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Real-time measurement of cholesterol secreted by human hepatocytes using a novel microfluidic assay

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Abstract

The use of microfluidics has become widespread in recent years because of the use of lesser resources such as small size, low volume of reagents, and physiological representation of mammalian cells. One of the advantages of microfluidic-based cell culture is the ability to perfuse culture media which tends to improve cellular health and function. Although measurement of cellular function conventionally is carried out using well-plates and plate readers, these approaches are insufficient to carry out in-line analysis of perfused cell cultures because of mismatch between volumes and sensitivity. We report the development of a novel microfluidic device and assay that is carried out under perfusion, in-line to measure the cholesterol secreted from a human hepatocyte tissue-chip. The heart of the assay is the unique implementation of enzymatic chemistry that is carried out on a polystyrene bead. Using this approach, we successfully measured cholesterol secreted by the perfused human hepatocytes.

Introduction

Owing to the rising rates of obesity and cardiovascular diseases in the US, there is a significant focus of the research enterprise and the pharmaceutical industry to develop more effective therapies. Microfluidic cell culture platforms have the potential to become an important tool in drug discovery due to their ability to utilize lesser resources than conventional cell culture techniques [1–3]. The recent increase in the use of tissue-chip models aims to mimic the in vivo physiology. These microfluidic tissue-chips are used because they provide the cells with a more physiological microenvironment such as perfusion and use a smaller number of cells and lower volume of reagents, which lowers costs [4–10]. Perfusion, on one hand, applies fluidic shear stress to cells which improves their morphology, differentiation, and health [11–13], while on the other, supplies fresh nutrients to the cells which improves cellular health and function [14],[15]. Perfusion also brings forth an important issue of measuring cellular function. In the ideal case, the analysis of perfused media would be conducted in-line, in real-time which would eliminate the need for collecting the samples for off-line analysis [16–18]. However, there are very limited examples of in-line measurement of cellular secretions that can be integrated with microfluidic tissue-chip systems.

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Cellular secretions comprise different classes of molecules that include hormones, proteins, and biomolecules such as cholesterol. In recent years, various analytical techniques such as electrospray ionization mass spectrometry [19], electrophoresis [20], fluorometric detection using either photomultiplier tube or fluorescent microscope [21],[22], colorimetric detection [23],[24], ELISA [8], and electrochemical sensors [25] have been incorporated in microfluidic devices to quantify cellular secretions. One of the primary objectives of combating obesity is to lower the cholesterol levels [26–28]. As a molecule discovered hundreds of years ago, cholesterol is typically measured using an enzyme-based chemistry in which an oxidation reaction is catalyzed by Horse Radish Peroxidase (HRP) to generate either a colorimetric or fluorometric signal. Cholesterol secreted by cells is typically measured either by using radioactive or fluorescent label for cholesterol efflux assays [29] or by conducting enzymatic assays on the collected culture medium [30]. The measurement of cholesterol in microfluidics has been carried out by using biosensors that use electrophoresis [31], colorimetry [24], and amperometry [32],[33], however, in most cases, the measurement was not carried out in-line for cellular analysis [24],[31–33]. Since enzymatic assays do not offer the same selectivity as an ELISA, implementation in microfluidics for in-line measurement has generally been difficult. One exception is recent elegant work in which glycerol secreted from mouse adipocytes was measured under perfusion, however, the use of a multilayered device increased the complexity.

Since liver is a major site of cholesterol synthesis [1], [2], [29], regulation of hepatic cholesterol is the target of several drugs collectively called statins [3], [26], [34]. Although liver tissue-chips have been used for applications in drug discovery [15], [35], there have been very few reports of in-line measurement of hepatic function [8], and no reports of in-line measurement of cholesterol secretions. Due to these limitations in measuring in-line secretions of liver tissue-chips, all functional analysis is carried out off-chip.

