# DIFFERENTIATION OF EMBRYONIC STEM CELLS INTO NEURAL LINEAGES IN AN ALGINATE ENCAPSULATION MICROENVIRONMENT

By

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ABSTRACT OF THE DISSERTATION

Differentiation of Embryonic Stem Cells into Neural Lineages in an Alginate Encapsulation Microenvironment

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Cell replacement therapies, using renewable stem cell sources, hold tremendous potential to treat a wide range of degenerative diseases. Although many studies have established techniques to successfully differentiate stem cells into different mature cell lineages using growth factors or extracellular matrix protein supplementation in both two and three-dimensional configurations, their practicality is limited by lack of control, low yields of differentiated cells and oftentimes, heterogeneous cell population outcomes. In order to address these issues, we have previously established a murine embryonic stem cell alginate-poly-l-lysine microencapsulation differentiation system. The three-dimensional alginate microenvironment maintains cell viability, is conducive to ES cell differentiation to hepatocyte lineage cells, and sustains differentiated cellular function. In addition, hepatocyte function was contingent upon aggregate formation within the alginate

microbeads. The present studies were designed to determine the feasibility of adapting the alginate encapsulation technique to neuronal lineage differentiation. The results of our studies indicate that by incorporating the soluble inducer, retinoic acid into the permeable microcapsule system, cell aggregation was decreased and neuronal lineage differentiation enhanced. In conjunction with the mechanical and physical characterization of the alginate crosslinking network, we have determined that 2.2% alginate microencapsulation can be optimally adapted to both hepatocyte and neuronal differentiation from embryonic stem cells. However, differentiation could be directed away from the hepatocyte and towards the neural lineage by lowering initial seeding density and physical cell-cell aggregation blocking, even in the absence of RA. This study promises to offer insights into targeting cellular differentiation towards both endodermal and ectodermal cell lineages, and could potentially be generalizable and adaptable to the differentiation of other stem cell types given the correct inducible factors and material properties.

## Acknowledgement and Dedication

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# Chapter 1: Introduction

## 1.1 Thesis Overview and Specific Aims

Pluripotent embryonic stem cells represent a promising renewable cell source to generate a variety of differentiated cell types, including hepatocytes, neurons and cardiovascular lineage cells. The emergence of stem-cell based therapy for treating central nervous system disorders relies on the efficient generation of large numbers of functional stable neural lineage cells, which offer several approaches for therapeutic and disease models. These stem cell-derived progenitor cells can potentially be transplantable and serve as a therapeutic strategy by replacing damaged or diseased cells, facilitating regeneration by providing neuroprotective or growth factors and/or providing permissive substrate for regenerative endogenous cells.

Therefore, an understanding of how ES cells differentiate into neural lineage cells *in vitro* is essential for developing and refining transplantation strategies for central nervous system repair, as well as for understanding the mechanisms underlying neurogenesis. Many investigators have designed embryonic stem cell differentiation strategies using biochemical (i.e. soluble growth factors) and mechanical (i.e. substrate gradient, cell-matrix interaction) stimuli, most commonly in a two-dimensional culture configurations. Although many techniques have successfully differentiated stem cells into different mature cell lineages, their practicality is limited by lack of controllability and scalability during the differentiation process. In most cases, the differentiation environments are supplemented with an array of lineage specific growth factors designed to only induce

one specific differentiated cell type. However, despite the introduction of lineage specific differentiation protocols, a heterogeneous population usually arises. Therefore, in order to control differentiated cell output, a more complete understanding of the factors that regulate lineage commitment must be defined.

In this dissertation work, we have established a three-dimensional alginate microencapsulation differentiation system to investigate neural lineage differentiation control. Murine embryonic stem (ES) cells are encapsulated within microbeads of a biocompatible alginate polymer. As a permeable barrier, soluble factors and biomolecules can diffuse through, and microcapsule properties can be controlled and altered. Differentiated stem cells can be easily recovered by depolymerizing the alginate beads. In addition, the alginate bead culture system may also be scaled up. We have demonstrated in previous studies that the alginate microenvironment maintains cell viability, is conducive to ES cell differentiation to hepatocyte lineage cells, and sustains differentiated cellular function. However, it is unclear if alginate encapsulation can be expanded to include other lineages and if so, what are the factors that can differentially regulate lineage specific differentiation.

To address the issues outlined above, the specific aims of this dissertation are as follows.
Specific Aim 1: To adapt alginate encapsulation to induce ES cells to differentiate into cells of other germ layer lineages, specifically neural lineage cells.
Specific Aim 2: To investigate the role of material properties on regulating neural differentiation.

Specific Aim 3: To investigate the role of intracapsular cell interaction on regulating neural differentiation.

### 1.2 Significance and Motivation

According to the National Institute of Neurological Disorders and Stroke, there are more than 600 neurological disorders, among which spinal cord injury (SPI), Parkinson's disease (PD), Alzheimer's disease (AD), and amyotrophic lateral sclerosis (ALS) are the most well known. It is estimated that approximately 50 million Americans are affected by neurodegenerative diseases every year. Neurodegenerative diseases could cause gradual and progressive loss of neural cells, which leads to nervous system dysfunction, and cost the US economy billions of dollars in providing direct health care to the patients.

Because of the limited regenerative capacity of the adult CNS, previous researches have been primarily focused on reducing secondary degeneration following the injury. Studies on stem cell-based therapeutic and disease models hold tremendous potential in their potential application for therapy of diseases and injuries of CNS. Undifferentiated stem cells cannot be directly used in regenerative therapies due to teratoma formation after transplantation. Therefore, they need to be pre-differentiated into desired cell lineages, which may then integrate into the transplant tissue (Brustle et al., 1999; Chen et al., 1995; Dinsmore et al., 1996; Liu et al., 2000). Committed progenitors derived from stem cells can be transplanted into the injured nervous system as a therapeutic strategy, and may serve a therapeutic role a number of ways as outlined in Figure 1.1. They may provide trophic support to host cells, slow a degenerative process, facilitate axonal growth or glial

function, secrete neurotransmitters deficient in the host cells, differentiate into oligodendrocytes and myelinate host axons, or differentiate into neurons and glia cells and either form neuronal connections across disconnected populations or replace damaged neuronal circuits (Kerr et al., 2006). In early 2009, the U.S. Food and Drug Administration (FDA) granted permission to proceed with the first human clinical trial to treat acute spinal cord injury using GRNOPC1 in its Phase I trials, which contains hESC-derived oligodendrocyte progenitor cells. It has been shown that transplantation of hESC-derived OPCs results in the integration of transplanted cells (i.e. survived, migrated, and spread from the site of implantation into the adjacent white matter), differentiation into mature oligodendrocytes, formation of compact myelin by transplanted cells as evidenced by the thin myelin sheaths relative to the axon diameter, and increased locomotor recovery in animal models of acute spinal cord injury (Nistor et al., 2005; Keirstead et al., 2005).

In addition, high-purity populations of ESC derivates will also prove useful in screens for conventional drug discovery, as well as pharmacokinetic/pharmacodynamic studies and toxicology. To elucidate the conditions that direct differentiation of ESCs to specific cell populations, it is essential to understand the local biochemical and mechanical microenvironment that stem cell sense, and to assess the factors and interactions that are can control stem call fate.

## 1.3 Overview of Approach, Prior Work in the Field and Selection of Methods

### 1.3.1 Stem Cells as a Source for Central Nervous System Repair

Selection of a candidate cell type for clinical cell replacement therapy requires several key considerations, such as the availability of the cell type, proliferative ability of the cell type to obtain the quantities necessary for clinical demands, genetic stability of the cell type, and the purity of the differentiated cell population.

Unlike other cells in the body, stem cells have several unique characteristics regardless of their source: they are capable of dividing and renewing in vitro indefinitely; they can remain in an undifferentiated state under specific factors and conditions; and they can give rise to specialized cell types that form the tissues in the body. Adult stem cells can typically differentiate into the cell types of their tissue of origin. In addition, research over the last several years has raised the possibility of adult stem cell plasticity: i.e. cells from one tissue may also be able to give rise to cell types of a completely different tissue. However in general, adult stem cells are rare in mature tissue. Olfactory ensheathing cells meet some of the requirements listed above, however like adult stem cells, it is not clear if this cell type can be expanded to sufficient quantities for use in cell replacement therapy.

Embryonic stem (ES) cells, on the other hand, can be self renewed for long periods. They are isolated from the blastocyst inner cell mass from Day 3 to Day 5 embryos (Evans et al., 1981; Martin et al., 1981). The addition of the cytokine, leukemia inhibitory factor (LIF), allows ES cells to retain an undifferentiated state. Upon removal of LIF, the cells can be differentiated into cells of all three germ layers, and ultimately toward all cell types in the body. Due to the important concern that large numbers of cells are needed for

stem cell replacement therapies, embryonic stem cells have emerged as a solution to solve the cell source limitation problem. Pluripotent embryonic stem cells offer the possibility of a renewable cell source to treat diseases like Parkinson's and Alzheimer's diseases, spinal cord injury, heart disease, diabetes, etc. In addition to its application in cell replacement, generation of mature cell types from stem cells could also support basic research and provide materials for pharmacological and toxicity testing to improve the safety and efficacy of new drugs (O'Neill et al., 2005). In animal models of acute spinal injury, hESC-derived oligodendrocyte progenitor cells have demonstrated the ability to integrate into the host tissue, further differentiate into mature oligodendrocytes, form compact myelin, and increase locomotor recovery. The first clinical trial to treat acute human spinal cord injury using similar approach is also in its Phase I development period.

It is worth mentioning that, recent groundbreaking work in inducing reprogramming of mouse and human somatic cells into induced pluriopotent stem (iPS) cells have been drawing tremendous attention in this area (Yu et al., 2007; Engler et al., 2006). They are artificially derived from non-pluripotent cells, typically adult somatic cells such as fibroblasts, following direct reprogramming to pluripotency by ectopic expression of four transcription factors (*Oct4*, *Sox2*, *Klf4* and *Myc*).

Reprogrammed human somatic cells, which are patient specific, exhibit the essential characteristics of ES cells, which have the ability to differentiate into any cell type. These iPS cells can differentiate in vivo and in vitro into several types of cells, contribute to the development of the germ line. Two recent studies demonstrate the initial characterization

of neurodegenerative disease patient-derived iPS cell cultures as proof of concept for the utility of this technology. Dimos et al. obtained fibroblast lines from two elderly siblings with early and late manifestations of ALS, caused by disease-associated mutation in the superoxide dismutase (SOD1) gene (Dimos et al., 2008). Patient fibroblasts were transduced with Yamanaka group's four-gene cocktail. These iPS cell lines appeared to be pluripotent in vitro, as they could be spontaneously differentiated into representative phenotypes of the three embryonic germ cell layers. Furthermore, spinal motor neuron specification was recapitulated using retinoic acid and sonic hedgehog. The study from Ebert et al. addressed spinal muscular atrophy, typically caused by genetic mutation in the survival motor neuron-1 (SMN1) gene (Ebert et al., 2008). Skin fibroblasts from a single patient and control (his unaffected mother) were transduced with a cocktail of lentiviral constructs (Oct 4, Sox2, Nanog, LIN28), leading to the generation of iPS cell lines, which could be differentiated to a variety of cell types, including cells with spinal motor neuron phenotype that harbor the SMN1 mutation. iPS cell-derived motor neurons were cultured for an additional 2 weeks and reduction in the accumulation and size of these cells was reported in patient cultures versus the normal control. In addition, the patient iPS cells displayed a predicted deficiency in nuclear SMN protein aggregates. Treatment of the cells with either of two known chemical inducers of SMN protein expression, valproic acid or tobramycin, suppressed this phenotype. Although certain limitations remain, including tumorigenesis through viral integration of expression vectors into the host genome and low efficiency of induction (about 0.1%), they could suppress patient iPS cell-associated phenotypes of repair of the underlying genetic

mutation and offer a potentially powerful approach to neurodegenerative disease modeling.

Of the cell types discussed above, ESCs currently show the greatest potential for a wide range of cell replacement strategies. In this dissertation, we ultimately chose a murine embryonic stem cell line ES-D3 (ATCC, Manassas, VA) as our model. However, it is our hope that the approaches and conclusions derived from this work will ultimately be applicable to developing differentiation strategies toward neural lineages, for a variety of stem cell sources.

### 1.3.2 Differentiation of Embryonic Stem Cells

As mentioned previously, the ability of embryonic stem cells to adopt multiple fates makes them prime candidates for current cell replacement studies. However, undifferentiated ES cells may cause teratoma formation *in vivo*. The potential use of ES cells to replace functional loss of particular tissues heavily depends on efficient differentiation protocols to derive lineage specific progenitor cells. For example, experimental models of demyelination are associated with several neurological pathologies and trauma such as spinal cord injury, multiple sclerosis, brain injury and stroke. Endogenous or transplanted mature oligodendrocytes are not capable of remyelination. Recent studies suggest that cell therapy using oligodendrocyte progenitor cells (OPCs), such as human embryonic stem cell (hESC) derived OPCs, promote remyelination and improve locomotor outcomes in animal models (Keirstead et al., 2006). Hence, an understanding of how ES cells differentiate into neural lineage cells *in vitro* is

essential for developing and refining transplantation strategies for central nervous system repair, as well as for understanding the mechanisms underlying neurogenesis.

Stem cell maintenance and differentiation are governed by unique local microenvironments (Engle et al., 2006). Identifying specific cues in the microenvironments will provide new tools with which to promote the differentiation of stem cells into particular cell types as shown in Table 1.1. The most commonly used approach for neural differentiation from ES cells involves utilizing all-trans retinoic acid (RA), in conjunction with embryoid bodies (EBs) formation prior to the RA treatment. The EB structure recapitulates certain aspects of early embryogenesis with the appearance of lineage specific regions similar to that found in the embryo. Following 2-4 days in suspension culture, primitive endoderm cells start to form on the surface of EBs and epiblast like cells inside. Continued culture of EBs results in the appearance of mesodermal and endodermal cell types. However, EBs exhibit stochastic differentiation into a variety of cell lineages. Treatments with morphogens/growth factors and/or use particular culture systems are necessary to achieve a directed differentiation and/or selective expansion of a specific lineage. For neural differentiation which occurs during early embryonic development, EBs are usually treated with morphogens such as retinoic acid as early as 4 days after their formation (Bain et al, 1995). Mouse ES cells treated with this protocol yield 38% of neuronal cells upon differentiation. When EBs are cultured in suspension for 4 days and then plated on an adhesive substrate in the presence of FGF2 in a serum free ITSF medium (DMEM/F12 medium supplemented with insulin, transferrin, selenium, and fibronection), 80% of replated cells differentiated into nestin-

positive neural precursor cells after 6-8 days of selection and expansion. Withdraw of FGF2 induces spontaneous differentiation into various neurons and glia. In addition to methods involving formation of EB formation, other techniques for promoting neural differentiation of ES cells include using noggin treatment (O'Shea et al., 1995) and N2 supplemented ITSF medium in a monolayer culture configuration (Yang et al., 2003). Although these methods are capable of generating considerably high percentage of neurons and glia cells after selection and expansion process, they generally have a low cell yield due to the serum free culture condition in defined ITSF medium. Direct differentiation of ES cells can also be achieved when co-culture with chick dorsal root ganglia (Shimizu et al., 2005), PA6 cells (Kawasaki et al, 2002) and conditioned media from glial cell (Cheng et al., 2005) and mesoderm-derived cell lines. However, neurotrophic factors present in the conditioned media or secreted from co-cultured cell line are not defined, and differentiation mechanisms and signaling pathway through these factors are not well understood in general.

Nevertheless, the majority of neural differentiation protocols developed so far yield a mixed population of cells. It is ideal, under some conditions, to isolate the desired cell subpopulations from the mixture for further applications, which can be achieved by immunoseparation techniques based on known neural cell-surface epitopes. Rao's group has used immunopaning and fluorescence-activated cell sorting (FACS) to select NCAM-positive neuronal restricted progenitors and A2B5 positive glial-restricted progenitors (Rao et al., 1997). Fleischmann's group used an EB mediated retinoic acid protocol, and

target the GFP reporter gene under the control of nestin, another neural precursor marker (Fleischmann et al., 2002).

Although considerable progress has been made in recent years to demonstrate neural lineage differentiation from ES cells, several significant obstacles must be overcome before this research translates to the cell-based therapies and clinical applications. Most protocols require culture at very low densities and cell-cell/matrix interactions for neuronal differentiation; however, the mechanisms underlying neuronal lineage commitment induced by these techniques remain unclear. Most studies use defined differentiation environments to target one specific cell type, but oftentimes a heterogeneous population arises without further cell sorting. Furthermore, the need to culture the cells at low density to achieve neuronal differentiation limits the cell output for potential transplantation strategies. The enormous potential of cell therapies relies on the generation of large functionally stable and homogenous differentiated cell populations. In animal models of acute spinal cord injury mentioned previously, it was shown that 24 separate animal studies required the production of more than five billion OPC cells. Due to the important fact that large numbers of cells are needed for cell-based therapies, generating a reliable cell source for large-scale manufacturing has also emerged as an important challenge in the field.

In order to control differentiated cell output, a more complete understanding of the factors that regulate lineage commitment must be defined. In this dissertation study, we will establish a differentiation system that could simultaneously control multiple

parameters for different lineage differentiation, and hopefully be scaled up for future cell transplantation purposes. We will adapt a murine embryonic stem cell line (ES-D3), ATCC) as our model, in order to identify and implement strategies to direct specific cell lineage differentiation.

