 50k, and 75k SH-SY5Y cells/well were embedded in 1% alginate hydrogel.

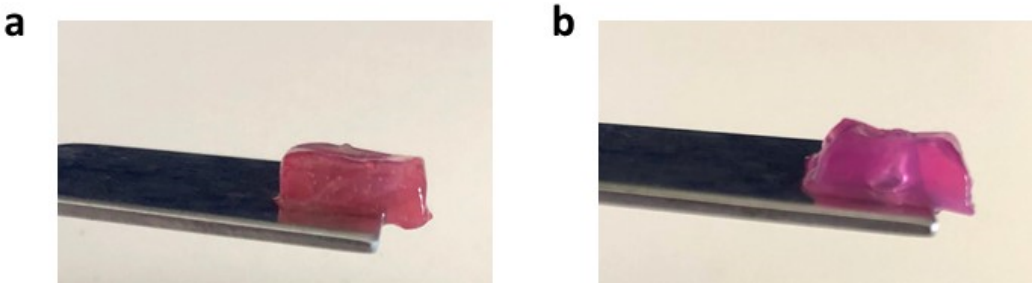


Figure 3.16 1% alginate hydrogels. a. extracted, b. commercial.

to understand live/dead cells. Acridine orange (AO) is a cell-permeable dye, while propidium iodide (PI) is an impermeable DNA-binding dye [40, 46]. In other words, AO stains live cells, whereas PI stains dead or dying cells. To understand cell survival and death, hydrogels were stained with acridine orange (AO) and propidium iodide (PI), and fluorescence images were taken for 7 days. The 2^{nd}, 4^{th}, and 7^{th} days are

shown in Figure 3.17 and Figure 3.18.

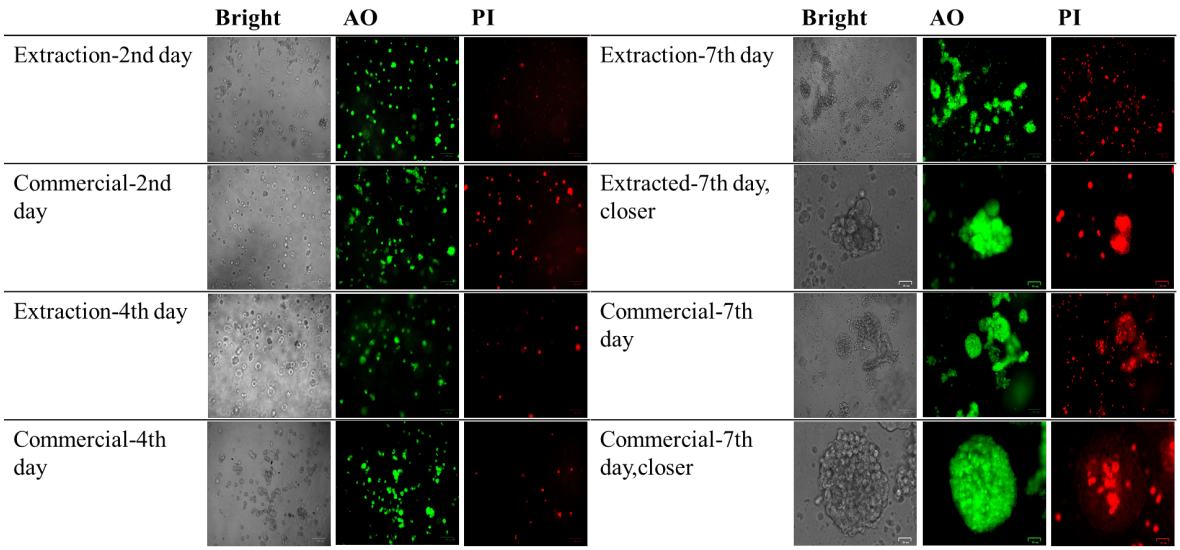


Figure 3.17 Fluorescence imaging of NSC-34 cells, images were taken on 2^{nd}, 4^{th}, 7^{th} day after embedding, extracted, and commercial. Scale bar: 100 µm, for closer 25 µm.

AO and PI stains have successfully stained the cells in the hydrogel; after the 4^{th} day in both extracted and commercial alginate, motor neuron cells migrated to each other and formed sphere-like shapes. These shapes were mostly located in the hydrogels' lowest level, where it was the closest to the floor of the well. On the 7^{th} day, the PI stained more than other days because cellular debris was increased with time.

Unlike the NSC-34 cell line, the SH-SY5Y cells did not migrate to each other to form sphere-like structures. PI stains density increase with time like NSC-34.

Cross-linking is formed between guluronic acid and calcium ions to obtain the alginate hydrogel. Adding calcium chelators, EDTA gives a chance to dissolve hydrogel and release cells [6, 21]. After fluorescence imaging, the percentage of dead cells into living cells was calculated to dissolve hydrogel with 0.5M EDTA, and cells counted after trypan blue staining. The rate of dead/live cells was not significantly different between extracted or commercial alginate hydrogels (shown in Figure 3.19 and 3.20).

Two biological replicates of NSC-34 cells were counted for every day after embed-

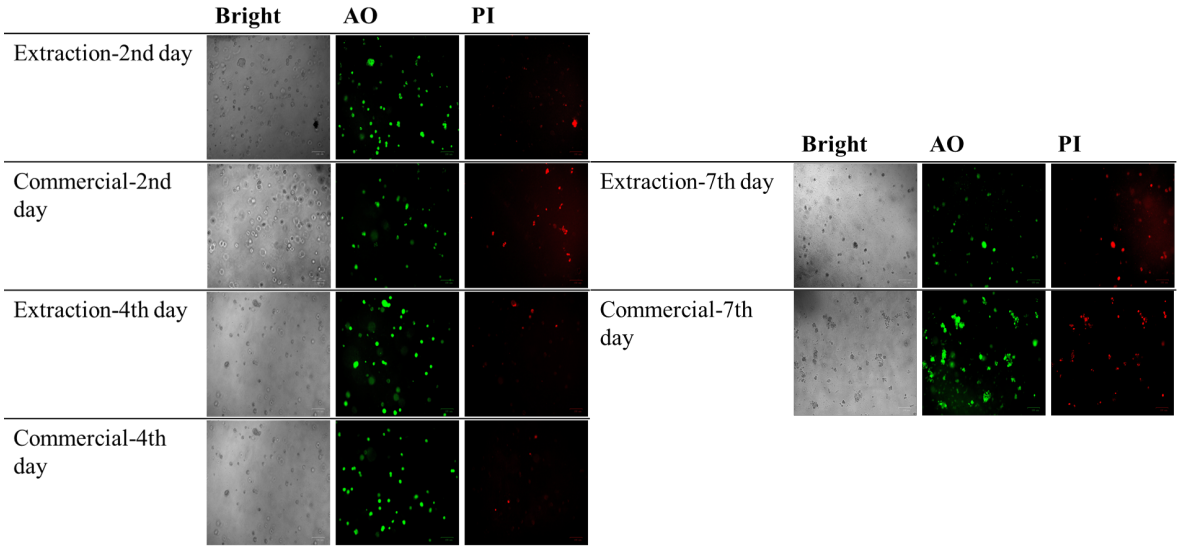


Figure 3.18 SH-SY5Y fluorescence imaging was taken on the 2^{nd}, 4^{th}, and 7^{th} day after embedding. Scale bar: 100 µm.

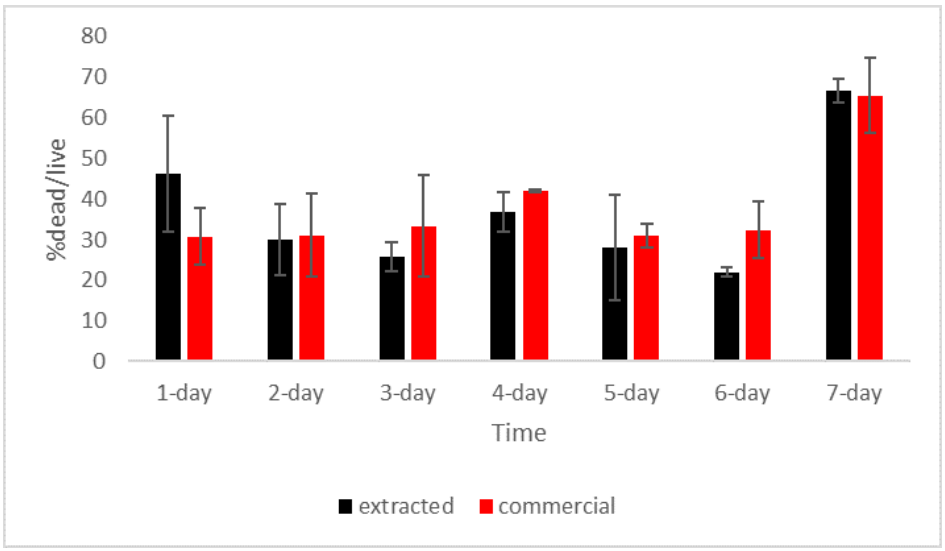


Figure 3.19 Dead/live cell percentages of NSC-34 cells for 7 days. Error bars indicate standard deviation.

ding. The means of these two replicates were taken, and dead cells were proportioned to live cells for both extracted and commercial ones. Standard deviations of samples were calculated with the same method; instead of taking mean, the standard deviation calculations were done. The percentage of dead/live cells decreased for 3 days, which means living cell numbers increased more than dying cell numbers. On the 4^{th} day, the increase of dying cells was more than living cells. 7^{th} day had the highest dead/live ratio; the living cells and dead cells almost had the same amount between each other.

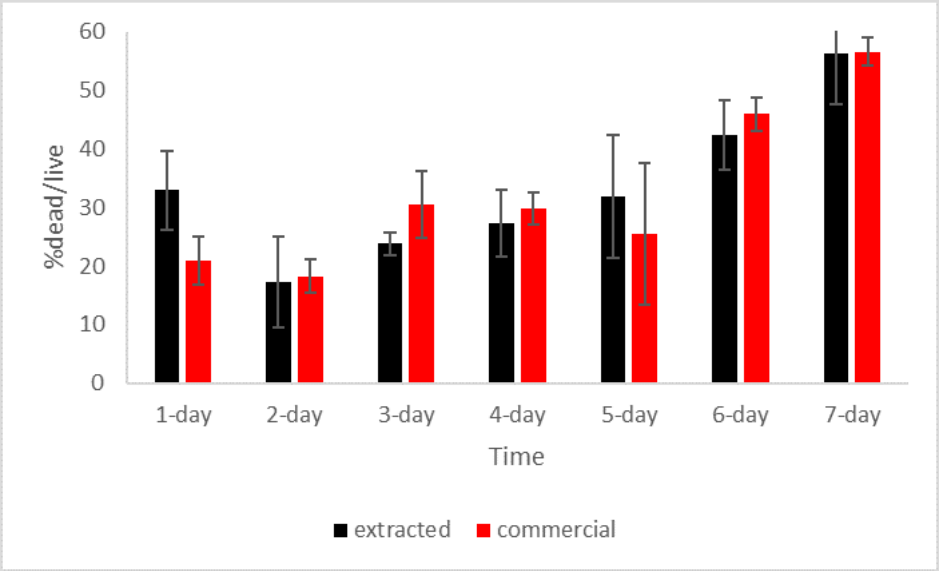


Figure 3.20 Dead/live cell percentage of SH-SY5Y cells for 7 days. Error bars indicate standard deviation.

