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Alginate Encapsulation Parameters Influence the Differentiation of Microencapsulated Embryonic Stem Cell Aggregates

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Abstract

Pluripotent embryonic stem cells (ESCs) have tremendous potential as tools for regenerative medicine and drug discovery, yet the lack of processes to manufacture viable and homogenous cell populations of sufficient numbers limits the clinical translation of current and future cell therapies. Microencapsulation of ESCs within microbeads can shield cells from hydrodynamic shear forces found in bioreactor environments while allowing for sufficient diffusion of nutrients and oxygen through the encapsulation material. Despite initial studies examining alginate microbeads as a platform for stem cell expansion and directed differentiation, the impact of alginate encapsulation parameters on stem cell phenotype has not been thoroughly investigated. Therefore, the objective of this study was to systematically examine the effects of varying alginate compositions on microencapsulated ESC expansion and phenotype. Pre-formed aggregates of murine ESCs were encapsulated in alginate microbeads composed of a high or low ratio of guluronic to mannuronic acid residues (High G and High M, respectively), with and without a poly-L-lysine (PLL) coating, thereby providing four distinct alginate bead compositions for analysis. Encapsulation in all alginate compositions was found to delay differentiation, with encapsulation within High G alginate yielding the least differentiated cell population. The addition of a PLL coating to the High G alginate prevented cell escape from beads for up to 14 days. Furthermore, encapsulation within High M alginate promoted differentiation toward a primitive endoderm phenotype. Taken together, the findings of this study suggest that distinct ESC expansion capacities and differentiation trajectories emerge depending on the alginate composition employed, indicating that encapsulation material physical properties can be used to control stem cell fate.

Keywords

microencapsulation; alginate; stem cells; bioprocessing

Introduction

Stem cells, including pluripotent embryonic stem cells (ESCs), have tremendous potential as tools for regenerative medicine and drug discovery. However, there are many challenges to overcome before stem cell-derived therapies can be made broadly available, including the lack of processes to manufacture viable and homogenous cell populations of sufficient numbers (Kirouac and Zandstra, 2008). Currently, the large scale production of mammalian cells typically occurs in suspension bioreactors, which impart hydrodynamic forces that can adversely affect stem cell viability and influence their phenotype (Gareau et al., 2012; Leung et al., 2011; Sargent et al., 2010; Wolfe and Ahsan, 2013). Stem cells are very sensitive to environmental stimuli, as external signals in the culture environment provide cues that determine whether stem cells continue to self-renew or differentiate into specific cell types. Because most current bioprocesses are designed around suspension culture systems, investigation into systems to culture anchorage-dependent stem cells in suspension is critical for translating process technology and for achieving the high cell densities required for therapeutic doses (Kehoe et al., 2010; Serra et al., 2012).

One approach to the challenge of scalable bioprocessing is to encapsulate stem cells in hydrogels, such as alginate, to better control microenvironmental cues and enable suspension culture. Microencapsulation has been used for decades to protect enclosed cells from the host immune system upon transplantation, but it can also shield cells from hydrodynamic shear forces found in bioreactor environments and prevent agglomeration of stem cell aggregates while permitting diffusion of nutrients and oxygen through the encapsulation material (Wilson and McDevitt, 2013). Several investigations of ESC microencapsulation, mostly with single cells, have been previously performed with alginate capsule formulations (Serra et al., 2011; Siti-Ismail et al., 2008; Wang et al., 2006), and directed differentiation has been achieved, in most cases through soluble factor addition, toward osteogenic (Hwang et al., 2009; Tang et al., 2012), cardiac (Bauwens et al., 2005; Jing et al., 2010), hematopoietic (Dang et al., 2004; Rahman et al., 2010), neural (Li et al., 2011b), pancreatic (Chayosumrit et al., 2010; Wang et al., 2009), and hepatocytic lineages (Fang et al., 2007; Maguire et al., 2005). Although most previous studies have examined the encapsulation of single ESCs, aggregates of ESCs are also important to investigate for a number of reasons. First, using pre-formed aggregates provides a consistent initial size that can be compared directly to unencapsulated controls, which can also be cultured in suspension. Though microencapsulation-based protocols have been developed to form multicellular aggregates (Magyar et al., 2001; Sakai et al., 2008; Wang et al., 2006), using microwell formation (Ungrin et al., 2008) may provide more consistency so that the impact of encapsulation parameters on cell phenotype is not confounded by the additional parameters of aggregate size or the aggregation kinetics (Kinney et al., 2012). Furthermore, microencapsulation of aggregates has been previously determined to improve cell viability (Serra et al., 2011), as ESCs generally require maintenance of intercellular adhesions for optimal survival (Watanabe et al., 2007). Finally, culture as 3D aggregates can initiate differentiation of ESCs as embryoid bodies (EBs) (Bratt-Leal et al., 2009; Doetschman et al., 1985), providing a platform to study the impact of microencapsulation parameters on differentiation trajectory.