### 1.3.3 2D vs. 3D Differentiation of ES Cells

Most of the *in vitro* cell studies of embryonic stem cell populations are experimentally designed using very hard surfaces with constant characteristics, despite the fact that cells reside, proliferate and differentiate *in vivo* within complex 3D microenvironments. Even though EBs have a three dimensional structure, terminal differentiation of EBs is usually conducted in 2D configuration. Besides EB mediated differentiation, most of the studies investigating stem cell differentiation have been performed on 2D coated plate or a variety of biomaterials/extracellular matrices, which does not mimic the *in vivo* physiological microenvironment and may result in inefficient and heterogeneous cell population arise. Recent tissue engineering studies have indicated significant differences in the differentiation profile of ESCs when cultured in a 3D microenvironment vs. 2D (Tanaka et al., 2004; Hwang et al., 2006). Maintenance and differentiation of ES cells in 3D culture may potentially promote cell-cell and cell-material interaction and their properties can be varied to promote differentiation of cells into specific lineages. 3D scaffolds for tissue engineering serve numerous functions and their role during tissue development is dependent upon specific properties of the chosen biomaterials. 3D culture systems have proven to enhance osteogenic (Garreta et al., 2006), hematopoietic (Liu et

al., 2005), chondrogenic (Hwang et al., 2006; Hwang et al., 2006) and neural (Willerth et al, 2006) differentiation.

Properties of 3D differentiation configuration, such as mechanical integrity, optimal fluid transport, and delivery of biomolecules, are critical parameters that may potentially influence cell adherence, organization and differentiation during tissue development. More importantly, based on our own experience, ES cells are less sensitive to their culture environment in the 3D alginate microbeads as compared to EB or monolayer mediated 2D differentiation system. In order to have a better control of mechanical properties of the material and minimize regulatory/manufacturing difficulties, we ultimately chose a controllable 3D alginate microencapsulation technique in this dissertation study.

### 1.3.4 Alginate Microencapsulation

Alginates are widely used in food and pharmaceutical industries. As biomedical materials, alginate exhibits a great biocompatibility (i.e. low undesirable immune response), significant mechanical resistance and biodegradability. Alginate has been considered as one of the most promising biomaterials for immunoisolation of allogeneic and xenogeneic cells and tissues (Zimmermann et al., 2005). It consists of blocks of co-polymers of 1,4-linked b-D-mannuronic acid (M) and a-L-guluronic acid (G) (Rowley et al., 1999). When multiple G-block regions are aligned side by side, a diamond-shaped hole results. Unlike Poly-D-mannurate, Poly-L-guluronate prefers intra-molecular

hydrogen bonding between the carboxyl group and the 2-OH group. Cations, such as $Ca^{2+}$, can replace this hydrogen bonding and zip the guluronate chain.

The microcapsules, coated with poly-L-lysine to stabilize the polymer, allow soluble factors and biomolecules to diffuse through (Tobias et al., 1999; Maysinger et al., 2005). The encapsulation system implemented an electrostatic droplet approach. The summation of gravity force and electrostatic force equals to the surface tension of the microcapsules (Poncelet et al., 1998). Therefore the microcapsule sizes can be manipulated by the voltage applied and alginate concentration. In addition microcapsule properties can also be controlled through variations in the encapsulation process (i.e. alginate concentration, alginate composition M/G ratio, PLL concentration, and cell seeding density), which discretely control key culture parameters. Differentiated stem cells can also be easily recovered by depolymerizing the alginate beads (Orive et al., 2006). These unique characteristics allow the successful use of alginate for three-dimensional cell cultures. The interactions existing between the alginate matrix and the embedded cells could also play a major role in their morphological and physiological behavior.

Our lab has previously demonstrated that the alginate microenvironment maintained cell viability, was conducive to ES cell differentiation to hepatocyte lineage cells, and maintained differentiated cellular function (Maguire et al., 2006; Maguire et al., 2007) In this dissertation, we will take advantage of the permeable microcapsule system, and investigate the feasibility of using alginate encapsulation to induce ES cells to differentiate into cells of other germ layer lineages, specifically neural lineage cells. We

will investigate and further improve differentiation efficiency by regulating intracapsular cell-material and/or cell-cell interactions. Based on a more complete understanding of the identified differentiation control parameters, the differentiation process could be potentially scaled up and adaptable to serve as a transplantable cell source for cell-based therapy for treating neurodegenerative diseases.

# Chapter 2: Control of Neural Differentiation from Embryonic Stem Cells in an Alginate Encapsulation Microenvironment

## 2.1 Introduction

Pluripotent embryonic stem cells represent a promising renewable cell source to generate a variety of differentiated cell types. Successful stem cell differentiation strategies to specific functional cell lineages offer the possibility utilizing a renewable cell source to treat devastating conditions such as Parkinson's and Alzheimer's diseases, spinal cord injury, heart disease, diabetes, etc (Dinsmore et al, 1996; Cao et al., 2002). In addition to their applications in cell replacement, generation of mature cell types from stem cells could also provide materials for pharmacological and toxicological testing to improve the safety and efficacy of new drugs (O'Neill et al., 2004). However their potential in therapeutic and pharmaceutical applications relies on the generation of large functionally stable and homogenous differentiated cell populations. Although many investigators have described techniques to successfully differentiate stem cells into different mature cell lineages using growth factors or extracellular matrix protein supplementation (Ying et al., 2003; Okabe et al., 1996; Kitazawa et al., 2005; Tian et al., 2005; Bain et al., 1995), most commonly in conjunction with embryoid body formation, their practicality is limited by lack of control and scalability during the differentiation process. Furthermore, in most cases, the differentiation environments are supplemented with an array of lineage specific growth factors, which although designed to induce only one specific differentiated cell type, nevertheless usually generate heterogeneous populations. Therefore, in order to control differentiated cell output, a more complete understanding of the factors that regulate lineage commitment needs to be defined.

In an effort to promote ES cell commitment to specific lineage in a controllable and scalable manner, we and others have previously utilized alginate microencapsulation-mediated differentiation approaches, and demonstrated cell viability and spontaneous induction of hepatocyte lineage commitment (Maguire et al., 2006; Maguire et al., 2007). During this process, murine embryonic stem (ES) cells are encapsulated within microbeads of a biocompatible alginate polymer. Soluble factors and biomolecules can diffuse through the permeable barrier, microcapsule properties can be controlled and altered, and differentiated stem cells can be easily recovered by depolymerizing the alginate beads. However, it is unclear if alginate encapsulation can be expanded to include other lineages and if so, what are the factors that can differentially regulate lineage specific differentiation. In the present work, we sought to investigate the feasibility of using the scalable and controllable alginate microenvironment culture system to induce ES cells to differentiate into cells of other germ layer lineages, specifically neural lineage cells. In this chapterwe assessed whether incorporating soluble inducers and manipulating capsule parameters could differentially regulate and target cellular differentiation towards neuronal cell lineages using this system.

## 2.2 Materials and Methods

### 2.2.1 ES Cell Culture

All cell cultures were incubated in a humidified 37$^{o}$C, 5% $CO_2$ environment. The ES cell line D3 (ATCC, Manassas, VA) was maintained in an undifferentiated state in T-75 gelatin-coated flasks (Biocoat, BD-Biosciences, Bedford, MA) in Knockout Dulbecco's

modified Eagles medium (Gibco, Grand Island, NY) containing 15% knockout serum (Gibco), 4 mM L-glutamine (Gibco), 100 U/mL penicillin (Gibco), 100 U/mL streptomycin (Gibco), 10 mg/mL gentamicin (Gibco), 1,000 U/mL ESGRO$^{TM}$ (Chemicon, Temecula, CA) and 0.1mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). Media was changed every two days until plates were confluent. ES cultures were split and passaged every 6 days. Following media aspiration, cells were washed with 10 mL of phosphate buffered solution (PBS) (Gibco), detached using 3 mL of trypsin EDTA (Gibco) for 3 minutes, and subsequently the addition of 12 mL of Knockout DMEM. Cells were then replated in gelatin-coated T-75 flasks at a density of 1 million cells/mL and only passages 10 through 25 were used in the experiment. In order to induce differentiation, cells were cultured in Iscove's modified Dulbecco's medium (Gibco) containing 20% fetal bovine serum (Gibco), 4 mM L-glutamine (Gibco), 100 U/mL penicillin (Gibco), 100 U/mL streptomycin (Gibco), 10 mg/mL gentamicin (Gibco) in the presence or absence of $10^{-7}$M trans-retinoic acid (Sigma-Aldrich).

### 2.2.2 Alginate Encapsulation

Alginate solution was prepared by dissolving 2.2 g of alginic acid sodium salt (MW: 100,000–200,000 g/mol, G Content: 65%–70%, Sigma-Aldrich) in 100 mL of $Ca^{2+}$ free DMEM (Gibco), using a heated magnetic stir plate at a temperature of 65$^{o}$C. The solution was then filtered using a 45 μm syringe filter (Fisher Scientific, Pittsburg, PA). To create the cell-alginate mixture, 1 mL aliquot of cell suspension with a seeding density of $5\times10^{7}$ cells/mL was added to 9mL of either 1.2%, 1.7%, 2.2% or 2.5% (w/v) alginate solution to yield a final cell seeding density of $5\times10^{6}$ cells/mL. This solution was transferred to a 10

mL syringe, and was connected to a syringe pump (KD Scientific, MA). Alginate beads were generated using an electrostatic bead generator (Nisco, Zurich, Switzerland) at a flow rate of 40 mL/h, and an applied voltage of 6.4 kV. The beads were extruded into a 200 mL bath of $CaCl_2$ (100 mM), containing 145 mM NaCl, and 10 mM MOPS (all from Sigma-Aldrich) and were left to polymerize for 10 min at room temperature as outlined in Figure 2.1 (Maguire et al., 2006). Beads were then transferred to a tissue culture treated T-25 flask. The $CaCl_2$ solution was removed using a 5 mL pipette, and the beads were washed with 5mL of HEPES (Gibco). The HEPES was removed and the beads were resuspended in 5 mL of poly-L-lysine (PLL) (Sigma-Aldrich, MW 68,600 g/mol) (0.05% w/v) for 2 min. The PLL was then gently removed, replaced with HEPES to wash the beads and the beads were ultimately resuspended into 5 mL of IMDM medium (Invitrogen, Carlsbad, CA) media with and without $10^{-7}$M trans-retinoic acid (Bain et al., 1995). Media was changed at days 4, 8, 12, 16 and 20 post encapsulation.

### 2.2.3 Depolymerization and Cell Recovery

Alginate microcapsules were washed with PBS and then incubated with $Ca^{2+}$ free IMDM medium (Invitrogen) containing 100 mM sodium citrate, 10 mM MOPS and 27 mM NaCl (all from Sigma-Aldrich) for 30 minutes at 37$^{o}$C to induce depolymerization. The released cells were centrifuged at 1,200 rpm for 6 minutes, the sodium citrate solution was aspirated, and the cell pellet was washed with PBS buffer twice, and responded in cell specific media. The cells were counted using the trypan blue method.

### 2.2.4 Characterization of Mechanical Properties

The local mechanical properties of polyacrylamide substrates were quantified by measuring the elastic modulus using spherical inclusions. A small steel ball with a diameter of 1/16" was suspended in 500 μL of gel. A calibrated magnetic force was applied to the ball and the displacement was measured with a video microscope. The calibration was performed by measuring the voltage required by the electromagnet to hold the steel ball submerged in water against the force due to gravity using a calibrated electromagnet, and the displacements of the bead were measured using a calibrated electromagnet, from which the elastic modulus E was calculated using equation

$$E = \frac{\rho}{2\pi R_0}\left(\frac{F}{\delta}\right)$$

where δ is the displacement, $R_0$ is the radius of the bead and ρ is a geometrical factor, which for the infinite medium with perfect bonding is 1.0. In general, ρ is a complicated function of Poison's ratio and the parameters characterizing the boundaries. From the direct compression experimental tests, the value of ρ for our experimental setup was found to be 0.9674 (Li et al., 2008)

To measure the compressive modulus, bulk alginate gels were prepared in 50 mL conical tubes and cut into a cylindrical disks of 30 mm diameter and 20 mm thickness with flat and parallel surfaces. Compression testing was performed at room temperature using an Instron-5542 (Instron Corporation, Canton, MA) instrument (Kuo et al., 2001). The compressive strain was set to a maximum 40% and the crosshead speed was 5mm/min. Compressive stress (MPa), strain (%), extension (mm) and load (N) were recorded using Merlin materials testing software (Instron Corporation, Canton, MA, USA). A total of 5 samples were tested for each concentration and the average was used to generate a force

versus displacement curve. A linear fit was imposed on the curves from which a linear correlation factor was determined.

### 2.2.5 Alcian Blue Staining

A modified alcian blue staining protocol was adapted (Powell et al, 1982). Empty microcapsules were incubated with 10 mg/mL alcian blue dye at pH 5.6 in 0.3M $MgCl_2$ solution for 24 hours, and washed three times for 10 minutes in deionized water. Bright field images were acquired using an Olympus IX 81 and Olympus digital camera. 10 z-sectional images were taken for each microcapsule with a step size of 5 μm. 3D surface view images were generated and crosslinking density was measured using Olympus Microsuite imaging analysis software. Three experiments incorporated an analysis of 10 beads per experiment.

### 2.2.6 Intracapsular Immunofluorescent Staining

Encapsulated cells were washed with PBS (Gibco) and fixed on days 4, 8, 12, 16 and 20 with 4% paraformaldehyde to evaluate surface proteins or together with 0.25% Triton-X to evaluate intracellular protein expression. Cells were incubated with A2B5 (1:250, Chemicon), neurofilament 160 kD (1:200, Chemicon), GFAP (1:50, Abcam, Cambridge, MA) and O1 (1:500, R&D Systems, Minneapolis, MN) overnight at $4^{o}C$ in PBS buffer containing 1% normal goat serum for surface proteins or with together with 0.25% Triton-X for intracellular proteins. The microcapsules were washed three times for 10 minutes in PBS buffer, and then treated with secondary antibody, FITC-conjugated goat anti-rabbit IgG or anti-mouse IgM (both 1:500, Invitrogen) for 2 hours at room

temperature. Normal immunoglobulin served as control for non-specific antibody binding, whose fluorescence intensity was subtracted from overall intensity of the antibody expression.

## 2.3 Results

In order to induce differentiation of large numbers of differentiated cells, an alginate microencapsulation culture system was developed as previously described (Maguire et al., 2006; Maguire et al., 2007). In this chapter, we investigated the feasibility of adapting this alginate microencapsulation system to induce differentiation of other germ layer lineages, specifically neural lineage cells, using a murine embryonic cell line ES-D3.

### 2.3.1 Physical and Mechanical Characterizations of Alginate Microcapsules

To have a better understand of the structure of alginate microcapsules, we first examined the mechanical and physical properties of the materials, i.e. microcapsule size, stiffness and crosslinking density.

The encapsulation system implemented an electrostatic droplet approach as shown in Figure 2.1. The summation of gravity force and electrostatic force equals to the surface tension of the microcapsules (Poncelet et al., 1998). Therefore our encapsulation setup could generate a variety of capsules sizes for different purposes by varying parameters such as alginate concentrations and electrostatic force applied. When we varied the alginate concentrations, it resulted in various bead diameters of 336.67±9.53 μm, 551.62±8.77 μm and 612.19±37.63 μm using 1.2%, 1.7% and 2.2% (w/v) alginate

concentrations respectively at an applied voltage of 6.0 kV as shown in Figure 2.2. Using 2.2% alginate concentration, the size of alginate microcapsules can be further reduced when increasing the voltage applied from 6.0 kV to 6.4 kV. By integrating the original setup with a voltage accelerator, we could further decrease the microcapsule size to around 100 μm if necessary.

In order to probe the three dimensional alginate microenvironment and the mechanical cues underlying the differentiation process, we examined the mechanical properties of alginate microcapsules at varying alginate concentrations. We adapted both the bead test (for local mechanical property assessment) and compression test (for bulk mechanical property assessment) to determine the rigidity of the alginate microcapsules. The mechanical properties of the alginate microbeads were initially characterized using 1/16" spherical magnetic beads as described previously (Li et al., 2008). A calibrated magnetic force was applied to the ball and the displacement measured with a video microscope. Force calibration was performed using the method described by Lin et al. A small volume of water (about 50 μL) was placed in a 0.6 mL microcentrifuge tube. A steel bead of 1/16" diameter was immersed in the water. Using the linear positioning stage, the distance between the pole of the magnet and the sphere was varied from about 1 mm to 3 mm in steps of 0.5mm. At each step, electrical power in excess of that required to hold the bead vertically against the pointed tip of the microcentrifuge tube was applied. The power was then decreased by voltage decrements of 0.01 V (the maximum resolution of the digital power supply) until the bead was released. The final voltage was recorded as

the amount necessary to produce a magnetic field capable of holding the bead in equilibrium against the gravitational force, $F_g$, given by

$$F_g = m_b g = [(\rho_{steel} - \rho_{water})V_{sphere}]g$$

where $m_b$ is the buoyant mass of the sphere, ρ is density ($\rho_{steel}$ = 8420 kg/m$^3$ and $\rho_{water}$ = 1000 kg/m$^3$), $V_{sph}$ is the volume of the sphere, and g is the acceleration due to gravity 9.81 m/s$^2$. For the particular beads used, the force was $1.906\times10^{-5}$ N. For any given distance between a spherical bead and an electromagnet, the force applied to the bead by the magnetic field is dependent on the current or voltage supplied to the magnet. Since the magnet resistance is constant, current and voltage are proportional.

$$F = a(x)V^2$$

where a(x) is a normalization constant that depends on the distance x between the sphere and the pole of the solenoid, and V is the applied voltage. The function a(x) was found by fitting a second-order curve to the calibration data. Upon application of a known voltage, the distance x was measured and substituted into the best-fit equation generated to obtain the constant a. The force on the bead can then be calculated.

Using the mechanical bead method, we found that variation of alginate concentration produced microbeads with stiffness of 3.70, 7.35 and 13.13 kPa for 0.5%, 1.1% and 2.2% alginate concentrations respectively in Figure 2.3. However, this method was sufficient for alginate gels up to 2.2%. As the concentration of alginate monomer (with constant G/M ratio) continued to increase, the maximum magnetic force we applied was not big enough to cause displacement of the 1/16” steel ball. Therefore, the stiffness of 2.5% alginate gels was evaluated by standard compression tests. The elastic modulus

determined by both compression and bead tests showed remarkable dependence on alginate concentration. Furthermore, the moduli were consistent in both tests, suggesting homogeneity of the alginate encapsulation microenvironment.

