The Application of Alginate Scaffolds for Three-Dimensional Cell Culture

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Introduction

There is growing recognition of the need to incorporate the three-dimensional (3D) cell culture paradigm towards in vitro toxicology models. The liver is a major target of toxic chemicals, the use of primary hepatocytes would be ideal. However, in standard hepatocyte monolayer culture, cells adopt a flat morphology that is accompanied by a rapid loss of liver-specific functions. This loss of function presents a significant barrier for predictive toxicology studies in which the time scale for the toxic response may be significantly longer than the time scale for the loss of liver-specific function. It is thought that this loss of tissue phenotype is largely due to the inability of conventional two dimensional (2D) cell culture models to sufficiently replicate the physiological architecture of the liver microenvironment, thereby lacking the appropriate cues necessary to maintain tissue-specific function. The use of 3D supporting templates that promote more in vivo-like cell interactions is crucial to developing more predictive models. When hepatocytes are induced to form multicellular spheroidal aggregates they demonstrate greater retention of liver-specific activities. Various structural studies have reported that hepatocyte spheroids demonstrate polarized cell morphology via tight junctions and bile canaliculi-like structures (1, 2). Functional analyses revealed that multicellular spheroids exhibit in vivo levels of expression of albumin and glucokinase (3), functional bile canaliculi (4), stable liver-related gene expression, and increased cytochrome P450 activity (5). Newer models that foster the controlled and consistent formation of such spheroids will be essential tools in identifying and understanding cellular responses to chemicals. We introduce here the use of the AlgiMatrix™ 3D Culture System for these purposes.

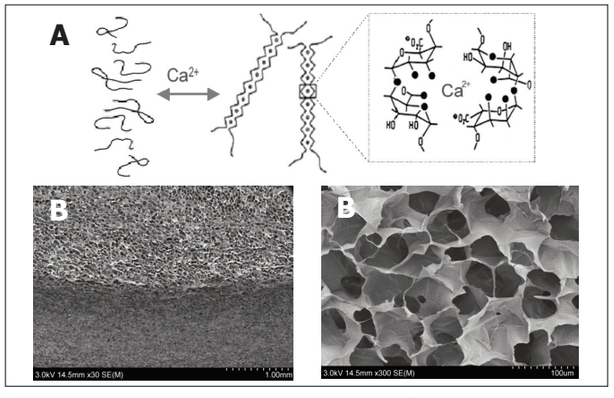
Methodology

Cell culture: Briefly, hepatocytes (primary rat hepatocytes or C3A human hepatoblastoma cells) 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. AlgiMatrix™ plates were incubated in 5% CO_2 and 37°C.

CYP 1A2 induction: Cells were induced with 3-methylcholatherene (3-MC, a CYP1A2 enzyme inducer) on days 3 and 4. On day 5, cells were assayed for CYP1A2 activity and viability. CYP1A2 enzyme activity levels were measured by the relative fluorescence of ethoxyresorufin (enzyme substrate) at excitation of 530nm and emission of 590nm. Cell viability was determined by resazurin relative fluorescence at an excitation of 560nm and emission of 590nm.

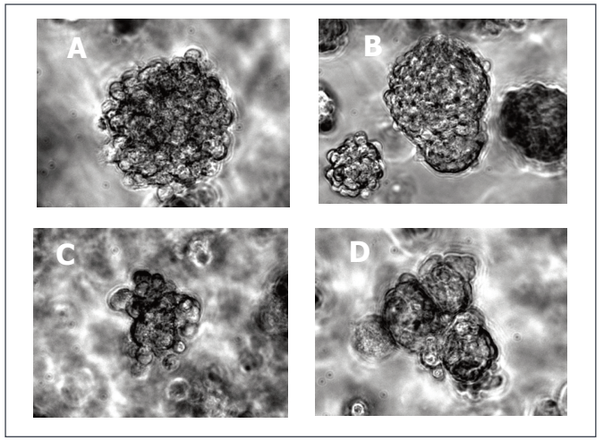
Scanning Electron Microscopy (SEM): For SEM, scaffolds were cut into segments with a scalpel both horizontally and vertically. Samples were sputter-coated with an ultrathin (100 angstrom) layer of gold and viewed at a voltage of 5 kW.