In this paper, we present a unique implementation of HRP based enzymatic chemistry using enzyme immobilized beads to quantify cholesterol in-line, real-time from microfluidic perfusion-based human hepatocytes. The basic premise behind our approach is the novel implementation of the enzymatic assay chemistry on a polystyrene bead such that the entire reaction is carried out under perfusion. The catalyst, horseradish peroxidase (HRP) was immobilized on the polystyrene beads, which converted resazurin into resorufin in presence of hydrogen peroxide that was generated from the oxidation of cholesterol (Figure 1). The microfluidic assay device has mixing channels for mixing the components of the assay with the perfusate from the hepatocytes (Figure 2). The fluorescent signal is developed on the beads under flow as the solution in immediate vicinity of the enzyme immobilized beads turns fluorescent. This gives the beads green fluorescent appearance which was captured using a high-speed camera which was connected to a fluorescence microscope and quantified (Supplementary Figure 1). This is the first time a continuous assay for real-time in-line on-chip measurement of cholesterol from human hepatocytes under perfusion has been developed. A microfluidic assay which can monitor the cholesterol secretion in real-time in-line can be an important analytical tool in studies designed to understand the link between varying metabolic states and related disorders as well as in drug discovery and toxicity.

Experimental

1. Reagents

Eagle's Minimum Essential Medium (EMEM) was purchased from Quality Biological (Gaithersburg, MD). Fetal bovine serum (FBS), Pencillin-Streptomycin, and Amplex Ultra Red were purchased from Thermo Fisher (Waltham, MA). Fibronectin and biotin-HRP were purchased from Sigma (St. Louis, MO). Collagen Type I was purchased from Massachusetts General Hospital (Boston, MA). Dow Corning 184 Sylgard silicone elastomer kit was purchased from Ellsworth Adhesives (Germantown, WI). Polystyrene streptavidin beads were purchased from SpheroTech (Lakeforest, IL). StabilGuard was purchased from Surmodics (Eden Prairie, MN). Hydrogen peroxide and cholesterol fluorometric detection kits were obtained from Cayman Chemicals (Ann Arbor, MI). Simvastatin was purchased from Acros Organics (Geel, Belgium).

2. Hepatocyte culture

Human hepatocellular carcinoma cells, HepG2 (HB-8065), were obtained from ATCC (Manassas, VA) and cultured as per standard cell culture protocols. The cells were maintained in EMEM 90% v/v, FBS 10% v/v, and Penicillin-Streptomycin 1 % v/v at 37⁰ C and 5% CO₂. The medium was changed for the flask cultures every two days and the cells were passaged using 0.05% v/v trypsin-EDTA solution when 80% confluency was reached. For microfluidic cell cultures, the glass surface of the cell chamber of the single chamber microfluidic device was coated with 50 µg/ml fibronectin and 0.125 mg/ml Collagen Type I. The cells were then seeded as 3000 cells/µl in the microfluidic device and cultured for up to one week in EMEM 90% v/v, FBS 10% v/v, and Penicillin-Streptomycin 1 % v/v at 37⁰ C and 5% CO₂. The medium for microfluidic cell cultures was changed daily. The health and viability of the microfluidic cell cultures was determined by live/dead and nuclear staining (Figure 5). The nuclei and cells after staining were imaged using fluorescence microscope and the cell count was determined using NIH Image J image analysis software.

3. Immobilizing HRP on streptavidin beads

The polystyrene beads (SpheroTech, Lake Forest, IL) functionalized with streptavidin were washed thrice in 0.01% v/v Tween-20 in 1X PBS. The beads were then conjugated with biotin-HRP through an overnight incubation and stored in StabilGuard. The concentration of Biotin-HRP was determined by the binding capacity of each bead. The unbound sites for streptavidin were blocked by StabilGuard. Before each use, the beads were washed with 1X PBS to ensure there were no unbound biotin-HRP molecules in the solution to produce background fluorescence.

4. Microfluidic chip fabrication

The microfluidic device had three inlets: one for cellular perfusate (which would contain the cholesterol), one for cholesterol oxidase together with the HRP-immobilized beads, and one for resazurin. The device consisted of two mixing channels – first for mixing the cellular perfusate with the cholesterol oxidase and the HRP-immobilized beads and the second for mixing the solution resulting from the first mixing channel with resazurin. The schematic in

Figure 2 shows the microfluidic device design. The microfluidic device layout was lithographically patterned with SU-8 photoresist on a silicon wafer. Using this wafer pattern as a mold, the devices were fabricated out of polydimethylsiloxane by soft lithography. Briefly, silicone elastomer was mixed with the curing agent in 9:1 ratio and poured over the silicon wafer. The mixture was then kept in the vacuum chamber for 3–4 hours for degassing to remove the air bubbles. After the PDMS looked clear of any air bubbles, the PDMS and the silicon wafer were baked at 70⁰ C overnight for the PDMS to set. The PDMS was removed from the silicon wafer and the individual patterns were cut into separate pieces and holes were punched for the individual inlets. The PDMS patterns were then bonded to pre-cleaned glass slides by treatment in a plasma cleaner (Harrick, Ithaca, NY) followed by a 10 min bake at 70⁰ C.