In addition to the rigidity of alginate crosslinking strands, we also characterized the spacing between crosslinkers in the gel network using alcian blue dye. Positively charged alcian blue molecules can bind to the anionic carboxyl and half-ester sulfate groups of alginate crosslinking strands, and form an insoluble blue precipitate. The staining with alcian blue dye allows the visualization of zones of the alginate microcapsules in which crosslinking strands are more concentrated. From the 3D surface view generated based on stacks of bright field images of the microcapsules, we found that alcian blue dye was aligned in one direction as shown in Figure 2.4 (Bottom). When we measured the spacing between crosslinkers in the alginate gel network, values of 32.8, 12.5 and 8.8 μm were measured for 1.2%, 2.2% and 2.5% microbeads respectively as shown in Figure 2.3 and Figure 2.5.

2.3.2 Assessment of Neural Specific Protein Expressions in the Presence of Retinoic Acid

It was demonstrated in previous studies that the alginate microenvironment maintained cell viability, was conducive to ES cell differentiation to hepatocyte lineage cells, and maintained differentiated cellular function (Maguire et al., 2006). As shown in Figure 2.6, under basal media conditions, expression of the hepatocyte marker, intracellular albumin, increased significantly after 8 days in the encapsulation culture. However, under this

condition, only less than 5% of the encapsulated cells express neural lineage markers GFAP, neurofilament and O1 as shown in Figure 2.8 (Bottom).

In the present study, we would sought to take advantage of the permeable microcapsule system, and investigate the feasibility of using alginate encapsulation to induce ES cells to differentiate into cells of other germ layer lineages, specifically neural lineage cells. Using a previously optimized encapsulation condition (i.e. 2.2% alginate and $5\times10^6$ cells/mL), experiments were designed to assess cell viability, proliferation and the expression of a wide array of neural special markers during a 20-day differentiation period in the presence of retinoic acid. To determine the optimal retinoic acid concentration for neural differentiation, we have previously adapted the hanging drop technique to generate EBs from the ES cells. In the presence of retinoic acid, we successfully induced 33% neurofilament expressing neuronal cells as early as Day 8. Based on the immunofluorescent intensity level of neurofilament marker, the optimal concentration of retinoic acid was found to be $10^{-7}$M (data not shown). In the retinoic acid induced system, differentiated cells expressed a variety of neural markers. Among these cells, about 60% expressed progenitor cell marker A2B5. In an effort to isolate committed subpopulation as a source of transplantable cells following nerve injuries, neuronal progenitor cells were isolated using two-color fluorescence-activated cell sorting (FACS) using A2B5 and PSA-NCAM antibodies on Day 8.

In alginate encapsulation mediated differentiation system, we first examined the cell proliferation in the presence and absence of retinoic acid. Encapsulated cells were

recovered on Days 4, 8, 12, 16 and 20 post encapsulation by depolymerizating the alginate microcapsules using sodium citrate, and cell number and viability were determined. As indicated in Figure 2.7, encapsulated cell numbers in the presence or absence of retinoic acid are similar and cell viability was greater than 95% in all cases (data not shown). The cell proliferation rates under both conditions exhibited biphasic kinetic properties, and ultimately reached a final density 3.5 times greater than the initial density under both conditions.

To further our analysis of lineage commitment within the alginate microenvironment, we examined individual cells within the encapsulated population using indirect immunofluorescence analysis with a panel of neural lineage specific antibodies, including A2B5 (progenitor marker), neurofilament 160kD (neuronal marker), O1 (oligodendrocyte marker) and GFAP (astrocyte marker). During development, neural precursors undergo sequential stages of differentiation characterized by expression of these distinct neural markers Specific antibody binding was assessed relative to a non-specific immunoglobulin control. The results of these experiments indicate progenitor marker A2B5 expression, after reaching maximal expression on Day 8, began to decrease. Furthermore, expression of later-stage markers, such as neurofilament, O1 and GFAP gradually reached their maximal levels on Day 12. In generally, neural marker expression cell sub-population decreased dramatically by the end of 16-day differentiation period. The maximum percentage of encapsulated cells positively stained for A2B5, NF 150kD, O1 and GFAP markers were 24.7%, 41.9%, 32.3% and 38.6% respectively following incubation in the presence of $10^{-7}$M retinoic acid. In contrast, less than 5% of

encapsulated cells stained positively for intracellular albumin, a hepatocyte lineage cell marker as shown in Figure 2.8. However, in the absence of any induction factor (i.e. under basal medium condition), while hepatocyte differentiation was favored with over 90% cells stained positive for albumin, only a very small fraction of the encapsulated cells expressed the markers listed above.

Next, we sought to regulate and target cellular differentiation towards neuronal cell lineages by manipulating capsule parameters. After assessing the neural specific markers in the previously optimized 2.2% condition, we also investigated the effects of different alginate concentrations, e.g. 1.2%, 1.7% and 2.5%, on neural differentiation in the alginate encapsulation microenvironment. As mentioned in 2.4.1, as we increase the alginate concentration, the mechanical properties of alginate gels gradually increase, whereas the crosslinking space decreases significantly. Variation of alginate concentration could produce microbeads with a variety of stiffness and crosslinking spacing. We compared the percent of encapsulated cells which stained positively for neural specific markers as summarized in Table 2.1. These experiments indicated 2.2% was the most favorable condition for all the neural markers that we examined.

**2.4 Discussion**

Stem cell proliferation and differentiation are governed by unique local microenvironments (Engler et al., 2006). Certain aspects of the microenvironment, especially the biochemical and mechanical environments, play critical roles in determining the lineage commitment of ES cells (Li et al, 2008; Engler et al, 2006; Philp

et al., 2005). Identifying specific cues in the microenvironments could provide new tools with which to promote the differentiation of stem cells into large numbers of functionally stable cell lineages. Here, we report the feasibility of using alginate microencapsulation to study the interaction among ES cells, their surrounding substrate network and ultimately differentiation pathway selection toward neural lineage cells.

Alginate microencapsulation has been extensively utilized to promote stem cell differentiation and maintain mature hepatocyte functions, most commonly through the formation of embryoid bodies (EB) in the microcapsules (Fang et al., 2007). In addition, as a biocompatible material, alginate scaffolds have also been implemented in promoting EB differentiation. We have previous described the use of alginate encapsulation technique to differentiate ES cells into hepatocyte lineage cells in the absence of growth factors. The current study was designed to evaluate whether the three-dimensional alginate encapsulation mediated differentiation environment could also be utilized to promote neural lineage specific differentiation from ES cells.

A variety of soluble factors have been previously used to induce neuronal differentiation from embryonic stem cells, including retinoic acid (Bain et al, 1995; Schuldiner et al., 2001), FGF-2 supplemented ITSFn medium (DMEM/F12 medium with insulin, transferrin, selenium, and fibronection) (Okabe et al, 1996), N2 supplemented ITS medium (DMEM/F12 medium with insulin, transferrin, selenium) (Ying et al., 2003) and conditioned media from chick dorsal root ganglia (Kitazawa et al., 2005) and glial cells (Tian et al., 2005). In particular, a study by Gottlieb's group demonstrated that 38%

differentiated cells expressed neurofilament marker in retinoic acid-mediated EB culture (Jones-Villeneuve et al., 1982). In the current study, we found that encapsulated ES cells remained viable in the presence and absence of retinoic acid supplementation. Following an initial lag-phase, they ultimately reached a cell density 3.5 times greater than the initial one. However, the cell density in general, was much lower in the encapsulation microenvironment as compared to those on 2D tissue culture treated plates. Although encapsulated cells continued to divide at a similar rate throughout the entire 20 day culture period, ES cells cultured in the absence of retinoic acid did not express neural lineage markers. The cell sub-population committed into neural lineages was less than 5% of the overall cell population, whereas in the presence of retinoic acid, the percentage of cells positively stained for A2B5, NF 150kD, O1 and GFAP markers were 24.7%, 41.9%, 32.3% and 38.6% respectively. Our studies demonstrated that neuronal lineage differentiation can be achieved within the alginate microenvironment with an efficiency comparable to traditional EB-mediated culture. However it is worth mentioning that alginate microenvironment is more desirable culture and differentiation environment as compared to conventional polystyrene culture surface, especially to murine embryonic stem cells. From our experience, when cultured in alginate microcapsules, ES cells are a lot less sensitive than in EB-mediated culture.

In addition to biochemical induction, the interactions existing between the alginate matrix and the embedded cells could also play a major role in their morphological and physiological behavior. In order to determine the influence of mechanical environments on stem cell commitment, which in our case can be modulated by alginate concentration,

we first characterized the elastic modulus E of alginate gels. Alginate gels consist of blocks of co-polymers of 1,4-linked b-D-mannuronic acid (M) and a-L-guluronic acid (G). The crosslinking reaction is triggered when cations, such as $Ca^{2+}$, replace the hydrogen bonding in guluronate chain as more than one G-block region align side by side. Keeping the G/M ratio constant, the microcapsule properties can be controlled by varying the concentration, and therefore alter the polymer gel's stiffness. By varying alginate monomer concentrations (G content: 65%–70%), we found that the stiffnesses ranged from 3.70 to 13.13 kPa for 0.5%, 1.1% and 2.2% alginate concentrations respectively using embedded magnetic beads. Furthermore, using a compression approach, the mechanical characterization can be extended to 2.5% alginate gels. In addition to the elastic resistance that a cell feels in the gel network, we could also modulate the crosslinking density of the alginate beads. Several specific staining molecules (e.g. calcon carboxylic acid, mureoide, methylene blue, alcian blue) that are negatively or positively charged interact with the gel network. These molecules allowed us to reveal the chemical interactions shown by the pattern coloration of the internal structure of the gel. We adapted a modified alcian blue staining protocol for measuring alginate crosslinking properties. The intensity of the staining with alcian blue dye allow the visualization of zones of the alginate microcapsules in which crosslinking strands are more concentrated. The experiment demonstrated that spacing of alginate crosslinking strands was 32.8, 12.5 and 8.8μm for 1.2%, 2.2% and 2.5% alginate microbeads respectively.

After assessing the neural specific markers in our previously optimized 2.2% condition, our next experiment was designed to determine the effects of various alginate

concentration and initial cell seeding densities on neural differentiation from ES cells. Using 1.2%, 1.7% and 2.5% alginate concentrations, neural specific marker expression followed a similar trend. However, with all alginate concentrations, neural progenitor marker A2B5 expression peaked on Day 8 and declined over time, while more mature markers reached their maximum expression on Day 16. By comparing the percent of cells positively staining for neural specific markers, we found that 2.2% was most conducive to ES differentiation to neural lineage cells. Overall, the mechanical and physical characterizations of alginate cells could allow us to have a better comprehension of the cellular behavior on encapsulated microenvironment.

In summary, we have established a neural differentiation system using an alginate microencapsulation technique. It was shown that encapsulated ES cells with and without retinoic acid supplementation, following an initial lag-phase, ultimately reached a final cell density 3.5 times greater than the initial seeding density. However, only with retinoic acid treatment, a significant number of encapsulated cells express A2B5 (neural progenitor marker), neurofilament 150kD (neuronal marker), O1 (oligodendrocyte marker) and GFAP (astrocyte marker), as determined by immuneflorescence staining. The highest yield was observed between Day 8 and Day 12 of the encapsulation period. In conjunction with the mechanical and physical characterization of the alginate crosslinking network, we have determined that 2.2% alginate microencapsulation can be optimally adapted to neuronal differentiation from embryonic stem cells. However, we are not able to sustain neural marker expression after 12 days post encapsulation by supplementation of soluble inducer and varying alginate concentrations to alter cell-

material interactions. In the following chapter, we would continue to investigate the roles of cell-cell interaction during neural differentiation process in the encapsulation microenvironment.

# Chapter 3: Regulation of Neural Lineage Embryonic Stem Cell Commitment via Cell Aggregation

## 3.1 Introduction

The development of implantable progenitor cells for cell based therapy in CNS repair is challenged by efficient generation of ES cell-derived neural lineage cells. In the previous chapter, we evaluated the feasibility of using potentially scalable and controllable three-dimensional alginate microenvironment culture system to induce neural differentiation from ES cells. Using this system, we assessed whether incorporating soluble inducers and manipulating capsule parameters, i.e. alginate concentration, could differentially regulate and target cellular differentiation towards neuronal cell lineages. So far, we have demonstrated that the alginate microenvironment maintains cell viability, is conducive to ES cell differentiation to neural lineage cells by incorporating chemical inducer retinoic acid. By comparing the percent of cells positively staining for neural specific markers, we found that 2.2% was most conducive to ES differentiation to neural lineage cells. However, neural lineage expressions after reaching maximum level on Day 12 decrease significantly afterwards with a starting condition of 5 million cells/mL. In the following chapter, we would continue to investigate the roles of cell-cell interaction during neural differentiation process in the alginate encapsulation microenvironment.

Previous studies from our laboratory have demonstrated that the spontaneous hepatocyte differentiation is mediated by cell-cell aggregation in the encapsulation microenvironment. Both cell aggregation and hepatocyte functions, such as urea and

albumin secretion, as well as increased expression of cytokeratin 18 and cyp450 7a1, occur concomitantly with surface E-Cadherin expression (Maguire et al., 2007). In the present study, we sought to modulate cell aggregation process through lowing initial cell seeding density and physically blocking cell aggregation. We found that neural specific markers, i.e. neurofilment, O1 and GFAP expression, can be induced and sustained in the absence of retinoic acid up to 20 days post differentiation at a lower seeding density. Although lowering the initial seeding density could improve neural specific marker expressions in the absence of retinoic acid, the need to culture the cells at low density to achieve neural differentiation limits the number of cells that could potentially be obtained for transplantation strategies. To overcome this, we also designed an antibody blocking experiment. Using the original cell seeding density, cell-cell contact could be disrupted by anti-E-Cadherin, which binds to the cell surface adhesion molecule. By blocking the cell aggregation using anti-E-Cadherin antibody, hepatocyte functions diminished, while encapsulated cells increased neuronal marker expression at a later stage of the encapsulation, even in the absence of retinoic acid. Therefore, as a result of chemical inducer, intracapsular cell-material and/or cell-cell interactions, we are capable of targeting cellular differentiation to both hepatocyte and neuronal cell lineages. These studies could potentially be expanded to more fully address alginate microenvironment regulation of ES cell differentiation.

## 3.2 Materials and Methods

### 3.2.1 ES Cell Culture

All cell cultures were incubated in a humidified 37$^{o}$C, 5% $CO_2$ environment. The ES cell line D3 (ATCC, Manassas, VA) was maintained in an undifferentiated state in T-75

gelatin-coated flasks (Biocoat, BD-Biosciences, Bedford, MA) in Knockout Dulbecco's modified Eagles medium (Gibco, Grand Island, NY) containing 15% knockout serum (Gibco), 4 mM L-glutamine (Gibco), 100 U/mL penicillin (Gibco), 100 U/mL streptomycin (Gibco), 10 mg/mL gentamicin (Gibco), 1,000 U/mL ESGRO$^{TM}$ (Chemicon, Temecula, CA) and 0.1mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). Media was changed every two days until plates were confluent. ES cultures were split and passaged every 6 days. Following media aspiration, cells were washed with 10 mL of phosphate buffered solution (PBS) (Gibco), detached using 3 mL of trypsin EDTA (Gibco) for 3 minutes, and subsequently the addition of 12 mL of Knockout DMEM. Cells were then replated in gelatin-coated T-75 flasks at a density of 1 million cells/mL and only passages 10 through 25 were used in the experiment. In order to induce differentiation, cells were cultured in Iscove's modified Dulbecco's medium (Gibco) containing 20% fetal bovine serum (Gibco), 4 mM L-glutamine (Gibco), 100 U/mL penicillin (Gibco), 100 U/mL streptomycin (Gibco), 10 mg/mL gentamicin (Gibco) in the presence or absence of $10^{-7}$M trans-retinoic acid (Sigma-Aldrich).

### 3.2.2 Alginate Encapsulation

Alginate solution was prepared by dissolving 2.2 g of alginic acid sodium salt (MW: 100,000–200,000 g/mol, G Content: 65%–70%, Sigma-Aldrich) in 100 mL of $Ca^{2+}$ free DMEM (Gibco), using a heated magnetic stir plate at a temperature of 65$^{o}$C. The solution was then filtered using a 45 µm syringe filter (Fisher Scientific, Pittsburg, PA). To create the cell-alginate mixture, 1 mL aliquot of cell suspension with a seeding density of $5\times10^{7}$ cells/mL was added to 9mL of either 1.2%, 1.7%, 2.2% or 2.5% (w/v) alginate solution to

yield a final cell seeding density of $5\times10^6$ cells/mL. This solution was transferred to a 10 mL syringe, and was connected to a syringe pump (KD Scientific, MA). Alginate beads were generated using an electrostatic bead generator (Nisco, Zurich, Switzerland) at a flow rate of 40 mL/h, and an applied voltage of 6.4 kV. The beads were extruded into a 200 mL bath of $CaCl_2$ (100 mM), containing 145 mM NaCl, and 10 mM MOPS (all from Sigma-Aldrich) and were left to polymerize for 10 min at room temperature as outlined in Figure 2.1 (Maguire et al., 2006). Beads were then transferred to a tissue culture treated T-25 flask. The $CaCl_2$ solution was removed using a 5 mL pipette, and the beads were washed with 5mL of HEPES (Gibco). The HEPES was removed and the beads were resuspended in 5 mL of poly-L-lysine (PLL) (Sigma-Aldrich, MW 68,600 g/mol) (0.05% w/v) for 2 min. The PLL was then gently removed, replaced with HEPES to wash the beads and the beads were ultimately resuspended into 5 mL of IMDM medium (Invitrogen, Carlsbad, CA) media. Media was changed at days 4, 8, 12, 16 and 20 post encapsulation.

