Three-Dimensional Hydrogel Encapsulated Embryonic Stem and Carcinoma Cells as Culture Platforms for Cytotoxicity Studies

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The utility of alginate hydrogels for three-dimensional (3-D) culture of mouse embryonic stem cells (mESCs) and future development of 3-D stem cell culture-based in vitro screens of toxicity is described. Using alginate hydrogels of various stiffness, we first evaluated the impact of substrate modulus on mESC viability, proliferation, as well as expression of pluripotency and germ-layer markers and observed that low concentration alginate hydrogels (0.5% and 1% alginate) were most suitable for long-term culture of mESCs. These results were not unique to mESCs; long-term viability and proliferation of mouse embryonic carcinoma cells (mECCs) was also best supported by similar conditions. Finally, we determined cytotoxic responses of alginate encapsulated cells to commercially available chemicals and interestingly observed similar responses for mESCs and mECCs, thereby suggesting that mECCs can predict stem cell responses to chemicals. These studies will facilitate future design of optimal stem cell-based platforms of organ-specific and developmental toxicity. *AIChE J*, 61: 3180–3184, 2015
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Introduction

Cell encapsulation in hydrogels is a useful approach for many tissue engineering and biomedical applications.[1,2] Their distinct advantage lies in providing a three-dimensional (3-D) microenvironment that facilitates cellular interactions more mimicking of the *in vivo* situation, which promotes cells to grow into tissue-like structures. In the context of embryonic stem cell (ESC) culture, hydrogel encapsulation has not only facilitated long-term feeder free culture of ESCs[3,4] but also their successful differentiation into various lineages.[5,6] An additional advantage of using hydrogels is the ability to vary the matrix stiffness by simply changing the concentration of either the monomer or crosslinker. Several studies have already used this property to demonstrate the role of two-dimensional (2-D) substrate stiffness in directing cell fate decisions.[7–10] For example, using polyacrylamide hydrogel substrates of varying stiffness, Engler et al. demonstrated the ability of matrix stiffness to direct lineage specification of mesenchymal stem cells (MSCs).[8] Such polyacrylamide hydrogel-based platforms have been also used to explore the role of 2-D substrate stiffness on ESC self-renewal and neuronal differentiation.[10] Stiffness-dependence of cell fates has also been demonstrated in 3-D;[11,12] for example, using neural stem cells encapsulated in alginate hydrogels, Banerjee et al. showed that neural stem cell proliferation and differentiation was sensitive to 3-D substrate modulus varied over two orders of magnitude.[11]

The ability to culture cells in 3-D hydrogel matrices that mimic one or more properties of the *in vivo* microenvironment is also advantageous to the development of *in vitro* platforms for screening new chemical entities. The use of hydrogels for the encapsulation of cancer cells for drug discovery and toxicity screening is already well reported in literature.[13–15] Recent works suggest that human cancer cells cultured in alginate hydrogels are more sensitive to test chemicals relative to those cultured on standard tissue culture plastic substrates.[15,16] Moreover, an improved correlation between *in vivo* LD_{50} values and *in vitro* IC_{50} values obtained using alginate-encapsulated human HepG2 cells relative to those obtained using conventional 2-D monolayer cultures of HepG2 was also observed.[15] Such hydrogel-based 3-D cell culture platforms have also been shown to be easily scalable and amenable to high-throughput screening.[17]

In addition to applications in tissue engineering and regenerative medicine, recent research also describes the use of stem cells to develop alternative methods for organ-specific toxicity. For example, it has already been established that