Figure 1. AlgiMatrix™ Scaffold Properties



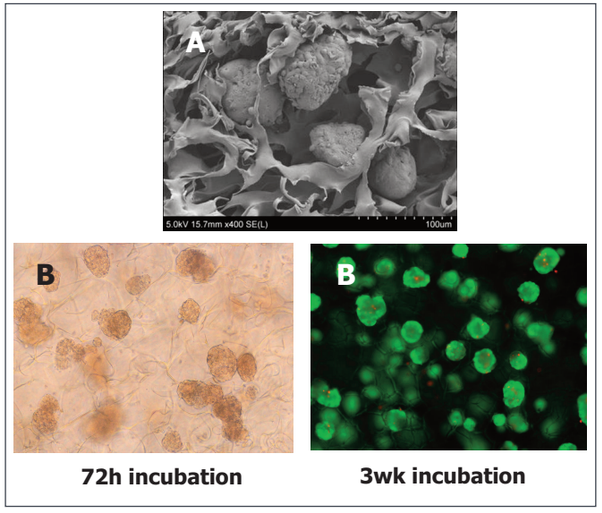
A) Alginate is a polysaccharide that is extracted from seaweed. This linear unbranched polymer is composed of 1,4-linked β-D-mannuronic (M) and α-L-guluronic acid (G) residues. Alginates contain three types of sequences: polyguluronic (GGGGG), polymannuronic (MMMMM), and alternating (GMGMG). Ca^{2+} ions cause polyguluronic regions to dimerize to form hydrogels with an egg-box like conformation. B) Scanning Electron Microscope (SEM) image analysis reveals pore diameters that range from 50 to 150µm.

Figure 2. AlgiMatrix™ Spheroid Formation



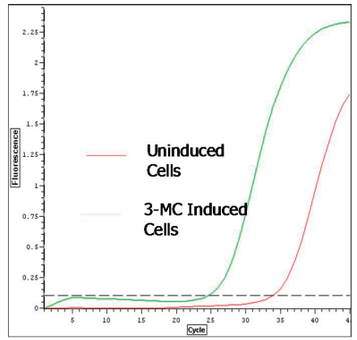
A) U87 glioblastoma cells (6.3×10⁴) and B) C6 glioma (5×10⁵) were seeded in AlgiMatrix™ and maintained for 7 days. C) DU145 prostate cancer cells (5×10⁵) and D) MCF7 breast cancer cells (2×10⁵) were seeded in AlgiMatrix™ and cultured for 3 days. Images courtesy of Dr. Barry Hudson, Columbia University.

Figure 3. AlgiMatrix™ C3A Cell Culture



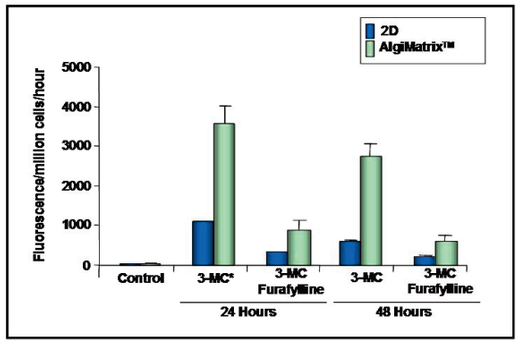
A) C3A (human hepatoblastoma) cells were seeded (2×10⁶ cells/well) into a 6-well AlgiMatrix™ plate and maintained for 3 weeks with media changes every 72h. SEM analysis reveals compact multicellular spheroidal aggregates. B) Phase-contrast image shows C3A spheroid formation present within 72h post cell seeding. Live/dead staining (2µM Calcein AM, 4µM Ethidium homodimer-1) indicates a significant number of viable hepatocytes present following 3 weeks in culture.

Figure 4. Induction of CYP 1A2 Gene Expression



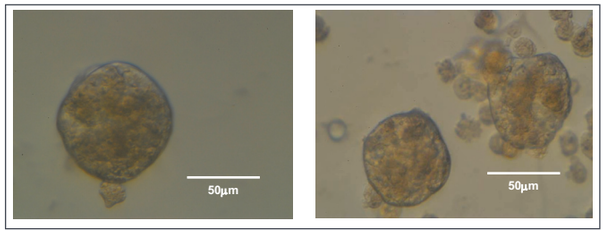
C3As were cultured in AlgiMatrix™ for 4 days in the absence and presence of 3-MC (a CYP1A2 enzyme inducer). The AlgiMatrix™ sponge was then dissolved in a dissolution buffer and the cell pellet recovered. RNA was extracted prior to cDNA synthesis and CYP1A2 gene expression quantitated by qRT-PCR.

