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Merging Microfluidics with Micro-titre Technology for More Efficient Drug Discovery

Nicole V. Tolan, Luiza I. Genes, and Dana M. Spence

Michigan State University, Lansing, MI 48824

Abstract

Detecting multiple components from a single red blood cell (RBC) sample within a flow-based system and in less than 20 minutes will enable improved *in vitro* determinations of drug efficacy and cellular response to administered drugs. Here, an example of an improved *in vitro* measurement involving iloprost, a pharmaceutical reported to improve blood flow, has been determined by incorporating multiple cell types onto a single device. The method allows fluid flow to address individual rows of wells contained within an 18-well microfluidic array that serves as a precursor to a 96-well micro-titre plate device. The ability to better mimic the *in vivo* circulation by incorporating the flow of blood components, coupled with simultaneous detection and laboratory automation in place for micro-titre plates, suggests that the microfluidic array presented here will allow for improved mechanistic drug research studies. Utilizing fluorescence microscopy, concentrations of multiple metabolites present within the RBC can also be determined using the microfluidic array. The current progress towards using this device for personalized medicine is presented here.

Keywords

Microfluidic; Micro-titre Plate; Vasodilation; Adenosine Triphosphate; Nitric Oxide; ADMET

Introduction

Collectively, the advantages associated with microfluidic technologies and micro-titre plate automation may enable advancements in biotechnology associated with improved mimics of *in vivo* processes. For example, the ability to integrate a flow-based and multicellular system with already available micro-titre plate technology will enable the properties of absorption, distribution, metabolism, excretion, and toxicity (ADMET) of drug candidates to be analyzed during drug research on a more realistic platform.¹⁻³ The Food and Drug Administration's Center for Drug Evaluation and Research has reported that it requires anywhere from 0.8 to 1.7 billion dollars for one drug candidate to reach the clinical trial phase⁴, or phase III, of the drug approval process. It was also determined that 92% of drug candidates that enter into phase I fail before reaching the market due to issues related to the properties of ADMET. Many aspects of the drug discovery process, including the mechanism of action of certain drugs, solubility of the drug candidate, and toxicity studies involving the candidate could be examined in a multicellular system that involves actual blood flow prior to the drug candidate entering into the clinical trial phase of development. This would allow researchers to discover any unforeseen complications earlier in the drug development stage, thereby reducing the number of drugs that reach clinical trials only to fail and add to the cost associated with testing drug candidates that subsequently fail.

Current techniques for drug development utilizing micro-titre plate technology have the advantage of incorporating commercial automation capabilities for high-throughput

screening. Complex biological sample preparation, introduction of various sample volumes simultaneously and integrating various instrumentation for sample separation, identification, and quantification are various tasks that can be performed with automated instruments. Due to the acceptance of a standard dimension for the micro-titre plate, numerous instruments have been developed on this scale leading to various advancements in the automation and detection methods for this technology.

Although automation of micro-titre plate technology can reduce the time required to perform redundant analyses, there are limitations of such systems when applied to drug discovery. For example, current micro-titre plate technology results in a static system that is unable to mimic the flow-based vascular system observed *in vivo*. Processes such as absorption, metabolism, and excretion of drugs within a biological system cannot be *accurately* examined without incorporating a mimic of blood flow. This is due to certain events being stimulated *in vivo* by flow-based shear. Also, the current plate design limits the ability to incorporate both adherent and non-adherent cells such as those found within the vascular system (endothelial cells, white and red blood cells, platelets, etc.). This limits the ability to realistically mimic and monitor the toxicity of a drug candidate on a particular cell-type of interest while simultaneously monitoring the affect on non-targeted cell-types. The current micro-titre plate platform is also unable to overcome the complications associated with matrix interferences that are inherent with a complex biological sample such as blood.

Exploiting the benefits of soft lithography techniques and the rapid replica molding associated with poly(dimethylsiloxane) (PDMS), the multi-dimensional microfluidic array presented here is fabricated in a relatively short period of time. Also, the ability to prepare numerous replica molds from a single master wafer reduces the overall cost per device. In 1998, the Whitesides group⁵ published their research demonstrating the ability to fabricate microscale total analysis systems (μ TAS) using PDMS. This flow-based system allows for optical transparency, permeability to gasses, and provides a non-toxic environment for cell growth.⁶ Various manipulations such as pneumatic valving⁷, sample injection techniques⁸, and sample separation⁹ are all possible using such PDMS-based devices. Moreover, the microscale dimensions of the channels of these microfluidic devices approximate resistance vessels *in vivo*, allowing for PDMS-based devices to provide a more realistic mimic of an *in vivo* environment, while including the necessary sample preparation techniques all on-chip.