The counting method was the same as the other cell line; two biological replicates were used. Unlike NSC-34, the SH-SY5Y cells started to die after the 2^{nd} day under these conditions, the death rate increasing over the days (Figure 3.20).

3.3.1 Fresh and one-week-old alginate & old and secondly filtered

Fresh alginate solutions were prepared from lyophilized samples for each experimental setup, whereas the one-week-old alginate solutions were also used to compare shelf-life of dissolved alginate hydrogels in 3D cultures of NSC-34 cells. In addition to these, cells were seeded on the alginate hydrogel in Appendix D.2. Only one of the replicates was shown in Figure 3.21 (the other replicates can be found in Appendix D.1)

After the 3^{rd} day, the pigmentation of old alginate was increased; therefore, although no significant difference in cellular survival was observed, preparing fresh alginate solutions appeared to be more suitable for tissue culture applications, particularly in the case of imaging.

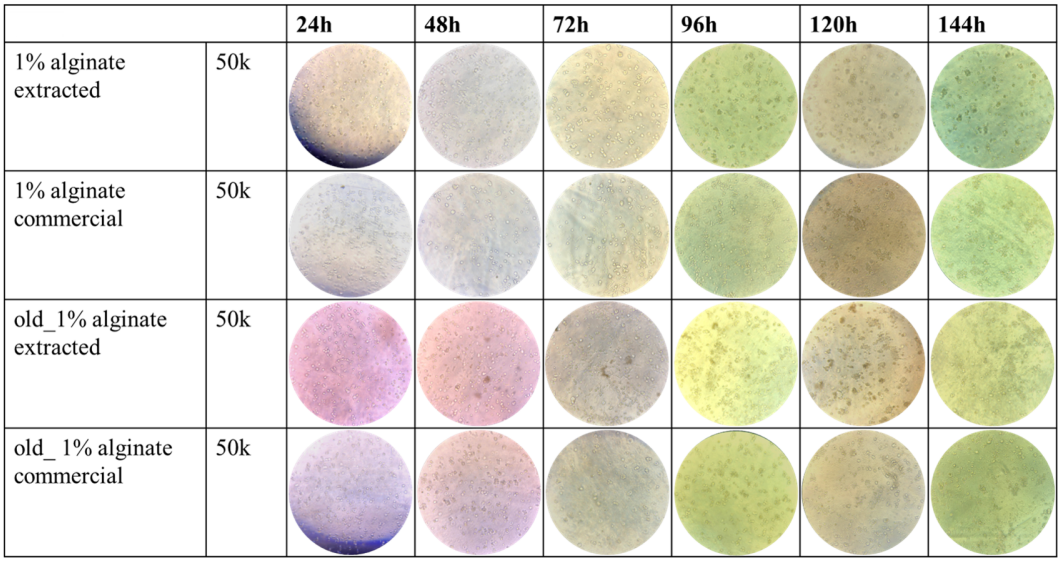


Figure 3.21 NSC-34 cells were embedded into hydrogels prepared from fresh and one-week-old alginate extracts.

The one-week-old alginate solutions were filtered with a 0.22 µm syringe filter to decrease pigmentations, and the experiment was set with fresh and old and second filtered alginate (Figure 3.22).

The pigmentation problem with extracted alginate was found to decrease with the extra filtering step (twice-filtered alginate), and similar to fresh alginate hydrogels, there were no significant differences in cellular behavior between twice-filtered and fresh (filtered once) alginate (Figure 3.22). However, qualitatively fresh alginate hydrogels were found to give a better background for imaging purposes.

3.3.2 Alginate/collagen hydrogel

To increase cellular attachment, the collagen was used for 1:1, 1:2, and 2:1 alginate/collagen mixtures for the NSC-34 cell line to address whether better cell attachment and axonal outgrowth can be achieved (Figure 3.24).

Initially, wells were coated with collagen in order to determine optimum coating conditions. Collagen coating was done with the concentration of 1.6 µg/mL for a 96-well plate; cells were seeded on collagen-coated wells and regular wells for 50,000

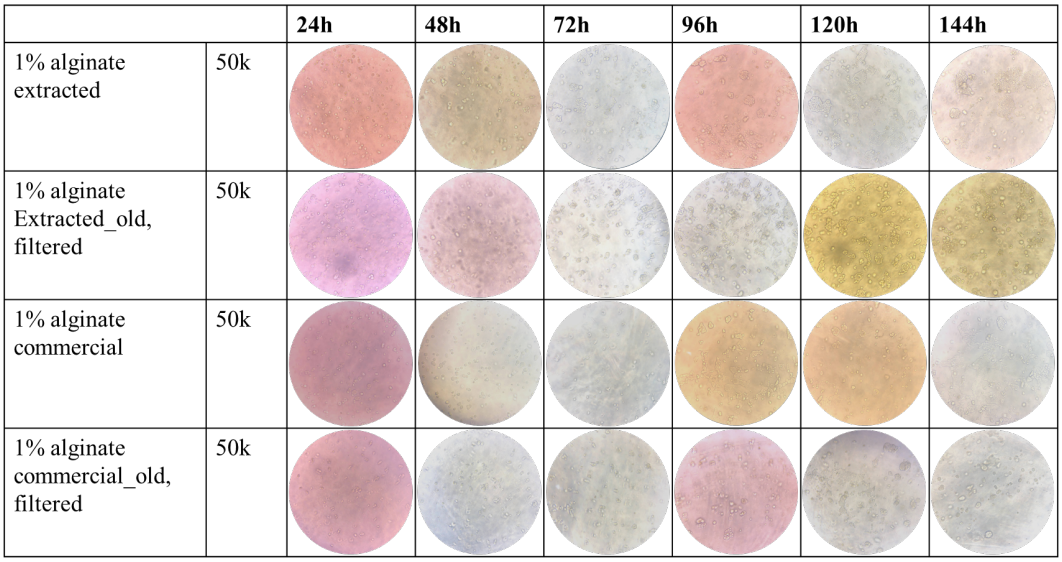


Figure 3.22 Cells were embedded in hydrogels prepared with 1 week-old alginate, twice-filtered alginate, and fresh alginate (filtered only once) with extracted samples, as well as commercial alginate as control.

cells/well (Figure 3.23). The cells seeded on collagen-coated wells proliferated faster than non-coated ones, and they reached confluency on the 3^{rd} day while cells seeded on regular well advanced on the 4^{th} day.

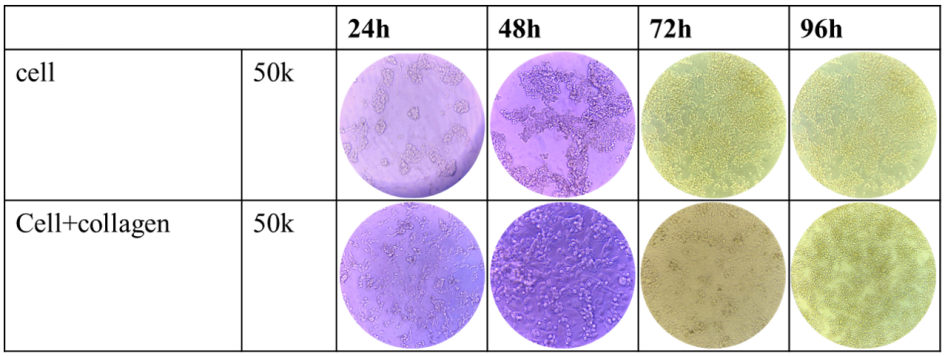
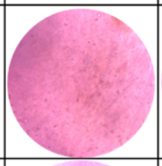
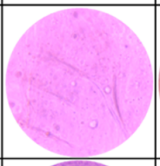
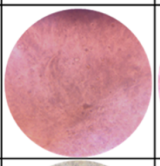
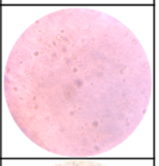
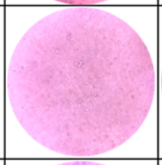
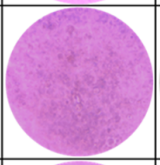
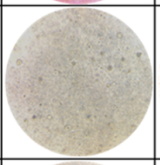
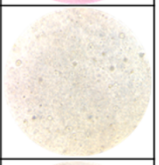
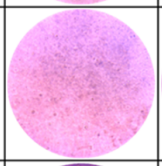
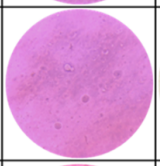
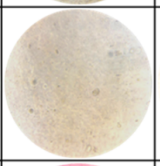
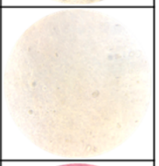
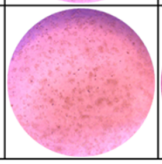
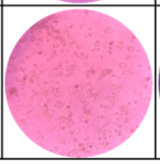
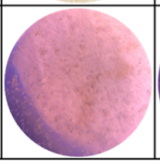
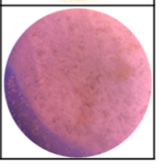


Figure 3.23 NSC-34 cells were seeded on plate and collagen coating well for 5×10^4 cells/cell.

For collagen-alginate mixed hydrogels, the initial concentration of collagen was used at 3 mg/mL and final collagen concentrations were 0.5 mg/mL, 0.67 mg/mL, and 0.33 mg/mL to obtain 1:1, 1:2, 2:1 alginate/collagen ratios, while alginate concentration was 1% for all conditions.

Cells in 2:1 alginate/collagen hydrogels seemed healthier than other concentrations, especially in extracted alginate samples. During collagen optimization, increasing

a

		24h	48h	72h	96h
1% alginate extracted	50k				
1% alginate extracted 1:1 collagen	50k				
1% alginate extracted 1:2 collagen	50k				
1% alginate extracted 2:1 collagen	50k				

b

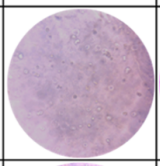
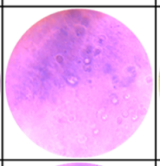
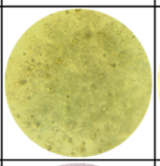
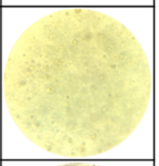
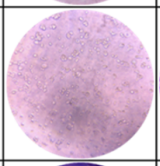
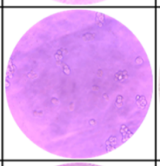
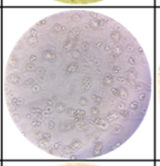
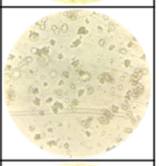
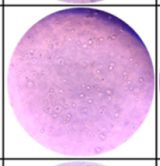
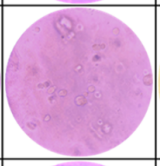
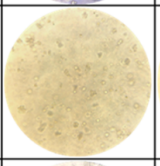
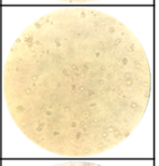
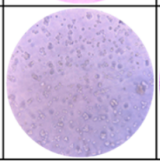
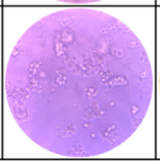
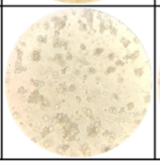
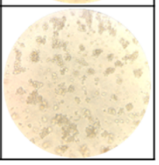
		24h	48h	72h	96h
1% alginate commercial	50k				
1% alginate commercial1:1 collagen	50k				
1% alginate commercial 1:2 collagen	50k				
1% alginate commercial 2:1 collagen	50k				

Figure 3.24 Alginate/collagen mixtures hydrogel with different collagen concentrations: a. extracted alginate was mixed with collagen in the ratio of 1:1, 1:2, 2:1, b. commercial alginate was mixed with collagen at the rate of 1:1, 1:2, 2:1.

cell attachment or cellular growth was not observed.

