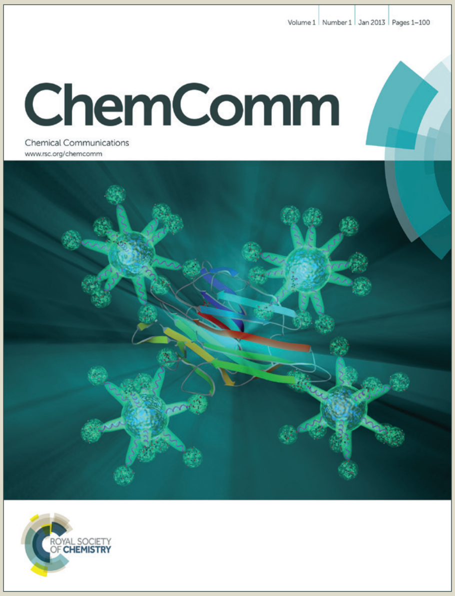
ChemComm

Accepted Manuscript


Volume 1 | Number 1 | Jan 2013 | Pages 1–100
ChemComm
Chemical Communications
www.rsc.org/chemcomm
ROYAL SOCIETY OF CHEMISTRY

This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

ChemComm

Dynamic Article Links►

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

COMMUNICATION

A novel multi-layer microfluidic device towards characterization of drug metabolism and cytotoxicity for drug screening

Qin Wu,[†abc] Dan Gao,[†bc] Juntong Wei,[abc] Feng Jin,[b] Weiyi Xie,[abc] Yuyang Jiang,*[abd] Hongxia Liu*[bc]

Received (in XXX, XXX) XthXXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

A novel multi-layer microfluidic device was developed for characterization of drug metabolism in human liver microsomes (HLMs) and their cytotoxicity on cells. The results demonstrated that this platform is robust for low levels of compounds and shows potential for high-throughput drug screening in the drug development.

In the past decades, the number of new chemical entities (NCEs) and screenable drug targets has been increased quickly. But only a very few of them can succeed. Over 40% of marketing candidate drugs terminated because of the undesirable biological consequences of drug metabolism and induced toxicity.[1] Thus, the early prediction of the metabolic fate and their toxicity is the essential and important part of the drug development process. However, the conventional methods for drug metabolism and cytotoxicity assays needed be performed separately on different platforms. And they have several disadvantages as follows: (1) cells are grown in a static macroscale environment that greatly differs from the *in vivo* biological environment;[2] (2) the drug metabolites should be manually collected for off-line measurements which cost a long time;[3] (3) requiring large amounts of cells, reagents, and complicated sample pretreatment.

Recently, microfluidic technology has attracted an increasing interest since it provides several advantages such as low reagent consumption,[4] easy control,[5] rapid analysis, miniaturization,[6] high integration and high throughput.[7] Microfluidics has been regarded as an excellent platform for cell-based assays,[8] such as drug metabolism,[9] cell interactions,[10,11] cytotoxicity assays,[12] single cell analysis,[13] and so on. More importantly, characterization of drug metabolism and cytotoxicity assays on a single device can be realized which will greatly speed up the process of drug development. Cytochrome P450 enzymes, a large and diverse group of enzymes, play a central role in drug metabolism and more than 75% of human drug enzymatic reactions are catalyzed by them.[14] Human liver microsomes (HLMs) are commonly used as the carrier of P450 and have been proved to be a good strategy for chip-based drug metabolism. Several researches have reported that HLMs could be entrapped in biocompatible materials like poly(ethylene) glycol (PEG) hydrogels[15,16]. However, the encapsulation of HLMs inside PEG required ultraviolet photopolymerization which might affect the enzymatic activity. For cytotoxicity assays, the majority systems were based on rigid two dimensional (2D) substrates. But the 2D culture systems cannot fully match the *in vivo* cellular microenvironment. Many studies have shown that higher levels of cellular specific functions could be retained better in three dimensional (3D) microenvironment, such as polarity, spatiotemporal chemical gradients and mechanical microenvironments of living organs,[17,18] which made experimental results more reliable and accurate. However, there are few reports about the characterization of drug metabolism and cytotoxicity assays on a single microfluidic device, and these reported methods have significant drawbacks. For example, Ma *et al.*[19] reported an integrated microfluidic device for the characterization of drug metabolites and cytotoxicity assays simultaneously by capillary electrophoresis (CE). However, CE cannot characterize the structure of the metabolites and fails to determine the quantity of the metabolites. Mao *et al.*[15] carried out the drug metabolism directly detected by mass spectrometry and cytotoxicity assays during one experiment, but this method needed at least two separate samples which would cost unnecessary time and regents.