While encapsulation has been explored as a tool for stem cell expansion and directed differentiation, the impact of alginate encapsulation parameters on stem cell phenotype has not been systematically examined. Alginate is a biocompatible polymer purified from brown seaweed that is commonly used to encapsulate mammalian cells due to its relative abundance and mild cross-linking requirements. Alginate is composed of two anionic monomers, α -L-guluronic acid (G) and β -D-mannuronic acid (M), which are arranged in both homopolymeric regions (GG blocks and MM blocks) and mixed monomeric regions (MG blocks) (Haug et al., 1966). Cross-linking occurs at G residues through binding of divalent cations such as calcium, therefore alginates with higher G content and longer GG blocks produce stiffer beads due to greater cross-linking than alginates with higher M content. Because native alginate is non-adhesive and therefore cannot interact directly with cells, the physical and chemical properties of the polymer dictate the relationship with the enclosed cells. A handful of studies have examined the impact of G and M content on cell proliferation, metabolism, and secretion. Experiments with murine insulinoma bTC3 cells found that alginates with high G content inhibited cell growth, leading to decreased metabolic and secretory activity (Stabler et al., 2001). Alternatively, studies of encapsulated neural stem cells observed improved secretion of neurotrophic factors when the cells were cultured in alginate with high G content, citing poor capsule stability in alginate with high M content (Purcell et al., 2009). Despite such examples of reported differences based on G and M content, most studies do not specify the composition of alginate species used, thus provoking questions about the potential influence of the encapsulation material utilized on the phenotype of the enclosed cells.

An additional microencapsulation property that can be varied is the presence of a coating surrounding the capsule, typically with a polycation like PLL, which has historically served as an added barrier to the host immune system upon transplantation by decreasing the permeability of larger molecules, such as antibodies, into and out of the bead (Lim and Sun, 1980). However, coating with PLL also confers additional mechanical stability (De Castro et al., 2005; Strand et al., 2003; Thu et al., 1996) which can be beneficial for maintaining capsule integrity over time in culture. Many studies have described issues with single cells escaping or leaking out of the capsules (Chang et al., 1994; Cheng et al., 2006; Jing et al., 2010; Paji -Lijakovi et al., 2007), an issue which may be ameliorated through PLL coating. However, the issue of cell escape may be increased when encapsulating pre-formed aggregates due to their large size and potential proximity to the bead edge, and it has yet to be determined whether addition of PLL is sufficient to prevent escape in this context, a question also relevant for the encapsulation of other aggregated cells.

Overall, the inconsistency in the composition of alginate and use of a coating layer in previous studies with microencapsulated cells raises questions about whether disparate outcomes result from differing material compositions. The potential impacts of microencapsulation material on cell phenotype are particularly relevant for the culture of ESCs due to their particular sensitivities to the surrounding physical microenvironment (Li et al., 2011a). Thus, the objective of this study was to systematically examine the impact of varying alginate compositions on microencapsulated ESC expansion and phenotype. Aggregates of murine ESCs were pre-formed and encapsulated in alginate microbeads composed of a high or low ratio of G to M residues, with and without a PLL coating,

providing four distinct alginate bead compositions for analysis. Mechanical characterization of each bead composition was performed, as well as analysis of encapsulated cell aggregate viability, morphology, and phenotype via gene and protein expression. The results of this study revealed distinctions in the growth rate, morphology, and differentiation trajectories of ESCs in each of the encapsulated formats over 14 days of culture, suggesting that alginate bead composition is an important parameter to consider when designing microencapsulation-based expansion and directed differentiation protocols.

Methods

Embryonic Stem Cell Culture

Murine ESCs (D3 cell line; chosen due to its widespread use) were cultured on tissue culture treated polystyrene dishes (Corning Inc., Corning, NY) adsorbed with gelatin (0.1% solution in diH₂O). Undifferentiated ESC culture media consisted of Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Herndon, VA) supplemented with 15% fetal bovine serum (Hyclone, Logan, UT), 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin (Mediatech), 2mM L-glutamine (Mediatech), 1× MEM non-essential amino acid solution (Mediatech), 0.1 mM 2-mercaptoethanol (Fisher Scientific, Fairlawn, NJ), and 10³ U/mL of leukemia inhibitory factor (LIF) (ESGRO, Chemicon, Temecula, CA). Cultures were replenished with fresh media every other day and passaged prior to reaching 70% confluence.

ESC Aggregate Formation and Culture

A single cell suspension of undifferentiated ESCs was obtained through dissociation of monolayer cultures with 0.05% trypsin-EDTA (Mediatech). Defined, serum-free N2B27 media (Ying and Smith, 2003) was used for all aggregate cultures and consisted of DMEM/F12 (50/50) medium (Gibco, Grand Island, NY) supplemented with N2 (Gibco), 25 µg/L bovine serum albumin (BSA), 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin (Mediatech), 2mM L-glutamine (Mediatech), all combined 1:1 with Neurobasal medium (Gibco) supplemented with B27 (Gibco). N2B27 basal media has been widely used by several groups for a number of different applications, including undifferentiated ESC culture (Ying et al., 2008), differentiation of ESCs to insulin-producing cells (Wang et al., 2009), and hematopoietic differentiation of ESCs (Rahman et al., 2010). Additionally, neural ectoderm differentiation is considered the “default” pathway for ESC differentiation (Smukler et al., 2006) in the absence of any exogenous factors, thus N2B27 media is an appropriate choice for basal media to examine ESC differentiation. Aggregation of ESCs was achieved by centrifugation (200 rcf) of ESCs into 400 µm square polydimethylsiloxane (PDMS) micro-wells (Aggrewell™, Stem Cell Technologies, Vancouver, Canada) as previously reported (Kinney et al., 2012; Ungrin et al., 2008). The cell seeding density yielded approximately 500 cells per individual well. The ESCs were incubated in the wells for approximately 20 h in serum-free N2B27 culture media to allow for aggregate formation. The resulting aggregate population was either immediately transferred to suspension culture (approximately 1,500 aggregates in 10 mL of serum-free culture media) for the unencapsulated condition or transferred after subsequent encapsulation. Aggregates were cultured in sterile 100 × 15 mm bacteriological grade

polystyrene Petri dishes (BD, Franklin Lakes, NJ) and maintained on rotary orbital shakers (Carpenedo et al., 2007) at ~45 rpm. A 90% media exchange was performed every 3 days following gravity-induced sedimentation of the aggregates in 15mL conical tubes. Suspension cultures were maintained for up to 14 days of differentiation.