After decided to alginate/collagen ratio, the experiments with collagen were set with 3 biological replicates for both NSC-34 and SH-SY5Y cell lines (Figure 3.26 and

Figure 3.28). Cell controls were also included in these experiments (Figure 3.25 and Figure 3.27).

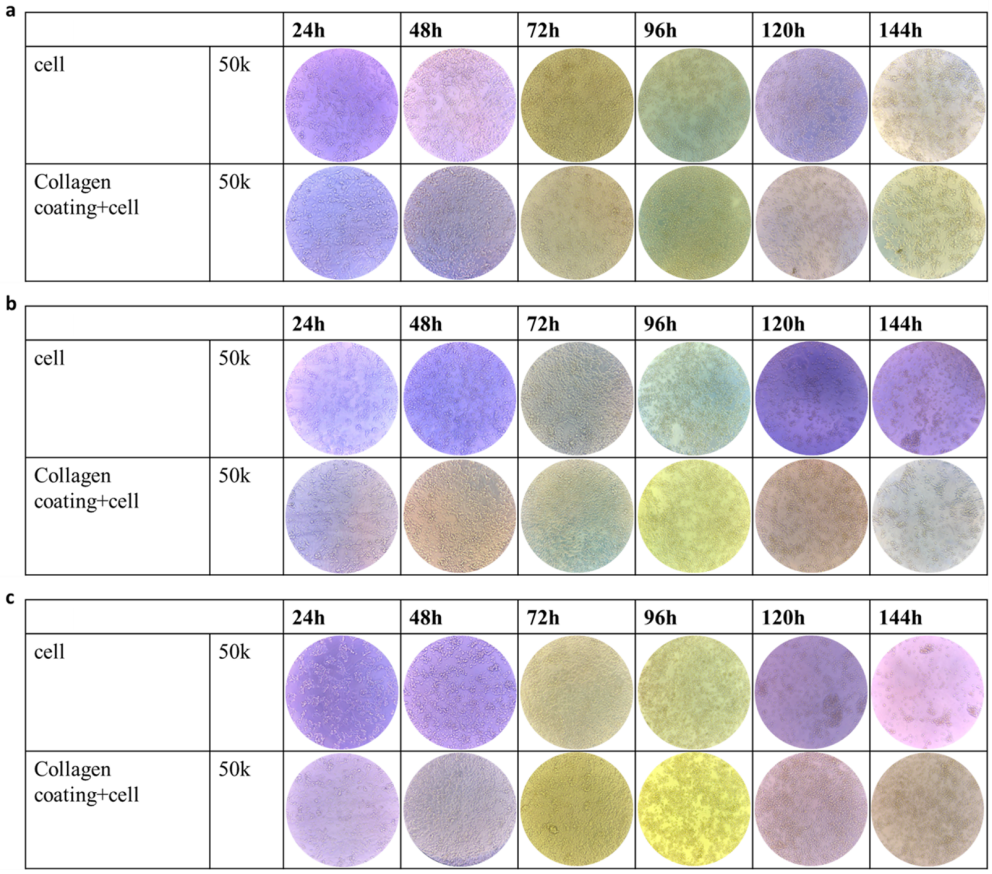


Figure 3.25 NSC-34 cell controls for embedded cells in alginate/collagen hydrogel. a. first biological replicate, b. second biological control, c. third biological control.

Images were taken for 6 days, and the situation of confluency did not change with replicates; all were the same as the previous set (Figure 3.25).

When NSC-34 cells embedded within alginate/collagen hydrogels, it was observed that cell migration was decreased with adding collagen, but axonal growth was observed neither with alginate nor alginate/collagen hydrogels.

SH-SY5Y cells were embedded with alginate/collagen hydrogel with the same ratio of NSC-34 cell line; 2:1. 75,000 cells/well were embedded, and the same number of cells were seeded with and without collagen-coated surfaces to compare the collagen effects on the surface (Figure 3.27) and inside the hydrogels (Figure 3.28).

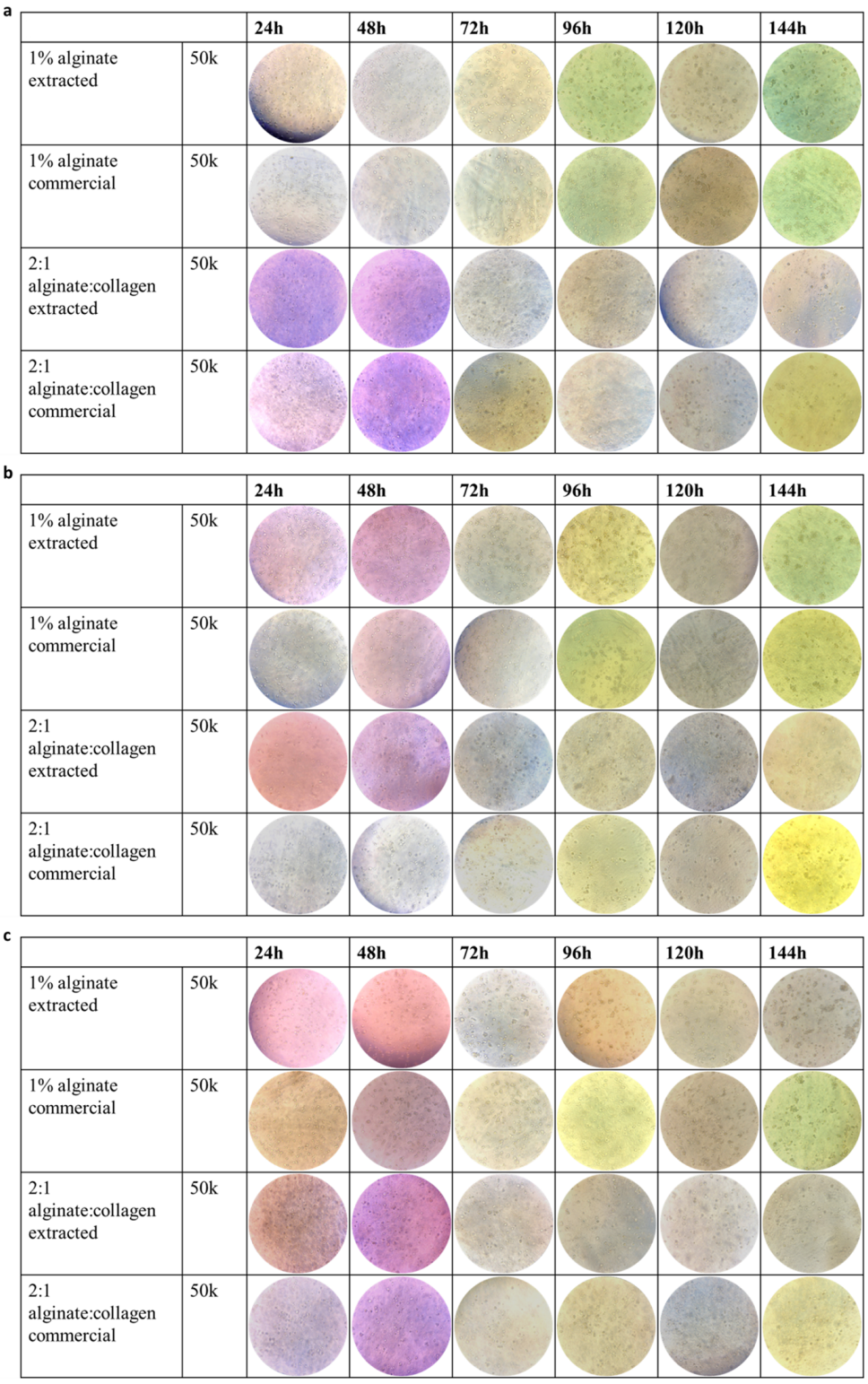


Figure 3.26 Collagen mix with 2:1 ratio (alginate/collagen), NSC-34 cells embedded with alginate and alginate/collagen hydrogels. a. first replicate, b. second replicate, c. third replicate.

Cells reached confluency in collagen-coated surfaces after the 3^{rd} day, and for non-coated surfaces, the confluency of SH-SY5Y was on the 4^{th} day because of the

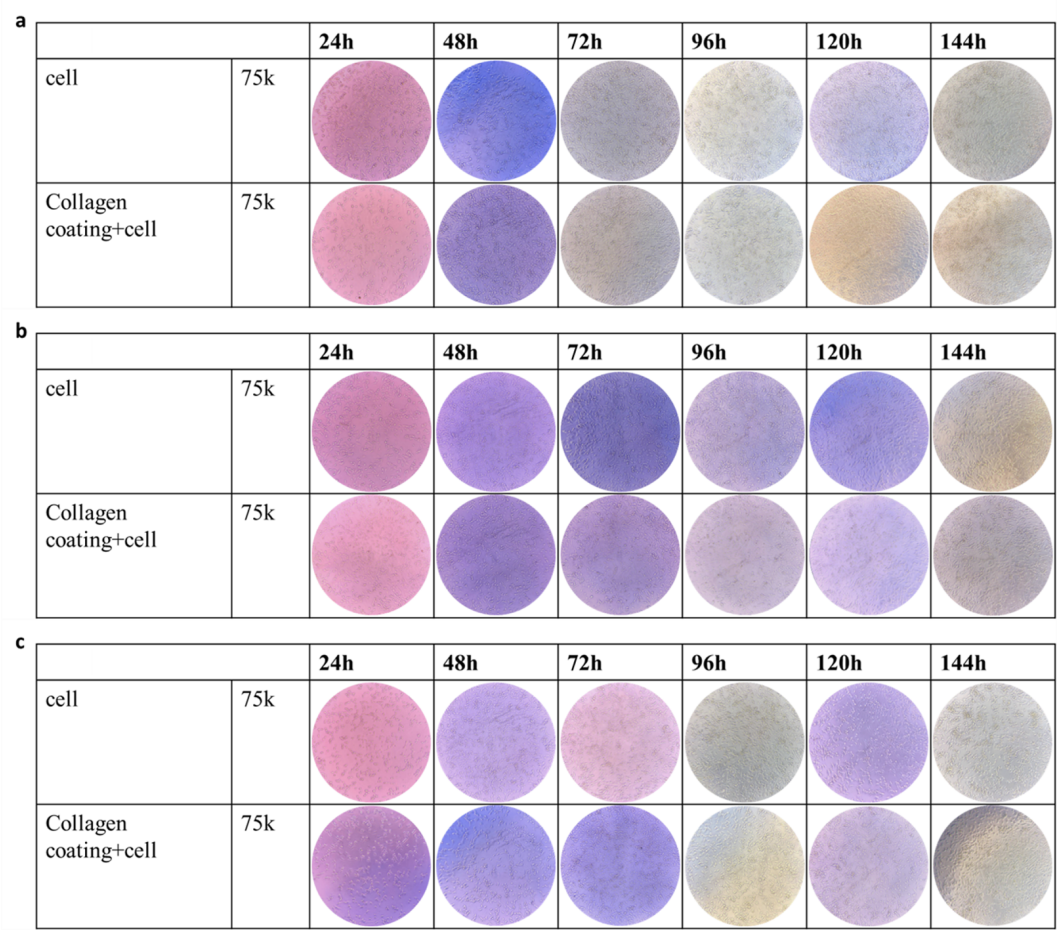


Figure 3.27 Three biological replicates of SH-SY5Y were seeded on well with and without collagen coating. a. first biological replicate, b. second biological control, c. third biological control.

doubling time of SH-SY5Y. The same number of cells were embedded in alginate, and alginate/collagen hydrogels, and images were taken for 6 days (Figure 3.27).