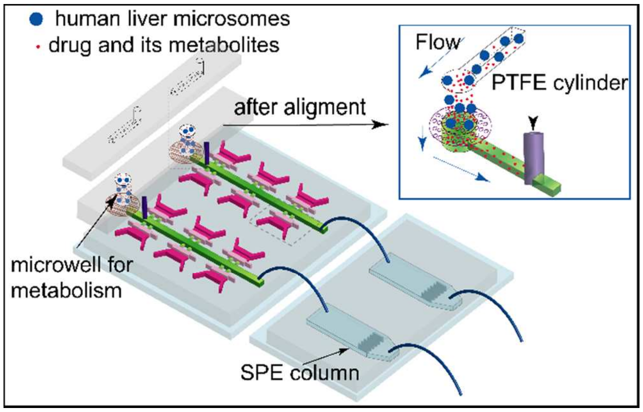


Fig. 1 Schematic drawing of the integrated microfluidic device containing bioreactor for drug metabolism, 3D cell cultivation modules, and sample pretreatment module prior to ESI-Q-TOF MS detection. The enlarged inset shows the layer-by-layer aligned microstructures.

Given the limitations of the current methods and combined with some new technologies related to drug metabolism and cytotoxicity assays, we presented an integrated microfluidic device with three functional parts for drug metabolism in human liver and high-throughput cell cytotoxicity screening in 3D-culture systems (Fig. 1). One part containing a microwell sandwiched between the upper PDMS sheet and a PC membrane with 0.4 μm diameter pores was used for loading HLMs. The pores on the PC membrane were sufficiently small to prevent HLMs flow through and large enough to permit molecular transport. The second part was composed of main channel, cell culture chambers and culture medium channel connected with lower height channels for 3D cell culture and drug stimulation (Fig. S1, ESI†).[19].The minor connecting channels were designed with a height of 10 μm, which is smaller than the diameters of mammalian cells, in order to assist in the prevention of cells from leakage to other channels during cell loading. The third part was an integrated micro-solid phase extraction (SPE) channel for sample cleanup and concentration prior to mass spectrometric analysis. The designed micropillar arrays with 30 μm wide intervals at the end of the channels were able to trap all the packing materials (Fig. S3, ESI†).

With this device, we have the capability to test drug metabolism and cytotoxicity assays during the same experiment. Flavopiridol (FLAP), a cyclin-dependent kinase (Cdk) inhibitor,[20] is selected as a model drug to verify the feasibility of our established platform. FLAP was metabolized with HLMs in the reaction region of microfluidic device and then the metabolites were injected into the main channel and diffused into the 3D cell culture chamber to incubate with cells. MCF-7, MCF-10A, HepG2 and QSG7701 cells cultured in 3D culture system were chosen for the cytotoxicity assays. After stimulation, the metabolites could be directly detected online with an electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF MS) after micro-SPE pretreatment. In addition, the established platform has the potential for parallelization, which provides the ability for high throughput drug screening to accelerate the drug development progress.