### 3.2.3 Depolymerization and Cell Recovery

Alginate microcapsules were washed with PBS and then incubated with $Ca^{2+}$ free IMDM medium (Invitrogen) containing 100 mM sodium citrate, 10 mM MOPS and 27 mM NaCl (all from Sigma-Aldrich) for 30 minutes at $37^oC$ to induce depolymerization. The released cells were centrifuged at 1,200 rpm for 6 minutes, the sodium citrate solution was aspirated, and the cell pellet was washed with PBS buffer twice, and responded in cell specific media. The cells were counted using the trypan blue method.

### 3.2.4 Intracapsular Aggregate Size Determination

Microcapsules were sampled from the tissue culture treated T-25 flasks and transferred to 24 well plate on the analysis days 4, 8, 12, 16 and 20. Bright field images were acquired using an Olympus IX70 microscope and an Olympus digital camera. For each microcapsule, 5 z-sectional images were taken at 50 μm intervals to avoid multiple quantification of the same aggregate, for a total depth of 250 μm. Image quantification was conducted using Olympus Microsuite imaging analysis software.

### 3.2.5 Antibody Blocking Experiments

To prevent the formation of aggregates, E-Cadherin (BD Biosciences) antibody was added at a concentration of 0.5 μg/mL and 1 μg/mL to a 5 mL culture sample of microcapsules for 4, 8, 12, 16 and 20 days. As a control for non-specific blocking of cell adhesion molecules a mouse $IgG_{2a}$ at 0.5 μg/mL and 1 μg/mL (BD Biosciences) was utilized in a separate 5 mL sample of microcapsules.

### 3.2.6 Intracapsular Immunofluorescent Staining

Encapsulated cells were washed with PBS (Gibco) and fixed on days 4, 8, 12, 16 and 20 with 4% paraformaldehyde to evaluate surface proteins or together with 0.25% Triton-X to evaluate intracellular protein expression. Cells were incubated with A2B5 (1:250, Chemicon), neurofilament 160 kD (1:200, Chemicon), GFAP (1:50, Abcam, Cambridge, MA) and O1 (1:500, R&D Systems, Minneapolis, MN) overnight at $4^{o}C$ in PBS buffer containing 1% normal goat serum for surface proteins or with together with 0.25% Triton-X for intracellular proteins. The microcapsules were washed three times for 10

minutes in PBS buffer, and then treated with secondary antibody, FITC-conjugated goat anti-rabbit IgG or anti-mouse IgM (both 1:500, Invitrogen) for 2 hours at room temperature. Normal immunoglobulin served as control for non-specific antibody binding, whose fluorescence intensity was subtracted from overall intensity of the antibody expression.

To detect cell surface adhesion molecule E-Cadherin, encapsulated cells were incubated with FITC-conjugated mouse anti-mouse E-Cadherin antibody (1:500, BD Biosciences), or mouse $IgG_{2a}$ (1:500, BD Biosciences) as an isotype control. They were then both washed with PBS three times for 10 minutes. For both stains, fluorescent images were acquired with an Olympus IX70 microscope and an Olympus digital camera using an excitation filter of 515nm. Image quantification was conducted using Olympus Microsuite imaging analysis software.

## 3.3 Results

### 3.3.1 Assessment of Cellular Aggregation during Neural Lineage Differentiation

Our previous studies indicated that spontaneous differentiation of hepatocyte lineage cells within the alginate microbeads was correlated with aggregation formation. Therefore we assessed the role of cellular aggregation during neural lineage differentiation. Although encapsulated cells continued to divide at a similar rate throughout the entire 20 day culture period in the presence of retinoic acid supplementation, they showed markedly decreased cell aggregation relative to non-supplemented cells. In contrast, differentiating cells cultured in basal media configuration continued to aggregate. To quantitate the

cellular aggregation in the encapsulation microenvironment, we estimated the aggregation size (e.g. diameter of cell aggregates) at different depth in the microcapsules as shown in Figure 3.1. It was shown that, under basal medium condition, the cell aggregate size doubled at the end of 16 day encapsulation culture period. As compared to retinoic mediated condition, aggregation sizes did not increase significantly up to Day 12 in the encapsulation culture.

To further quantify the cellular aggregation process, we also measured the expression of E-Cadherin of encapsulated ES cells. E-Cadherin is a surface adhesion molecular that plays an important role in cell aggregation. As shown in Figure 3.2, the expression of E-Cadherin was greatly up-regulated under basal media conditions. Cell surface adhesion molecule E-Cadherin expression also correlated with previous studies on gene expression of encapsulated cells under basal medium condition using DNA microarray as shown in Table 3.1, in which hepatocyte differentiation was favored. Both cell aggregation and hepatocyte functions, such as intracellular albumin expression, urea and albumin secretion, as well as increased expression of cytokaretin 18 and cyp450 7a, occur concomitantly with surface E-Cadherin expression (Maguire et al., 2007). However, in the presence of retinoic acid, encapsulated cells had much lower expression of E-Cadherin.

### 3.3.2 Effects of Initial Seeding Density on Neural Differentiation

Based upon the cell aggregation and E-Cadherin studies, we hypothesized that differentiation towards neural lineage cells could possibly be modulated by controlling

cell aggregation in the encapsulation microenvironment even in the absence of retinoic acid. We first evaluated the effects of initial seeding density on cell aggregation and neural differentiation in the alginate microenvironment. A remarkably decrease in cell aggregation was observed when lowering the initial seeding density. When ES cells were encapsulated at 5 and 10 million cells/mL, cells started to form small aggregates as early as Day 8 as shown in Figure 3.3. While in 1 million cells/mL condition, cell aggregate size was much smaller, and it is almost indistinguishable to the RA supplemented condition.

Furthermore, the immunefluorescent staining demonstrated that lowering cell seeding density results in increased neural specific protein expressions. Like many other neural differentiation protocols, neural differentiation is in general favored when lower cell seeding densities were applied. Higher initial seeding densities (i.e. 10 million cells/mL) followed the similar trends as the 5 million cells/mL condition, with most of the neural marker expression diminished after 12 days post encapsulation (data not shown). While at lower initial seeding densities (i.e. 1 million cells/mL), neural specific markers, i.e. neurofilment, O1 and GFAP expression, can be induced and sustained in the absence of retinoic acid up to 20 days post differentiation. However, when we lowered the cell seeding density from 5 million cells/mL to 1 million cells/mL, we found that cell density also was significantly decreased after 20 days in encapsulation system as shown in Figure 3.4.

### 3.3.3 Control of Cellular Differentiation by Antibody Blocking

In addition to lowering cell seeding density to reduce cell-cell aggregation possibility, we also evaluated the effects of antibody blocking on cell aggregation and neural differentiation in the alginate microenvironment. 0.5 μg/mL of E-Cadherin and corresponding isotype antibodies were added to cultures to prevent the formation of aggregates as described in previous studies (Maguire et al., 2007). The net expression of neural specific markers was quantified by subtracting non-specific isotype antibody yields from cultures continuously exposed to E-Cadherin antibody blocking. As shown in Figure 3.5, cell aggregate size was significantly reduced following E-Cadherin blocking. Furthermore, by blocking the cell aggregation using 0.5 mg/mL anti-E-Cadherin antibody, intracellular albumin expression declined dramatically, while encapsulated cells continued to express neuronal markers A2B5, NF 150kD, O1 and GFAP marker at a later stage of the encapsulation even in the absence of retinoic acid in Figure 3.6. Encapsulated cell sub-population expressing neural specific markers, after reaching the maximum levels at Day 16 (Day 12 for neurofilament), plateaued by the end of 24-day culture period in the presence of E-Cadherin blocking antibody.

To further our analysis of E-Cadherin blocking for ES differentiation, we raised the concentration of E-Cadherin blocking antibody from 0.5 to 1 μg/mL. The results of intracapsular immunofluorescence indicated a similar functional trend during the first 12 days in the encapsulation microenvironment. However, as shown in Figure 3.6, the net expression of neural specific markers with1 μg/mL antibody blocking gradually increased during the differentiation process. Hepatocyte differentiation was complete diminished, while the neural marker expressions were sustained and enhanced. Therefore

disruption of cell aggregation in the alginate microcapsules induced and sustained neural specific differentiation even in the absence of retinoic acid, without compromising final cell output.

### 3.4 Discussion

Previous studies in our laboratory have demonstrated that the alginate microenvironment maintains cell viability, is conducive to ES cell differentiation to hepatocyte lineage cells, and maintains differentiated hepatocyte cellular function. In addition, hepatocyte differentiation is mediated by cell-cell aggregation in the encapsulation microenvironment. Both cell aggregation and hepatocyte functions, such as urea and albumin secretion, as well as increased expression of cytokeratin 18 and cyp450 7a1, occur concomitantly with surface E-Cadherin expression. Furthermore, by incorporating soluble inducers, such as retinoic acid, into the permeable microcapsule system, we demonstrate decreased cell aggregation and enhanced neuronal lineage differentiation with the expression of various neuronal specific markers, including neurofilament, A2B5, O1 and GFAP. We found that in the presence of retinoic acid, encapsulated cells showed markedly deceased cell aggregation and the size of individual and unaggregated cells was approximately 13μm throughout the entire 16 day differentiation period as shown in Figure 3.1. This cell size corresponds to the crosslinking spacing of 2.2% alginate gels, the optimal alginate concentration for differentiation induced to both neural and hepatocyte lineages (Maguire et al., 2006). It suggests that 2.2% alginate concentration can optimally adapted for embryonic stem cell differentiation in general in encapsulation mediated system. It is currently unclear whether physical structure contributes to metabolic transport, or other factors that may contribute differentiation control in the

microbeads. Nevertheless, our results suggest the possibility of modulating differentiated ES cell commitment via rigidity and crosslinking density of the alginate gel network.

In addition to significantly decreased cell aggregate size, only a small portion of retinoic acid treated cells expressed E-Cadherin, which is known for controlling the process of cellular aggregation. Although the effects of retinoic acid on embryonic stem cell aggregation are not fully understood, previous studies demonstrated more than 75% decrease in aggregate volume of embryonal carcinoma cell culture P19 culture treated in retinoic acid as compared untreated case (Jones-Villeneuve et al., 1982; Jones-Villeneuve et al., 1983). The decreased aggregation probably reflects accumulation of cells in the G1 phase of the cycle. However the volume decrease only affected the cells in which neurons and glia were destined to develop. Retinoic acid-treated RAC65 cells, another embryonal carcinoma cell line that differentiates into fibroblast-like cells, did not show such decrease in volume. These studies suggest the possibility that cell aggregate size might be associated with neural and glial progenitor cell commitments, especially during Day 8 and Day 12 where the highest yield was observed.

Based upon the cell aggregation and E-Cadherin studies, we hypothesized that differentiation towards neural lineage cells could possibly be modulated by controlling cell aggregation in the encapsulation microenvironment even in the absence of retinoic acid. A lot of existing neural differentiation protocols suggests culture ES cells at very low densities (Ying et al., 2003). It is likely to be associated with the Wnt/β-catenin signaling pathway for neural lineage commitment, which has been shown to be a cell-cell

(and/or cell-matrix) contact-regulated process that is involved in neurogenesis (Otero et al., 2003). Membrane bound β-catenin is associated with E-Cadherin junctions and bridge E-Cadherin to the cytoskeleton. When ES cells were induced to differentiation at high density in EB mediated culture, a greater proportion of β-catenin was phosphorylated on its N terminus. Phosphorylation of β-catenin targets it for ubiquitin-directed proteolysis, and inhibits nuclear β-catenin, therefore suggesting that β-catenin signaling is more active when ES cells are differentiated at low density. In our present studies, we have also evaluated the effects of initial seeding density on neural differentiation in the alginate microenvironment. The results of these studies suggest that neural differentiation can be induced and sustained in the absence of chemical inducers by lowering the initial cell seeding density. However the need to culture the cells at low density to achieve neural differentiation limits the number of cells that could potentially be obtained for transplantation strategies. Therefore an antibody blocking experiment, using anti-E-Cadherin antibody, was designed to prevent formation of cell aggregates. Previous studies have demonstrated enhanced neural differentiation at high seeding density without RA treatment by tranfecting ES cells with H2kd-E-Cadherin (i.e. dominant-negative E-Cadherin). H2kd-E-Cadherin has been shown to decrease endogenous levels of E-Cadherin, disrupt cell-cell contact, increase protein levels of β-catenin and therefore increase β-catenin signaling.

Our antibody blocking experiment demonstrated that with continuous E-Cadherin blocking, hepatocyte differentiation diminished, while encapsulated cells increased neuronal marker expression and reached a plateau at the end of 24 day culture period.

Although prevention of cell aggregation and concomitant neuronal differentiation can be achieved in the presence of the soluble inducer retinoic acid, the process cannot be sustained. Neural lineage markers decreased and albumin expression increased by day 12 post-differentiation induction and the re-emergence of the hepatocyte phenotype occurred concomitantly with cell aggregation. Based on the aggregation size study, once again small aggregates began to form after 12 days in the alginate microenvironment. To validate the aggregation-mediated hypothesis, we sought to further modulate cellular aggregation by increasing the concentration of blocking antibodies. In these experiments, we determined that, increasing the blocking antibody concentration resulted in further inhibition of aggregate size during the 24 day differentiation period. In addition, expression of neural lineage specific markers gradually increased during the differentiation process.

In summary, our results showed that retinoic acid mediated differentiation pathway selection may be modulated, at least in part, through cellular aggregation inhibition in the alginate microenvironment. Our study demonstrated that hepatocyte differentiation is mediated by cell-cell aggregation, and the aggregation size of encapsulated cells doubles over the 16 day culture period. Both cell aggregation and hepatocyte function increase concomitantly with surface E-Cadherin expression. In contrast, in the presence of retinoic acid, encapsulated cells show decreased cell aggregation and enhanced neuronal lineage differentiation with the expression of various neuronal specific markers. Furthermore, by lowering initial seeding density, we have demonstrated enhanced and more sustainable neural marker expression in the absence of retinoic acid. Further enhancement of

differentiation efficiency can be achieved by physically blocking aggregation using antibodies, in which hepatocyte functions diminished, and neural differentiation further improved even in the absence of the inducer without compromising cell numbers.

# Chapter 4: Conclusions and Future Applications

## 4.1 Summary of Studies

The ability of embryonic stem cells to adopt multiple fates makes them prime candidates for current cell replacement studies. The potential use of ES cells to replace functional loss of particular tissues heavily depends on efficient differentiation protocols to derive lineage specific progenitor cells. We have previously established a scalable and controllable alginate-poly-l-lysine microencapsulation differentiation system. This dissertation study was designed to determine the feasibility of adapting the alginate encapsulation technique to neuronal lineage differentiation. The results of our studies indicate that by incorporating the soluble inducer, retinoic acid into the permeable microcapsule system, cell aggregation was decreased and neuronal lineage differentiation enhanced. In conjunction with the mechanical and physical characterization of the alginate crosslinking network, we have determined that 2.2% alginate microencapsulation can be optimally adapted to both hepatocyte and neuronal differentiation from embryonic stem cells. However, differentiation could be directed away from the hepatocyte and towards the neural lineage by lowering the initial cell seeding density and physical cell-cell aggregation blocking, even in the absence of retinoic acid. This study promises to offer insights into targeting cellular differentiation towards both endodermal and ectodermal cell lineages, and could potentially be generalizable and adaptable to the differentiation of other stem cell types given the correct inducible factors and material properties.

## 4.2 Future Work

### 4.2.1 Neuronal Function Enhancement through Secondary Inducer

The kinetic profile of encapsulated cells suggested that the neural specific marker expression, after reaching its peak on Day 8 and 12, declined significantly after 16 days in the encapsulation microenvironment. Previous studies suggested that retinoic acid and secondary inducer such as neurotrophins act at distinct stages in neurogenesis. RA could first promote the acquisition of a neuronal fate, but is not capable of sustaining and maturating neural function. To further sustain the neuronal functions in the encapsulation microenvironment, we could investigate the possibility of utilizing secondary inducers in conjunction with retinoic acid supplementation. We have initiated some study using conditioned medium from primary rat hippocampus culture cultures in low-serum medium, which could induce a neuron-enriched population. In our preliminary studies, encapsulated cells, after 8 day treatment in retinoic acid, were cultured in conditioned medium from primary rat neuronal culture for another 6 days. The intracapsular staining demonstrated that the percent of differentiated cells positive for O1 marker increased to about 60% after 6 days in conditioned medium as shown in Figure 4.1. These studies suggested the possibility of secondary differentiation induction of neural lineages. Other possible candidates as secondary inducer include hedghehog signaling (Gratsch et al., 2002). Recent studies also show that sonic hedgehog (Shh) can act as a morphogen or mitogen, and promote motor neurons after initial induction by retinoic acid.

### 4.2.2 Co-effects of retinoic acid and E-Cadherin blocking

In Chapter 3, we have demonstrated that by blocking cellular aggregation using E-Cadherin antibody, we were able to increase neural marker expression, even in the absence of retinoic acid. At low seeding density without RA treatment, there is also a significant amount of neural differentiation at the end of 20 day encapsulation period, as demonstrated by expression of neurofilament, GFAP and O1. However, the yield is lower than the retinoic acid treated condition and antibody blocking condition. Although RA signaling is not required in β-catenin mediated neural commitment from ES cells, some of the previous studies suggest RA could potentiate β-catenin mediated neural differentiation by increasing the percentage of cells that are committed to the neural lineage (Otero et al., 2004). By combination of RA treatment and E-Cadherin blocking, the cell aggregation may potentially be inhibited and therefore enhance neural functions in the alginate microenvironment, as an alternative to secondary inducer such as conditioned medium and sonic hedgehog.

### 4.2.3 Cell Recovery and Scale up

One of the issues with using ES derived neural progenitor cells in therapeutic applications is the large cell mass needed. In animal models of acute spinal cord injury mentioned previously, it was shown that 24 separate animal studies required the production of more than five billion OPC cells. Due to the important concern that large numbers of cells are needed for cell-based therapies, generating a reliable cell source for large-scale manufacturing has also emerged as an important challenge in the field. To utilize neural progenitor cells generated from alginate encapsulation differentiation system as a transplantable cell source, they have to be recovered from alginate

microcapsules first. Moreover, in our previous studies, morphological quantification cannot be achieved in the microcapsule environment as cells are either in suspension and/or attached to the alginate micro-architecture. In either case cells in the capsule appear uniformly rounded, and do not exhibit the typical branched neural morphology.