Cellular behaviors did not change with collagen mixture in alginate hydrogels for SH-SY5Y; there was no significant cellular growth or axonal outgrowth.The only difference for both cell lines in alginate/collagen hydrogels appeared to be increased with hydrogel depth comparing with only alginate hydrogel. Collagen could affect the capturing on optical view from the hydrogel due to fibril structure characteristic of collagen.

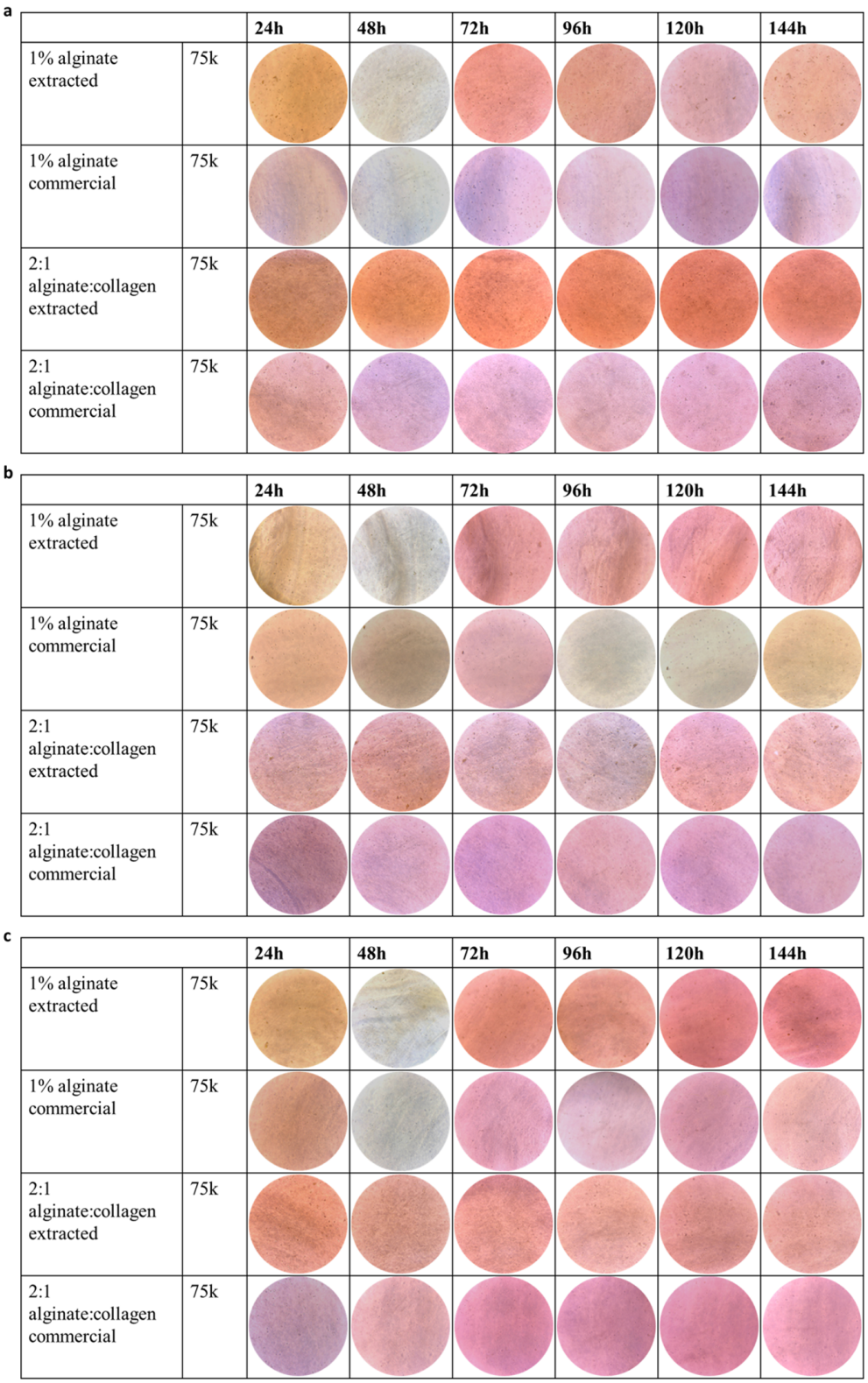


Figure 3.28 Collagen mixes with 2:1 ratio (alginate/collagen) SH-SY5Y cell line. a.first replicate, b. second replicate, c. third replicate.

3.3.3 Neuronal Differentiation in alginate hydrogels

NSC-34 cell line is a hybrid cell line originated in embryonic mouse motoneuron-enriched primary spinal cord and neuroblastoma, and it has the ability to differentiate into motor neurons using NGF and FGF [38, 47]. SH-SY5Y is a human neuroblastoma cell line, and it can be differentiated into human mature neurons with different methods. Differentiated SH-SY5Y cells have long and branch axons, slow proliferation rate [48,49]. In this part of the thesis, we wanted to address whether alginate hydrogels supported neuronal differentiation.

3.3.3.1 Differentiation with NGF for NSC-34 cell line. As per the previously optimized differentiation procedure from AXANLAB, NSC-34 was differentiated using 50 ng/mL NGF (differentiation medium); when adapting this protocol for alginate hydrogels, cells were treated by the medium with NGF prior to seeding on the hydrogel. On the 3^{rd} day, the cells started to differentiate (Figure 3.29) [38].

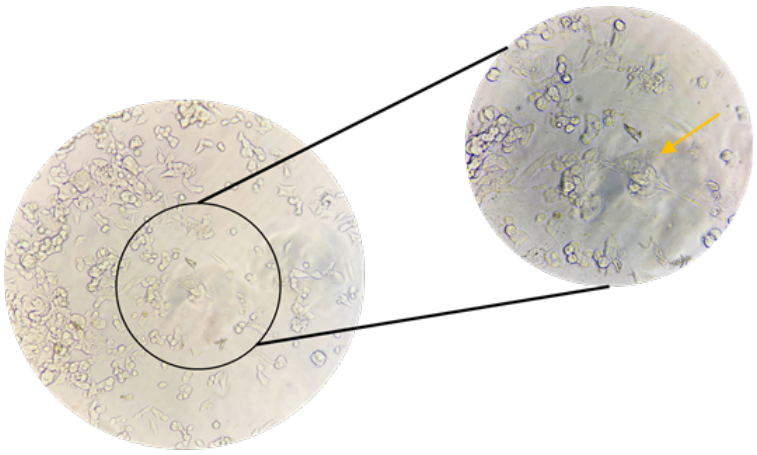


Figure 3.29 NSC-34 cells were differentiated after treatment with 50 ng/mL NGF, imaging on the 3^{rd} day. The image with the arrow was the closing presentation of the image. The yellow arrow shows the NSC-34 cell differentiated into motor-neuron.

After observed cell differentiation with NGF, the cells were embedded in alginate with complete DMEM, including 50 ng/mL NGF (Figure 3.31). Two biological replicates were used; cells were treated with NGF (Figure 3.30). The cells were seeded on a 96-well plate with a density of 50,000 cells/well.

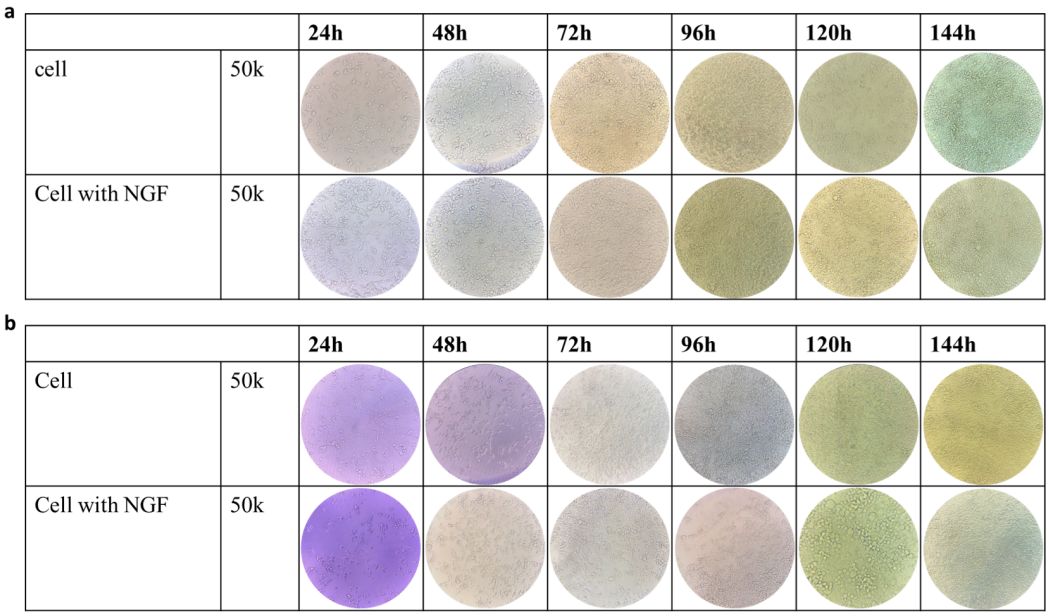


Figure 3.30 NSC-34 cell differentiation with 50 ng/mL NGF included DMEM. a. first replicate, b. second replicate.

NGF-treated cells started to differentiate on the 3^{rd} day, and after the 4^{th} day, the cellular differentiation was not observed due to over-confluency (Figure 3.30). The cells were embedded alginate as the same density and with DMEM including 50 ng/mL, NGF to achieve the same conditions with standard conditions.

The migration of cells increased with the addition of NGF; the clumps were increased earlier, about 2^{nd} to 3^{rd} day. It can be said from observations, and cellular debris was decreased with NGF for 6-day observations (Figure 3.31).

