Alginate Immobilization of Metabolic Enzymes (AIME) for High-Throughput Screening Assays

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Objective

To develop a platform to retrofit existing high-throughput screening assays with metabolic competence.

Introduction

The EPA's ToxCast program utilizes a wide variety of high-throughput screening assays (HTS) to assess chemical perturbations of molecular and cellular endpoints. A key limitation of many HTS assays used for toxicity assessment is the lack of xenobiotic metabolism which precludes the detoxification as well as toxic bioactivation of chemicals tested *in vitro*, thereby mischaracterizing the potential hazard posed by these chemicals. To address this deficiency, we have developed a platform to retrofit existing HTS assays with extracellular xenobiotic metabolism. We demonstrate the successful encapsulation of human hepatic S9 in alginate microspheres and the deployment of this platform to a cell-based oxidative stress assay.

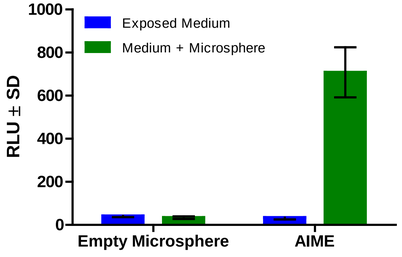


Figure 1: CYP3A4 is retained in the AIME microsphere
AIME microspheres were incubated in DMEM/1% FBS for 1 hour at 37ºC and then transferred to fresh DMEM/1% FBS containing a pro-luciferin substrate specific for CYP3A4 (luciferin-IPA). Luciferin-IPA was then added to the incubation medium and the assays were allowed to proceed for 10 minutes before detection of the luciferin metabolite.

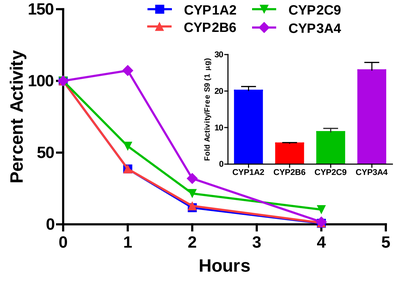
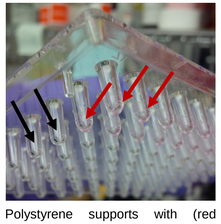


Figure 2: Encapsulated human hepatic S9 maintains measurable cytochrome P450 activity over time
AIME microspheres were incubated at 37ºC in DMEM/1% FBS for the indicated time. CYP-specific pro-luciferin substrates were then added and the reaction was allowed to proceed for 1 hour prior to detection of the luciferin metabolite. Data are normalized to activity at t=0. Inset: relative CYP activity at t=0. Values represent the mean ± SEM of three measurements.

Materials & Methods

Chemicals – All chemicals were purchased from Sigma-Aldrich and stock solutions prepared in DMSO.

Alginate Immobilization of Metabolic Enzymes (AIME) – Human hepatic S9 (BioreclamationIVT; Lot OUL) was encapsulated in alginate microspheres using a modification of a cell encapsulation protocol by Lee et *al*. (1). The microspheres were made using 10% S9 and attached to commercially available Nunc polystyrene replicators.

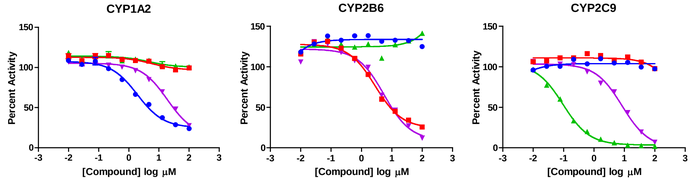


Polystyrene supports with (red arrows) and without (black arrows) AIME microspheres

Cytochrome P450 (CYP) Enzyme Activity – CYP enzyme activity was quantified using Promega P450-Glo kits with the following modifications: assays were performed in DMEM/1% FBS in order to replicate conditions in cell-based assays and were allowed to proceed for 1 hour. All assays included an NADPH regeneration system per the manufacturer's protocol.

Nrf2 Reporter Gene Assay with AIME - A Nrf2-activated reporter gene was used to assess oxidative stress induced by test chemicals with and without metabolic activity. Human hepatoma (HepG2-ARE/luc) cells containing a stably integrated Nrf2-responsive luciferase reporter gene have been previously described (2). Cells were plated at 40,000 cells/well in white, 96-well microplates and allowed to attach overnight at 37ºC. Post-attachment, cells were treated with a 54-compound library in 8-point concentration range covering 0.1-100 µM. (DMSO 0.5% (v/v) final concentration). An equal volume of a 2X NADPH regeneration solution was then added to all wells and an AIME lid added to experimental plates. Both control (standard lid) and experimental (AIME lid) plates were incubated for 20 hours at 37ºC. Luciferase activity was determined as previously described (2).

Cell Viability Assay – HepG2-ARE/luc cells were plated and treated as described above except a 12-point concentration range covering 0.01-100 µM was used. Cell viability was assessed using CellTiter-Fluor (Promega) according to the manufacturer's instructions.