The dimensions of minor connecting channels were firstly optimized to ensure drug permeate into the cell culture chambers within an apporpriate time. As shown in Fig. S5 (ESI†), three kinds of the minor connecting channels with different widths were designed and optimized. A fluorescence balance between cell culture chamber and middle main channel was observed in the device with two parallel connecting channels after 60 min. It is reasonable because of the larger width of the single minor connecting channel which allows the molecule to diffuse into the chambers more easily. When cell-agarose mixture was introduced into the cell culture chambers, the gas−liquid interface would form naturally (Fig. S6, ESI†). A theoretical calculation was performed to get that the surface tension induced pressure at the end positions of the minor channels where the gas−liquid interface existed is only 0.91 Pa. The pressure is so small compared to atmospheric pressure that cell-agarose mixture cannot leak into the minor channel.

Then the conditions of metabolites detection including extraction materials, elution flow rate and MS parameters were studied and confirmed. When FLAP metabolites were pretreated using the on-chip micro-SPE column (C18), the SPE procedures related flow rates have important impact on the response of system, including the relative mass spectrometry signal intensity, reproducibility, and lifetime of on-chip SPE and the ESI-Q-TOF MS combination system. 5 μL min^{-1} was finally used as the elution flow rate in consideration of the best detection sensitivity and the stability of the micro-SPE column. For characterization of FLAP and its metabolites, the mass spectral results were obtained under the mass range m/z 50-1000 in positive ion mode. As shown in Fig. 2b and 2c, FLAP with $[M+H]^+$ =402.1 and [glu-FLAP] with $[M+H]^+$ = 578.1 were detected, and their structures were further identified by MS/MS spectra. All the above results showed that FLAP and its metabolite could be well detected under these conditions.

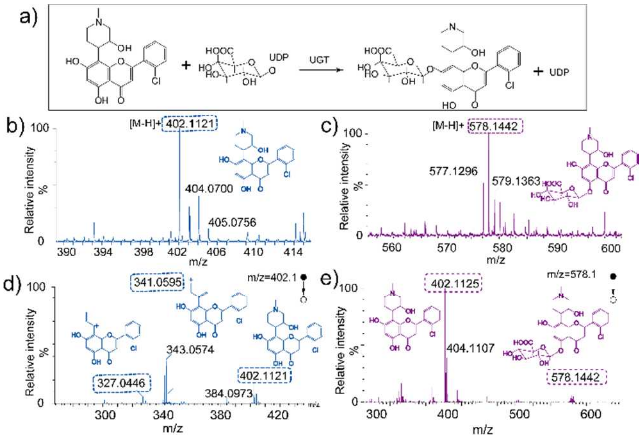


Fig. 2 a) The main metabolic pathway of FLAP in HLMs. Mass spectra of FLAP (b) and [glu-FLAP] (c) in metabolites. Their structures were determined with the MS/MS spectra (d) and (e). All the spectra were obtained in the positive ion mode.

Due to the flow rate might have influence on the vitality of HLMs, their vitality before and after assembly in the microfluidic device was evaluated by detecting the metabolism of 10 μM FLAP both inside and outside the chip. The results (Table S1, ESI†) of the metabolism were within experimental error and this demonstrated that compared with the recent reported approaches of encapsulating HLMs inside hydrogels, our physical immobilization method could maintain the activity of HLMs without any damage.

The metabolic rate of FLAP was further measured with different concentrations according to the fraction of FLAP remaining in the metabolites. The sum of the intensity of *m/z* 402.1 was used to analyse the remaining FLAP concentration (The relationship between molecular ion intensity and FLAP concentration was shown in Fig. S8, ESI†). As shown in Fig. S9, the metabolic rate of 20 μM FLAP was faster than the other two concentrations. The results indicated that the metabolic rate might have close relationship with the initial concentration of FLAP.