The aforementioned sample handling features of microfluidic devices can be seamlessly incorporated to the current dimensions of the 96-well plate. As illustrated in figure 1, fluid samples can address each individual well from sample inlets introduced at the top and bottom of the device. Samples are then directed under each well through channels that are terminated at a waste port adjacent to each column of wells. Immobilization of various cell lines and molecular probes in the wells would take advantage of the automation techniques already in place for 96-well plates, while simultaneously enabling the introduction of flow into the system. By integrating the microfluidic channels and keeping the same dimensions of the standard micro-titre plate, clinical diagnosis, disease progression, drug research and efficacy studies could now include the very relevant aspect of blood flow. Here, the potential of such a device is demonstrated with two examples; drug efficacy and their mechanisms of action, as well as the application of this device to personalized medicine.

Experimental

Construction of the microfluidic array

The microfluidic array is composed of two individual PDMS layers, irreversibly thermocured around a polycarbonate membrane. Blood components are hydrodynamically pumped through the bottom layer within microfluidic channels that are 200 μ m wide by 100

μm in depth. The top layer is comprised of an array of 18 wells, each 1/8" in diameter, that are separated from the channels by a polycarbonate membrane having pores that are 1 μm in diameter. Samples and standards are pumped at a rate of 1.0 μL/min through the underlying channels for approximately 15 minutes, allowing the metabolites of interest (e.g., ATP) derived from the red cells in the flowing solution to interact with either cultured endothelial cells (iloprost studies) or a fluorescence probe (metabolite studies) immobilized within each well.

Measurement of Fluorescence Intensity

After capturing the fluorescence images of the array, the pixel intensities were measured for each individual well and correlated to the particular analyte of interest. When determining the affect of iloprost on the RBCs, the fluorescence observed in each well is a result of the intracellular nitric oxide (NO) produced by the cultured bovine pulmonary artery endothelial cells (bPAECs). These cells (bPAECs) are immobilized within each well and loaded with DAF-FM DA, an intracellular fluorescence probe for NO. The fluorescence intensities were performed with an Olympus IX71 inverted fluorescence microscope using a mercury-arc lamp source; the excitation and emission filtering employed a 4',6-diamidino-2-phenylindole (DAPI) filter cube with excitation and emission wavelengths of 372 nm and 456 nm, respectively.

To analyze the RBC sample for intracellular nicotinamide adenine dinucleotide phosphate (NADPH) concentrations, an important metabolite in the maintenance of the antioxidant system of the RBC, the wells are filled with a resazurin reaction mixture that becomes fluorescent once reduced in the presence of NADPH. The entire microfluidic array can be imaged in a single snap shot by taking advantage of the low magnification capabilities of the Olympus MVX fluorescence stereo microscope. The MVX microscope utilizes a mercury-arc lamp fitted with a resorufin filter cube with excitation and emission wavelengths of 577 nm and 620 nm, respectively.

Results and Discussion

Drug efficacy and mechanisms of action

Recent advancements in drug discovery have shifted to a more mechanistic approach. The development of a device that includes multiple cells types in a single channel, coupled with the fluid flow forces that are found within the circulation, results in a tool that more closely mimics cell-to-cell communication that may exist in the bloodstream. For instance, overcoming hypertension through vasodilation (resulting from the communication between multiple cell types) can now be investigated more closely using an *in vitro* platform.

The RBC has been implicated as a determinant in hypertension, a complication associated with various diseases including diabetes, cystic fibrosis, and cardiovascular disease. In this construct, as shown in figure 2, a release of ATP occurs when RBCs are subjected to such stimuli as mechanical deformation¹⁰ or pharmaceutical agents such as iloprost¹¹. The released ATP can migrate to the endothelial cell wall lining the blood vessel, resulting in the activation of the purinergic (P_{2Y}) receptor found on the membrane of the endothelial cell. Upon activation of the P_{2Y} receptor, and in the presence of calcium dependant calmodulin (Ca²⁺-CaM), nitric oxide synthase (NOS) will convert L-arginine to L-citrulline with the production of NO as a by-product, ultimately resulting in smooth muscle relaxation and vasodilation.

Our group and others have shown that RBCs from diabetic patients release less ATP in comparison to RBCs from healthy, non-diabetic controls, when these cells are subjected to mechanical deformation.¹² This is thought to be due, in part, to a reduction in the ability of

the cells to fight off oxidant insults, resulting in decreased membrane flexibility when subjected to oxidant stressors. A decrease in glucose 6-phosphate dehydrogenase (G6PD) activity results in abnormally lowered levels of the non-enzymatic antioxidant, glutathione (GSH).¹³ With a stiffened cellular membrane, the amount of deformation-induced ATP release from the RBCs of diabetic patients is significantly decreased, which may lead to insufficient endothelium-derived NO. This decrease in NO is thought to result in reduced vasodilation and, ultimately, increased hypertension. The ability to monitor cellular processes involving RBCs and other cells (such as the endothelium) will enable the microfluidic array to be used as a clinical diagnostic tool that mimics the *in vivo* system.