5. Hydrogen peroxide measurement using the bead-based assay

The bead-based assay was first tested by generating a standard curve to measure hydrogen peroxide. Hydrogen peroxide solutions were prepared in 0, 0.039, 0.078, 0.156, 0.313, 0.625, 1.25, 2.5, 5, and 10 μ M concentrations. The working solution for the assay contained 10 μ M Amplex Ultra Red and HRP immobilized beads. In a 96-well plate, 50 μ l of the hydrogen peroxide standards and 50 μ l of the enzymatic working solution were added to each well. The signal generated was detected using SpectraMax M2 microplate reader (Molecular devices, Sunnyvale, CA). The values obtained were plotted against those obtained by measuring the hydrogen peroxide samples through a commercial hydrogen peroxide kit (without the beads) as per the protocol provided with the kit.

6. Cholesterol measurement using the bead-based assay

The bead-based assay was then tested for measuring cholesterol. Cholesterol standard solutions were prepared in 0, 25, 50, 75, 100, and 150 μ M concentrations. Working solutions for the assay contained 10 μ M Amplex Ultra Red, 50 μ l/ml cholesterol oxidase enzyme, and HRP immobilized beads. In a 96-well plate, 50 μ l of the cholesterol standards and 50 μ l of the enzymatic working solution were added to each well. The values obtained were plotted against those obtained by measuring the cholesterol samples through a commercial cholesterol kit (without the beads) as per the protocol provided with the kit.

7. Cholesterol measurement in devices using the bead-based assay

A standard curve was obtained for different cholesterol concentrations in perfusion in the microfluidic assay. Cholesterol standards were prepared at 0, 25, 50, 75, 100, 150, and 200 μ M concentrations. Cholesterol was introduced through inlet 1 of the device. HRP immobilized beads and cholesterol oxidase were infused through inlet 2 and Amplex Ultra Red solution through inlet 3. The flow rates for all the assay reagents and standard solutions were 5 μ l/hr. Images and videos of the fluorescent beads were captured near the outlet using a high-speed camera (Hamamatsu Photonics, Japan) and Nikon Ti epifluorescence microscope. The fluorescent intensity of the beads was characterized by using NIS AR Elements software (Nikon) and plotted against the cholesterol concentrations. For in-line detection of cholesterol secreted from the hepatocytes, EMEM 90% v/v, FBS 10% v/v, and Penicillin-Streptomycin 1 % v/v media was perfused over the cells at 5 μ l/hr. The cells were maintained at 37⁰ C and 5% CO₂. The perfusate from the cell chamber in the microfluidic

device was directly connected to the assay device to inlet 1 (Figure 2) and the rest of the assay was carried out similar to the cholesterol standard curve conducted in the assay device with channel dimensions $50\text{ }\mu\text{m} \times 75\text{ }\mu\text{m} \times 100\text{ }\mu\text{m}$ (width X height X length).

8. Effect of simvastatin on cholesterol secretion

Simvastatin is a drug used to treat dyslipidemia and lower cholesterol in patients with cardiovascular disorders. For assessing the effect of simvastatin on the hepatocytes, the cells were first seeded in a 24-well plate and cultured for 24 hours. After a wash, the cells were cultured under serum-free EMEM with $10\text{ }\mu\text{M}$ simvastatin for 0, 1.5, 6, and 18 hours. The media was collected and stored at -80°C . For simvastatin treatment in the microfluidic devices, the cells were washed and cultured under serum-free EMEM with $10\text{ }\mu\text{M}$ simvastatin. The cultured were incubated with the treatment medium for 18 hours before cholesterol was measured using the bead-based assay. The 18-hour time-point was selected as per the findings from the well plate study which showed the maximum drop in cholesterol secretion.

9. Statistics

All measurements were made from a minimum of three replicates. Error bars were plotted based on standard deviation from multiple measurements.