Cell recovery from alginate gels can be efficiently achieved using sodium citrate and/or alginate lyase. In the future, extending the effects of maintenance in secondary culture such as utilizing extracellular matrix for recovered cells can provide researchers with a more sustainable and readily available cell source.

### 4.3 Conclusions

In conclusion, we have demonstrated that alginate microencapsulation could potentially provide a scalable system to control embryonic stem cell differentiation into neural lineage cells through retinoic acid supplementation, lowering initial cell seeding density and physical disrupt cell-cell contact using anti-E-cadheirn antibody blocking. As a result of the chemical inducer, intracapsular cell-material and/or cell-cell manipulation, we are able to target cellular differentiation to neural cell lineages. Through these studies, we have established a promising approach to modulate neural lineage differentiation from renewable embryonic stem cells, in a mechanically defined culture environment. Ultimately cellular encapsulation of ES cells may provide a controllable approach to generate large numbers of differentiated cells for a variety of clinical and pharmaceutical applications.

**Table 1.1:** Embryonic stem cell differentiation protocols.

| | Differentiation Configuration | Neural Differentiation | Hepatocyte Differentiation |
|---|---|---|---|
| **Biochemical Cues** | Embryoid bodies (EB) | ▪ Retinoic acid (Bain, 1995)<br>▪ FGF-2 in ITSFn medium (Okabe, 1996) | ▪ Spontaneous (Hamazaki, 2001; Novik, 2006)<br>▪ Growth factor and ECM protein supplementation (Hamazaki, 2001; Novik, 2006) |
| | Monolayer | ▪ N2 in ITS medium (Ying, 2003)<br>▪ Noggin and chordin (O'Shea, 2002) | ▪ Sodium butyrate (Sharma, 2006) |
| | Co-Culture | ▪ Chick dorsal root ganglia conditioned medium (Kitazawa, 2005)<br>▪ Glial cell conditioned medium (Tian, 2005)<br>▪ Co-culture with PA6 cells (Kawasaki, 2006) | ▪ Co-Culture with nonparenchymal cells (Soto-Gutierrez, 2007)<br>▪ Collagen sandwich with hepatoctyes (Cho, 2008) |
| **Mechanical Cues** | Extracellular matrix | ▪ Laminin (Pierret, 2007)<br>▪ Fibronectin (Parashurama, 2008)<br>▪ Collagen (Pierret, 2007; Novik, 2008) | |
| | Synthetic polymers | ▪ Polyacrylamide (Engler, 2006; Li, 2008)<br>▪ HEMA/DMAEMA (Kroupova, 2006)<br>▪ PLGA/PLA (Levenberg, 2003) | |
| | Natural materials | ▪ Alginate (Fang, 2007; Maguire, 2006; Magyar, 2001) | |

**Table 2.1:** Comparison of percent positive cells in the medium with and out retinoic acid supplementation.

| **Neural Specific Markers** | **A2B5** | **O1** | **NF-160kD** | **GFAP** |
|---|---|---|---|---|
| | Progenitor Marker | Oligodendrocyte Marker | Neuronal Marker | Astrocyte Marker |
| **Basal** | <5% | <5% | <5% | <5% |
| **1.2% + RA** | 13.3 ± 1.7% | 30.6 ± 5.5% | 27.5 ± 4.2% | 18.1 ± 2.1% |
| **1.7% + RA** | 9.3 ± 1.2% | 26.7 ± 4.1% | 24.5 ± 3.8% | 15.6 ± 0.8% |
| **2.2% + RA** | 24.7 ± 1.3% | 32.3 ± 1.0% | 41.9 ± 2.6% | 38.6 ± 1.9% |
| **2.5% + RA** | 16.7 ± 2.1% | 28.6 ± 2.7% | 33.7 ± 3.2% | 25.3 ± 1.9% |

Error bar represents standard error of the mean.

**Table 3.1:** Microarray analysis of encapsulated cells

| Description | Abbrev. | Expression ratio | P-value |
|---|---|---|---|
| Cytokeratin 18 | CK-18 | 1.20 | 0.0002 |
| Cadherin 17 | CDH17 | 1.04 | 0.025 |
| Connexin 26 (Gap junction membrane channel protein beta 2) | GJB2 | 1.08 | 0.009 |
| Connexin 32 (Gap junction membrane Channel protein beta 1) | GJB1 | 3.06 | 0.001 |
| **E-Cadherin (Cadherin 1)** | **CDH1** | **1.93** | **0.030** |
| E-Selectin | SELE | 1.50 | 0.040 |

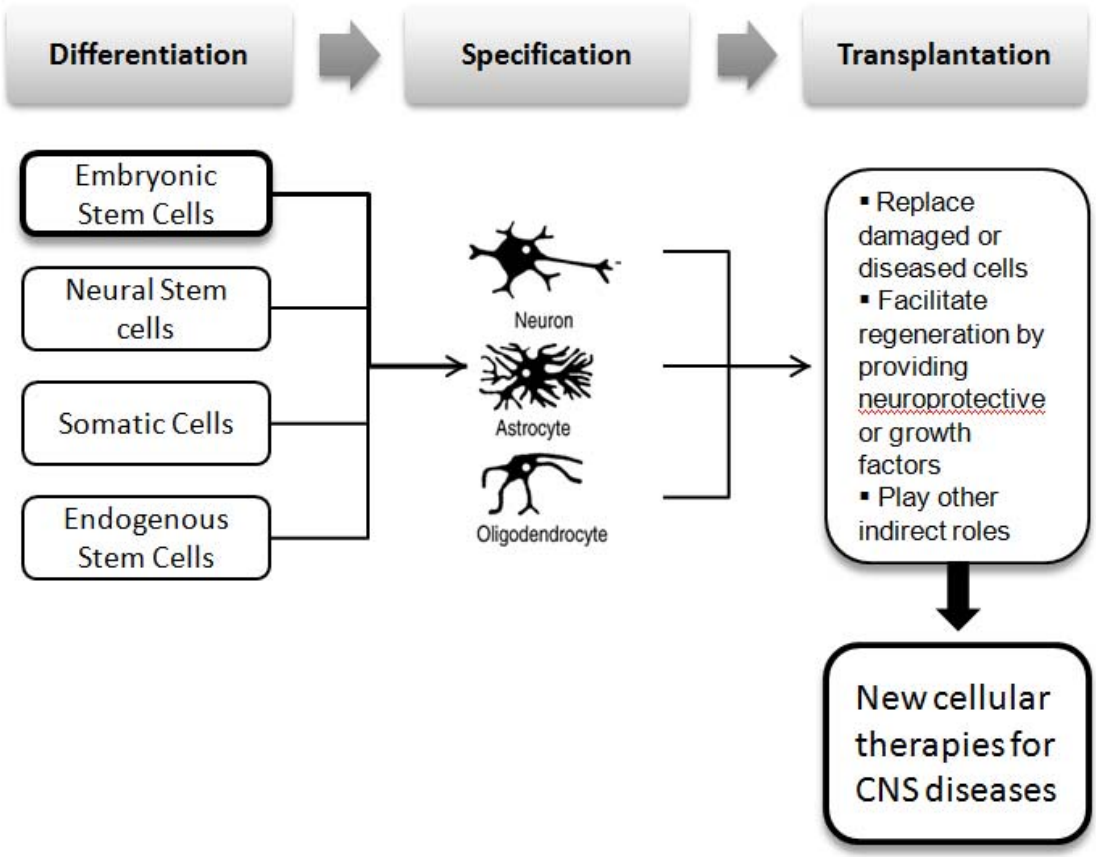


**Figure 1.1:** Differentiation of stem cells into neural lineage cell type and application in cellular therapies in central nervous system repair.

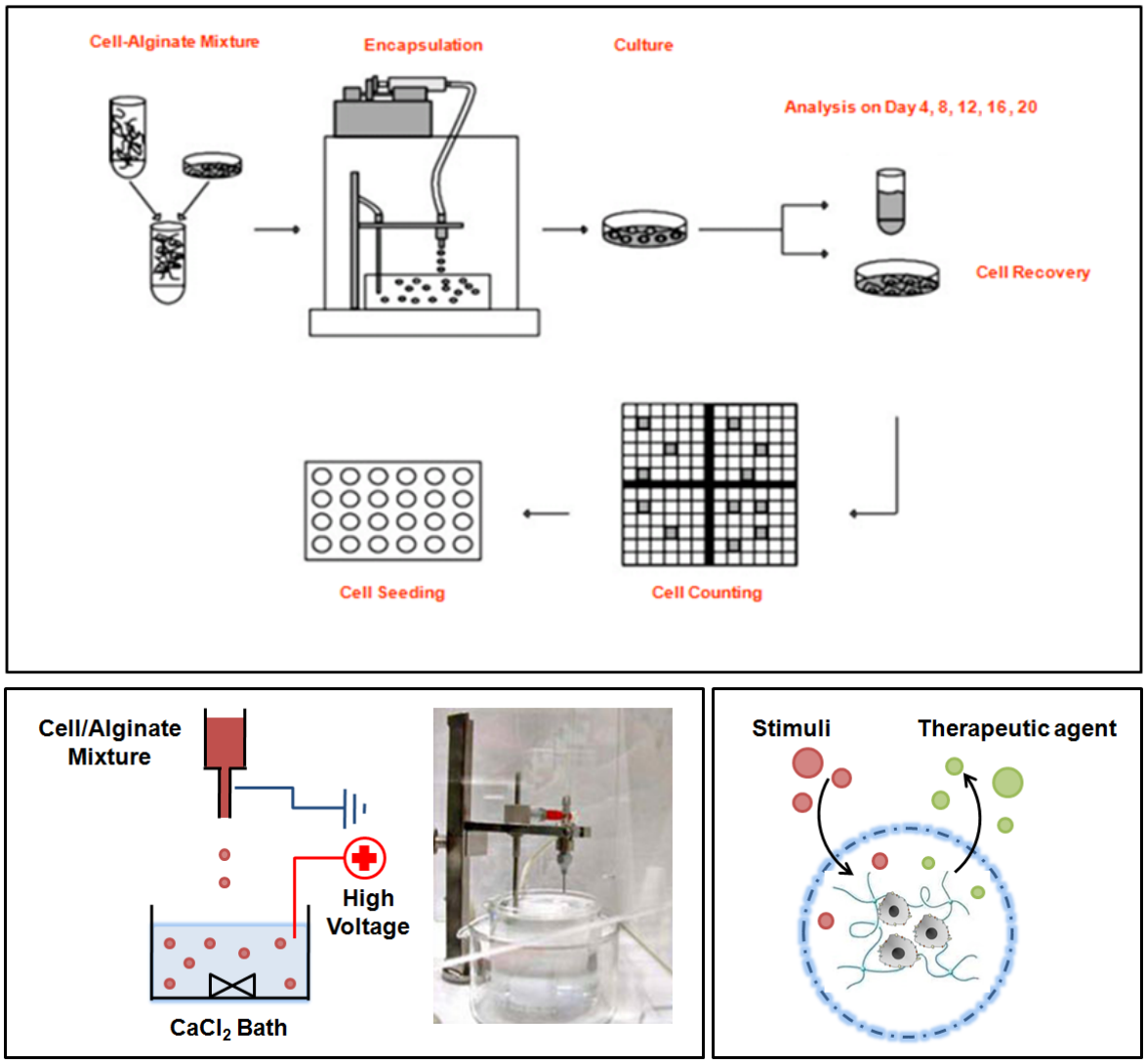


**Figure 1.2:** Illustration of alginate microencapsulation technique.

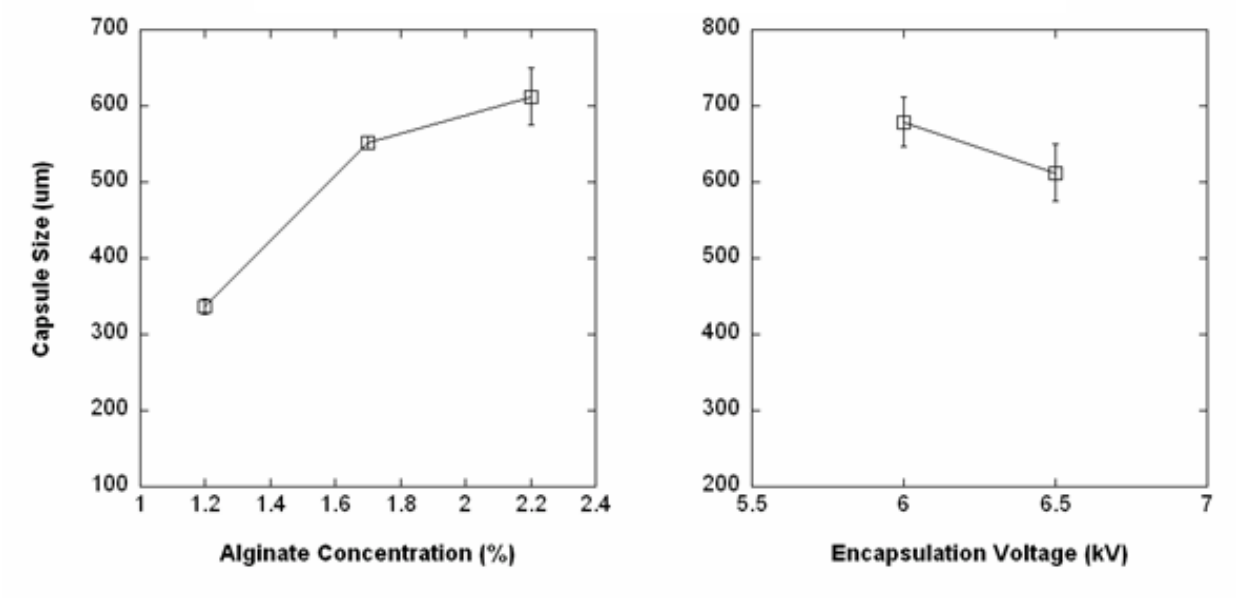


**Figure 2.2:** Capsule sizes at different alginate concentrations and encapsulation voltages.

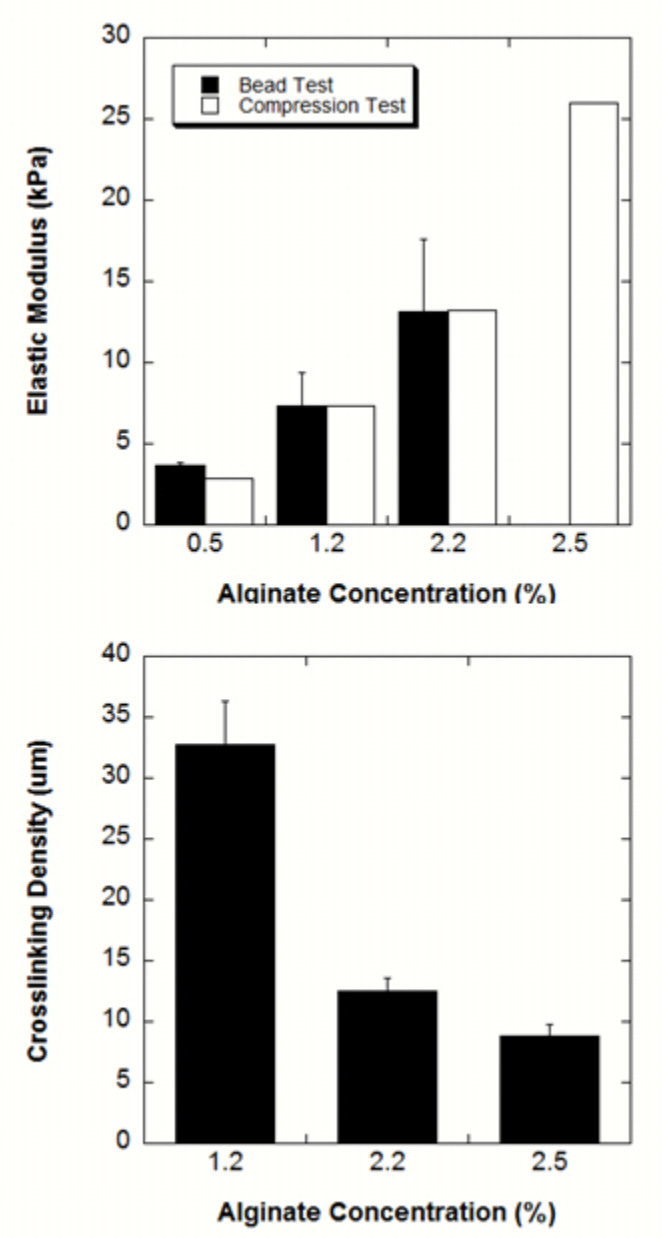


**Figure 2.3: (Top)** Characterization of mechanical properties of alginate gels at various concentrations. **(Bottom)** Characterization of crosslinking density of alginate gels at various concentrations.