To demonstrate the potential of such a multicellular device to mimic *in vivo* stimulation of NO by RBC-derived ATP, RBCs were incubated in the presence and absence of iloprost. Iloprost is a therapeutic agent that is thought to induce vasodilation by increasing the amount NO produced by endothelial cells¹¹ which is known to induce smooth muscle relaxation.¹⁴⁻¹⁶ However, it has also been shown that this drug increases the ATP release from RBCs when these cells were incubated with 1 μ M iloprost for 15 minutes and compared to RBCs in the absence of iloprost.¹⁷ This increase in RBC-derived ATP resulted in a 39% increase in the intracellular fluorescence due to the NO produced within the immobilized bPAECs.

Our group has previously examined the necessity of a flow system as it applies to drug efficacy and mechanisms of action; specifically we have determined the affect of flow on ATP release from RBCs either incubated with pentoxifylline (Trental) or iloprost.¹⁸ It was determined that the ability of Trental to increase the ATP release from a RBC sample was dependent upon the RBC being subjected to flow-induced deformation. In the absence of flow, it was established that Trental did not significantly increase the concentration of ATP released from the RBCs. Therefore, only by monitoring this drug in a flow system could the possible mechanism of action (ATP release) be determined. Furthermore, unpublished results in our group have shown that hydroxyurea, a current treatment for patients with sickle cell disease,^{19, 20} also behaves in the same manner, requiring mechanical deformation of the RBC to substantially increase the release of ATP from the RBC.

Personalized medicine

The ability to utilize the microfluidic device described here for personalized medicine may lead to improvements in the manner by which drugs are administered to patients. For example, it is anticipated that the microfluidic device shown in figure 1 may lead to higher success rates of prescribing the right drug for the right patient on the first attempt. Specifically, in order to prescribe the most effective drug and at an effective dosage, the microfluidic array will allow clinicians to actually monitor the affect on various cell-types even before the drug is administered to the patient. For example, this device has been used by our group to determine the concentration of NADPH found within a single RBC sample. This quantitative determination of NADPH, which is a major determinant in the antioxidant status of the cell and is a required metabolite for the reduction of the disulfide form of glutathione (GSSG) to produce GSH (figure 3), is performed in a single step.

A single fluorescence image (figure 4) can be used to simultaneously provide the information necessary to construct a standard calibration curve as well as quantify the concentration of NADPH contained in a single RBC sample. The microfluidic array employed here significantly reduces the time required to make multiple measurements when compared to a single measurement detection method. Sample preparation and fabrication of the microfluidic array aside, the actual time required for the entire analysis including the incubation required for the NADPH in solution to react with the fluorescent probe immobilized within each well, is approximately 20 minutes. This analysis will allow further

insight as to the cause for a reduced antioxidant status of the RBC on an individual basis, leading to a more specific diagnosis and possibly, an improved method of treatment.

Conclusions

Most, if not all, *in vitro* methods involving drug discovery do not incorporate the flow of blood found *in vivo*. By integrating flow into a high throughput and automated platform, drug efficacy that is dependent upon shear-induced deformation (such as Trental) can now be accurately analyzed using a controlled *in vitro* platform. As outlined in table 1, the combination of features found in microfluidic technologies and micro-titre based measurements are utilized with the microfluidic array that is described in this work. Many of the advantages of microfluidic devices are associated with the ability to integrate a more lab-on-a-chip approach onto a single device such as the ability to perform on chip cell culture, sample injection and separation, and incorporate gaseous interfaces and valving techniques. The automation and various technologies already available for micro-titre plates will allow for this microfluidic array to handle simultaneous and variable sample volumes all by maintaining the currently accepted dimensions of micro-titre plates. The microfluidic array holds great possibilities for high-throughput and automated clinical testing and research by combining all of the advantages listed for each system.

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Figure 1. Flow-based microfluidic 96-well plate fabricated from two individual PDMS layers thermocured around a polycarbonate membrane.

Figure 2. Signal-transduction pathway of iloprost-stimulated vasodilation resulting from RBC-derived ATP release and endothelium-derived NO production.

Figure 3.
Illustration of the link between glucose metabolism and GSH levels within the RBC, affecting the overall antioxidant status of the cell.

Figure 4.

(a) Fluorescence image of the microfluidic array used to measure standard solutions and a single 7% lysed RBC solution simultaneously, to determine intracellular concentrations of NADPH and (b) the resulting calibration curve.

Table 1

Comparison of various aspects associated with microfluidic and micro-titre plate technologies and the combination of these advantages associated with the microfluidic array.

	Microfluidic Technologies	Micro-titre Plate Technologies	Microfluidic Array
Multiple Detection Schemes	✓	✓	✓
Multicellular Capabilities	✓		✓
High Throughput Analysis		✓	✓
Simultaneous Detection		✓	✓
Flow-based System	✓		✓
Current Clinical Diagnostics Tool		✓	✓
μL to nL Volumes	✓		✓
Lab-on-a-Chip Integration	✓		✓
Automation		✓	✓