Alginate Sponges as Physiologically Relevant Culture Environments for Primary Rat Hepatocytes

Alexandria Sams[1], Zhensheng Li[1], Jonathan Jackson[2], Jason Wright[3], Marie Gonzalez[3], Jeanette Hill[2], and Mark Powers[1]
Invitrogen Corporation [1]7335 Executive Way • Frederick, MD 21704 [2]4301 Emperor Blvd • Durham, NC 27703 [3]1624 Headway Circle • Austin, TX 78754

Abstract#: 1964
Poster Board: 629

INTRODUCTION

Mammalian cell culture platforms have traditionally focused on optimizing specific targets of interest such as growth, productivity, or protein expression. While such approaches have enabled fundamental biological discoveries over the past several decades, they are typically incompatible with replicating *in vivo* cellular function. It is thought that this is in large part due to the inability of such systems to provide cultured cells with the proper physiological context. Recapitulating key aspects of the hierarchical environments in which cells reside in vivo -- mechanical, chemical, spatial, architectural, etc. -- has been shown to allow cells to function in a more functionally relevant manner. Approaches include cellular co-culture, perfusion bioreactors, and architecturally and chemically relevant biomaterials. Within our group, the design and development of extracellular matrices and scaffolds for the three-dimensional (3D) growth of cells is a particular area of focus.

One such material is AlgiMatrix®, a lyophilized alginate sponge scaffold that drives intercellular interactions through the architectural properties of the interstitial pores. This novel scaffold is a platform technology to facilitate the creation of multicellular spheroidal structures: because cells do not significantly interact with alginate, they are able to interact more readily with one another. The technology is of particular interest in the field of liver biology, as hepatocellular spheroids have been shown to maintain expression of key hepatic markers, but are traditionally somewhat cumbersome to culture and maintain. For the purposes of these studies, primary rat hepatocytes were cultured in 24-well AlgiMatrix® plates and assessed for cell morphology, viability and functional parameters relative to standard culture formats.

MATERIALS & METHODS

Cell culture: Briefly, hepatocytes (primary Sprague-Dawley rat or C3A human hepatoblastoma) were seeded by pipetting a concentrated cell suspension on top of the dry AlgiMatrix® scaffold. Following the complete absorption of media into the scaffold an additional volume of culture medium was added to each well. C3A cells were cultured in serum-containing media. Primary rat hepatocytes were cultured in serum-free media. Seeding concentrations and culture periods are detailed in each figure caption. All cultures were incubated in 5% CO_2 & 37℃ with daily medium changes. For collagen-Geltrex™ sandwich cultures, 24-well plates were coated with 30µg/mL collagen (Invitrogen, Carlsbad, CA) and primary rat hepatocytes (PRH) were seeded (0.375×10^6 cells/well). At ~5h post seeding serum-free media containing Geltrex™ (0.35mg/mL) (Invitrogen, Carlsbad, CA) was added to each well. Cultures were incubated in 5% CO_2 & 37℃ with daily medium changes.

Caspase-3 Activity Determination: The quantification of caspase-3 proteolytic activity was assessed using a commercial kit (ApoTarget™ Caspase-3 Fluorometric Protease Assay, Invitrogen, Carlsbad, CA). The substrate is composed of a caspase-3 cleavage site and a fluorophore (ex: 400nm, em: 505nm).

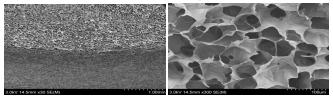
CYP450 induction: At 48h post-seeding PRH were induced with either 3-Methylcholanthrene (3-MC, a CYP1A inducer), Phenobarbital (PB, a CYP2B inducer), or Pregnenolone-16α-carbonitrile (PCN, a CYP3A inducer) for 3 consecutive days. On day 5 1A-induced cells were exposed to 10µM 7-ethoxyresorufin and 10µM dicumarol for 30min at 37℃ & 5% CO_2. CYP1A activity was determined by accumulation of the resorufin metabolite (ex: 560, em: 590). On day 5 2B-induced cells were exposed to 10µM pentoxyresorufin and 10µM dicumarol for 120min at 37℃ & 5% CO_2. CYP2B activity was determined by accumulation of the resorufin metabolite (ex: 560, em: 590). On day 5 3A-induced cells were exposed to 200µM testosterone for 14min at 37℃ & 5% CO_2. 6β-hydroxytestosterone was quantified by LC-MS/MS analysis using a Micromass® VG Quattro II & a YMC-ODS-AQ® column (Waters, Milford, MA).