Aggregate Microencapsulation

Two different medium viscosity alginates were used in this study, ultrapure MVG (Pronova, Oslo, Norway) which contains greater than 60% G residues (High G) and ultrapure MVM (Pronova) which contains greater than 50% M residues (High M). Alginate solutions were prepared at 1.5 wt% in calcium-free DMEM (Gibco) and autoclaved for sterilization. Once the solutions were cooled to 37°C, aggregates were re-suspended in the sterile alginate at a density of 12,000 aggregates per mL of alginate (approximately 6×10^6 cells/mL). An electrostatic bead generator (Nisco, Zurich, Switzerland) was utilized for encapsulation. The alginate solution was extruded through a 400 μ m nozzle using a syringe pump at a flow rate of 6 mL/h and dropped into a 100 mM calcium chloride (EMD) hardening bath. After encapsulation, the beads were rinsed with serum-free media 3 $\times$, and half of the beads from each alginate type were coated with 0.05% PLL (MW 15,000–30,000; Sigma, St. Louis., MO) for 2 min prior to three additional media rinses. All encapsulated aggregates were cultured in serum-free N2B27 media on a rotary orbital shaker as previously described.

Mechanical Characterization of Microbeads

Alginate beads of both High G and High M were formed (without cells) and some coated with PLL as described. The beads were maintained in serum-free (N2B27) media on a rotary orbital shaker to simulate conditions experienced during culture. At Days 1, 7, and 14 following formation, the mechanical properties of six alginate beads of each type were assessed through micron-scale parallel-plate compression using a CellScale MicroSquisher and the associated SquisherJoy software (Baraniak et al., 2012). The upper compression plate of a 302.8 μ m cantilever compressed the samples at 40% strain over a period of 40 s, held at constant deformation for 10 s, and released over a period of 40 s to document the magnitude of hysteresis. To determine the Young's modulus, Hertzian theory for non-adhesive elastic contact was used to fit a linear regression line to 30 data points above and below the 20% strain data point on the plot of force (F) versus deformation^{2/3} (d). The resultant slope in addition to the initial radius (R) of the bead was used to calculate the Young's modulus of the sample (E), as described by the following standard expression:

$$F = \frac{4}{3} E \times R^{1/2} \times d^{2/3}$$

During testing, all samples were held in a PBS fluid bath (pH 7.4, 0.1 g/L Ca^{2+} and 0.1 g/L Mg^{2+}).

Cell Number Quantification

Samples were collected from each group at Days 4, 7, 10, and 14 of differentiation. Encapsulated aggregates were released from beads through 10 min incubation with TrypLE™ (Invitrogen, Carlsbad, CA) for PLL-coated beads only, trituration, and 5 min

incubation with 55 mM sodium citrate (Sigma) for all beads, as previously described (Jing et al., 2010). The cells were centrifuged at 200 rcf for 5 min and rinsed 3 × with PBS. Cells from all conditions were pelleted at 375 rcf for 4 min followed by supernatant removal and storage at −20°C. A CyQUANT Cell Proliferation Assay Kit (Molecular Probes, Inc., Eugene, OR) was used to determine cell number, with a standard curve created using undifferentiated ESCs that were counted using a hemocytometer. The fluorescence was read using a Synergy H4 plate reader (Biotek, Winooski, VT). The net growth rate (μ_{net}) was determined by examining change in cell density (X) over given periods of time (t) using the following standard expression:

$$\frac{dX}{dt} = \mu_{\text{net}} X$$

Viability Staining and Imaging

Cell viability was assessed at Day 14 of differentiation using a LIVE/DEAD kit (Molecular Probes, Inc.). Samples were incubated in PBS containing 0.1 μM calcein AM and 8 μM ethidium homodimer-1 at 4°C for 1 h. The samples were washed with PBS, transferred to a glass-bottomed 24-well plate, and immediately imaged using a Zeiss LSM 700-405 confocal microscope (Carl Zeiss, Inc., Jena, Germany).

Histological Analysis

Encapsulated and unencapsulated aggregates were sampled at Days 4, 7, and 14 of differentiation, rinsed with PBS, fixed in 4% paraformaldehyde for 30 min with rotation at room temperature, rinsed with PBS, and stored at 4°C. Fixed aggregates were embedded in Histogel (Richard-Allen Scientific, Kalamazoo, MI), processed via a series of graded ethanol and xylene rinses, and embedded in paraffin. Paraffin-embedded samples were sectioned at a thickness of 5 μm using a rotary microtome (Microtom HM310). For histological analysis, sections were de-paraffinized through a series of xylene and graded ethanol concentrations, followed by staining with hematoxylin and eosin (H&E). Stained sections were imaged using a Nikon Eclipse 80i equipped with a SpotFlex digital camera (Diagnostic Instruments, Sterling Heights, MI).

Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

Encapsulated aggregates were released from beads through 10 min incubation with TrypLE™ (Invitrogen) for PLL-coated capsules only, trituration, and 5 min incubation with 55 mM sodium citrate (Sigma) for all beads. The cells were centrifuged at 200 rcf and rinsed 3× with PBS to remove residual PLL. RNA was extracted from the aggregates with the RNeasy Mini kit (Qiagen, Inc., Valencia, CA). The RNA (300 ng/sample) was converted to complimentary DNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) and analyzed using real time PCR (MyIQ cycler, Bio-Rad). Forward and reverse primers for *18s*, *Oct4*, *Nanog*, *Pax6*, *AFP*, *MLC-2v*, and *Flk1* were designed with Beacon Designer software (sequences and conditions are given in Table I) and purchased from Invitrogen. *Oct4* and *Nanog* gene expression were calculated with respect to undifferentiated ESC expression

levels as previously described (Pfaffl, 2001). *Pax6*, *AFP*, *MLC-2v*, and *Flk1* concentrations were determined using a standard curve and normalized to *18s* expression levels.