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cardiomyocyte- and hepatocyte-like cells derived from ESCs have the potential to serve as *in vitro* models of cardiotoxicity and hepatotoxicity, two leading causes of late stage drug attrition.[18] ESC-based platforms have also been proposed as alternative approaches to embryotoxicity or developmental toxicity testing.[19,20] However, in order for such stem cell based approaches to provide meaningful data, it is essential that the *in vitro* cultures sufficiently replicate the *in vivo* stem cell environment. As highlighted earlier, recent research has already established the utility of hydrogels in developing 3-D feeder-free cultures of stem cells that capture one or more properties of the *in vivo* stem cell microenvironment. In this article, we investigate the role of the mechanical properties of alginate hydrogels on the growth, viability, and pluripotency of embryonic stem cells in 3-D and demonstrate its potential use as a 3-D stem cell culture platform for toxicity studies. We chose alginate for its easily controllable mechanical properties, biocompatibility, and high porosity and large pore size ($\sim$100 nm) that allows efficient diffusion of low molecular substrates.[21–23] Moreover, alginate-based scaffolds have been shown to maintain the functional activities of a variety of mammalian cell types, including stem cells.[24–26] Using the mouse embryonic stem cell (mESC) line E14TG2a as our pluripotent stem cell model, we determined the optimal alginate concentrations for encapsulating mESCs and evaluated the sensitivity of alginate-encapsulated mESCs to test chemicals with a wide range of IC_{50} values. We also carried out these studies using the P19 mouse embryonic carcinoma cell (mECC) line, the malignant counterpart of mouse embryonic stem cells.[27] P19 cells are pluripotent and their differentiation mechanisms appear to be similar to those of normal embryonic stem cells, which has led to the use of P19 cells as a model for *in vitro* differentiation.[28,29] It would, therefore, be of interest to determine if 3-D cultures of mECCs can similarly be used as a suitable model for cytotoxicity studies; the cytotoxic responses of mECCs encapsulated in alginate were compared to those of mESCs in alginate. This study represents the first step toward establishing hydrogel-based 3-D cultures of stem cells for testing the effects of a variety of toxins, including those responsible for developmental and organ-specific toxicity.

Materials and Methods

Cell culture and encapsulation in alginate beads

E14TG2a mES cells (ATCC) were maintained on 0.1% gelatin-coated tissue culture flasks in high glucose DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 1000 U/mL LIF, and 1% penicillin-streptomycin. P19 mEC cells (ATCC) were maintained in αMEM supplemented with 7.5% FBS, 2.5% FCS, 1% nonessential amino acids, 2 mM L-glutamine, and 1% penicillin-streptomycin. The cell culture dishes were incubated at 37 °C in a humidified 5% CO_2 atmosphere. Subsequently, the cells were encapsulated in alginate (Sigma Aldrich) by the extrusion method using a syringe and 25G needle (the alginate beads were ca. 2.2 mm in diameter, see Supporting Information Figure 1). Alginate solutions (sterile filtered using 0.45 μm microfilter) and cell suspensions in culture media were mixed gently to yield final cell concentrations of 2.5×10^6 cells/mL and final alginate concentrations of 0.5%, 1.0%, and 1.5% w/v. Alginate beads containing the cells were crosslinked in 100 mM $CaCl_2$ solution and washed three times with PBS, before suspending in the culture media. Alginate-encapsulated cells were maintained in the conditions described above for up to 7 days.

Mechanical analysis of alginate hydrogels

Alginate samples of different concentrations were characterized using rheometry in a MCR302 rotational rheometer (Anton Paar) with parallel plate geometry. Frequency sweep measurements of the alginate samples crosslinked using 100 mM were carried out at constant amplitude and temperature (25 °C) to determine the shear storage modulus, G' and the shear loss modulus, G''. G' is related to the elastic behavior of a viscoelastic material and describes its stiffness and G'' is related to the viscous behavior of a viscoelastic material. First, amplitude sweeps at a constant frequency of 1 Hz were performed to ensure that the measurements were carried out in the linear viscoelastic regimes for the alginate samples. Dynamic sweep tests over frequencies ranging from 0.1 to 100 Hz were then recorded in the linear viscoelastic regimes (strain amplitude = 0.01) for determining G' and G''. The shear storage and loss modulus of noncrosslinked alginate solutions were also determined as controls.

Cell viability and proliferation analysis and toxicity assays

Live/dead analysis was performed on the cells encapsulated in alginate beads at day 7 using Calcein AM (Invitrogen)/Propidium Iodide (PI) (Sigma) staining assay; the final concentrations used for cell staining were 2 μM Calcein AM and 4 μM PI. Stained cells were observed and images were captured using a fluorescence microscope (Axiovert 200M, Zeiss) on day 7 at 10X magnification. Quantitative cell proliferation analysis was performed on Day 7 using the WST-8 cell proliferation assay (Cell Counting Kit-8, Dojindo Molecular Technologies), as per the manufacturer's instructions. Stock solutions of the test chemical compounds, acrylamide, cadmium chloride, and quinidine (obtained from Sigma Aldrich) for the toxicity assays were made in DMSO. The test chemical concentration (selected based on previous literature) were as follows: acrylamide (0.01–100 mM), cadmium chloride (0.1–1000 μM), and quinidine (0.1–1000 μM). Cells encapsulated in alginate were exposed to the chemicals for 48 h before being assayed for viability using the WST-8 assay; all measurements were performed using six different concentrations in triplicate.