Results and Discussion

1. Microfluidic device and assay chemistry

The overall assay chemistry of the bead-based enzymatic assay for cholesterol and its microfluidic assay format can be explained as per Figure 1. The biotinylated HRP is conjugated on the streptavidin beads by exploiting the simple biotin-streptavidin binding chemistry. The HRP immobilized beads act as the catalyst for the oxidation reaction between hydrogen peroxide (generated after the oxidation of cholesterol) and the fluorophore resazurin as well as provide a solid substrate that localizes the signal. The beads with HRP and cholesterol oxidase were introduced before the resazurin dye through separate inlet to prevent resazurin oxidation in the mixture. This ensured that the signal was generated only after cholesterol was oxidized to hydrogen peroxide and not by spontaneous oxidation of the dye. The reaction results in a green fluorescence on the beads which is measured by fluorescence microscopy (Supplementary Figure 1).

2. Comparison between conventional and bead-based enzymatic assays

To test the working principle of the bead-based assay, we constructed a standard curve for hydrogen peroxide in standard well-plates. The samples contained two groups: known concentration of hydrogen peroxide with (1) HRP without beads and resazurin as would be in a conventional assay (Supplementary Figure 2), and (2) HRP immobilized on the beads and resazurin (Supplementary Figure 3). The enzyme-bead group had a linear standard curve with an excellent agreement ($R^2 = 0.9913$) with the conventional assay (Figure 3A). The lowest concentration used for the comparison was $0.039\text{ }\mu\text{M}$ or 39 nM of hydrogen peroxide, which corresponded to the detection limit of the conventional assay – the bead-based assay could detect hydrogen peroxide at this concentration. This suggests that our bead-assay can

function as well as the conventional assay for quantifying hydrogen peroxide generated even in low concentrations. Reactions catalyzed by HRP follow the Michaelis-Menten enzyme kinetics model [36],[37] which can be summarized by the following conditions: (1) if the substrate concentration is lower than the enzyme concentration, then the rate of the reaction varies linearly with the substrate concentration. This is also known as first-order reaction. As the concentration of the substrate becomes more than that of the enzyme, the rate of the reaction becomes independent of the concentration of the substrate, becoming a zero-order kinetic reaction. In other words, no additional product will form due to enzyme being saturated by the substrate. HRP catalyzed reactions have a linear trend if the substrate is at a lower concentration than the enzyme; as the substrate (hydrogen peroxide) concentration reaches a point in the solution (20 μM) which saturates the enzyme, no additional product (resorufin) will be produced. Therefore, the limit of detection for the HRP based enzymatic assays is determined by the point of saturation for HRP beyond which the linearity of the assay is lost. We observed this in our experiments. As the measurements approached the concentration 20 μM of hydrogen peroxide, there was a lot of variation in the intensity values leading to a higher standard deviation than the lower concentrations. This could be due to the saturation of the signal as maximum product is formed and point of saturation of HRP is approached.

To test whether cholesterol can be measured with the bead-based assay, we constructed a standard curve. The samples contained two groups: known concentration of cholesterol with (1) HRP without beads and resazurin as would be in a conventional assay (Supplementary Figure 4), and (2) HRP immobilized on the beads and resazurin (Supplementary Figure 5). The enzyme-bead group had a linear standard curve with an excellent regression with the conventional assay ($R^2 = 0.9880$) (Figure 3B). This showed that the bead-based enzymatic assay was comparable to the conventional assay.

By conducting both the assays, conventional and bead-based, using a microplate reader we observed that both the assays are comparable. Plate based conventional assay works in a confined space of the well plate. The fluorescent signal in the form of fluorescent solution then is read by the photomultiplier tube. However, the plate assay would not be suitable for microfluidic measurements as the assay is not confined and is in fact under flow. The need for immobilizing HRP on a solid substrate arises when adopting the assay in a microfluidic format. Our bead-based assay addresses this issue.

3. In-line cholesterol measurement using bead-based enzymatic assay

To test the bead-based enzymatic assay in the microfluidic format, we first established a standard curve for cholesterol in the microfluidic assay format and then used it to estimate the concentration of cholesterol secreted by the hepatocytes cultured under perfusion. We obtained a standard curve for cholesterol concentrations ranging from 0–200 μM and observed a linear trend. Before quantifying cholesterol in the perfusion based hepatocyte cultures we conducted the assay in the device using the same medium composition used for hepatocyte maintenance but without the cells or cellular secretions to see if there is any interference or false positive signal on the beads. We did not observe any fluorescence on the beads using only maintenance medium. We then quantified cholesterol from hepatocytes

under perfusion and the cells secreted cholesterol in the range of 150–170 μ M or 7.5–8.5 nM/cell (Figure 4).