Immunofluorescent Staining

Encapsulated and unencapsulated aggregates were sampled at Days 4, 7, and 14 of differentiation, rinsed with PBS, fixed in 4% paraformaldehyde for 30 min with rotation at room temperature, rinsed with PBS, and stored at 4°C. Fixed aggregates were embedded in Histogel (Richard-Allen Scientific) and subject to graded sucrose and OCT infiltration prior to rapid freezing in a dry ice-ethanol bath and storage at -80°C. OCT-embedded samples were sectioned at a thickness of 10 µm using a CryoStar NX70 cryostat and allowed to dry at room temperature. Each section was surrounded using a PAP hydrophobic barrier pen and rinsed with PBS 3 × for 5 min. The slides were blocked and permeabilized with 3% donkey serum and 0.05% Triton X-100 for 45 min at room temperature. After rinsing with PBS 2 × for 5 min, primary antibody solution diluted in blocking buffer (3% donkey serum in PBS) was added and incubated overnight at 4°C. Primary antibodies against OCT-4 (Santa Cruz Biotechnology sc-8628; goat polyclonal; 1:100), AFP (Dako A000829-2; rabbit polyclonal; 1:100), and α-SMA (Dako M0851; mouse monoclonal; 1:100) were used. Following overnight incubation, slides were rinsed with PBS 3 × for 5 min and incubated with secondary solutions diluted in blocking buffer (1:1000 AlexaFluor® 488 donkey anti-goat, 1:1000 Alexa Fluor 488 donkey anti-rabbit, and 1:1000 Alexa Fluor 488 donkey anti-mouse) for 1 h at room temperature. Slides were rinsed with PBS 3 × for 5 min and incubated with Hoechst dye (1:100) for 10 min at room temperature. Following a final PBS rinse, coverslips were mounted with Fluoromount-G (SouthernBiotch, Birmingham, AL) and sealed with clear nail polish. The slides were imaged using a Zeiss LSM 700-405 confocal microscope (Carl Zeiss, Inc.).

Statistics

All experiments were performed with replicate samples from independent conditions ($n = 6$ for mechanical testing, $n = 3$ for cell counts, $n = 5$ for PCR). The data is represented as the mean of the independent replicates, and the error bars represent the standard error of the mean. Before performing statistical analysis, data were normalized using a Box-Cox power transformation to normalize data variance. Two-way ANOVA was calculated between different conditions (unencapsulated, High G, High G + PLL, High M, and High M + PLL) and time points, followed by post hoc Tukey analysis to determine significant differences ($P < 0.05$). All statistical analysis was performed using SYSTAT software.

Results

Characterization of Alginate Beads and Aggregate Encapsulation

Embryonic stem cell (ESC) spheroids were encapsulated in alginate with a high (High G) or low (High M) ratio of guluronic acid to mannuronic acid. Additionally, a portion of the beads were coated with PLL, creating High G + PLL and High M + PLL beads in addition to uncoated beads. The seeding density of spheroids in alginate gave rise to a small number (<15%) of empty beads, with the majority of beads (>71%) containing one to three spheroids, consistent with the expected Poisson distribution assuming an average rate of two

aggregates per capsule (Fig. 1A). The average bead diameter ($\sim 734 \mu\text{m}$) was relatively uniform for all of the bead compositions examined over the 14-day culture period (Fig. 1B).

The bulk mechanical properties of each bead type were determined by parallel plate compression testing. The Young's modulus ranged from 5–18 kPa (Fig. 1C) and was consistently greater in the PLL-coated beads, likely due to the increased mechanical stability provided by the external polyelectrolyte layer. The uncoated High G alginate was significantly stiffer than the uncoated High M alginate at Days 1 ($P = 0.011$) and 7 ($P < 0.001$) following formation, consistent with previous literature reports that alginate containing a higher proportion of guluronic acid residues enhances the mechanical strength of the hydrogels (Uludag et al., 2000). The modulus remained constant over the course of the culture period for each composition tested, indicating that the beads were physically stable for at least 2 weeks when cultured dynamically without enclosed cells in standard culture media.

Although the beads appeared similar after 1 week, unencapsulated spheroids (Fig. 1D) and aggregates within each bead type (Fig. 1E–H) exhibited distinct morphologies depending on the type of alginate used. All of the encapsulated spheroids appeared smaller than unencapsulated spheroids, with those in High G and High G + PLL remaining spherical (Fig. 1E and F) and those in High M and High M + PLL appearing more ovoid (Fig. 1G and H).

ESC Escape From and Proliferation Within Alginate Beads

Aggregate escape from the alginate beads was observed as early as 4 days post-encapsulation for aggregates within the High M alginate (Fig. 2A–E). After 14 days in culture, aggregate escape was more frequent, with many of the initially encapsulated aggregates no longer residing within the alginate beads (Fig. 2F–J). In general, the High G alginate contained the aggregates more effectively than the High M alginate, with the addition of the PLL coating improving retention. The High G + PLL formulation maintained all spheroids within beads (Fig. 2K), while the uncoated High M only retained $\sim 4\%$ of the aggregates within beads after 14 days.