An initial concentration of 5 μM was chosen to evaluate the cytotoxicity on different cell types. After 5 μM FLAP was metabolized for different times in five parallel devices, the metabolites were then injected into the downstream to let the drug solutions permeate to the cell culture chambers. The chosen cell lines MCF-7, MCF-10A, HepG2 and QSG7701, represent key organs as liver and breast which FLAP will act on after metabolization. The main channels and the minor connecting channels can mimic blood flow using culture medium. Then the whole microfluidic device can partly reproduce the dynamics of complex multi-organ interactions *in vitro*. These cells were cultured in 3D system for three days and the viability of cells was well to proceed the next cytotoxicity assays (Fig. S7, ESI†). After drug exposure for 12 h, a Live/Dead staining solution was introduced to test cell viability. Fig. 3 shows that for the same concentration of FLAP and its metabolites, the viability of MCF-7 cells was obviously inhibited. These data indicated that FLAP could significantly inhibit the viability of MCF-7 cells, but not to the normal breast. And the hepatotoxicity is also nearly ignorable from the result of the cytotoxicity to HepG2 and QSG7701 cells.

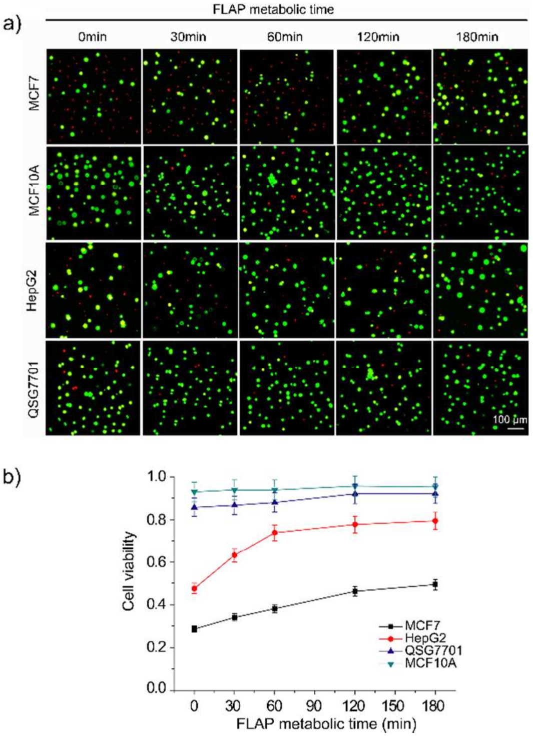


Fig. 3 Analysis of cell viability in the microfluidic device after FLAP metabolites treatment and fluorescence staining. a) Fluorescence images show 3D cultured cells stained by Live/Dead assay kit. b) Cell viabilities of the four kinds of cells after treated with FLAP metabolites under different metabolic time were quantified. The experiments were repeated for three times.

The result was in accordance with the fact that MCF-7 cells were FLAP targeted on. Compared with the MTT assays performed on 96-well plates (Table S2-S4, ESI†), the obtained cell viabilities of 3D cultured MCF-7 cell in our microfluidic device was a little higher than 2D culture mode and the differences of other cells are not obviously, which is in accordance with the previously reported studies.[21]

Conclusions

In summary, this work focused on performing cell viability assay and drug metabolism characterization using a microfluidic device directly coupled to ESI-Q-TOF MS. This integrated microfluidic system contained metabolite generation, sample pretreatment, online detection with ESI-Q-TOF MS and incubation with cultured cells to evaluate metabolism-induced cytotoxicity. Compared with conventional microfluidic drug-screening assay methods, this platform can achieve the study of drug metabolism and cytotoxicity assay on a single device semiquantitatively and qualitatively. In addition, the use of the PC membrane to immobilize the HLMs has no damage to its activity which can ensure the accuracy of the results. This method can also be used to study the pharmacological kinetics in real time on living cells. We envisaged that the developed microfluidic device could be a potential useful tool for drug screening at the early stages of drug development to help accelerate the drug development process.

This work was supported by China Ministry of Science and Technology under Contract (No. 2012ZX09506001-010), the National Natural Science Foundation of China (Nos. 21272134, 21172129) and the National Youth Science Foundation (No. 21305074).