Compound	Mol. Wt. (g/mol)	Targeted P450	IC50 Free S9 (µM)	IC50 AIME (µM)
Furafylline	260.25	1A2	2.39	1.92
Thio-TEPA	189.22	2B6	7.46	2.86
Tienilic Acid	331.17	2C9	.053	.096
Ketoconazole	531.43	3A4	.086	0.12

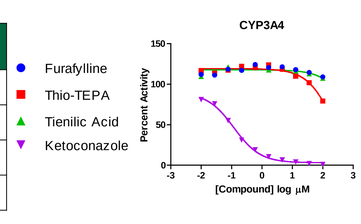


Figure 3: Compounds of varying molecular weight produce similar CYP inhibition IC50 values in both encapsulated and free human hepatic S9
AIME microspheres and 1 µg free S9 were treated with known CYP inhibitors and incubated in DMEM/1% FBS and CYP-specific pro-luciferin substrates for 1 hour at 37ºC followed by detection of the luciferin metabolite. Data are expressed as a percent activity of the DMSO control.

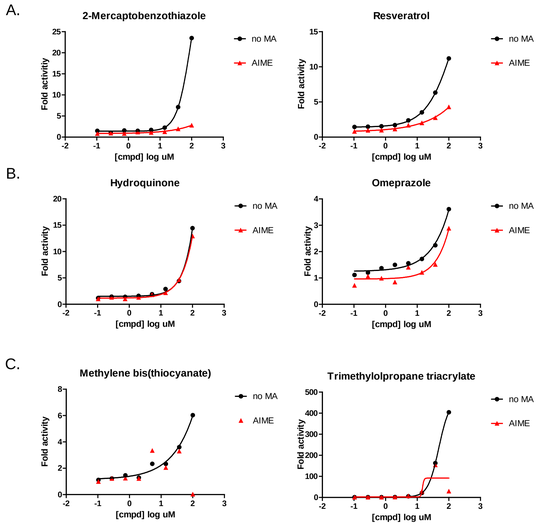


Figure 4: Nrf2-dependent oxidative stress reporter gene assay demonstrates differences in response using the AIME platform
(A) monotonic decreased effect; (B) no effect; (C) non-monotonic decreased effect. See Materials and Methods for details. MA: metabolic activation

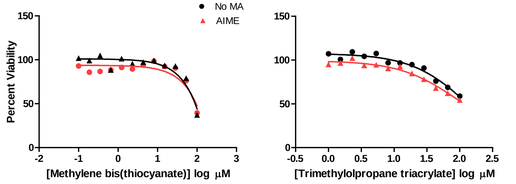


Figure 5: Non-monotonic effect observed in the Nrf2-dependent oxidative stress reporter gene assay cannot be explained by cell viability alone
HepG2-ARE/luc cells were treated as described in the Materials and Methods section. Data are normalized to DMSO control. MA: metabolic activation

Results & Conclusions

- We have successfully produced functional AIME microspheres compatible with cell-based assay conditions in a 96-well plate.
- Although the exact pore size of the AIME microspheres is currently unknown, small molecules as large as ketoconazole (MW = 531) can freely enter the microsphere while CYP3A4 (MW = 57 kDa) is confined to the microsphere.
- Passive diffusion of CYP inhibitors as well as various pro-luciferin substrates and their resulting luciferin metabolite demonstrates small molecule permeability into and out of the AIME microsphere. This is a necessary criteria for retrofitting existing HTS assays with metabolic competence.
- AIME microspheres encapsulating human hepatic S9 demonstrate a variety of CYP activities (ranging from 5 to 25 µg of free S9 equivalent) which are relevant to xenobiotic metabolism. This activity is detectable over several hours using P450-Glo assays.
- AIME-mediated metabolic activity had a pronounced effect on chemically-induced Nrf2 activation. A screen of 54 test compounds using a cell-based Nrf2 assay showed that metabolism detoxified most of the Nrf2-activating compounds; however, methylene bis(thiocyanate) and trimethylolpropane triacrylate demonstrated a unique non-monotonic response that could not be explained by cytotoxicity.

Future Directions

- Optimization of an appropriate negative protein control for the AIME platform in cell-based and cell-free assays, e.g. heat-inactivated S9
- Identification and quantitation of reference metabolites using analytical chemistry techniques to validate metabolic capacity of the AIME platform
- Determination of AIME microsphere pore size and passive diffusion characteristics
- Functional validation of AIME platform using training set of chemicals known to be detoxified and bioactivated by various human liver CYP enzymes
- Deployment of the AIME platform to additional cell-free and cell-based assays with expanded set of ToxCast chemicals

References

1. Lee, DW, Yi SH, Jeong SH, Ku B, Kim J, Lee, M-Y. (2013) Sens. Actuators, B 177, 78-85.
2. Simmons, SO, Fan, C-Y, Yeoman, K, Wakefield, J and Ramabhadran R. (2011) Curr. Chem. Genomics 5, 1-12