3.3.3.2 Differentiation with FGF for NSC-34 cell line. The cells were exposed to medium with FGF 10 ng/mL, a previously used procedure in AXANLAB [38]. The first step was to ensure cell differentiation with FGF like NGF experiments; the differentiation period was longer than NGF; it took 4 to 5 days to differentiate (Figure 3.32).

The cells were embedded in alginate hydrogel with a differentiation medium, including 10 ng/mL FGF, for three biological replicates (Figure 3.34). Cells were

seeded to plate to control cellular differentiation (Figure 3.33).

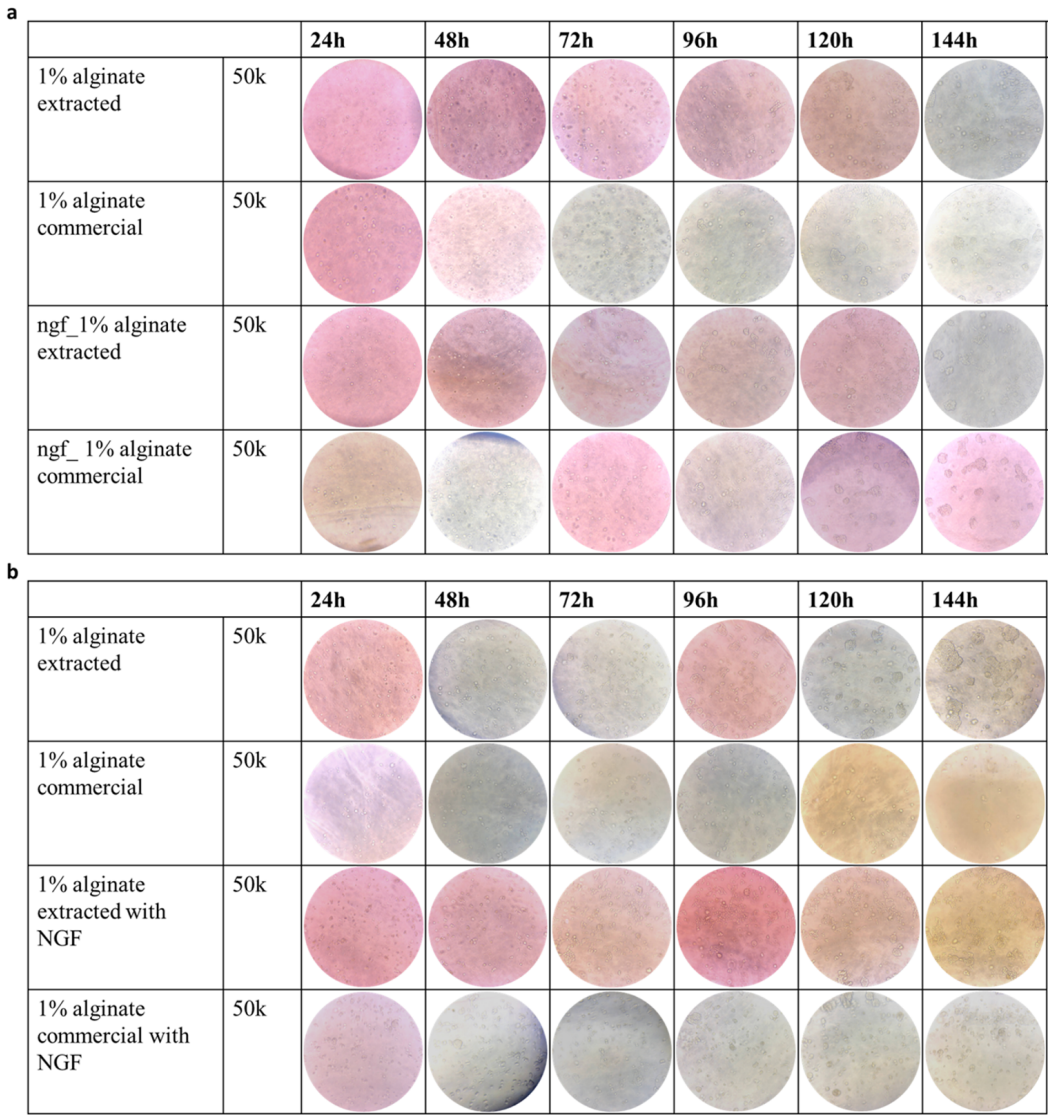


Figure 3.31 NSC-34 cells embedded with & without included NGF. a. first biological replicate, b. second biological replicate.

The cell differentiation was observed on the 4^{th} day (Figure 3.33), and cells embedded in alginate were imaged simultaneously (Figure 3.34).

The cellular migration was decreased with using FGF, and cell clusters were not observed. The cellular lifetime was increased as well (Figure 3.34).

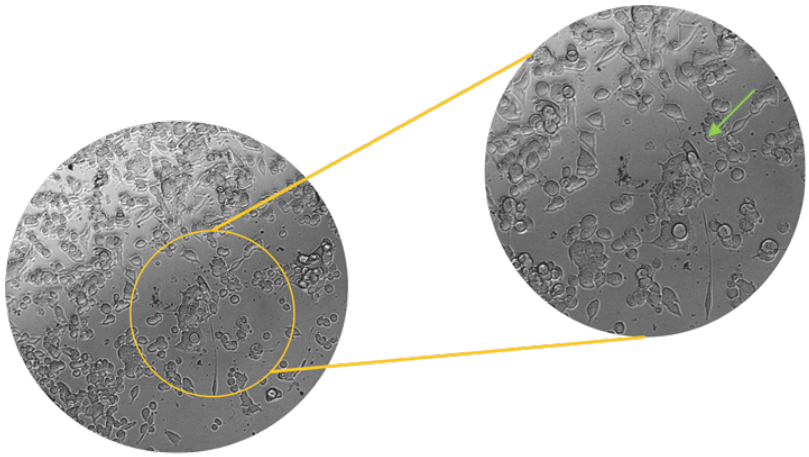


Figure 3.32 NSC-34 cells were differentiated with 10 ng/mL FGF—the image on the 4^{th} day. The image with the arrow was the closing presentation of the image. The green arrow represents the NSC-34 cell to differentiate into motor-neurons after FGF treatments.

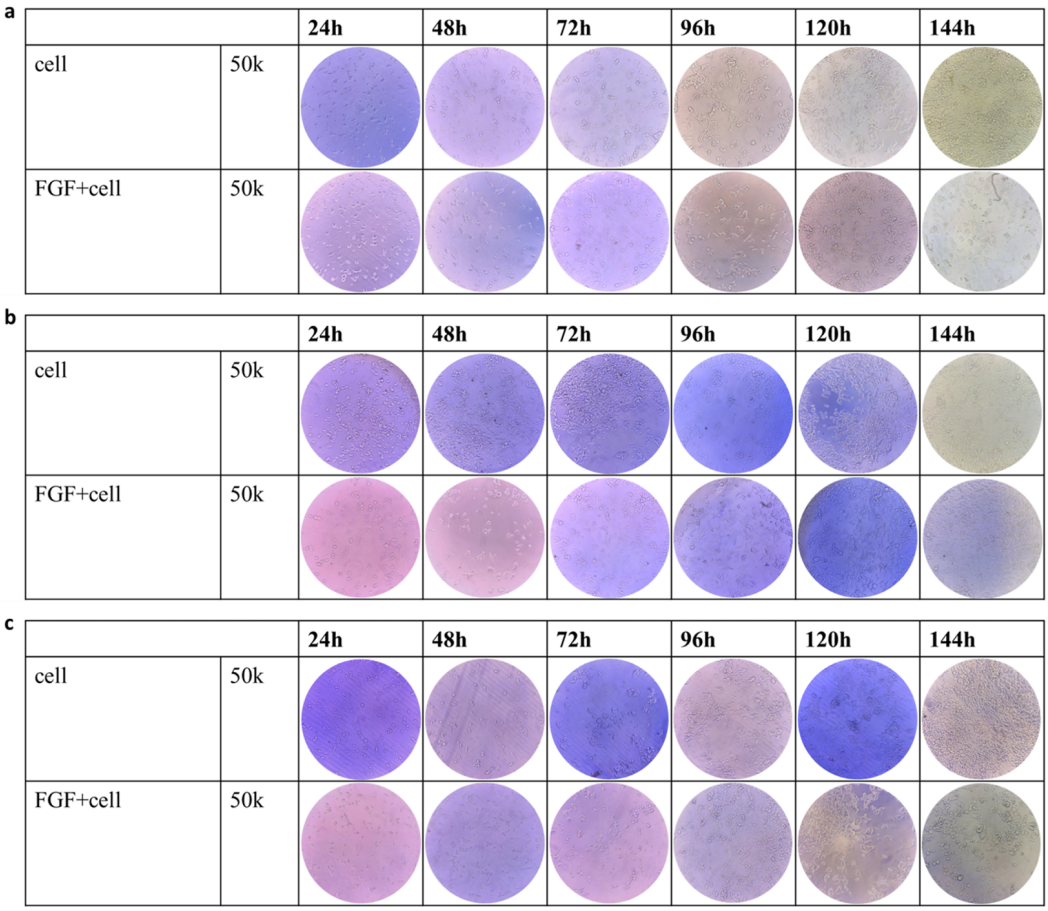


Figure 3.33 NSC-34 cell line differentiation using FGF controlled. a. first replicate cells with medium and medium including FGF, b. second replicate with DMEM and DMEM with FGF, c. third replicate of cells with medium and medium with FGF.