Glutathione Determination: The detection of intracellular levels of reduced glutathione (GSH) was assessed using a commercial kit (GSH-Glo™ Glutathione Assay, Promega, Madison, WI) that generates a luminescent signal based on the conversion of a luciferin derivative into luciferin in the presence of glutathione.

Scanning Electron Microscopy (SEM): For SEM, scaffolds were cut into segments with a scalpel both horizontally and vertically. Samples were sputter-coated with an ultra thin (100 angstrom) layer of gold and viewed at a voltage of 5 kW.

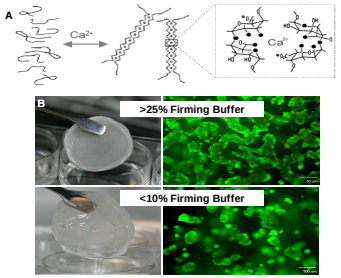
RESULTS

1. Scaffold Properties



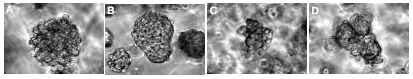
AlgiMatrix® scaffolds are made from alginate (a polysaccharide extracted from seaweed) cast in multiwell plates and lyophilized to optimize pore formation. These SEM images of sponges removed 96 well plates reveal interconnected pores with diameters ranging from 50 to 150µm. Cells residing in the inert alginate pores are able to interact with one another (rather than with the alginate biomaterial) to form 3D cellular structures.

2. Tunable & Dissolvable



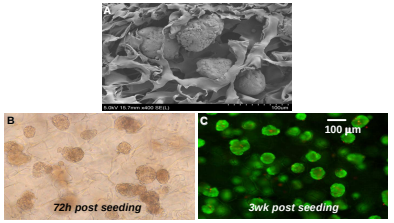
A) Alginate is a linear unbranched polymer composed of 1,4-linked β-D-mannuronic and α-L-guluronic acid residues. Ca^{2+} ions cause polyguluronic regions to dimerize to form hydrogels. **B**) **AlgiMatrix® Firming Buffer** is a calcium based solution that is applied prior to or concurrent with cell seeding. The ratio of firming buffer to medium in the solution dictates the firmness of the sponge and allows for tunable control of compliance/stiffness (an important factor in dictating cellular phenotype). **AlgiMatrix® Dissolving Buffer** (a sodium citrate solution) chelates calcium to liberate cells from sponges.

3. Ready to Use for Diverse Cell Types



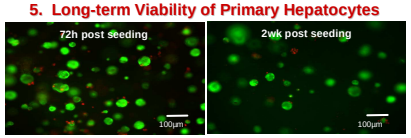
A) U87 glioblastoma cells (6.3×10^4) and **B**) C6 glioma (5×10^5) were seeded in AlgiMatrix® and maintained for 7 days. **C**) DU145 prostate cancer cells (5×10^5) and **D**) MCF7 breast cancer cells (2×10^5) were seeded in AlgiMatrix® and cultured for 3 days. Images courtesy of Dr. Barry Hudson, Columbia University.

4. Hepatocyte Spheroid Formation



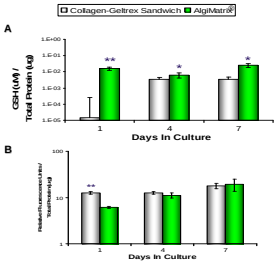
C3A (human hepatoblastoma) cells were seeded (2×10^6 cells/well) into a 6-well AlgiMatrix® plate and maintained for 3 wks with medium changes every 72h. **A**) SEM image illustrates coalescence of cells to form compact multicellular spheroids. **B**) Phase-contrast image shows spheroid formation within 72h post cell seeding. **C**) Live/dead staining (2µM Calcein AM, 4µM Ethidium homodimer-1) indicates a significant # of viable hepatocytes present at 3 wks in culture.

5. Long-term Viability of Primary Hepatocytes



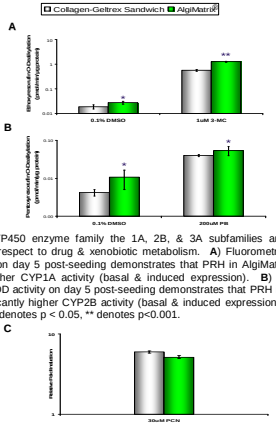
Freshly-isolated primary rat hepatocytes (Sprague-Dawley) were seeded (1×10^6 cells/well) in a 24-well AlgiMatrix® plate and maintained with daily medium changes. Cell morphology was optimized at 10% Firming Buffer for use in primary hepatocyte applications. Live/dead staining (8µM Calcein AM, 4µM Ethidium homodimer-1) indicates that the spheroids contain a significant fraction of viable cells, through at least 2 wks of culture.