The proliferation rates of the ESCs cultured in different configurations (Fig. 2L) diverged, with the unencapsulated spheroids having the highest initial net growth rate (0.38 day^{-1} vs. 0.12 day^{-1} for High G, 0.32 day^{-1} for High G + PLL, 0.24 day^{-1} for High M, and 0.22 day^{-1} for High M + PLL). After 14 days of culture, the number of cells in the unencapsulated aggregates was significantly greater than in the High G ($P = 0.016$), High G + PLL ($P < 0.001$), and High M + PLL ($P = 0.035$) conditions. Cell growth appeared most stunted in the cells within the stiffer High G and High G + PLL beads, though the beads coated with PLL exhibited overall similar growth trends to each other after Day 4. A marked increase in cell number was observed after the majority of encapsulated aggregates escaped from the beads (Day 7 for High M and Day 10 for High G), indicating that the cells proliferated more robustly when not encapsulated. Cell growth plateaued at a cell density of $\sim 1.8 \times 10^6 \text{ cells/mL}$, likely due to the volumetric capacity of this particular culture system and exhaustion of nutrients from the culture media.

Encapsulated ESC Viability and Aggregate Morphology

Cell viability was assessed following 14 days in culture for each of the encapsulation conditions for both encapsulated and escaped aggregates due to the incidence of spheroid escape. Viable cells were more prevalent when the aggregates were not within the beads (Fig. 3A–D), with non-viable cells present more frequently in the encapsulated aggregates. The non-viable cells tended to be localized to the more rounded region of aggregates, with more viable cells present in the elongated region of aggregates when present. In addition, more viable cells were observed in the beads coated with PLL.

The elongated morphology that was observed in some groups (Fig. 1D–E, Fig. 2E, Fig. 3E–H) prompted further examination into the morphological differences observed based on encapsulation conditions. At Day 4, the ESC aggregates were generally small and fairly round (Fig. 4A–C), although some elongated protrusions were observed in the High M and High M + PLL groups (Fig. 4D–E). The aggregates were densely packed without any morphogenic structures, suggesting that a primarily undifferentiated phenotype persisted. By Day 7 of differentiation, some cavitation was observed in the unencapsulated (Fig. 4F) and High M (Fig. 4I) conditions (which by Day 7 had only half of the spheroids within beads), suggesting that differentiation was progressing more rapidly in the unencapsulated and High M conditions. While the aggregates in the High G (Fig. 4G) and High G + PLL (Fig. 4H) alginate beads still appeared relatively round, more distinguished extension of the ovoid geometry occurred in the High M + PLL (Fig. 4J) condition. After 2 weeks in culture, more differentiated structures were formed in the unencapsulated (Fig. 4K) and High M (Fig. 4N) groups, including epithelial layer arrangement, small neural rosettes, and cavitation. The unencapsulated and High M conditioned aggregates appeared larger, consistent with the cell proliferation data (Fig. 2G) previously described. In contrast, aggregates encapsulated in the High G (Fig. 4L) and High G + PLL (Fig. 4M) beads continued to exhibit a smaller cross-sectional area, though more multicellular protrusions were observed than at earlier time points. The significantly elongated morphology in the High M + PLL condition at Day 14 (Fig. 4O) was present at similar levels to the High M + PLL at Day 7 and at greater frequency than any of the other experimental groups.

Alginate Compositional Influences on Stem Cell Phenotype

To determine what, if any, impact encapsulation in alginate with varying compositions had on stem cell differentiation, gene, and protein expression were examined over the course of 2 weeks. Relative to the undifferentiated ESC starting population, most conditions exhibited decreased gene expression of the pluripotency markers *Oct4* (Fig. 5A) and *Nanog* (Fig. 5B) over time. However, different temporal trajectories were observed, with the cells encapsulated in the High G and High G + PLL beads exhibiting significantly higher *Oct4* levels at Day 7 compared to cells in unencapsulated ($P < 0.001$ and $P < 0.001$, respectively), High M ($P < 0.001$ and $P < 0.001$), and High M + PLL ($P < 0.001$ and $P = 0.006$,) conditions. Furthermore, the cells within the High M and High M + PLL had significantly higher levels of *Oct4* expression when compared to the unencapsulated spheroids ($P < 0.001$ and $P < 0.001$, respectively), signifying that residence within the alginate beads, even temporarily, may delay differentiation. By Day 14, cells in the High G and High G + PLL beads continued to express higher levels of *Oct4* compared to the unencapsulated, High M, and High M + PLL

conditions. A similar trend was observed in the expression of *Nanog*, with cells in the High G alginate exhibiting higher levels compared to unencapsulated cells at Day 4 and aggregates in the High G and High G+PLL conditions having higher expression than the unencapsulated aggregates at Day 7. After 14 days, the cells in the High G + PLL beads maintained significantly increased expression over the unencapsulated and High M groups. The steady *Nanog* levels observed in the High G + PLL group, as opposed to the decrease observed in all other groups, was noteworthy since High G + PLL was the only condition to retain 100% of the spheroids over the entire 2-week period of culture.

Genes representative of the three germ lineages were also examined to determine if encapsulation conditions could impact differentiated phenotype(s), since culture as aggregates can lead to spontaneous differentiation as EBs. *Pax6*, an early ectodermal marker, was elevated in the unencapsulated, High M, and High M + PLL conditions compared to the High G and High G + PLL conditions at Day 7 (Fig. 5C) and increased in all groups by Day 14, with the High G + PLL and High M + PLL groups having significantly higher levels than the High M and unencapsulated groups. Investigation of alpha-fetoprotein (AFP) expression, an endodermal marker, revealed dramatic increases in the High M and High M + PLL groups (Fig. 5D), with significantly higher expression compared to all other groups observed both at Day 7 and 14 of differentiation.