4. Effect of simvastatin on cholesterol secretion in hepatocytes

Previous reports have shown that simvastatin decreases cholesterol secreted from hepatocytes by approximately 18% for 18-hour treated cells [34]. In the plate cultures, we observed a similar drop in the 18-hour treated wells (Supplementary Figure 6). Mirroring this response, we observed that the hepatocytes in the devices showed a drop of ~18% in cholesterol secreted by the cells that had been treated with simvastatin for 18 hours (Figure 6).

Conclusions

We have shown in-line measurement of cholesterol secreted by human hepatocytes cultured in a microfluidic tissue-chip under perfusion. As the flow of the medium was continuous in the perfusion-based cultures, the signal that was generated monitored the cholesterol secretion in-real time. We show that the fluorescent signal was directly proportional to the concentration of hydrogen peroxide generated during the reaction. Quantifying molecules in flowing solutions is often a challenge and requires special equipment such as a photomultiplier tube. One of the advantages of using a bead for the measurement was that it could be carried out by a fluorescence microscope. Our assay is unique in the following ways 1) the design for microfluidic assay device which can carry out an enzymatic reaction in real-time under perfusion and 2) the bead-based assay chemistry which localizes the fluorescent signal by carrying out the entire reaction on a bead. While the enzymatic fluorescent assay chemistry has been widely used for detection of biomolecules including cholesterol, this is the first time a polystyrene bead with immobilized HRP has been used for conducting the fluorogenic oxidation reactions for cholesterol detection in microfluidic format. The advantage of our assay device is that it makes possible in-line real-time monitoring of biomolecules that use assay chemistry similar to that of cholesterol – examples are glycerol, glucose, and fatty acids. Since in-line measurements enable temporal analysis of cellular secretions which can provide valuable insight into the signaling events our study opens the door for comprehensive in-line analysis of cells cultured in tissue-chips.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- [1]. Cohen DE, “Balancing cholesterol synthesis and absorption in the gastrointestinal tract.,” J. Clin. Lipidol, vol. 2, no. 2, pp. S1–3, 4 2008. [PubMed: 19343078]