Notes and references

[a] *Department of Chemistry, Tsinghua University, Beijing, 100084, China*
[b] *State Key Laboratory Breeding Base-Shenzhen Key Laboratory of Chemical Biology, Graduate School at Shenzhen, Tsinghua University, Shenzhen, 518055, China.*
[c] *Key Laboratory of Metabolomics at Shenzhen, Shenzhen, 518055, China. Fax/Tel: (+86) 755-26036035; E-mail: liuhx@sz.tsinghua.edu.cn*
[d] *Department of Pharmacology and Pharmaceutical Sciences, School of Medicine, Tsinghua University, Beijing, 100084, P. R. China. Fax/Tel: (+86)755-26036017; E-mail: jiangyy@sz.tsinghua.edu.cn*
† *These authors contributed equally to this work.*

† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

1. M. Y. Lee, C. B. Park, J. S. Dordick and D. S. Clark, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 983-987.
2. S. Zhang, *Nat. Biotechnol.*, 2004, **22**, 151-152.
3. Z. Teng, C. Yuan, F. Zhang, M. Huan, W. Cao, K. Li, J. Yang, D. Cao, S. Zhou and Q. Mei, *PLoS One*, 2012, **7**, e29647.
4. D. Gao, H. Liu, J. M. Lin, Y. Wang and Y. Jiang, *Lab Chip*, 2013, **13**, 978-985.
5. B. B. Xu, R. Zhang, X. Q. Liu, H. Wang, Y. L. Zhang, H. B. Jiang, L. Wang, Z. C. Ma, J. F. Ku, F. S. Xiao and H. B. Sun, *Chem. Commun.*, 2012, **48**, 1680-1682.
6. G. M. Whitesides, *Nature*, 2006, **442**, 368-373.
7. G. S. Du, J. Z. Pan, S. P. Zhao, Y. Zhu, J. M. den Toonder and Q. Fang, *Anal. Chem.*, 2013, **85**, 6740-6747.
8. D. Gao, H. Liu, Y. Jiang and J.-M. Lin, *Trends Anal. Chem.*, 2012, **35**, 150-164.
9. D. Gao, H. Wei, G. S. Guo and J. M. Lin, *Anal. Chem.*, 2010, **82**, 5679-5685.
10. M. J. Carroll, L. E. Stopfer and P. K. Kreeger, *Chem. Commun.*, 2013. DOI: 10.1039/c3cc47507g.
11. H. Wei, H. Li, S. Mao and J. M. Lin, *Anal. Chem.*, 2011, **83**, 9306-9313.
12. J. Wu, Q. Chen, W. Liu, Y. Zhang and J. M. Lin, *Lab Chip*, 2012, **12**, 3474-3480.
13. J. Tam, S. Salgado, M. Miltenburg and V. Maheshwari, *Chem. Commun.*, 2013, **49**, 8641-8643.
14. L. Vignati, E. Turlizzi, S. Monaci, P. Grossi, R. Kanter and M. Monshouwer, *Toxicology*, 2005, **216**, 154-167.
15. S. Mao, D. Gao, W. Liu, H. Wei and J. M. Lin, *Lab Chip*, 2012, **12**, 219-226.
16. J. C. Zguris, L. J. Itle, D. Hayes and M. V. Pishko, *Biomed. Microdevices*, 2005, **7**, 117-125.
17. D. Huh, G. A. Hamilton and D. E. Ingber, *Trends Cell Biol.*, 2011, **21**, 745-754.
18. M. C. Cushing and K. S. Anseth, *Science*, 2007, **316**, 1133-1134.
19. B. Ma, G. Zhang, J. Qin and B. Lin, *Lab Chip*, 2009, **9**, 232-238.
20. B. Hagenauer, A. Salamon, T. Thalhammer, O. Kunert, E. Haslinger, P. Klingler, A. M. Senderowicz, E. A. Sausville and W. Jager, *Drug Metab. Dispos.*, 2001, **29**, 407-414.
21. Xiaokang Li, Xinyong Zhang, Shan Zhao, Jingyu Wang, Gang Liu and Yanan Du, *Lab Chip*, 2013, DOI: 10.1039/c3lc51103k.