Spontaneous contractile beating was observed in approximately half of the unencapsulated aggregates and approximately one-third of the High M aggregates at Days 10 and 14, motivating examination into mesodermal cardiac differentiation. Expression of *MLC-2v*, an isoform of myosin light chain-2, was elevated in High M and High M+PLL conditions at Day 7 (Fig. 5E). Furthermore, increased *MLC-2v* expression was observed in the unencapsulated and High M groups at Day 14, consistent with the visually observed beating activity. In addition, expression of *Flk1*, which encodes for VEGF receptor-2 and is a common marker of primitive mesoderm differentiation, was increased in the unencapsulated, High M, and High M + PLL conditions at Day 7 (Fig. 5F), though only transient expression was observed, as levels were decreased in all groups by Day 14 compared to Day 7.

To determine spatial patterns of phenotype expression within encapsulated aggregates, samples from each group were immunostained for OCT-4, AFP, and alpha smooth muscle actin (α -SMA). Similar to what was observed in the gene expression data, the relative intensity of OCT-4 expression in the encapsulated groups (Fig. 6B–E) was elevated compared to the unencapsulated group (Fig. 6A) at Day 7. AFP was expressed most frequently in cells from the High M condition (Fig. 6I) at Day 14, with little to no expression detected in the other groups (Fig. 6G–H, J–K). In the High M conditions, AFP was commonly observed throughout the aggregates or in central localized regions surrounded by an epithelial layer, as displayed in Figure 6I. Expression of α -SMA was observed frequently at localized regions near the aggregate exterior in the unencapsulated (Fig. 6K) and High M (Fig. 6N) groups at Day 14, the same conditions in which spontaneous beating was observed. More diffuse expression of α -SMA was observed in the High G (Fig. 6L), High G + PLL (Fig. 6M), and High M + PLL groups (Fig. 6O), none of which exhibited spontaneous beating in culture.

Discussion

The findings of this study indicate that the composition of alginate beads can have a dramatic impact on the expansion and phenotype of microencapsulated ESCs. Examination of four different bead compositions (High G, High G + PLL, High M, and High M + PLL) yielded divergent phenotypes of the enclosed ESC aggregates. The addition of a PLL coating to alginate beads dramatically reduced the rate of cell escape from the microbeads, completely preventing escape in the case of the High G + PLL condition. Encapsulation generally retarded cell growth while also inhibiting the loss of pluripotency, particularly more so for cells cultured in the stiffer High G alginate than cells in the more flexible High M alginate. These results demonstrate that encapsulation material properties and format impact pluripotent stem cell expansion and differentiation.

To define the physical microenvironment experienced by the encapsulated ESCs, the elastic modulus was determined, and the values of the different alginate beads (5–18 kPa) were similar to previously reported literature values (Banerjee et al., 2009; Capone et al., 2013; Chan et al., 2011; Hwang et al., 2009; Yeatts et al., 2011). PLL-coated alginate beads were stiffer than the non-coated beads (Fig. 1C), consistent with the increased stability exhibited following polycation addition. However, the calculated bulk modulus values may result from the increased exterior strength and not accurately reflect the mechanical forces encapsulated cells experience within beads. Uncoated High G alginate was significantly stiffer (~1.3-fold) compared to the High M alginate, indicating that the cells within the High G beads may be experiencing a more constrained microenvironment than those in the High M beads. No significant decrease in modulus within the same group was observed over 2 weeks of culture, which was unexpected based on previous reports that bead stability decreases with time (Purcell et al., 2009; Shoichet et al., 1996; Thu et al., 1996) and the data presented here regarding aggregate escape from the beads (Fig. 2F).

The difference between the constant elastic modulus over time and the increasing frequency of aggregate escape may be due to the discrepancy between a bulk mechanical measure like the Young's modulus and the incidence of local fissures in the material that can create pathways for cell escape. The formation and propagation of local cracks in the beads is further supported by the correlation between the extended, ovoid morphology, and the frequency of escape (Fig. 2), indicating that the aggregates appear to grow and expand along the axis of weakness prior to escape. Although some previous reports of elongated, "lens-like" morphology of microencapsulated cells have been described (Capone et al., 2013; Magyar et al., 2001), no clear explanation for this phenomenon has been offered. The correlation between cell growth and cell escape is likely related to differences in gel mechanics, however, it is unclear whether stiffer alginate materials impede cell growth (and therefore the expansion of aggregates out of the bead) directly, or whether less stress is exerted on the stiffer alginate due to less net cell growth, leading to prolonged bead stability. It is also possible that local gradients in calcium due to cellular calcium use may contribute to the generation of local weak points in the alginate. In general, greater cell numbers and higher cell viability were observed in the conditions with more elongated aggregates, indicating that in cases where elongation was not observed, cell growth may have been physically impeded. Increased initial cell growth rates (Fig. 2L) and higher cell viability

(Fig. 3) were observed in the PLL-coated capsules, which is consistent with previous studies that directly compared PLL-coated and uncoated alginate capsules (Capone et al., 2013; Purcell et al., 2009). While the direct mechanism behind the differences observed in PLL-coated and non-coated beads is unknown, it is possible that the increased barrier to diffusion in the PLL-coated beads (Capone et al., 2013) could result in “trapping” of autocrine growth factors within the bead, leading to increased cell proliferation and viability. While others have found that encapsulation within alginate can slow cell proliferation (Banerjee et al., 2009; Markusen et al., 2006; Stabler et al., 2001), it is not universally observed, indicating that the diverse alginate compositions reported in the literature have yielded varying conclusions regarding the impact of microencapsulation on cell growth.