- [2]. Zhao B, Natarajan R, and Ghosh S, "Human liver cholesteryl ester hydrolase: cloning, molecular characterization, and role in cellular cholesterol homeostasis," *Physiol. Genomics*, vol. 23, no. 3, pp. 304–310, 11 2005. [PubMed: 16131527]
- [3]. Bonetti P, Lerman L, Napoli C, and Lerman A, "Statin effects beyond lipid lowering— are they clinically relevant?," *Eur. Heart J*, vol. 24, no. 3, pp. 225–248, 2 2003. [PubMed: 12590901]
- [4]. Young EWK and Beebe DJ, "Fundamentals of microfluidic cell culture in controlled microenvironments," *Chem. Soc. Rev*, vol. 39, no. 3, pp. 1036–1048, 2010. [PubMed: 20179823]
- [5]. Halldorsson S, Lucumi E, Gómez-Sjöberg R, and Fleming RMT, "Advantages and challenges of microfluidic cell culture in polydimethylsiloxane devices," *Biosens. Bioelectron*, vol. 63, pp. 218–231, 2015. [PubMed: 25105943]
- [6]. McCarty WJ, Usta OB, and Yarmush ML, "A Microfabricated Platform for Generating Physiologically-Relevant Hepatocyte Zonation," *Sci. Rep*, vol. 6, p. srep26868, 2016.
- [7]. Blagovic K, Kim LY, and Voldman J, "Microfluidic Perfusion for Regulating Diffusible Signaling in Stem Cells," *PLoS One*, vol. 6, no. 8, p. e22892, 8 2011. [PubMed: 21829665]
- [8]. Luan Q, Cahoon S, Wu A, Bale SS, Yarmush M, and Bhushan A, "A microfluidic in-line ELISA for measuring secreted protein under perfusion," *Biomed. Microdevices*, vol. 19, no. 4, p. 101, 12 2017. [PubMed: 29128921]
- [9]. Bricks T et al., "Development of a new microfluidic platform integrating co-cultures of intestinal and liver cell lines," *Toxicol. Vitro*, vol. 28, no. 5, pp. 885–895, 8 2014.
- [10]. Tatárová Z, Abbuehl JP, Maerkl S, and Huelken J, "Microfluidic co-culture platform to quantify chemotaxis of primary stem cells," *Lab Chip*, vol. 16, no. 10, pp. 1934–1945, 5 2016. [PubMed: 27137768]
- [11]. Tourovskaya A, Figueroa-Masot X, and Folch A, "Differentiation-on-a-chip: A microfluidic platform for long-term cell culture studies," *Lab Chip*, vol. 5, no. 1, p. 14, 12 2005. [PubMed: 15616734]
- [12]. Grayson WL et al., "Effects of initial seeding density and fluid perfusion rate on formation of tissue-engineered bone.," *Tissue Eng. Part A*, vol. 14, no. 11, pp. 1809–20, 11 2008. [PubMed: 18620487]
- [13]. Wan X et al., "Perfused Three-dimensional Organotypic Culture of Human Cancer Cells for Therapeutic Evaluation," *Sci. Rep*, vol. 7, no. 1, p. 9408, 12 2017. [PubMed: 28842598]
- [14]. Goral VN, Hsieh Y-C, Petzold ON, Clark JS, Yuen PK, and Faris RA, "Perfusion-based microfluidic device for three-dimensional dynamic primary human hepatocyte cell culture in the absence of biological or synthetic matrices or coagulants," *Lab Chip*, vol. 10, no. 24, p. 3380, 12 2010. [PubMed: 21060907]
- [15]. Hegde M et al., "Dynamic interplay of flow and collagen stabilizes primary hepatocytes culture in a microfluidic platform," *Lab Chip*, vol. 14, no. 12, pp. 2033–2039, 2014. [PubMed: 24770663]
- [16]. Vyawahare S, Griffiths AD, and Merten CA, "Miniaturization and Parallelization of Biological and Chemical Assays in Microfluidic Devices," *Chem. Biol*, vol. 17, no. 10, pp. 1052–1065, 10 2010. [PubMed: 21035727]
- [17]. Roper MG, Shackman JG, Dahlgren GM, and Kennedy RT, "Microfluidic chip for continuous monitoring of hormone secretion from live cells using an electrophoresis-based immunoassay," *Anal. Chem*, vol. 75, no. 18, pp. 4711–4717, 2003. [PubMed: 14674445]
- [18]. Clark AM, Sousa KM, Jennings C, MacDougald OA, and Kennedy RT, "Continuous-flow enzyme assay on a microfluidic chip for monitoring glycerol secretion from cultured adipocytes.," *Anal. Chem*, vol. 81, no. 6, pp. 2350–6, 3 2009. [PubMed: 19231843]
- [19]. Zhang JLQCJWY, "Qualitative and Quantitative Analysis of Tumor Cell Metabolism via Stable Isotope Labeling Assisted Microfluidic Chip Electrospray Ionization Mass Spectrometry," *Anal. Chem*, vol. 3, no. 84, pp. 1695–1701, 1 2012.
- [20]. Wang X, Yi L, and Roper MG, "Microfluidic Device for the Measurement of Amino Acid Secretion Dynamics from Murine and Human Islets of Langerhans," *Anal. Chem*, vol. 88, no. 6, pp. 3369–3375, 2016. [PubMed: 26891222]