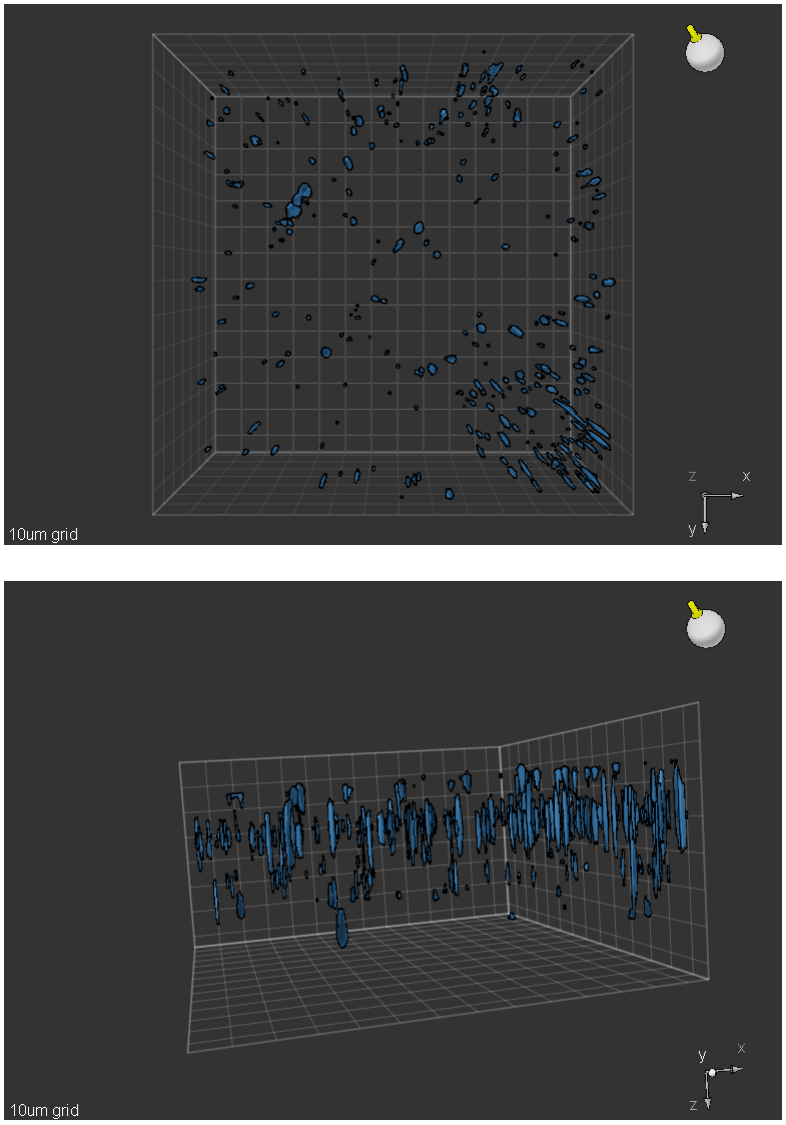


**Figure 2.4: (Top)** Top view and **(Bottom)** side view of crosslinging spacing of 2.2% alginate microcapsule with alcian blue staining. 3D Surface View created based on stack of 10 images at 60x using Slidebook.

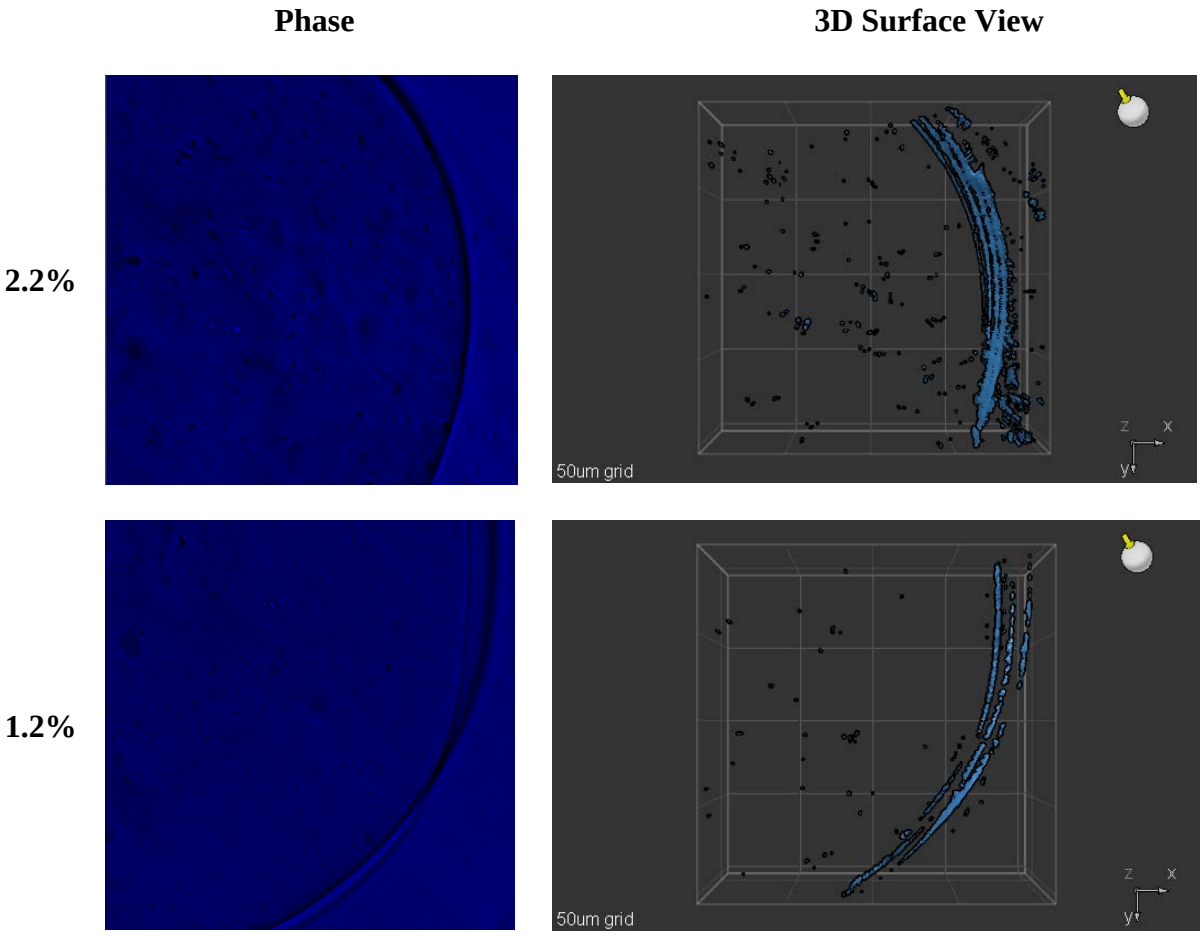


**Figure 2.5:** Phase images and 3D Surface View of crosslinging spacing with alcian blue staining at various alginate concentrations.

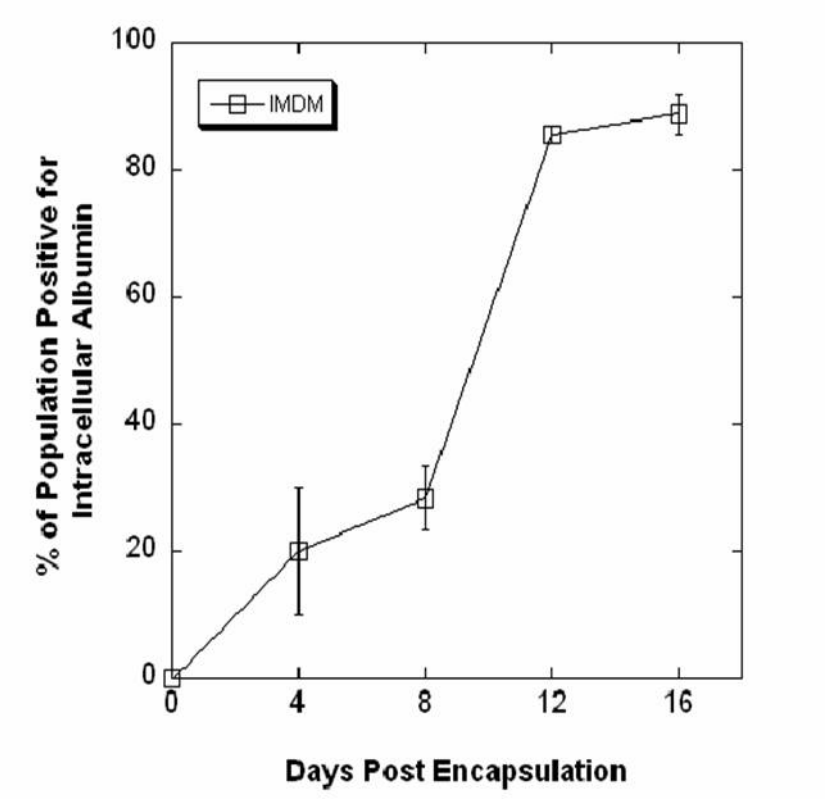


**Figure 2.6:** Kinetic profile of intracellular albumin expression in alginate microencapsulation differentiation system. ES cells were encapsulated in 2.2% (w/v) alginate at a cell seeding density of $5\times10^6$ cells/mL, and cultured in IMDM medium for 16 days. Error bar represents standard error of the mean.

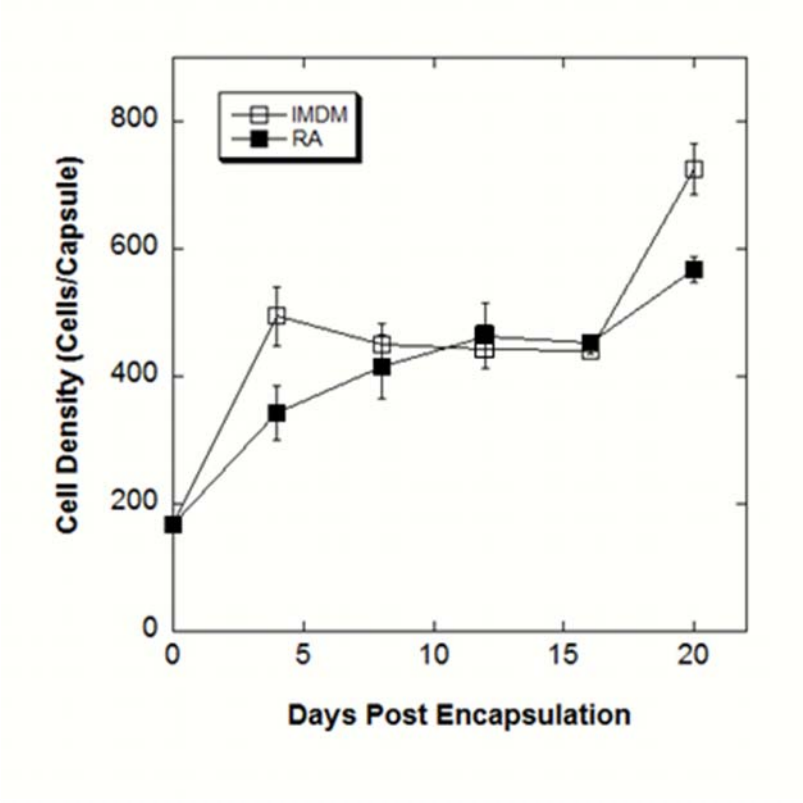


**Figure 2.7:** Kinetic profile of cell density in alginate microencapsulation differentiation system. ES cells were encapsulated in 2.2% (w/v) alginate at a cell seeding density of $5\times10^6$ cells/mL, and cultured in IMDM medium with and without $10^{-7}$ M trans-retinoic acid supplementation. Error bar represents standard error of the mean.

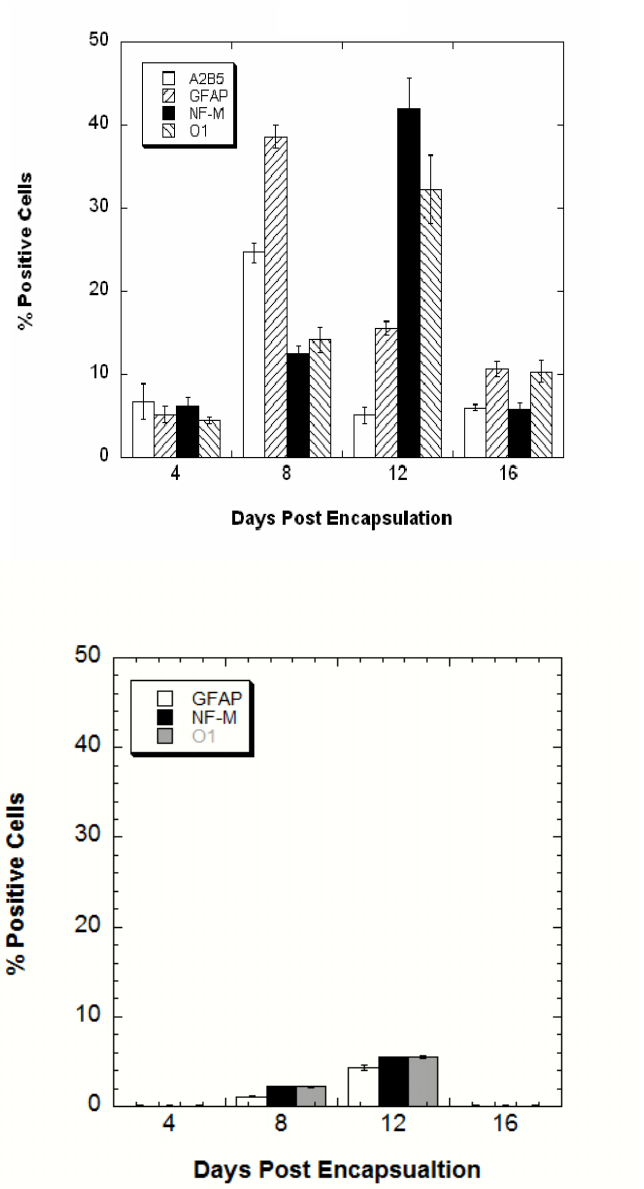


**Figure 2.8:** Kinetic profile of percent of positive population in 2.2% alginate microencapsulation differentiation system in the presence **(Top)** and in the absence **(Bottom)** of retinoic acid. ES cells were encapsulated at a cell seeding density of $5\times10^6$ cells/mL, and cultured in IMDM medium with and without $10^{-7}$ M trans-retinoic acid supplementation for 16 days. Error bar represents standard error of the mean.

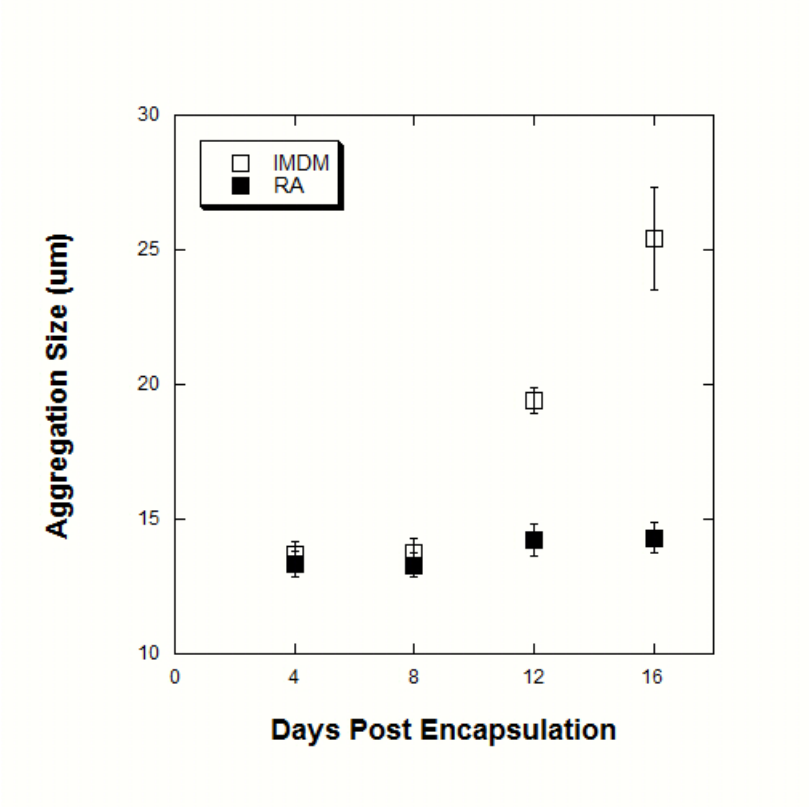


**Figure 3.1:** Kinetic profile of size of aggregate formation in alginate microencapsulation differentiation system. Time course of cell aggregates diameter. ES cells were encapsulated in 2.2% (w/v) alginate at a cell seeding density of $5\times10^{6}$ cells/mL, and cultured in IMDM with and without $10^{-7}$ M trans-retinoic acid supplementation.

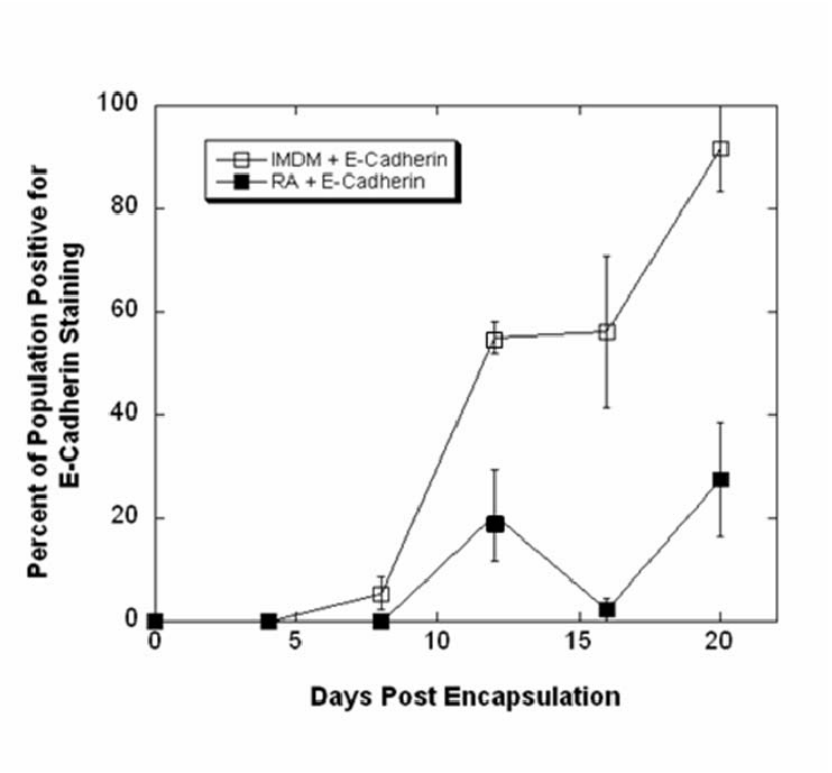


**Figure 3.2:** Expression of E-Cadherin in alginate microencapsulation differentiation system. Time course of percentage of encapsulated cells expressing cell adhesion molecule E-Cadherin. ES cells were encapsulated in 2.2% (w/v) alginate at a cell seeding density of $5\times10^{6}$ cells/mL, and cultured in IMDM with and without $10^{-7}$ M trans-retinoic acid supplementation. E-Cadherin intensity values were determined using intracapsular immunofluorescence techniques. Error bar represents standard error of the mean.