Quantitative PCR analysis

Quantitative real-time polymerase chain reaction (PCR) analysis was performed on day 7 samples of mESCs encapsulated in alginate hydrogels to compare expression levels of pluripotent and germ layer markers. RNA was extracted from the cell pellet using the Qiagen RNA extraction kit and quantified using UV-vis spectroscopy. The cDNA sample was obtained from the extracted RNA by reverse transcription following the manufacturer's protocol (Promega) using the Bio-Rad MyCycler machine. qPCR analysis was performed on Nanog, Oct-4, mBrachyury, Nestin, and mAFP. The housekeeping gene GAPDH served as the reference gene. The cDNA samples were added to a 96-well plate along with master mix containing forward and reverse primers, SYBR green master mix, and nuclease-free water. The iQ5 iCycler from BioRad was used and the samples were run under a two-step melt curve real time PCR protocol. The results obtained from

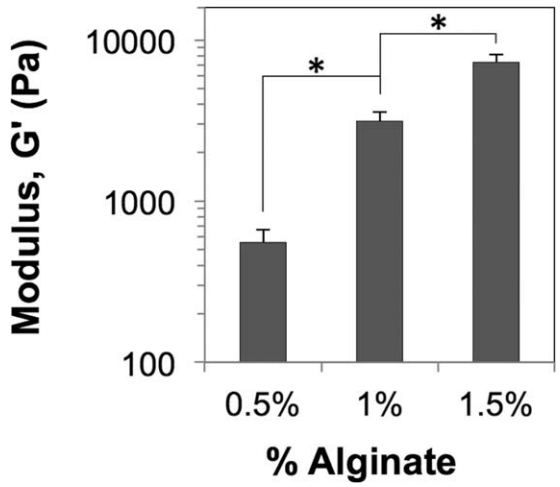

Figure 1. Rheological analysis of alginate hydrogels.

Hydrogel elastic modulus, G' of 0.5%, 1.0%, and 1.5% alginate hydrogels crosslinked using 100 mM $CaCl_2$ as characterized by rotational rheometry. *$P < 0.01$ for elastic moduli of hydrogels prepared using 0.5%, 1.0%, and 1.5% alginate, as determined by Student's unpaired two-tailed t test.

the machine were analysed using the delta CT method (normalized by GAPDH expression levels) and compared.

Results and Discussion

Impact of hydrogel modulus on viability and proliferation of mouse embryonic cells

It is well known that the mechanical properties of alginate hydrogels are highly dependent on the alginate concentration, with higher concentrations leading to improved mechanical stability and rigidity.[22,30] Therefore, to study the influence of 3-D hydrogel stiffness on the viability and proliferation of encapsulated cells, we first prepared hydrogel beads using 0.5%, 1.0%, and 1.5% alginate, while keeping the concentration of the Ca^{2+} crosslinker constant at 100 mM. Rheological characterization of the hydrogel samples showed that hydrogel modulus of alginate samples increased with increasing concentration of alginate, which was consistent with previous studies (Figure 1).

Next, we proceeded to evaluate the viability and proliferation of mouse embryonic stem cells (mESCs) encapsulated in 0.5%, 1.0%, and 1.5% alginate hydrogel beads over a 7-day culture. Since unmodified alginate is bioinert and does not support cell adhesion,[2,23] alginate-encapsulated mESCs formed EBs over this period. Live-dead staining experiments on day 7 indicated diminished viability of mESCs in 1.5% alginate (as seen by relatively high number of PI-stained cells) relative to those encapsulated in 0.5% and 1% alginate (Figure 2a). Similar observations, that is, low viability of nonadherent cells (e.g., leukemia cells) in 1.5% alginate hydrogels, have been made in previous studies.[31] In addition to the qualitative microscopy experiments, we also assessed cell proliferation using the WST-8 assay. The cell proliferation results showed that 0.5% and 1% alginate best supported the proliferation of mESCs, while 1.5% alginate was least favorable (Figure 2b). Proliferation experiments using mouse embryonic carcinoma cells (mECCs) yielded comparable results; mECCs proliferated more readily in 0.5% and 1.0% alginate (no statistically significant differences observed between 0.5% and 1% alginate) relative to 1.5% alginate (Figure 2b). These results are not entirely surprising, as higher alginate concentrations not only lead to increased hydrogel modulus that may constrain the rate of EB growth but can also contribute to reduced transport efficiencies of essential nutrients to cells encapsulated in the 3-D hydrogel culture microenvironment leading to reduced viability.[21,32]