- [21]. Dugan CE and Kennedy RT, "Measurement of Lipolysis Products Secreted by 3T3-L1 Adipocytes Using Microfluidics," *Methods Enzymol*, vol. 538, pp. 195–209, 2014. [PubMed: 24529440]
- [22]. Dugan CE, Cawthorn WP, MacDougald OA, and Kennedy RT, "Multiplexed microfluidic enzyme assays for simultaneous detection of lipolysis products from adipocytes.," *Anal. Bioanal. Chem*, vol. 406, no. 20, pp. 4851–9, 8 2014. [PubMed: 24880873]
- [23]. Hassan SU, Nightingale AM, and Niu X, "Continuous measurement of enzymatic kinetics in droplet flow for point-of-care monitoring," *Analyst*, vol. 141, no. 11, pp. 3266–3273, 2016. [PubMed: 27007645]
- [24]. de Souza FR, Alves GL, and Coltro WKT, "Capillary-Driven Toner-Based Microfluidic Devices for Clinical Diagnostics with Colorimetric Detection," *Anal. Chem*, vol. 84, no. 21, pp. 9002–9007, 11 2012. [PubMed: 23072590]
- [25]. Lee G et al., "Single Microfluidic Electrochemical Sensor System for Simultaneous Multi-Pulmonary Hypertension Biomarker Analyses," *Sci. Rep*, vol. 7, no. 1, p. 7545, 12 2017. [PubMed: 28790334]
- [26]. Stroes E, "Statins and LDL-cholesterol lowering: an overview," *Curr. Med. Res. Opin*, vol. 21, no. sup6, pp. S9–S16, 1 2005.
- [27]. Daniels TF, Killinger KM, Michal JJ, Wright RW Jr., and Jiang Z, "Lipoproteins, cholesterol homeostasis and cardiac health," *Int. J. Biol. Sci*, pp. 474–488, 2009. [PubMed: 19584955]
- [28]. Nemes K, Åberg F, Gylling H, and Isoniemi H, "Cholesterol metabolism in cholestatic liver disease and liver transplantation: From molecular mechanisms to clinical implications.," *World J. Hepatol*, vol. 8, no. 22, pp. 924–32, 8 2016. [PubMed: 27574546]
- [29]. Tsai AG, Williamson DF, and Glick HA, "Direct medical cost of overweight and obesity in the United States: a quantitative systematic review," *Obes. Rev*, vol. 12, no. 1, pp. 50–61, 7 2011. [PubMed: 20059703]
- [30]. Krueger WH et al., "Cholesterol-Secreting and Statin-Responsive Hepatocytes from Human ES and iPS Cells to Model Hepatic Involvement in Cardiovascular Health," *PLoS One*, vol. 8, no. 7, p. e67296, 7 2013. [PubMed: 23874411]
- [31]. Ruecha N, Siangproh W, and Chailapakul O, "A fast and highly sensitive detection of cholesterol using polymer microfluidic devices and amperometric system," *Talanta*, vol. 84, no. 5, pp. 1323–1328, 2011. [PubMed: 21641446]
- [32]. Wisitsoraat A, Sritongkham P, Karuwan C, Phokharatkul D, Maturos T, and Tuantranont A, "Fast cholesterol detection using flow injection microfluidic device with functionalized carbon nanotubes based electrochemical sensor," *Biosens. Bioelectron*, vol. 26, no. 4, pp. 1514–1520, 12 2010. [PubMed: 20727731]
- [33]. Ali MA et al., "Highly Efficient Bionzyme Functionalized Nanocomposite-Based Microfluidics Biosensor Platform for Biomedical Application," *Sci. Rep*, vol. 3, no. 1, p. 2661, 12 2013. [PubMed: 24071971]
- [34]. Mullen PJ, Lüscher B, Scharnagl H, Krähenbühl S, and Brecht K, "Effect of simvastatin on cholesterol metabolism in C2C12 myotubes and HepG2 cells, and consequences for statin-induced myopathy," *Biochem. Pharmacol*, vol. 79, no. 8, pp. 1200–1209, 4 2010. [PubMed: 20018177]
- [35]. Ware BR and Khetani SR, "Engineered Liver Platforms for Different Phases of Drug Development.," *Trends Biotechnol*, vol. 35, no. 2, pp. 172–183, 2 2017. [PubMed: 27592803]
- [36]. Johnson KA, Goody RS, Johnson KA, and Goody RS, "The Original Michaelis Constant: Translation of the 1913 Michaelis–Menten Paper," *Biochemistry*, vol. 50, no. 39, pp. 8264–8269, 10 2011. [PubMed: 21888353]
- [37]. Seong GH, Jinseok Heo A, and Crooks RM, "Measurement of Enzyme Kinetics Using a Continuous-Flow Microfluidic System," *Anal. Chem*, vol. 75, no. 13, pp. 3161–3167, 2003. [PubMed: 12964765]

Figure 1.
Schematic showing the implementation of the cholesterol enzyme assay on a polystyrene bead

Figure 2.
Microfluidic device showing the assay chemistry at the different sections

Figure 3.
Correlation plots - comparison between the relative fluorescence values of bead-based assay (X-axis) and conventional assay (Y-axis) for A) Hydrogen peroxide and B) Cholesterol

Figure 4.
Standard curve for measuring cholesterol in the microfluidic device

Figure 5. Live/dead and nuclear imaging for Hepatocytes in microfluidic device. Green fluorescent cells indicate live cells with blue nuclei. Red fluorescence indicates dead cells. A) Day 1, B) Day 7, and C) Percent Cell Viability

Figure 6.
Comparison of cholesterol secreted by perfused human hepatocytes with and without simvastatin treatment