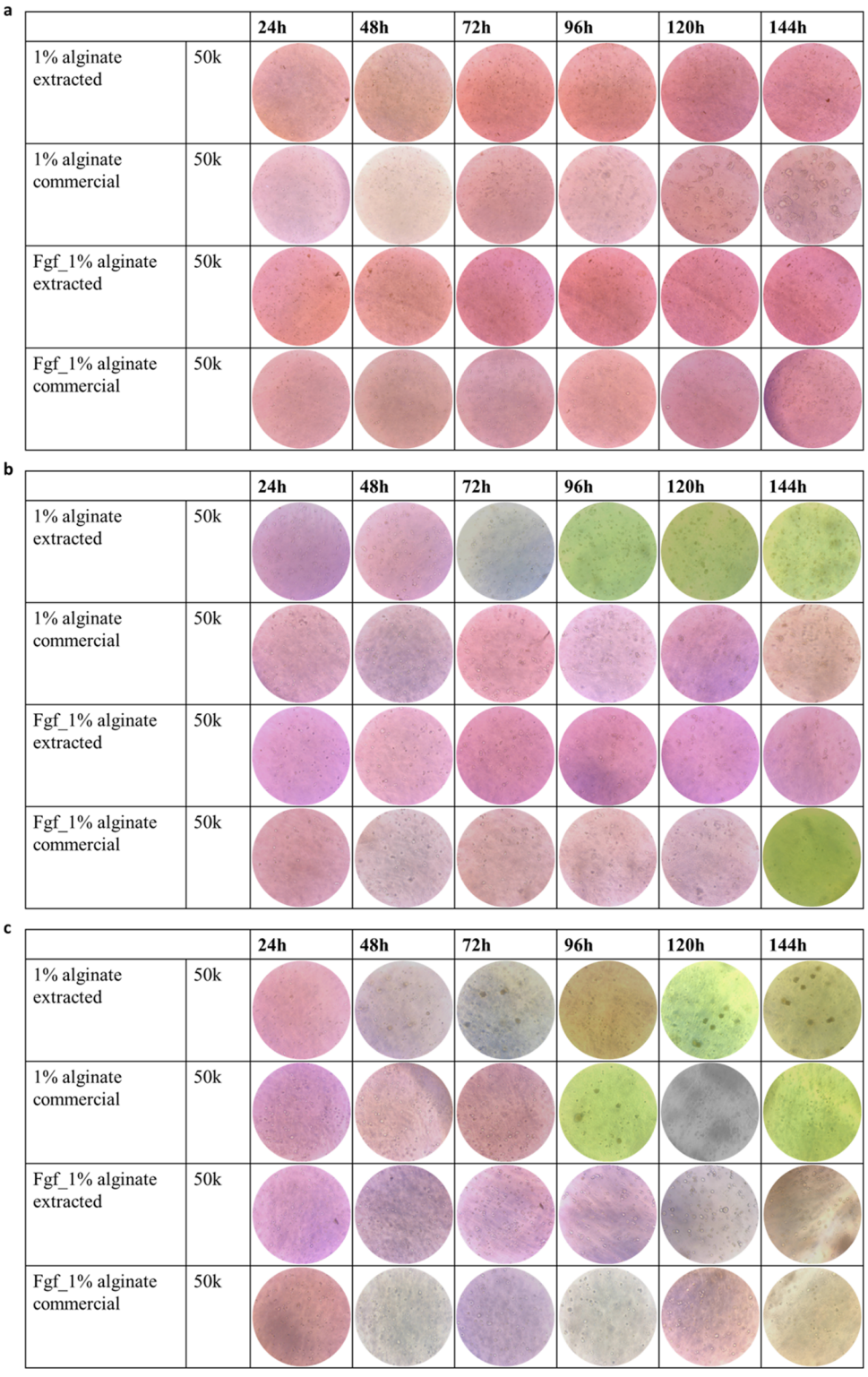


Figure 3.34 NCS-34 cells embedded with & without FGF included medium. a. first replicate, b. second replicate, c. third replicate.

3.3.3.3 Differentiation with serum withdrawal and RA for SH-SY5Y.

The differentiation method of neuroblastoma cells was different from motor neurons, de-

scribed in 2.2.11. Cells embedded with medium including 1% FBS, 1% pen/strep, and 10 µM RA for 7 days (Figure 3.37). For differentiation, cell medium was changed with DMEM including 1% FBS and 1% pen/strep on the 2^{nd} day, the next day; the medium was exchanged with DMEM including 1% FBS, 1% pen/strep, and 10 µM RA. The medium was refreshed every two days after adding RA. To control neuron differentiation, cells were seeded on the plate (Figure 3.35) [38].

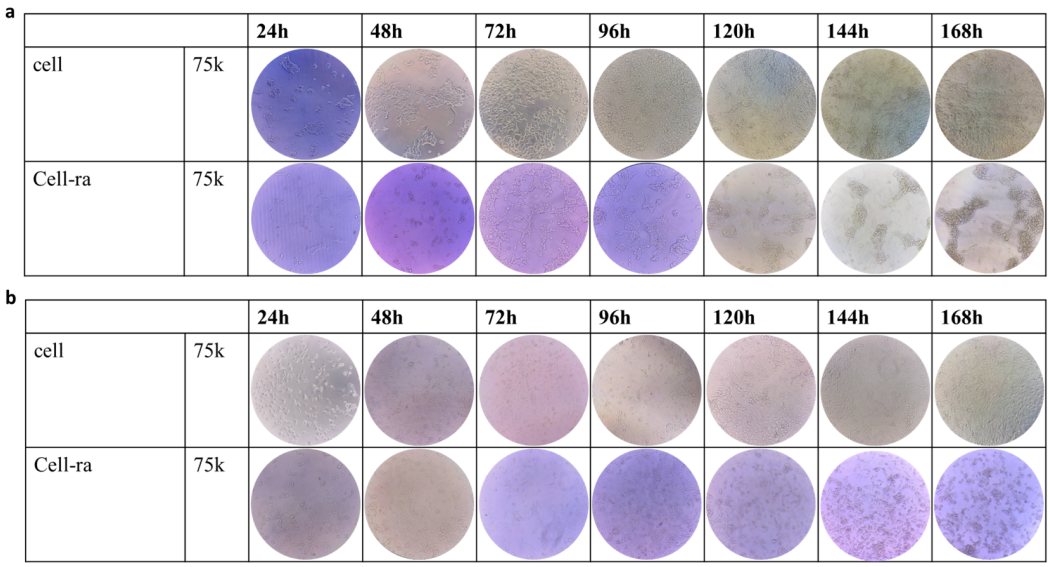


Figure 3.35 SH-SY5Y cell seeding to control cellular differentiation outside of the hydrogel. a. first replicate, b. second replicate.

Cellular differentiation in 2D enviroment was not successful for this cell line (Figure 3.35); after adding the medium, including RA, cells started to die. Even though cells were not healthy with this condition, embedding was done with the same conditions. Cells were imaged with ZOE fluorescent cell microscopy to see the exact shapes of the cells (Figure 3.36).

Serum withdrawal and RA treated cells within hydrogel were not healthy like cells on the surface; no cellular differentiation, axonal outgrowth, or cellular growth were observed. The differentiation of SH-SY5Y cells within the hydrogel was not successful.

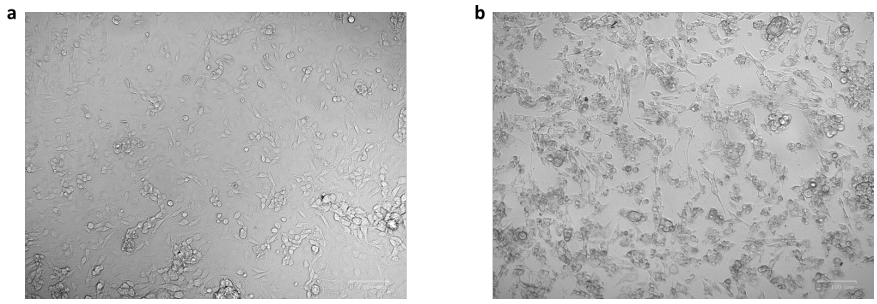


Figure 3.36 SH-SY5Y cells were investigated with ZOE; images were taken on the 6^{th} day after seeding. a. cells with normal conditions, b. cells with RA treatment. Scale bar: 100 µm.

a

		24h	48h	72h	96h	120h	144h	168h
1% alginate extracted	75k							
1% alginate commercial	75k							
ra_1% alginate extracted	75k							
ra_1% alginate commercial	75k							

b

		24h	48h	72h	96h	120h	144h	168h
1% alginate extracted	75k							
1% alginate commercial	75k							
ra_1% alginate extracted	75k							
ra_1% alginate commercial	75k							

Figure 3.37 SH-SY5Y cells were embedded in hydrogels and hydrogels including RA for 7 days. For embedding with medium including RA, the medium exchanges were similar with usual embedding methods but with medium with RA. a. first replicate, b. second replicate.

4. DISCUSSION

In this study, we have investigated the potential of alginate extracted from seaweed originally present in Tuzla as the growth medium for the differentiated NSC-34 and SH-SY5Y cell lines. The alginate was extracted within the scope of the OBEK platform for 3D cell culture applications. The mannuronic acid and guluronic acid composition on extracted and commercial alginate were found to be similar in chemical characterization studies, and they were both found to be guluronic acid-rich. According to FTIR results, the expected gelation characteristics were the same for extracted and commercial alginate. The chemical components percentages of extracted alginate are similar to literature values extracted from *Cystoseira barbata* and commercial alginate. The fluorescence spectroscopy was shown that there were no significant phenolic compounds, so impurities in both samples. The percentage of these two samples'purity was similar, and they were ready to use without any purification steps [30].

Alginate can have low viscosity, medium viscosity, and high viscosity; the numbers to distinguish alginate as viscosity characteristic are <240 mPas, 240-3500 mPas, and >3500 mPas, respectively [29]. According to this information, extracted and commercial alginate had low viscosity characteristics after the viscometer results, and they both have a similar density. Extracted and commercial alginates have a high flow rate and low molecular weight due to low viscosity properties [29, 50].

Alginate is known to be biodegradable. To investigate whether the extracted alginate sample in our study exhibited similar degradability profiles, degradation tests were performed. Degradation results showed that which alginate solution, extracted or commercial, old or fresh, once or twice filtered, used for gelation was not essential considering the release. Degradation of alginate hydrogels was increasing with time, and after the 10^{th} day, due to lack of liquid, the hydrogel started to dry. The degradation of alginate may cause the color change of the medium.

Characterization analysis showed that extracted alginate and commercial alginate could be used for comparison due to similar structures in all aspects: the chemical composition, purity, viscosity, and degradation.

During alginate concentration optimization, it was observed that the increases with concentration caused increasing pigmentations, and this pigmentation affected cell debris in extracted alginate. Because of that, the alginate concentrations were decided to reduce by 1% and 0.5%. Due to hydrogel quality in 0.5% alginate hydrogel, the optimization was done with a 1% alginate hydrogel. Also, the color of the extracted alginate solution was darker than the commercial solution. Therefore, increasing pigmentation can be caused by the extraction method. For further experiments, alginate extraction could be obtained with fewer pigments.

Meanwhile, cell density was optimized, considering imaging and cell behavior. In the alginate, cells were optimized to 10^5 cells/well for NSC-34 cell line for 24-well plate and 96-well plate experiments; the cells density was estimated to 5×10^4 cells/well, while SH-SY5Y cell density was optimized to 7.5×10^4 cells/well. The extraction method, the collected season of algae, type of algae could affect the alginate properties.

Non-modified alginate hydrogel does not support neurite growth; additionally, neurons tend to form spheroids [51]. Fluorescence imaging results showed that NSC-34 cells migrated towards each other and formed spheroid-like structures, which is assumed to be a survival response in relatively low cell attachment in alginate. Our qualitative observations suggest that these cells survive better as spheroids than single cells, which could be due to paracrine signaling within the spheroid structure. Also, the spheroid structure formed on the edges and surface-closer areas of the hydrogel, which could have lower stiffness, gives cells some living chance to form spheroids. The cells' morphology did not change after embedding; it was a round-shape from the beginning to the end of the experiments, suggesting these neuronal model cells, which are known to have few surface receptors that can interact with alginate, did not properly attach to hydrogels. In some cases, the cellular extensions were observed during movements, or cells were getting bigger with time differently from 2D cultures.

On the other hand, SH-SY5Y cells did trend to form clusters but not as big as NSC-34 cells did, at least for 7 days. Cells in alginate hydrogel were in round-shape form from embedded to end; no cellular growth was observed. When cell seeding density is too low, adherent cells may be inclined to clustering toward growth factors secreted by nearby cells through a paracrine signaling pathway. With diffusion, these growth factors reached the neighboring cells, as the migration and clustering of NSC-34 and SH-SY5Y could happen [52].