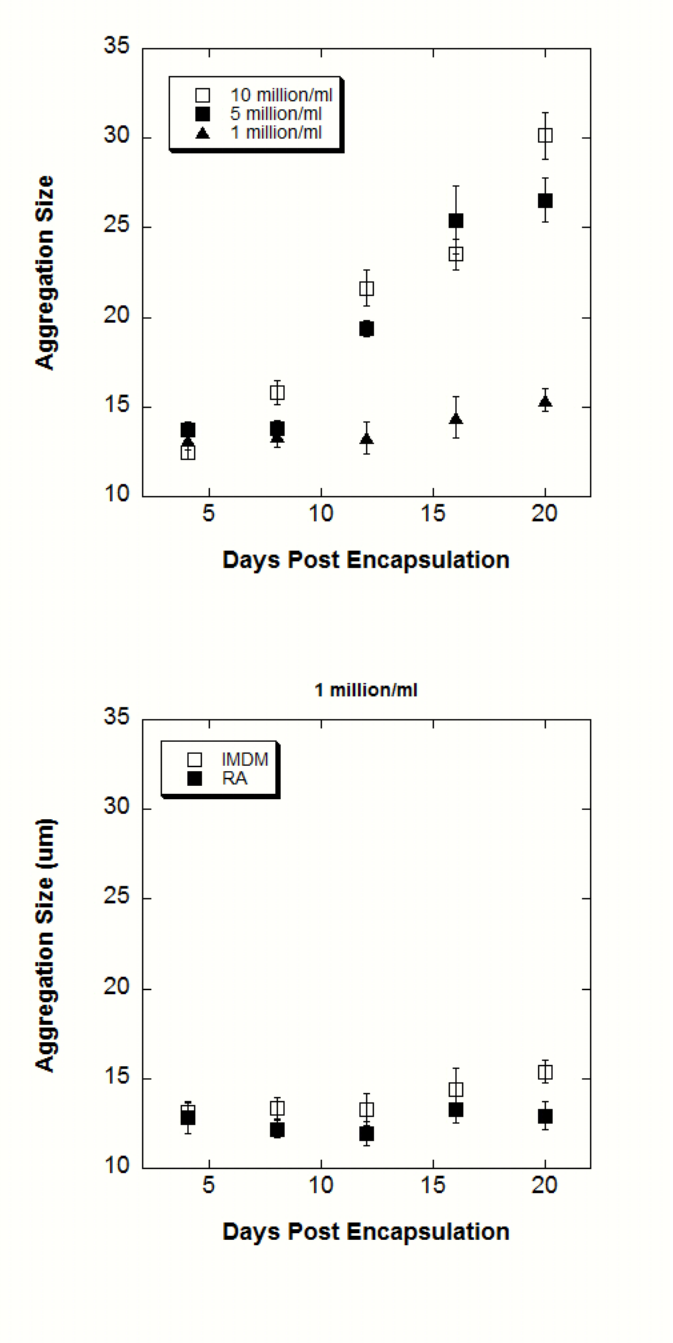


**Figure 3.3: (Top)** Kinetic profile of size of aggregate formation in alginate microencapsulation differentiation system at varying initial seeding density. ES cells were encapsulated in 2.2% (w/v) alginate at cell seeding density of $1\times10^6$ ,$5\times10^6$ and $10\times10^6$ cells/mL, and cultured in IMDM medium. **(Bottom)** Kinetic profile of size of

aggregate formation in alginate microencapsulation differentiation system at $1\times10^{6}$ cells/mL, and cultured in IMDM medium with and without $10^{-7}$ M trans-retinoic acid supplementation.

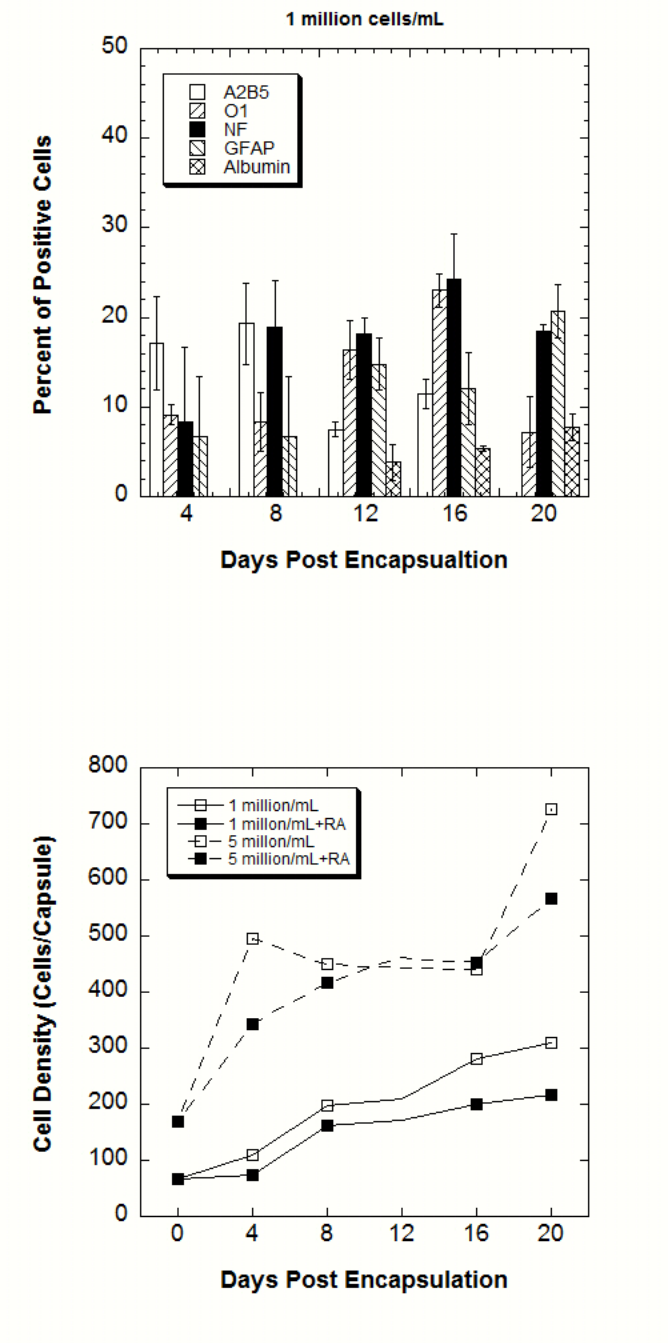


**Figure 3.4: (Top)** Kinetic profile of percent of positive population and cell density in 2.2% alginate microencapsulation differentiation system at lower initial seeding density. ES cells were encapsulated at a cell seeding density of $1\times10^6$ cells/mL, and cultured in IMDM medium for 20 days. **(Bottom)** Kinetic profile of cell density at varying initial

seeding density. ES cells were encapsulated in 2.2% (w/v) alginate at a cell seeding density of $1\times10^6$ and $5\times10^6$ cells/mL, and cultured in IMDM medium with and without $10^{-7}$ M trans-retinoic acid supplementation. Error bar represents standard error of the mean.

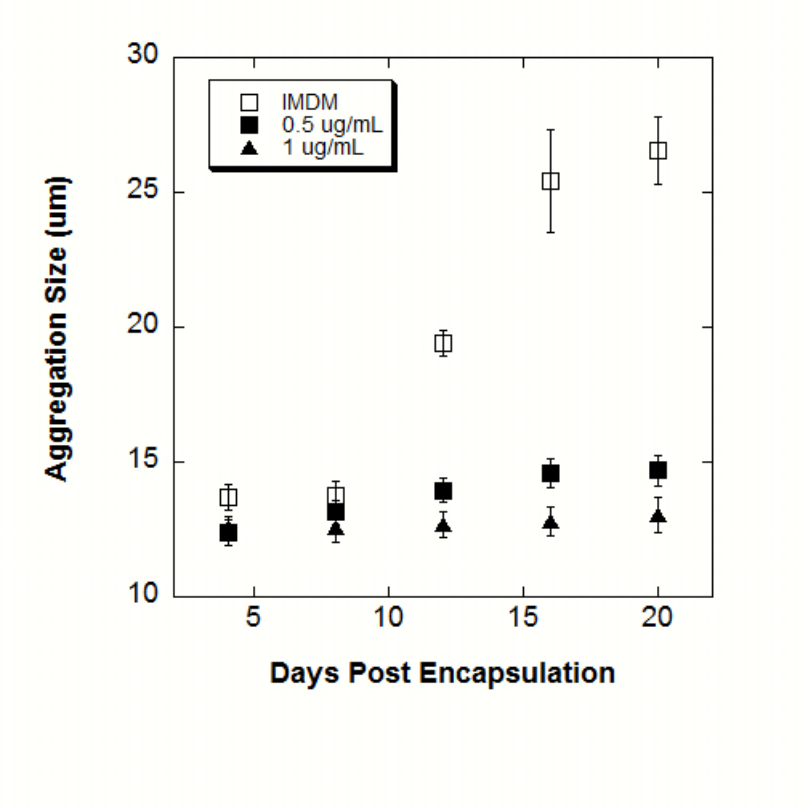


**Figure 3.5:** Kinetic profile of size of aggregate formation in alginate microencapsulation differentiation system with antibody blocking. Time course of cell aggregates diameter. ES cells were encapsulated in 2.2% (w/v) alginate at a cell seeding density of $5\times10^6$ cells/mL, and cultured in IMDM with and without 0.5 and 1 µg/mL E-Cadherin antibody blocking.

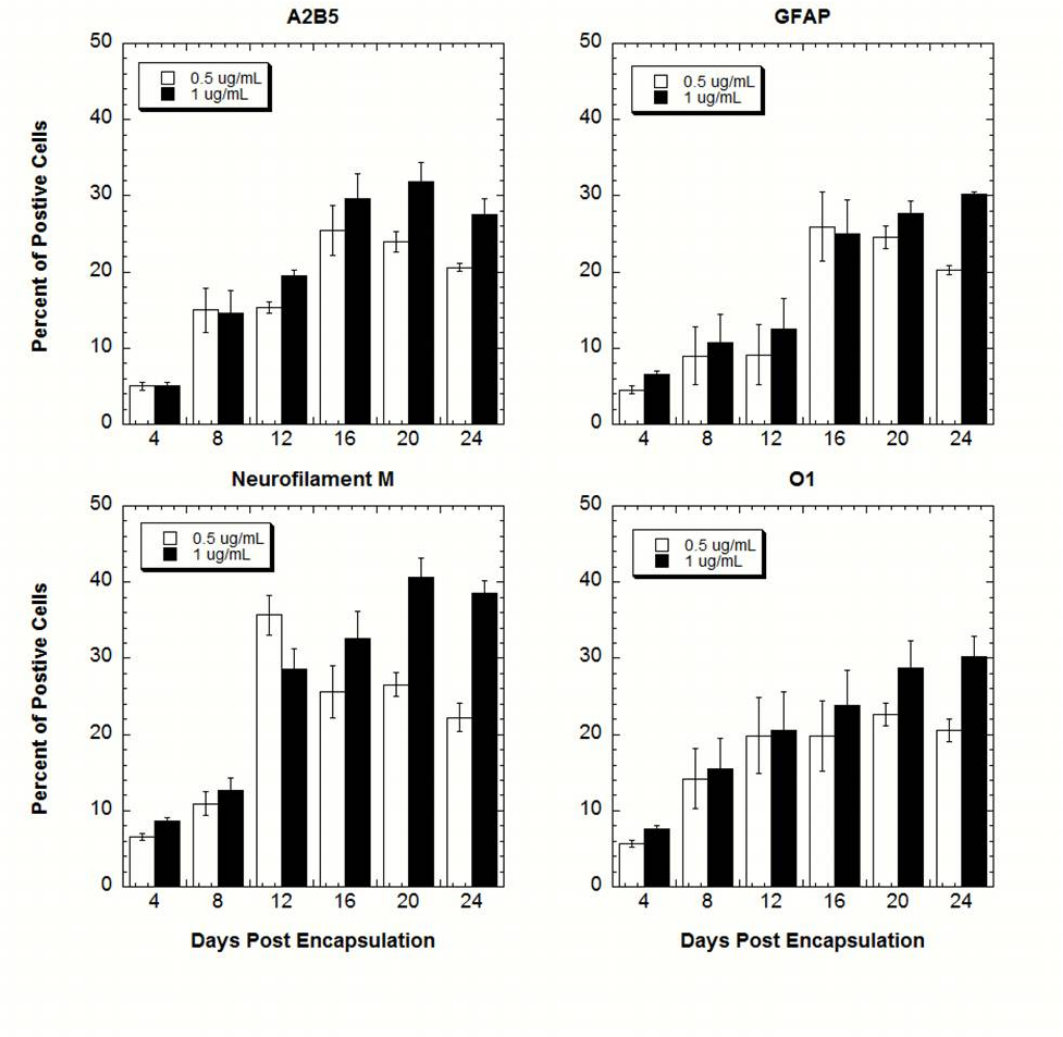


**Figure 3.6:** Kinetic profile of percent of positive population with E-Cadherin antibody blocking in alginate microencapsulation differentiation system. Time course of percentage of encapsulated cells expressing A2B5, neurofilment 160kD, O1 and GFAP antibodies. ES cells were encapsulated in 2.2% (w/v) alginate at a cell seeding density of $5\times10^6$ cells/mL, and cultured in IMDM medium with 0.5 and 1 μg/mL of E-Cadherin antibody blocking. Fluorescent intensity values of four markers were determined using intracapsular immunefluorescence techniques. Error bar represents standard error of the mean.

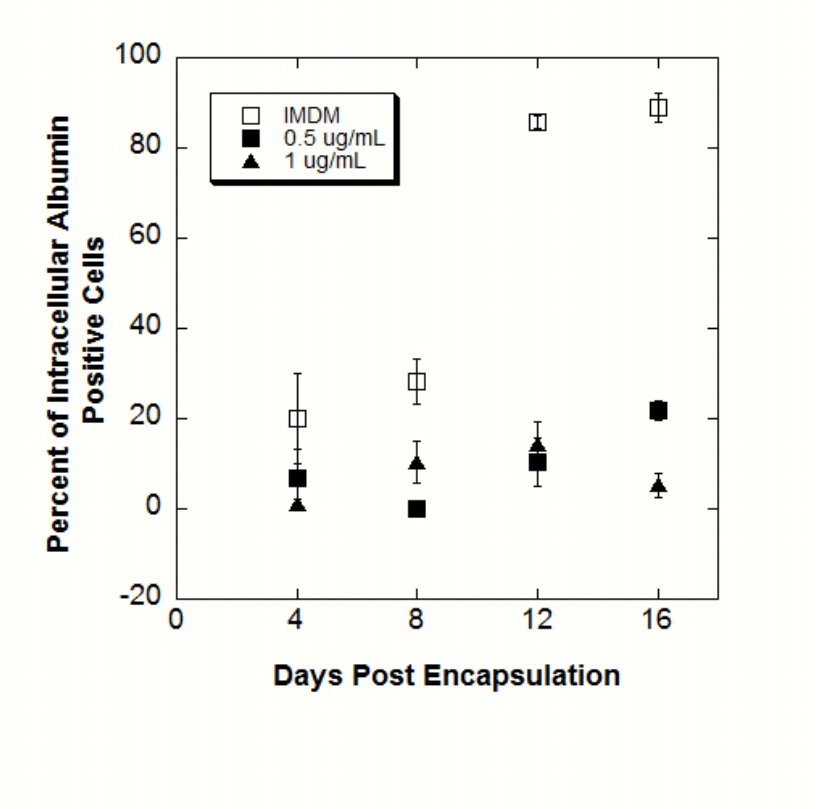


**Figure 3.7:** Kinetic profile of percent of positive population of intracellular albumin expression with E-Cadherin antibody blocking in alginate microencapsulation differentiation system. ES cells were encapsulated in 2.2% (w/v) alginate at a cell seeding density of $5\times10^6$ cells/mL, and cultured in IMDM medium alone and with 0.5 and 1 µg/mL of E-Cadherin antibody blocking. Fluorescent intensity values of albumin expression were determined using intracapsular immunefluorescence techniques. Error bar represents standard error of the mean.

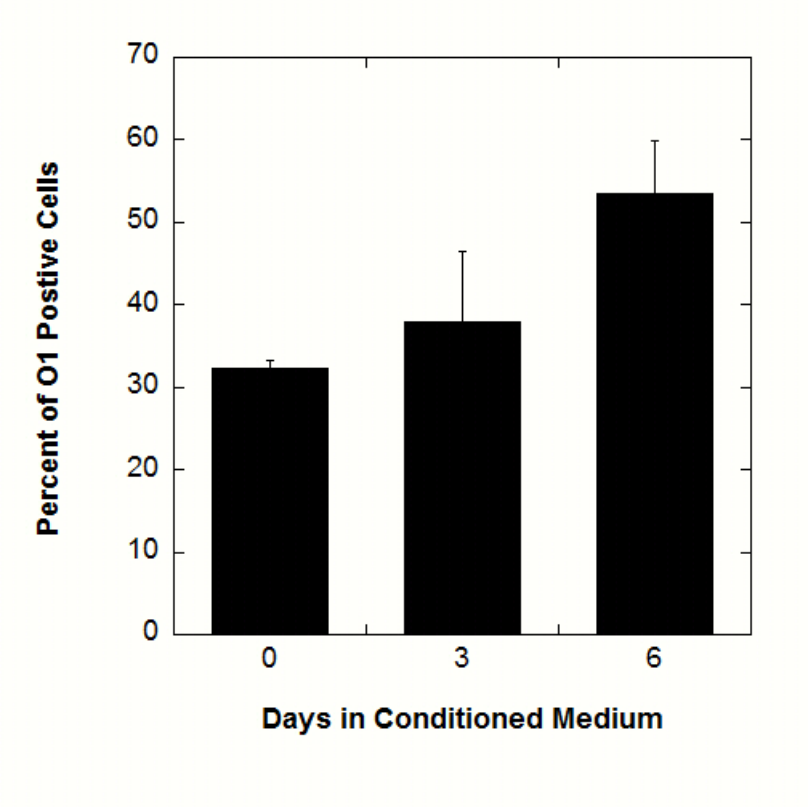


**Figure 4.1:** Kinetic profile of percent of positive population of O1 expression in primary neuronal conditioned medium. ES cells were encapsulated at 2.2% and cell seeding density of $5\times10^{6}$ cells/mL, and cultured in IMDM medium with $10^{-7}$ M trans-retinoic acid supplementation for 8 days, followed by another 6 days in conditioned medium.

