Polystyrene Scaffolds: A Novel Tool For *In Vitro* Three-Dimensional Cancer Models

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Abstract

It is well known that three-dimensional (3D) cultures can more accurately represent physiological cell responses, and that two-dimensional (2D) *in vitro* cell culture systems fall short in predicting tumor cell behavior *in vivo.* Major shortcomings of 2D cell culture systems include altered cell morphologies, reduced aggression, and an inaccurate representation of the dynamic 3D cellular environment experienced by cells *in vivo.* These 2D cell culture obstacles become even more deleterious to research when performing drug screenings or studying disease pathogenesis. Hence, cancer research demands a more biologically relevant *in vitro* model system that can improve drug discovery success rates to combat rising rates of cancer and other diseases. Moreover, an accurate and reproducible *in vitro* model will accelerate the drug discovery process and significantly reduce development costs. To this end, we have engineered a novel transparent 3D polystyrene (PS) scaffold to meet the emerging demand. We hypothesize that these 3D PS scaffolds are a superior 3D *in vitro* model for tumor pathogenesis research and drug discovery and development. To test our hypothesis we cultured and observed the growth of two cancer cell lines, human epithelial MCF-7 cells and HepG2 hepatocarcinoma cells, on both 2D PS-tissue culture plates (TCP) and 3D PS scaffolds (3D Insert™-PS). Observation by an inverted light microscope indicated that MCF-7 and HepG2 cells cultured on the 3D PS scaffolds formed cell aggregates along scaffold fibers and within pores, whereas cells cultured on the 2D PS-TCPs adopted a phenotype characteristically seen in cells growing in flat monolayers. Proliferation time-course assays performed with MCF-7 and HepG2 cells demonstrated that growth rates and metabolic activities of cells cultured on 3D scaffolds were significantly enhanced at numerous time points when compared with cells cultured on 2D TCPs, as determined by MTT assay. These data suggest that 3D Insert™-PS scaffolds provide an improved *in vitro* culture environment. The susceptibility of both 2D and 3D cultured MCF-7 and HepG2 cells to anticancer drugs was assessed by tamoxifen (TAM) [10^{-6} M and 10^{-5} M] and methotrexate (MTX) [25 µM and 100 µM] treatments, respectively. The cellular cytotoxic response to TAM and MTX was measured by MTT and LDH assays and was found to be significantly lower in MCF-7 and HepG2 cultured on 3D scaffolds compared with cells cultured in 2D TCPs. Taken together, these data indicate that cancer cells cultured on 3D Insert™-PS scaffolds are more robust and resistant to anticancer drug treatments than cells cultured on traditional 2D TCPs. Furthermore, 3D Insert™-PS scaffolds do not have the same limitations encountered with traditional 2D cell culture. In conclusion, using 3D Insert™-PS scaffolds as *in vitro* 3D tumor models will provide a superior 3D cell culture environment for cancer research and the evaluation of new anticancer drugs.

Materials and Methods

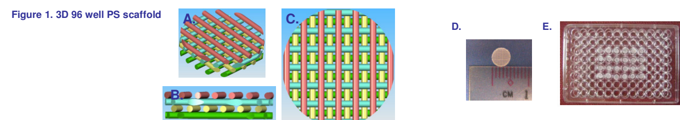
Cells

MCF-7 and HepG2 cells were purchased from ATCC (Manassas, VA) and cultured in Minimum Essential Medium (Gibco) containing 10% fetal bovine serum, 2 mM L-glutamine, 10 mM NEAA, 1 mM sodium pyruvate, 100 IU/ml Pen-Strep, and 0.01 mg/ml bovine insulin (Sigma Aldrich). Cultures were maintained in 5% CO_2 at 37ºC.