Depending on time, the cells started to die in both extracted and commercial alginate hydrogels, suggesting this is not a toxic impurity complication due to extraction procedure, but a neuron-specific response to specifically alginate. After the 5^{th} day, the dead cell number and live-cell number became almost similar. It was an expected result, but it can be said that until the 5^{th} day, the cell number increased in both cell lines. There was a proliferation in embedded SH-SY5Y and NSC-34 cell lines.

To increase and to mimic the ECM, collagen was mixed with alginate hydrogel to investigate whether cellular attachment and thus survival could be improved. Three different final concentrations of alginate/collagen ratios were tried, but none of them was found to support neurite growth or cell proliferation in either cell line (Figure 3.26 and Figure 3.28). The cells did not look like in alginate hydrogel when the collagen concentration increased (Figure 3.24). Increasing stiffness of hydrogels may have made the gel harder to survive; it was reported in the literature that alginate/collagen composite hydrogel supports neurogenesis in cortical neurons [53]. According to Adamson and colleagues in collagen hydrogels, NSC-34 cells proliferate but do not have neurite extension [54]. For collagen hydrogels, collagen concentration can be caused by decreasing or increasing cell proliferation depending on cell type [55, 56]. According to Desai and colleagues, they did not see any significant difference for SH-SY5Y cell line proliferation in 2D and 3D culture for collagen [55]. The collagen affects different cell type profile, and alginate does not support cellular growth to gather this information. Combining these two materials also did not support cell proliferation or neurite extension for NSC-34 and SH-SY5Y cell lines. Different types of cells can be tried to see proliferation or neurite extension. The cells were not counted in alginate/collagen

hydrogel due to a lack of sufficient amount of collagen; for further experiments, the proliferation rate can be calculated to understand exact numbers. Although the counting was not applied, it can be said that there was not cellular proliferation observed during experiments for both cell lines.

For NSC-34, due to its hybrid conformation, it is possible to differentiate to form longer neurites. The expectation was to achieve neurite extensions in the hydrogel. For this purpose, 50 ng/mL NGF was used. The migration of the embedded period became shorter than in previous experiments. Spheroid-like structures were formed in a shorter time, as well. NGF is an essential molecule for nervous systems; it regulates proliferation, cellular survival, and differentiation to extend axons. Differentiation to nerve cells plays a vital role in repairing, regeneration [57, 58]. After cell embedding with NGF differentiation medium, cells formed clusters in a short time.

FGF is known to regulate both proliferation and differentiation under different conditions [52]. Upon FGF addition, the migration and clustering of the cells were decreased compared to NGF differentiation, which may indicate that these cells respond better to FGF or that this growth factor is required for survival and/or attachment to alginate hydrogel. Cellular survival time was also increased with the addition of NGF and FGF. The cells were not differentiated to a great extent in hydrogels, but cells embedded within hydrogels appeared much better in the presence of growth factors.

RA is an active vitamin A derivative, and there are vital roles in the nervous system, including cell growth, patterning development, and differentiation [59,60]. After adding the medium with RA to cells, instead of differentiating, we have observed that cells exhibited poor survival, and in the hydrogel, no migration or proliferation was apparent. The serum withdrawal and retinoic acid differentiation of SH-SY5Y cells is well-established in AXANLAB; however, the hydrogel environment may not be suitable for this differentiation protocol and may need to be further optimized. On the other hand, the brain-derived neurotrophic factor (BDNF) addition after RA differentiation may give better results in terms of survival of the SH-SY5Y neuron-like cells in the hydrogel.

5. CONCLUSION

In summary, alginate that was extracted from Tuzla province appeared to exhibit similar chemical composition to commercial counterparts and, in general, was suitable for 3D cell culture applications. Many studies in the literature were carried out with stem cells, which do not necessarily require the attachment. Alginate hydrogels do not appear to be best suited for neuronal differentiation experiments unless coupled with FGF treatment because cell lines used in this study are dependent on ECM interactions. Other modifications of alginate may also be necessary to improve cell embedding, survival, and differentiation.

5.1 List of publications produced from the thesis

(i) Characterization of a 3D Neuronal-Culture Using Alginate Hydrogels and Optimize for Neuronal Survival and Axon Growth, B. Dalbayrak, E. Sonmez, H. Kurt, M. I. Hosoglu, I. Kucuk, H. Saybasili, I. Kurnaz, " *Natural & Applied Sciences Journal, IDUNAS*, vol.3/8, special issue, pp. 71–80, 2021.

(ii) Nöron Yaşamının Optimum Olması İçin Aljinat Hidrojeli İle 3B Nöron Hücre Kültürü Oluşturma, B. Dalbayrak, E. Sonmez, H. Kurt, M. I. Hosoglu, I. Kucuk, H. Saybasili, I. Kurnaz, " *18. Ulusal Sinirbilim Kongresi*", Özet Kitabı, 2020

APPENDIX A. EXTRACTION METHOD

The extraction starts with pre-extraction from alginate in algae to alginic acid. Neutralization is done by using sodium carbonate (Na_2CO_3) or sodium hydroxide (NaOH). The next step is precipitation; this step can be done using calcium ions (Ca^{2+}) or hydrochloric acid (HCl). If calcium ions are preferred for precipitation, the product would be calcium alginate, and this calcium alginate can be turned into alginic acid by using HCl. The next step is to use Na_2CO_3 to obtain sodium alginate (Figure A.1) [21].

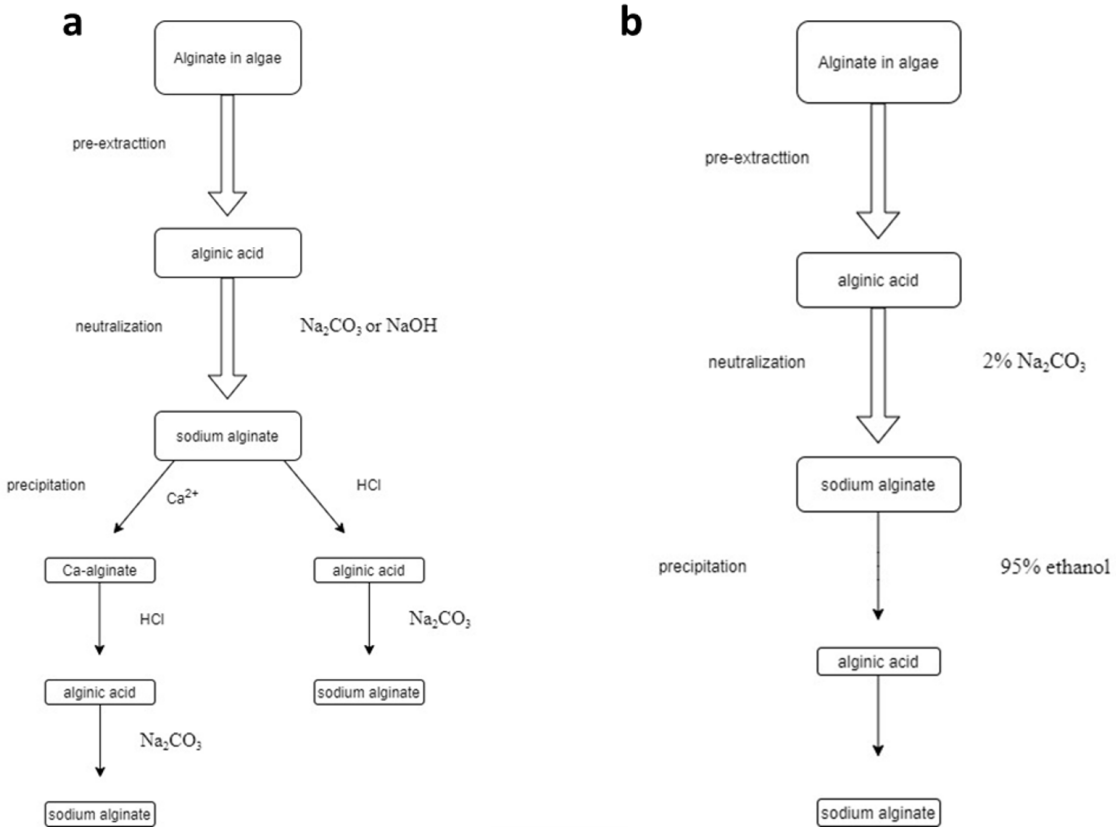


Figure A.1 Alginate extraction methods a. in literature b. the extraction method for using alginate in this thesis.

The extracted alginate used for this thesis was extracted from a Master's student, Habibe Kurt. The extraction was started with dried *Cystoseira barbata*. The dried algae were taken and grounded to have powder form. Powder algae were put

into 0.1M HCl, and the solution was placed on the magnetic stir overnight at 500 rpm, room temperature. Samples were divided into 50 mL centrifuge falcons and centrifuged at 500 rpm, 4°C for 15 minutes. Supernatants were discarded, and distilled water was added into the pellets and centrifuged at 500 rpm, 4°C, for 15 minutes. Supernatants were removed, and pellets were dissolved with Na_2CO_3, and solutions were placed on the magnetic stir at 250 rpm, 60°C for 3 hours. The solution was precipitated with 95% ethanol in a 1:1 ratio. To achieve the solid part, the liquid portion was filtered, and the sodium alginate was lyophilized.

APPENDIX B. CHARACTERIZATION

B.1 FT-RAMAN Results

To promote the FTIR results, the extracted and commercial alginate samples were examined with FT-RAMAN. The results showed that the transmission percent for both samples were similar to others. There were not done any calculations with FT-RAMAN results (Figure B.1); these results were used to verify the FTIR results.

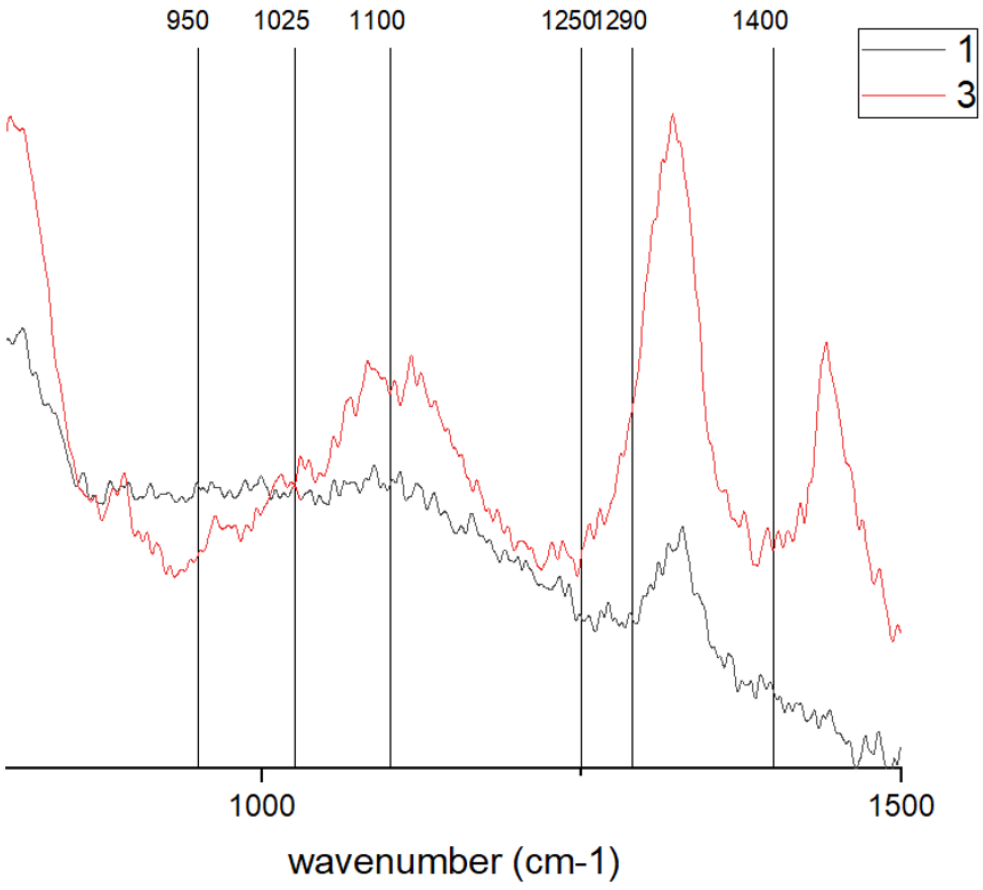


Figure B.1 FT-RAMAN results of extracted, black line, and commercial, red line, alginates.