**Bibliography**

Bain, G., et al., *Embryonic stem cells express neuronal properties in vitro.* Dev Biol, 1995. **168**(2): p. 34257.

Basu, S. and S.T. Yang, *Astrocyte growth and glial cell line-derived neurotrophic factor secretion in three*
*dimensional polyethylene terephthalate fibrous matrices.* Tissue Eng, 2005. **11**(5-6): p. 940-52.

Bienaime, C., J.N. Barbotin, and J.E. Nava-Saucedo, *How to build an adapted and bioactive cell*
*microenvironment? A chemical interaction study of the structure of Ca-alginate matrices and their*
*repercussion on confined cells.* J Biomed Mater Res A, 2003. **67**(2): p. 376-88.

Brustle, O., et al., *Embryonic stem cell-derived glial precursors: a source of myelinating transplants.* Science, 1999. **285**(5428): p. 754-6.

Cao, Q., R.L. Benton, and S.R. Whittemore, *Stem cell repair of central nervous system injury.* J Neurosci
Res, 2002. **68**(5): p. 501-10.

Chen, U. and H. Mok, *Development of mouse embryonic stem (ES) cells: IV. Differentiation to mature T*
*and B lymphocytes after implantation of embryoid bodies into nude mice.* Dev Immunol, 1995. **4**(2): p.
79-84.

Cho, C.H., et al., *Homogeneous differentiation of hepatocyte-like cells from embryonic stem cells:*
*applications for the treatment of liver failure.* FASEB J, 2008. **22**(3): p. 898-909.

Clagett-Dame, M., E.M. McNeill, and P.D. Muley, *Role of all-trans retinoic acid in neurite outgrowth and*
*axonal elongation.* J Neurobiol, 2006. **66**(7): p. 739-56.

Clayton, H.A., et al., *The effect of capsule composition on the biocompatibility of alginate-poly-l-lysine*
*capsules.* J Microencapsul, 1991. **8**(2): p. 221-33.

Dean, S.K., et al., *Differentiation of encapsulated embryonic stem cells after transplantation.*
Transplantation, 2006. **82**(9): p. 1175-84.

Dhoot, N.O., et al., *Peptide-modified alginate surfaces as a growth permissive substrate for neurite*
*outgrowth.* J Biomed Mater Res A, 2004. **71**(2): p. 191-200.

Dimos, J.T., et al., *Induced pluripotent stem cells generated from patients with ALS can be differentiated*
*into motor neurons.* Science, 2008. **321**(5893): p. 1218-21.

Dinsmore, J., et al., *Embryonic stem cells differentiated in vitro as a novel source of cells for*
*transplantation.* Cell Transplant, 1996. **5**(2): p. 131-43.

Du, Z.W. and S.C. Zhang, *Neural differentiation from embryonic stem cells: which way?* Stem Cells Dev, 2004. **13**(4): p. 372-81.

Ebert, A.D., et al., *Induced pluripotent stem cells from a spinal muscular atrophy patient.* Nature, 2009.
**457**(7227): p. 277-80.
Engler, A.J., et al., *Matrix elasticity directs stem cell lineage specification.* Cell, 2006. **126**(4): p. 677-89.
Evans, M.J. and M.H. Kaufman, *Establishment in culture of pluripotential cells from mouse embryos.*
Nature, 1981. **292**(5819): p. 154-6.
Fang, S., et al., *Differentiation of embryoid-body cells derived from embryonic stem cells into hepatocytes in alginate microbeads in vitro.* Acta Pharmacol Sin, 2007. **28**(12): p. 1924-30.
Garreta, E., et al., *Osteogenic differentiation of mouse embryonic stem cells and mouse embryonic*
*fibroblasts in a three-dimensional self-assembling peptide scaffold.* Tissue Eng, 2006. **12**(8): p. 2215-27.
Gratsch, T.E. and K.S. O'Shea, *Noggin and chordin have distinct activities in promoting lineage*
*commitment of mouse embryonic stem (ES) cells.* Dev Biol, 2002. **245**(1): p. 83-94.
Hamazaki, T., et al., *Hepatic maturation in differentiating embryonic stem cells in vitro.* FEBS Lett, 2001.
**497**(1): p. 15-9.
Horak, D., et al., *Poly(2-hydroxyethyl methacrylate)-based slabs as a mouse embryonic stem cell support.*
Biomaterials, 2004. **25**(22): p. 5249-60.
Hwang, N.S., et al., *Effects of three-dimensional culture and growth factors on the chondrogenic*
*differentiation of murine embryonic stem cells.* Stem Cells, 2006. **24**(2): p. 284-91.
Hwang, N.S., et al., *Enhanced chondrogenic differentiation of murine embryonic stem cells in hydrogels*
*with glucosamine.* Biomaterials, 2006. **27**(36): p. 6015-23.
Jones-Villeneuve, E.M., et al., *Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells.* J Cell Biol, 1982. **94**(2): p. 253-62.
Jones-Villeneuve, E.M., et al., *Retinoic acid-induced neural differentiation of embryonal carcinoma cells.* Mol Cell Biol, 1983. **3**(12): p. 2271-9.
Keirstead, H.S., et al., *Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury.* J Neurosci, 2005. **25**(19): p. 4694-705.
Kitazawa, A. and N. Shimizu, *Differentiation of mouse embryonic stem cells into neurons using conditioned medium of dorsal root ganglia.* J Biosci Bioeng, 2005. **100**(1): p. 94-9.
Kroupova, J., et al., *Functional polymer hydrogels for embryonic stem cell support.* J Biomed Mater Res B Appl Biomater, 2006. **76**(2): p. 315-25.
Kuo, C.K. and P.X. Ma, *Ionically crosslinked alginate hydrogels as scaffolds for tissue engineering: part 1. Structure, gelation rate and mechanical properties.* Biomaterials, 2001. **22**(6): p. 511-21.

Lee, S.H., et al., *Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells.* Nat Biotechnol, 2000. **18**(6): p. 675-9.

Lenka, N., et al., *Quantitation and functional characterization of neural cells derived from ES cells using nestin enhancer-mediated targeting in vitro.* J Cell Sci, 2002. **115**(Pt 7): p. 1471-85.

Levenberg, S., et al., *Differentiation of human embryonic stem cells on three-dimensional polymer scaffolds.* Proc Natl Acad Sci U S A, 2003. **100**(22): p. 12741-6.

Li, L., et al., *Functional modulation of ES-derived hepatocyte lineage cells via substrate compliance alteration.* Ann Biomed Eng, 2008. **36**(5): p. 865-76.

Li, R.H., *Materials for immunoisolated cell transplantation.* Adv Drug Deliv Rev, 1998. **33**(1-2): p. 87-109.

Liu, H. and K. Roy, *Biomimetic three-dimensional cultures significantly increase hematopoietic differentiation efficacy of embryonic stem cells.* Tissue Eng, 2005. **11**(1-2): p. 319-30.

Liu, S., et al., *Embryonic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation.* Proc Natl Acad Sci U S A, 2000. **97**(11): p. 6126-31.

Lowry, W.E., et al., *Generation of human induced pluripotent stem cells from dermal fibroblasts.* Proc Natl Acad Sci U S A, 2008. **105**(8): p. 2883-8.

Maguire, T., et al., *Control of hepatic differentiation via cellular aggregation in an alginate microenvironment.* Biotechnol Bioeng, 2007. **98**(3): p. 631-44.

Maguire, T., et al., *Alginate-PLL microencapsulation: effect on the differentiation of embryonic stem cells into hepatocytes.* Biotechnol Bioeng, 2006. **93**(3): p. 581-91.

Magyar, J.P., et al., *Mass production of embryoid bodies in microbeads.* Ann N Y Acad Sci, 2001. **944**: p. 135-43.

Martin, G.R., *Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells.* Proc Natl Acad Sci U S A, 1981. **78**(12): p. 7634-8.

Mayer-Proschel, M., et al., *Isolation of lineage-restricted neuronal precursors from multipotent neuroepithelial stem cells.* Neuron, 1997. **19**(4): p. 773-85.

Maysinger, D., et al., *Microencapsulated ciliary neurotrophic factor: physical properties and biological activities.* Exp Neurol, 1996. **138**(2): p. 177-88.

Nistor, G.I., et al., *Human embryonic stem cells differentiate into oligodendrocytes in high purity and myelinate after spinal cord transplantation.* Glia, 2005. **49**(3): p. 385-96.

Noll, E. and R.H. Miller, *Regulation of oligodendrocyte differentiation: a role for retinoic acid in the spinal cord.* Development, 1994. **120**(3): p. 649-60.

Novik, E., et al., *Maintenance and augmentation of hepatocyte-specific function in ES derived hepatocyte-like cells using collagen sandwich and SNAP.* Tissue Eng, 2008.

Novik, E.I., et al., *Embryoid body-mediated differentiation of mouse embryonic stem cells along a hepatocyte lineage: insights from gene expression profiles.* Tissue Eng, 2006. **12**(6): p. 1515-25.

Okabe, S., et al., *Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro.* Mech Dev, 1996. **59**(1): p. 89-102.

O'Neill, A. and D.V. Schaffer, *The biology and engineering of stem-cell control.* Biotechnol Appl Biochem, 2004. **40**(Pt 1): p. 5-16.

Orive, G., et al., *Biocompatibility of alginate-poly-L-lysine microcapsules for cell therapy.* Biomaterials, 2006. **27**(20): p. 3691-700.
Otero, J.J., et al., *Beta-catenin signaling is required for neural differentiation of embryonic stem cells.* Development, 2004. **131**(15): p. 3545-57.
Parashurama, N., et al., *Activin alters the kinetics of endoderm induction in embryonic stem cells cultured on collagen gels.* Stem Cells, 2008. **26**(2): p. 474-84.
Philp, D., et al., *Complex extracellular matrices promote tissue-specific stem cell differentiation.* Stem Cells, 2005. **23**(2): p. 288-96.
Pierret, C., et al., *Elements of a neural stem cell niche derived from embryonic stem cells.* Stem Cells Dev, 2007. **16**(6): p. 1017-26.
Powell, K.R., et al., *Quantitation of acidic capsular polysaccharides by Alcian blue binding.* Anal Biochem, 1982. **119**(1): p. 31-7.
Rowley, J.A., G. Madlambayan, and D.J. Mooney, *Alginate hydrogels as synthetic extracellular matrix materials.* Biomaterials, 1999. **20**(1): p. 45-53.
Schuldiner, M., et al., *Induced neuronal differentiation of human embryonic stem cells.* Brain Res, 2001. **913**(2): p. 201-5.
Seidenfaden, R., A. Krauter, and H. Hildebrandt, *The neural cell adhesion molecule NCAM regulates neuritogenesis by multiple mechanisms of interaction.* Neurochem Int, 2006. **49**(1): p. 1-11.
Sharma, N.S., et al., *Sodium butyrate-treated embryonic stem cells yield hepatocyte-like cells expressing a glycolytic phenotype.* Biotechnol Bioeng, 2006. **94**(6): p. 1053-63.
Soto-Gutierrez, A., et al., *Differentiation of mouse embryonic stem cells to hepatocyte-like cells by co-culture with human liver nonparenchymal cell lines.* Nat Protoc, 2007. **2**(2): p. 347-56.
Takahashi, J., T.D. Palmer, and F.H. Gage, *Retinoic acid and neurotrophins collaborate to regulate neurogenesis in adult-derived neural stem cell cultures.* J Neurobiol, 1999. **38**(1): p. 65-81.
Tanaka, H., et al., *Chondrogenic differentiation of murine embryonic stem cells: effects of culture conditions and dexamethasone.* J Cell Biochem, 2004. **93**(3): p. 454-62.
Tian, H.B., et al., *Efficient differentiation of embryonic stem cells into neurons in glial cell-conditioned medium under attaching conditions.* Acta Biochim Biophys Sin (Shanghai), 2005. **37**(7): p. 480-7.
Tobias, C.A., et al., *Grafting of encapsulated BDNF-producing fibroblasts into the injured spinal cord without immune suppression in adult rats.* J Neurotrauma, 2001. **18**(3): p. 287-301.
Tobias, C.A., et al., *Alginate encapsulated BDNF-producing fibroblast grafts permit recovery of function after spinal cord injury in the absence of immune suppression.* J Neurotrauma, 2005. **22**(1): p. 138-56.
Wichterle, H., et al., *Directed differentiation of embryonic stem cells into motor neurons.* Cell, 2002. **110**(3): p. 385-97.
Willerth, S.M., et al., *Optimization of fibrin scaffolds for differentiation of murine embryonic stem cells into neural lineage cells.* Biomaterials, 2006. **27**(36): p. 5990-6003.
Yang, I.H., C.C. Co, and C.C. Ho, *Spatially controlled co-culture of neurons and glial cells.* J Biomed Mater Res A, 2005. **75**(4): p. 976-84.

Ying, Q.L., et al., *Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture.* Nat Biotechnol, 2003. **21**(2): p. 183-6.

Yu, J., et al., *Induced pluripotent stem cell lines derived from human somatic cells.* Science, 2007. **318**(5858): p. 1917-20.

Zimmermann, H., et al., *Towards a medically approved technology for alginate-based microcapsules allowing long-term immunoisolated transplantation.* J Mater Sci Mater Med, 2005. **16**(6): p. 491-501.

# CURRICULUM VITAE

## L U L U  L I

### Education and Special Training

2004-2009 Ph.D., Chemical and Biochemical Engineering
Graduate School-New Brunswick
Rutgers, The State University of New Jersey
New Brunswick, New Jersey

2002-2004 M.Phil., Bioengineering
Hong Kong University of Science and Technology
Clear Water Bay, Kowloon, Hong Kong

1999-2002 B.Eng. with *First Class Honour*, Chemical Engineering
Hong Kong University of Science and Technology
Clear Water Bay, Kowloon, Hong Kong

1998-1999 Undergraduate Prep Study, Chemistry
Fudan University
Shanghai, China

### Academic Research Experience

2005-2009 Department of Biomedical Engineering, Rutgers, The State University of New Jersey, Piscataway, NJ
Doctoral Student in the Laboratory for Tissue and Cellular Engineering Laboratory

- Developed alginate microencapsulation technique to direct multiple-lineage stem cell differentiation
- Investigated mechanisms of microenvironment and soluble factors on stem cell differentiation

2006-2009 Department of Biomedical Engineering, Rutgers, The State University of New Jersey, Piscataway, NJ
Doctoral Student in the Laboratory for Biomechanics Laboratory

- Developed novel DNA-based biomaterials for stem cell research
- Investigated effects of substrate compliances on hepatocyte differentiation

2002-2004 Department of Chemical Engineering, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong
Master Student in the Laboratory for Electroanalysis and Microsystem Laboratory

- Developed electrochemical-based methods for DNA/PCR amplicons detection

- Investigated polymer and substrate modification for DNA immobilization

**Publications**

Li L, Davidovich AE, Schloss JM, Chippada U, Schloss RR, Langrana NA, Yarmush ML. Neural Lineage Differentiation Within Alginate Microbeads is Regulated by Preventing Embryonic Stem Cell Aggregation (in review)

Li L, Sharma N, Chippada U, Jiang X, Schloss R, Yarmush ML, Langrana NA. Functional Modulation of ES-Derived Hepatocyte Lineage Cells via Substrate Compliance Alteration. Annals of Biomedical Engineering 2008; 36(5): 865-76.

Li L, Cai H, Lee TMH, Barford J, Hsing IM. Electrochemical Detection of PCR Amplicons Using
Electroconductive Polymer Modified Electrode and Multiple Nanoparticle Labels. Electroanalysis 2004; 16: 81-87.

Lee TMH, Li L, Hsing IM. Enhanced Electrochemical Detection of DNA Hybridization Based on Electrode Surface Modification. Langmuir 2003; 19: 4338-4343.

**Professional Presentations**

Li L, Schloss R, Langrana NA., Yarmush ML, Effects of encapsulation microenvironment on embryonic stem cell differentiation. ASME Summer Bioengineering Conference, Marco Island, FL, 2008.

Li L, Chippada U, Jiang X, Schloss R, Yurke, Bernard, Yarmush ML, Langrana NA. Effects of Substrate Compliance on Hepatic Differentiation of Embryonic Stem Cells. BMES Annual Meeting, Chicago, IL, 2006.

Li L, Lee TMH, Cai H, Hsing IM. DNA-Based Bioaerosol Detection on a Microfabricated Device. AIChE Annual Meeting, San Francisco, CA, 2003.

**Honors and Fellowships**

1999- 2002 Hong Kong Jockey Club Fellowship for Outstanding Mainland Students
2000- 2001 HKUST Dean's List
2002- 2004 HKUST Postgraduate Fellowship
2006- 2007 New Jersey Commission on Spinal Cord Research Fellowship
- Received a 1 year, $30,000 fellowship grant from the New Jersey Commission on Spinal Cord Research titled "Electric Stimulation of Neural Differentiation from Embryonic Stem Cells"

2008 – 2009 Terner Scholarship