Effect of hydrogel modulus on pluripotency of mouse embryonic stem cells

Quantitative PCR results showed that stem cell properties of mESCs were not significantly affected by mechanical stiffness of alginate hydrogels used in this study. We observed similar expression levels of pluripotent markers, Nanog and Oct-4, in all alginate formulations, however, the batch-to-batch variability was significant for 1.5% alginate (Figure 3a). The expression of the germ layer markers, Nestin, Brachury T, and AFP, were also comparable for mESCs encapsulated in 0.5%, 1%, and 1.5% alginate, but again significant batch-to-batch variability was observed for 1.5% alginate hydrogels (Figure 3b). Therefore, from a process standpoint, the 1.5% alginate was least favorable for ESC 3-D culture because of low rates of cell proliferation and viability, as well as due to inconsistencies in the expression of pluripotency and germ layer markers for mESCs in 1.5% alginate. And, even though our data suggested that 0.5% alginate adequately supported 7-day culture

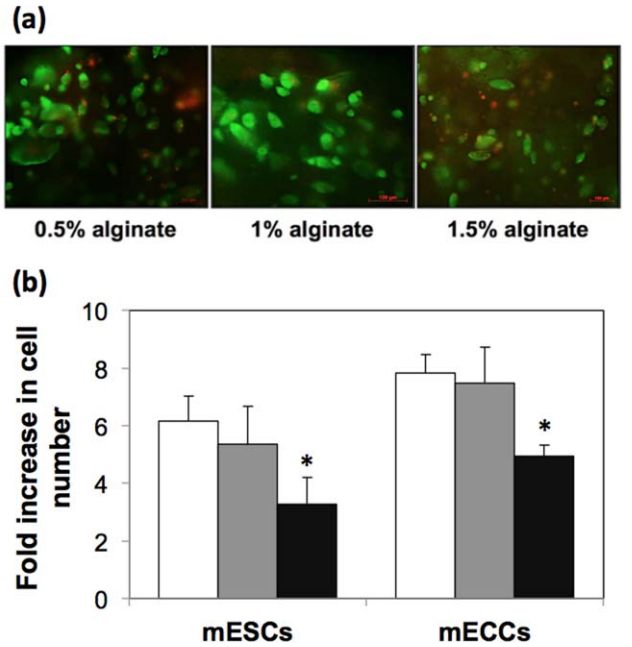

Figure 2. Cell viability and proliferation in alginate beads of varying stiffness.

(a) Fluorescent live/dead staining images using Calcein AM (green) and propidium iodide (red) of mESC EBs in 0.5%, 1.0%, and 1.5% alginate hydrogels. (b) Fold increase in cell numbers of mESCs and mECCs encapsulated in 0.5% (white bars), 1% (gray bars), and 1.5% (black bars) on day 7 relative to day 0. Error bars represent the standard deviation of three samples. *$P < 0.01$ for increase in cell numbers of mESCs and mECCs encapsulated in 1.5% alginate compared with those cultured in 0.5% and 1% alginate, as determined by Student's unpaired two-tailed t test. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

of mESCs and mECCs, low concentration alginate hydrogels tend to suffer from loss of gel integrity over long-term cultures. Considering all the factors, we concluded that 1% alginate hydrogel was most suitable for 3-D encapsulation of mouse embryonic cells.