6. Metrics of Cellular Stress: Primary Hepatocytes in AlgiMatrix® *vs.* Collagen-Geltrex™ Sandwich Culture



A) Intracellular levels of glutathione (GSH) are significantly higher for PRH maintained in AlgiMatrix® throughout the culture period. Depletion of glutathione (GSH), a critical antioxidant, can lead to cell death. Therefore, GSH levels are used to assess toxicological responses. **B**) At the earliest time point (1 day post seeding) caspase-3 activity is significantly greater in hepatocytes seeded in the collagen-Geltrex™ sandwich culture. Caspase-3 is a key mediator of apoptosis. n=6. Two sample t-test, * denotes p<0.05, ** denotes p<0.001.

7. Functional Performance: CYP450 Activity



Among the CYP450 enzyme family the 1A, 2B, & 3A subfamilies are particularly important with respect to drug & xenobiotic metabolism. **A**) Fluorometric analysis of EROD activity on day 5 post-seeding demonstrates that PRH in AlgiMatrix® maintain significantly higher CYP1A activity (basal & induced expression). **B**) Fluorometric analysis of PROD activity on day 5 post-seeding demonstrates that PRH in AlgiMatrix® maintain significantly higher CYP2B activity (basal & induced expression). n=6. Two sample t-test, * denotes p < 0.05, ** denotes p<0.001.

C) LC-MS/MS analysis of 6β-hydroxytestosterone formation on day 5 post-seeding indicates that PRH in AlgiMatrix® maintain similar fold induction of CYP3A activity compared to sandwich culture. n=8.

SUMMARY

- AlgiMatrix® is a chemically defined, highly porous (>90%) 3D scaffold; the hydrophilic nature of the material allows for rapid and efficient cell seeding and distribution.
- AlgiMatrix® stiffness can be controlled by the use of Firming Buffer (a calcium based solution). Cell recovery from AlgiMatrix® is achieved with the use of Dissolving Buffer, a non-enzymatic solution which dissolves the scaffold within a few minutes but leaves the cellular aggregates intact for further processing and/or analysis.
- Using various cell types we have demonstrated that cell aggregation within AlgiMatrix® leads to spheroid formation that is restricted by the fixed pore sizes (50-150µm) within the scaffold thereby preventing the development of hypoxic cores.
- We have demonstrated long-term viability using primary rat hepatocytes and C3A hepatoma cells. Cellular toxicity measurements demonstrate that the AlgiMatrix® culture environment promotes a healthier phenotype than the conventional sandwich culture.
- Our functional studies pertaining to CYP450 activity illustrate the superior capability of AlgiMatrix® to preserve liver-specific function and response in comparison to the conventional sandwich culture.

CONCLUSIONS

We have created a well-defined porous alginate matrix (AlgiMatrix®) comprising interconnected pores that act as localized compartments in which cells form reproducible spheroidal aggregates. Our liver biology studies demonstrate that this scaffold offers an efficient platform for the creation of functionally relevant hepatocellular spheroids relative to conventional culture methods. Cellular toxicity measurements (intracellular glutathione levels, caspase-3 activity) demonstrate that the AlgiMatrix® culture environment is less stressful for hepatocytes in comparison to conventional sandwich culture. Functional performance analysis reveals that primary hepatocytes in AlgiMatrix® can respond specifically and quantitatively to model CYP450 inducers. Collectively, these results suggest that this platform is a potentially useful predictive tool in xenobiotic metabolism and toxicology research. We conclude that 3D cell culture scaffolds such as AlgiMatrix® serve as a foundation for the creation of more physiologically relevant culture systems.

REFERENCES

1. Landry, J. et al. J Cell Biol. **101**, 914-923 (1985).
2. Peshwa, M. et al. In Vitro Cell Dev Biol Anim. **32**, 197-203 (1996).
3. Koide, N. et al. Biochem Biophys Res Commun. **161**, 385-391 (1989).
4. Abu-Absi, S. et al. Exp Cell Res. **274**, 56-67 (2002).
5. Brophy, C. et al. Hepatology. **49**, 578-586 (2009).
6. Glicklis, R. et al. Biotechnol Bioeng. **86**, 672-680 (2004).

ACKNOWLEDGEMENTS

We wish to thank Ed LeCluyse, Stephen Ferguson, and Cornelia Smith for helpful discussions regarding primary hepatocyte work in AlgiMatrix®.

Contact: alexandria.sams@lifetech.com