B.2 Surface Tension

1% (w/v) alginate solution was prepared in distilled water for both commercial and extracted alginates. Alginate solution was taken with the syringe of the device, and the measurements were done with KSV CAM 200 Contact Angle Meter.

The surface tension of extracted and commercial alginate was examined with contact angle devices. The surface tension of extracted alginate was 67.04 ± 0.01 mN/m; for commercial alginate, the number was 66.51 ± 0.12. After the student t-test was applied, the p-value was calculated ($p=0.000721$, $p<0.05$). Extracted and commercial alginate surface tensions were not significantly different.

B.3 Hydrogel Characterization with SEM

For scanning electron microscope imaging, alginate hydrogels were prepared with the same procedure as in 2.2.6. After alginate hydrogels were prepared, they incubated at 37°C, 5% CO_2, for two days. The medium was then removed, and the plate was placed in the vacuum oven at 30°C, 30 mbar for 24-hour for exsiccating. When alginate hydrogels were dried, the dried form of alginate was cut to have a section for SEM imaging. The dried hydrogels were not appropriate for successful results, so to make more extensive sections, alginate hydrogels were prepared in the 2 mL eppendorf for 1 mL alginate for 75k cells/eppendorf. Preparation was the same with others, but the amount of $CaCl_2$ was 1 mL, and washing steps were with 1 mL DPBS and medium. After two days of incubation with 1 mL of DMEM present medium was removed, and eppendorfs were left to dry at the vacuum oven at the same conditions but for 16-hour. The sections were arranged the same.

After alginate hydrogels were taken from the vacuum oven, the sections were prepared for SEM analysis (carried out in Gebze Technical University; courtesy of Instructor Ahmet Nazim). Figure B.2 represents the dried alginate samples. The

images taken by SEM were not conclusive (data are not shown).

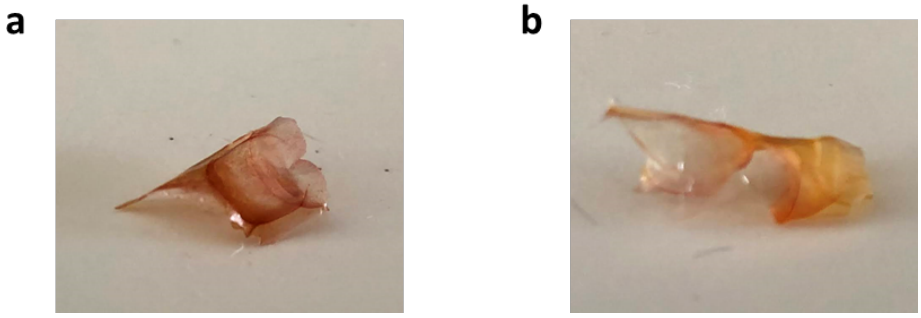


Figure B.2 Dried alginate hydrogels under vacuum oven. a. extracted alginate, b. commercial alginate.

Even different solutions were used to have SEM images; the results did not clear enough to present in this thesis. The reason might be that the drying procedure might affect the porous structure of alginate hydrogel. Because of that SEM investigation could be performed with freeze drying or other methods for drying to observe the hydrogel's porous structure.

APPENDIX C. CELL CULTURE

C.1 Cell Thawing

The cells are taken from a -80°C freezer or liquid nitrogen tank. For SH-SY5Y, after cell solution thawed in the cryovial, cell solution was added into the centrifuge tube with 10 mL DMEM, 4.5 g/L D-Glutamine, (+) pyruvate, 10% FBS, 1% pen/strep to decrease the DMSO effect. The cell solution was centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded, and 1 mL medium was added into the cell pellet to dissolve. Depending on the cell concentration, a T-25 flask, 40 mm dish, or 60 mm dish was used for cell seeding. The dissolved cell solution was added to the selected flask after adding the required amount of medium to this flask; 5 mL for T-25 and 60 mm dish, 1 mL 40 mm dish. When cell seeding was done, cells were placed into the incubator at 37°C with 5% CO_2. When the cells attached to the surface, the existent medium was exchanged with fresh medium.

After cell solution thawed, the cryovial content was directly placed in the proper flask after adding DMEM, 4.5 g/L D-Glutamine, (-) pyruvate, 10% FBS, 1% pen/strep to the flask for the NSC-34 cell line. The flask was replaced into the incubator at 37°C, 5% CO_2. 4-hour later, the medium was replaced with a fresh medium. If the cells did not attach to the surface, cells were incubated further, and the medium was changed after attachment.

C.2 Cell Freezing

For the SH-SY5Y cell line, after cells detached and centrifugated, cells were dissolved to achieve the concentration of 1.11×10^6 cells/mL. 900 µL of cell solution was placed in a cryovial then 100 µL DMSO was added to the cell solution. The cryovial was inducted into the -20°C freezer, then put into the -80°C freezer.

For NSC-34, the freezing medium was prepared with 60% medium, 30% FBS, and 10% DMSO. The cells were dissolved after centrifugation with a concentration of 10^6 cells/ mL using the freezing medium. 1 mL of cell solution in freezing medium was placed into the cryovial and put into the -20°C fridge for 2 hours, then transferred the cryovial into the -80°C freezer.

APPENDIX D. CELLULAR SURVIVAL WITH ALGINATE

D.1 Alginate Hydrogels Comparison Between Old and Fresh & Commercial and Extracted

Two more biological replicate results of Figure 3.21 (Figure D.1).

b

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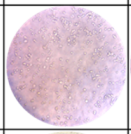
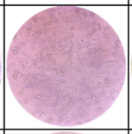
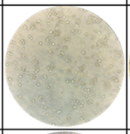
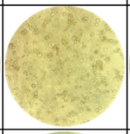
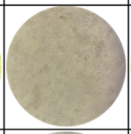
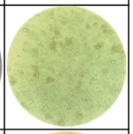
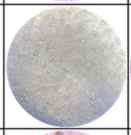
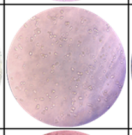
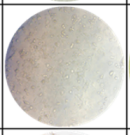
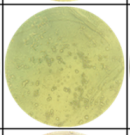
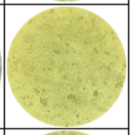
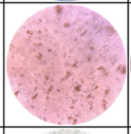
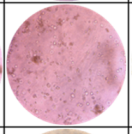
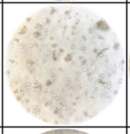
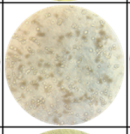
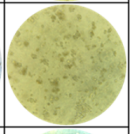
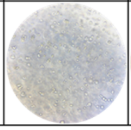
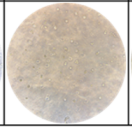
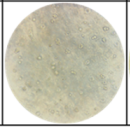
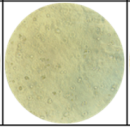
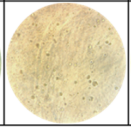
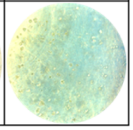
1% alginate extracted	50k						
1% alginate commercial	50k						
old_1% alginate extracted	50k						
old_ 1% alginate commercial	50k						

c

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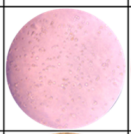
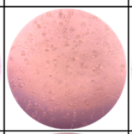
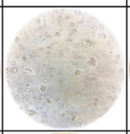
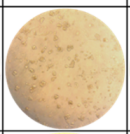
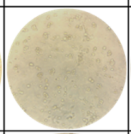
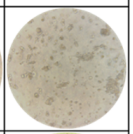
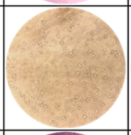
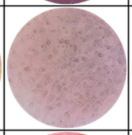
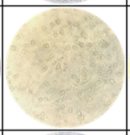
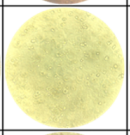
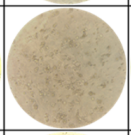
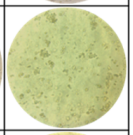
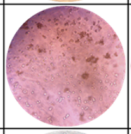
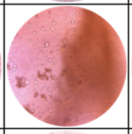
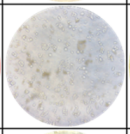
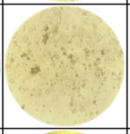
1% alginate extracted	50k						
1% alginate commercial	50k						
old_1% alginate extracted	50k						
old_ 1% alginate commercial	50k						

Figure D.1 NSC-34 cells were embedded with old and fresh alginate hydrogels. b and c are represented different replicates; figure a is Figure 3.21.

D.2 Cell Seeding on Alginate Hydrogel

1% of alginate hydrogels were prepared with the same procedure but without cells, and the NSC-34 cells were seeded on the alginate. The results were imaged for 6 days (Figure D.2).

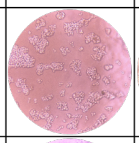
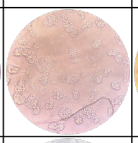
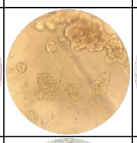
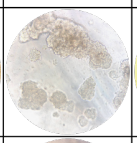
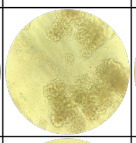
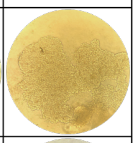
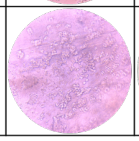
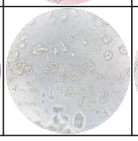
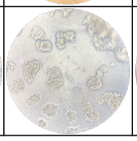
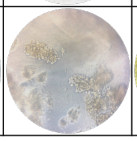
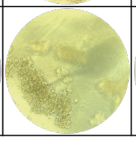
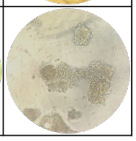
		24h	48h	72h	96h	120h	144h
Alginate surface coating-extracted	50k						
Alginate surface coating-commercial	50k						

Figure D.2 NSC-34 cells were seeded on alginate hydrogels.

The cellular extensions did not form on alginate hydrogels, and they adhered to each other and form clusters to survive.

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