In addition to proliferative and morphologic changes, differences in phenotype were observed for cells from the different encapsulation conditions, implying that the bead configuration can impact the differentiation trajectory of the entrapped cells. Generally, the longer that cells were entrapped within the beads without escape, the more prolonged the expression of pluripotency markers was observed. The High M group, which had the highest incidence of aggregate escape, exhibited pluripotency marker expression similar to the unencapsulated aggregates, whereas the cells in the High G + PLL group, which contained the aggregates for the entire 14 days of culture, had the highest expression of both *Oct4* and *Nanog* at Day 14. In the High G + PLL group, *Nanog* expression remained relatively unchanged from undifferentiated ESC levels even as *Oct4* expression decreased, indicating that regulation of *Nanog* may be occurring independently from the other pluripotent transcription factors (Muñoz et al., 2012; Faunes et al., 2013; Navarro et al., 2012). Persistent OCT-4 signal in the encapsulated conditions was also observed via immunostaining (Fig. 6A–E). The correlation between lower cell growth and high expression of the pluripotency markers appears somewhat counterintuitive given that undifferentiated cells are commonly believed to proliferate at a faster rate. However, recent work has demonstrated that inhibiting cell division of hESCs led to increased NANOG levels (Wu and Tzanakakis, 2012), implying that a similar mechanism of action may be responsible for the high *Oct4* and *Nanog* levels observed in the groups which grew more slowly. Previous reports have indicated that microencapsulation can be used to prevent or delay differentiation of both single ESCs (Siti-Ismail et al., 2008; Wang et al., 2006) and ESC aggregates (Serra et al., 2011), which is interesting given that aggregation of ESCs is often deemed to be an important step in many differentiation protocols and indicates that differentiation within alginate hydrogels alters the kinetics and trajectory of differentiation. Therefore, when directed differentiation within alginate is desired, the presence of additional cues, either soluble or co-entrapped within the material matrix, may be critical for efficient ESC differentiation.

Along with changes in the loss of pluripotency, modulation of differentiation lineage commitment was also observed depending on the bead configuration, which has been the focus of few investigations thus far. The most striking difference was the increased propensity of cells within High M beads to differentiate toward an endodermal lineage, evidenced by AFP gene (Fig. 5D) and protein (Fig. 6I) expression. In some cases, the AFP+ cells were localized to regions surrounded by an epithelial-looking layer, similar to

structures observed via histology (Fig. 4N). Interestingly, even though 96% of the aggregates in the High M condition escaped the bead prematurely and therefore were actually unencapsulated at Day 14 of differentiation, dramatic differences in AFP were observed between the High M group and the unencapsulated group, indicating that the earlier encapsulation may have primed the differentiation of the entrapped cells toward an endodermal fate. The other major phenotypic distinction observed was the high frequency of spontaneous contractile beating in the unencapsulated and High M cultures, and the complete lack of beating in the other conditions. *MLC-2v* levels were elevated in the unencapsulated, High M, and High M + PLL conditions (Fig. 5E), and staining for alpha smooth muscle actin (α -SMA) exhibited strong localized expression in the unencapsulated and High M groups. There are several hypotheses for the increased incidence of cardiac differentiation which may not be directly related to encapsulation parameters. First, culture of EBs under rotary orbital suspension has been previously implicated in inducing cardiomyogenic differentiation (Sargent et al., 2009), and the unencapsulated condition was directly exposed to the hydrodynamic fluid shear throughout the culture period. Due to the early escape of cells from the beads (Fig. 2F), the High M group was also exposed to hydrodynamic forces for a more prolonged duration than the other encapsulated conditions that were shielded within the beads for a longer time period. A second hypothesis is based on the recent finding that high lactate culture environments lead to preferential survival of cardiomyocytes (Tohyama et al., 2012). Since the unencapsulated and High M conditions consistently had higher cell densities than the other conditions (Fig. 2G), there were possibly higher levels of lactate present in the culture media. Finally, while effort was put forth to control for initial aggregate size, the aggregates in the unencapsulated and High M groups resulted in larger diameter EBs. Previous work investigating the impact of aggregate size on differentiation has observed that larger aggregates have a greater tendency to undergo cardiac differentiation (Bauwens et al., 2008; Hwang et al., 2009; Mohr et al., 2010). Overall, these results suggest that sustained encapsulation impedes cardiomyogenic differentiation, which along with sustained expression of pluripotency markers, signifies that encapsulation is a capable platform for maintenance and/or expansion of ESCs in a less differentiated state.

Overall, this study establishes the significant impacts of alginate bead composition on the expansion and phenotype of encapsulated pluripotent stem cells (PSCs). Alginate beads with a high ratio of guluronic acid residues maintain a less differentiated phenotype and addition of a PLL coating to the High G alginate can prevent cell escape from the bead while maintaining cell viability. Additionally, employing alginate with a high ratio of mannuronic to guluronic acid residues induced differentiation toward an endodermal lineage. Future work with human pluripotent stem cells (hPSCs) may provide further insight into the impact of encapsulation parameters on hPSC behavior and determine whether alginate microencapsulation is a feasible expansion and/or differentiation platform for hPSC bioprocessing. Overall, the findings of this study suggest that selecting different alginate compositions for PSC expansion or differentiation provides a facile approach to control stem cell fate, as distinct phenotypes may be yielded by different material compositions and hydrogel properties.

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Figure 1.

Characterization of microbead configurations. ESC aggregates (500 cells/aggregate) were encapsulated in alginate with a high (High G) or low (High M) ratio of guluronic acid to mannuronic acid with or without a poly-L-lysine (PLL) coating. The seeding density of spheroids in alginate generally followed the expected Poisson distribution and gave rise to a small number (<15%) of empty beads, with the majority of beads containing one or two spheroids (A). The average bead diameter ($734 \pm 41 \mu\text{m}$) remained unchanged for the different types of beads and time in culture (B). The Young's modulus of each bead type (C) was determined through parallel plate compression testing and was consistently greater in the PLL-coated beads. Representative phase images of unencapsulated control spheroids (D) and aggregates within each bead type (E–H) 1-week following formation reveal beads of similar appearance, but indicate morphologically distinct encapsulated aggregates. Scale bar = $100 \mu\text{m}$ for (D–H). Significant ($P < 0.05$) increases over unencapsulated (*), High G (#), High G + PLL (\$), High M (+), and High M + PLL () are denoted.