Figure 5. CYP 1A2 Induction



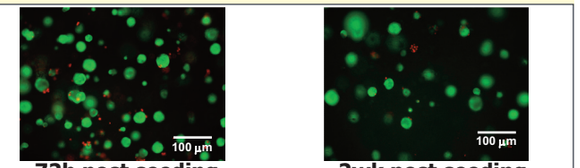
C3A hepatocytes were seeded (1.2×10⁶ cells/well) into both a 24-well AlgiMatrix™ plate and a tissue culture treated polystyrene plate (2D) and cultured for 4 days with daily medium changes. Each culture was untreated or induced with 3-MC (a CYP1A2 enzyme inducer) for 24h and 48h in the absence or presence of inhibitor (furafylline, 10µM).

Figure 6. Primary Hepatocyte Morphology



Freshly-isolated primary rat hepatocytes (Sprague-Dawley) were seeded (1×10⁶ cells/well) into a 24-well AlgiMatrix™ plate and maintained for 6 days with daily medium changes. Phase contrast microscopy reveals compact spheroids in which individual cells coalesce to form larger multicellular entities.

Figure 7. Primary Hepatocyte Viability



Freshly-isolated primary rat hepatocytes (Sprague-Dawley) were seeded (1×10⁶ cells/well) in a 24-well AlgiMatrix™ plate and maintained for 2 weeks with daily medium changes. Live/dead staining (8µM Calcein AM, 4µM Ethidium homodimer-1) indicates that the spheroids contain a significant fraction of viable cells.

Discussion

We have developed a well defined porous alginate matrix (AlgiMatrixTM) with interconnecting pores that are controlled in size (50-150µm) (Figure 1b). The alginate scaffold is a hydrophilic material that undergoes rapid wetting as cells are pulled in by capillary action and retained by the tortuous pore structure. Once inside the matrix cells do not bind to the alginate due to the highly hydrated anionic surface of the alginate material. The lack of cellular attachment to the matrix promotes increased cell-cell interactions that cause clustering within the matrix pores. The development of compact multicellular spheroidal aggregates in these pores is a major factor in increased cell survival and maintenance of differentiated function. We have observed that AlgiMatrix™ leads to the formation of spheroids in many different cell types for which 3D architecture provides a clear physiological relevance (Figure 2).

Our studies demonstrate that AlgiMatrix™ is a powerful tool to maintain hepatocytes with a more liver-like phenotype. Pore size is fixed within a range such that oxygen diffusion will not be rate limiting and therefore central hepatocytes are not at risk of becoming hypoxic (6). As a result, healthy, viable spheroids can be maintained, and are observed in long-term culture (3 weeks) (Figure 3). Functional analyses revealed enhanced CYP1A2 gene expression and enzyme induction compared to 2D monolayer culture (Figure 4 & Figure 5). We have also demonstrated that primary rat hepatocytes form compact, multicellular, and viable spheroids when cultured in AlgiMatrix™ (Figure 6 & Figure 7). We conclude that AlgiMatrix™ is a powerful platform for creating more physiologically relevant models for a variety of tissue types, including hepatocytes. It represents a significant step forward in bridging the in vitro/in vivo gap that many researchers encounter.

Conclusions

- AlgiMatrix™ is a chemically defined, animal-origin free, pharmaceutical grade matrix composed of alginate.
- AlgiMatrix™ is a highly porous (>90%) 3D scaffold; the hydrophilic nature of the material allows for rapid and efficient cell seeding and distribution.
- Using various cell types we have demonstrated that cell aggregation within AlgiMatrix™ leads to spheroid formation. Spheroid growth is restricted by the controlled pore sizes (50-150µm) within the scaffold thereby preventing the development of hypoxic interiors.
- Cell recovery from AlgiMatrix™ is achieved with the use of a non-enzymatic dissolution buffer which dissolves the scaffold within a few minutes. This process leaves the cellular aggregates intact in suspension for further processing and/or analysis.
- The AlgiMatrix™ culture environment promotes more physiologically relevant intercellular interactions. We have demonstrated long-term viability using primary rat hepatocytes and C3A hepatoma cells. Our functional studies pertaining to CYP1A2 gene expression and induction illustrate the superior capability of AlgiMatrix™ to preserve liver-specific function and response in comparison to conventional monolayer culture.
- Current efforts in our group are focused on characterizing the biotransformation of model toxic compounds using primary hepatocytes maintained in AlgiMatrix™. Thus, we expect to establish AlgiMatrix™ as an effective in vitro model for predictive hepatotoxicity.

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).