Cytotoxicity studies

Encouraged by the aforementioned results, which indicate that 1% alginate hydrogel beads are suitable for long-term viability and maintenance of mESCs and mECCs, we decided to investigate the utility of pluripotent mouse embryonic cells encapsulated in 1% alginate beads for toxicity screening. For this, we first tested the response of mESCs encapsulated in 1% alginate to various concentrations of the test chemicals—acrylamide, cadmium chloride and quinidine (Table 1). Since a limited number of studies have reported the use of 3-D cultures of cells, particularly stem cells, for toxicity evaluation, we compared our results to current literature data obtained for different cell types, including stem cells using both 2-D and 3-D culture conditions.[15,16,33–38] The IC_{50} values obtained using alginate-encapsulated mESCs were comparable to data from previous studies, thereby validating the use of alginate for development of toxicity testing screens for stem cells. Furthermore, our experiments also revealed that the sensitivity of mECCs to chemical toxins is comparable to that of mESCs (Table 1), and, therefore, mECCs can potentially be used to predict stem cell responses to chemicals.

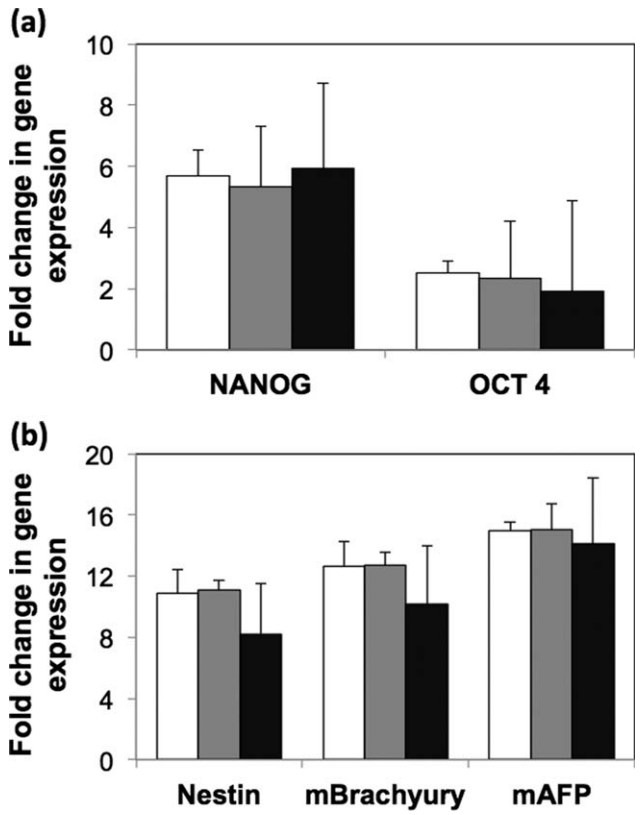

Figure 3. Influence of alginate modulus on mESC pluripotency.

Relative expression of (a) pluripotent markers, Nanog and Oct4 and (b) three germ layer markers, Nestin, mBrachyury, and mAFP in mESCs encapsulated in 0.5% (white bars), 1% (gray bars), and 1.5% (black bars) on day 7. Error bars represent the standard deviation of three samples.

Table 1. IC_{50} Values of mESCs and mECCs Encapsulated in 1% Alginate Hydrogels

	IC_{50} (μM)	
Chemical	mESCs	mECCs
Acrylamide	1949 ± 186	2212 ± 283
Cadmium	27 ± 7	19 ± 3
Quinidine	153 ± 22	239 ± 43

Conclusions

In this study, we show that encapsulation of ESCs in alginate hydrogels provides a favorable 3-D microenvironment that supports spontaneous EB formation and high cell densities mimicking the *in vivo* conditions. Using both qualitative microscopy and quantitative cell proliferation assays, we determined that the optimal concentration of alginate for the preparation of hydrogel beads that supported healthy cell growth and high viability of mESCs and mECCs in 3-D culture were identical (i.e., 1% alginate). Also, our results showed that while varying the stiffness of alginate hydrogels did not significantly impact the pluripotency of mESCs, significant batch-to-batch differences were observed for higher concentrations (1.5% w/v) of alginate. Finally, we demonstrated the utility of alginate-based 3-D cultures of mESCs and mECCs in developing *in vitro* screens of toxicity and found that the chemical sensitivity of both cell types in 3-D cultures were similar. Our future experiments will explore the potential of such 3-D cultures of mouse embryonic cells to evaluate the role of toxins on differentiation of stem cells into various lineages. We believe that these experiments will pave the way for the future development of 3-D stem cell culture-based platforms of developmental toxicity.

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