Polystyrene (PS) Scaffold Microfabrication (3D Insert™-PS)

Porous polystyrene scaffolds **(Figure 1)** were engineered using 3D Biotek's Precision 3D Micro-fabrication Technology. **Figure 1A-C** represents the four-layer structural design of 3D Insert™-PS scaffolds. Offset fibers make each of the PS scaffold's four distinct layers visible when viewing with an inverted light microscope. Uniquely, fiber diameter is controlled by nozzle diameter and spacing between fibers is controlled by a motion control system. This study utilized 96-well compatible PS scaffolds, 5.0 mm in diameter **(D)**, with 150 µm fiber diameter and 200 µm pore size configuration (PS1520) that fit into a standard 96-well tissue culture plate **(E)**. The total cell growth area of a 96-well 3D Insert™-PS1520 is 1.06 cm^2 compared to 0.32 cm^2 of total cell growth area in a 96-well 2D TCP. Before use, PS scaffolds are plasma treated and terminally sterilized by γ-radiation.

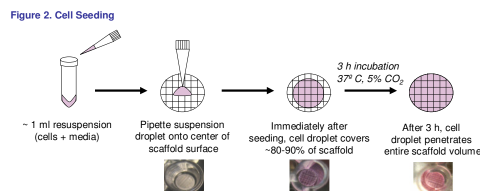
Figure 1. 3D 96 well PS scaffold



Cell Seeding and Culture

2D and 3D cell culture was carried out in treated and non-treated tissue culture plates, respectively. Cells were resuspended in 1 ml growth media before using 1x10⁴ cells/well in 200 µl (2D) and 1x10⁴ cells/scaffold in 20 µl (3D). To seed each scaffold, 20 µl of the cell suspension was slowly pipetted onto the top surface of each 3D Insert™-PS. To ensure high seeding efficiency, the cell suspension droplet was not allowed to contact the sides of the wells. After a 3 h incubation in 5% CO_2 at 37ºC, 180 µl of media was added to the 3D wells. After 24 h, scaffolds were gently transferred into new wells of a non-treated tissue culture plate, and fresh media was added. Media was changed every 48 h in 2D and 3D cultures until use **(Figure 2)**.

Figure 2. Cell Seeding



Cell Proliferation

Alamar blue (Invitrogen) and 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assays (Cayman Chemical) were performed according to manufacturer's instructions on cells in 2D TCPs and on 3D Insert™-PS.

Cytotoxicity Assays

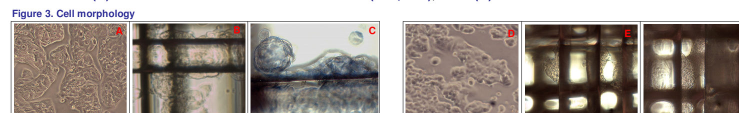
Tamoxifen (TAM) (Sigma, H7904) and Methotrexate (MTX) (Sigma, M9929) treatment began 24 h after 2D and 3D cell seeding. Cytotoxicity was measured at pre-determined days during the TAM and MTX treatment time-course by MTT and Lactase Dehydrogenase Activity Assays (LDH) (Cayman Chemical) according to manufacturer's indications.

Results

Cell Morphology

MCF-7 **(A-C)** and HepG2 **(D-F)** cells growing in 2D TCPs **(A, D)** and on 3D PS scaffolds **(B-C, E-F)** were imaged in real-time using an inverted light microscope **(Figure 3)**. Compared to cells cultured in 2D **(A, D)**, which grew in a characteristic monolayer, cells grown on 3D PS scaffolds **(B-C, E-F)** formed aggregates **(B, E-F)** and rounded cell structures **(C)** within the scaffold's 3D environment. 100X **(A-B, D-F)**, 200X **(C)**.