Figure 2.

Aggregate growth and escape from microbeads. After 4 days in culture, most aggregates remained encapsulated (A–E), with the exception of the High M group (D). At day 14 of culture, many of the initially encapsulated aggregates had escaped from the alginate (F–J), with only the High G + PLL formulation maintaining all spheroids within beads (K). The proliferation rates for the ESCs cultured in the different configurations (L) diverged, with the cell density in the unencapsulated condition significantly higher than the cell density in the High G, High G + PLL, and High M + PLL conditions. Significant ($P < 0.05$) increases over unencapsulated(*), High G (#), High G + PLL(\$), High M(+), and High M + PLL () are denoted. Scale bar = 500 μ m.

Figure 3.

Viability of encapsulated and escaped aggregates. Cell viability was assessed following 14 days in culture for each of the encapsulation conditions using a LIVE/DEAD assay and projection images were obtained via confocal microscopy. Due to the incidence of spheroid escape, both escaped (A–D) and encapsulated (E–H) aggregates were examined (when present). Viable cells, indicated in green, were more frequently observed when the aggregates were not within the beads, with non-viable cells, indicated in red, present at a higher frequency in the encapsulated aggregates. Cell viability appeared to be better maintained in the beads coated with PLL when compared to the non-coated counterparts. Scale bar = 500 μm .

Figure 4.

Histological analysis of aggregate morphology. Hematoxylin and eosin (H&E) staining of encapsulated and unencapsulated spheroids were examined at Days 4, 7, and 14 of differentiation. At Day 4, the aggregates were generally small, densely packed, and fairly circular (A–C), though some elongated protrusions were observed in the High M and High M + PLL groups (D, E). By Day 7 of differentiation, some cavitation was observed in the unencapsulated (F) and High M (I) conditions. The aggregates in the High G (G) and High G + PLL (H) alginates appeared relatively spherical, whereas more elongated aggregates were observed in the High M + PLL (J) condition. By Day 14, more organized structures were formed in the unencapsulated (K) and High M (N) groups, including epithelial arrangement, small neural rosettes, and continued cavitation, while elongated protrusions began to appear in the High G and High G + PLL groups (L,M) and continued to exist in the High M + PLL group (O). Scale bar = 100 μm.

Figure 5.

Gene expression analysis. Relative to the undifferentiated ESC starting population, most conditions exhibited decreased gene expression of the pluripotency markers *Oct4* (A) and *Nanog* (B) over time, however different temporal trajectories were observed, with encapsulated cells, particularly those in the High G and High G + PLL groups, maintaining *Oct4* and *Nanog* expression longer than the unencapsulated aggregates. *Pax6* was elevated in the unencapsulated, High M, and High M + PLL conditions over the High G and High G + PLL conditions at Day 7 (C) and increased in all groups by Day 14, with the High G +

PLL and High M + PLL expressing *Pax6* at higher levels than the High M and unencapsulated groups. Expression of alpha-fetoprotein (*AFP*) was increased in the High M and High M + PLL groups (**D**), with significantly higher expression compared to other groups observed both at Day 7 and 14 of differentiation. Expression of *MLC-2v* was elevated in High M and High M + PLL conditions at Day 7(**E**). *Flk1* was more highly expressed in the unencapsulated, High M, and High M + PLL conditions at Day 7 (**F**). Significant ($P<0.05$) increases over unencapsulated (*), High G (#), High G + PLL (\$), High M (+), and High M + PLL () are denoted.

Figure 6.

Immunostaining analysis. Frozen sections from each group were stained for OCT-4, AFP, and alpha smooth muscle actin (α -SMA) and counterstained for cell nuclei. The extent of OCT-4 expression was higher in the encapsulated groups (**B–E**) compared to the unencapsulated group (**A**) at Day 7. AFP was observed frequently in the High M condition (**I**) at Day 14, with little to no expression detected in the other groups (**G,H,J,K**). Expression of α -SMA was observed at localized regions in the unencapsulated (**K**) and High M (**N**) groups at Day 14. More diffuse expression of α -SMA of a lower relative intensity was observed in the High G (**L**), High G + PLL (**M**), and High M + PLL groups (**O**). Scale bar = 100 μ m.

Table 1

PCR primer sequences and annealing temperatures.

Gene	Forward sequence	Reverse sequence	Melt temperature (°C)
18s	CTCTAGTGATCCCTGAGAAGTTCC	ACTCGCTCCACCTCATCCTC	60.0
Oct4	CCGTGTGAGGTGGAGTCTGGAG	GCGATGTGAGTGATCTGCTGTAGG	60.0
Nanog	GAAATCCCTTCCCTCGCCATC	CTCAGTAGCAGACCCCTGTAAGC	60.0
Pax6	ACGGCATGTATGATAAACTAAG	GCTGAAGTCGCATCTGAG	58.0
AFP	CACACCCGCTTCCCTCATCC	TTCTTCTCCGTCACGCACTGG	59.0
MLC-2v	GACCATTCTCAACGCATTCAAG	CTTCTCCGTGGGTAATGATGTG	59.0
Flk1	GGCGGTGGTGACAGTATC	TGACAGAGGCGATGAATGG	64.3