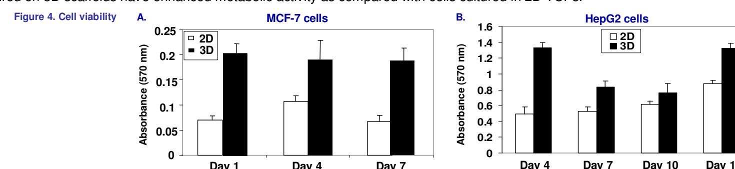
Figure 3. Cell morphology



Cell Viability

MCF-7 and HepG2 cells were seeded at concentrations of 1x10⁴ cells/96-2D well/96-3D PS scaffold. At various time-points, cell proliferation assays demonstrated that cells cultured on 3D Insert™-PS scaffolds are viable **(Figure 4)**. At all time points during the proliferation study, MCF-7 **(A)** and HepG2 **(B)** cells cultured on 3D scaffolds have enhanced metabolic activity as compared with cells cultured in 2D TCPs.

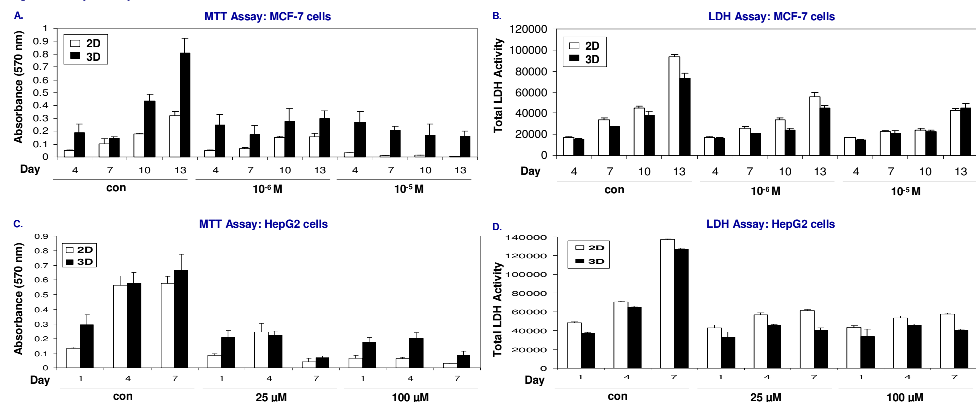
Figure 4. Cell viability



Cytotoxicity

Twenty-four hours after initial cell seeding at concentrations of 1x10⁴ cells/96-2D well/96-3D PS scaffold, media were changed to contain 10^{-6} M and 10^{-5} M TAM (MCF-7 cells), and 25 µM and 100 µM MTX (HepG2 cells). TAM and MTX, two chemotherapeutic agents, were used to compare the cytotoxic response of MCF-7 and HepG2 cells, respectively, cultured on 2D TCPs with cells cultured on 3D PS scaffolds **(Figure 5)**. Fresh media, containing 10^{-6} M and 10^{-5} M TAM, and 25 mM and 100 mM MTX, was added fresh every 48 h until assay. MTT **(A, C)** and LDH **(B, D)** assays were performed at various days throughout the treatment time-course. In response to TAM and MTX treatments, 3D cell cultures show less cytotoxicity when compared with 2D cell cultures.

Figure 5. Cell cytotoxicity



Conclusion

This study demonstrates that novel 3D Insert™-PS scaffolds can be used to create superior *in vitro* tumor models. Cells cultured on these PS scaffolds exhibit a unique morphology as well as the ability to differentiate into 3D structures that are not formed in 2D monolayer cultures. Furthermore, MCF-7 and HepG2 cells cultured on 3D Insert™-PS have greater cell viability and an increased resistance to cytotoxic effects than cells cultured on 2D TCPs. Taken together, these data suggest that using 3D Insert™-PS scaffolds in pathogenesis studies may more effectively recreate an *in vivo* microenvironment and imitate a tumor's true physiological response. Furthermore, 3D Insert™-PS scaffolds are easy to use and compatible with common 2D assays. In conclusion, 3D Insert™-PS scaffolds provide a model system for a better understanding of cancer cell organization, proliferation, and the evaluation of different anticancer drugs.

Acknowledgements

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