Immune System On-a-Chip

Microfluidics-based Approaches to Study Cellular Interactions under In Vivo Mimetic Environment

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Dedication

For my dad, Bong Woon Kim, my mom, Jin Sook Kwon, and my brother, Jinhyuk Kim
Abstract

The human immune system is a complex network of molecules, organelles, cells, tissues, and organs - there are an uncountable number of interactions and transformations interconnecting all the system components. In addition to such biochemical components, pressure, flow, morphology and location of all of such biophysical components also play an important role in the human immune system. While technical difficulties have frequently limited consideration of the immune system component interactions, some state-of-the-art analytical techniques have revealed distinct cellular behaviors that occur only in vivo or in vivo-mimetic environments. These types of findings inspire analytical/bioanalytical chemists to provide new tools to mimic such interactions occurring in the human body and facilitate better understanding of the human immune system.

Use of microfluidics in analytical/bioanalytical science is a relatively recent development but provides significant potential for such purpose. Well-designed microfluidic approaches can reveal both biophysical and biochemical aspects of human in vivo systems with quick and temporally, spatially, and chemically resolved analyses of in vitro assays. The aim of this thesis is to enable the study of cellular behaviors in physiologically relevant environments using microfluidics. Chapter 1 of this thesis provides a brief review of microfluidics-based innovations in cellular biology studies, and the following chapters focus on biophysical (e.g. flow, Chapter 2), biochemical (e.g. multiple chemical signals, Chapter 3), and higher level biological networks (e.g. cell-cell interaction, Chapter 4) and describe how microfluidics-based approaches advance our
current understanding of cellular behaviors. Furthermore, this thesis suggests a potential future direction of microfluidics-based assay platforms by incorporating a powerful detection scheme, surface-enhanced Raman scattering (SERS), into a microfluidic platform (Chapter 5).

Fluid flow is ubiquitous in the human body, and thus, the first part of the thesis focuses on the flow-induced biophysical impact on cellular behaviors. The biological model herein is the human blood vessel, and the cytotoxicity of nanoparticles under varying flow conditions is investigated in the context of its impact on cellular viability and adhesion/aggregation behaviors. Mesoporous silica nanoparticles are used as the model nanoparticles and human endothelial cells and platelets are used as the model cell types to mimic human blood vessels. Compared to traditional cytotoxicity assays performed under static conditions, mesoporous silica nanoparticles show higher and shear stress-dependent toxicity to endothelial cells under flow conditions. Furthermore, nanoparticles slightly compromise platelet adhesion to endothelial cells at low nanoparticle doses; however, high nanoparticle doses significantly increase the number of platelet adhesion events, leading to higher probability for uncontrolled platelet actions (e.g. clot formation in vivo). Interestingly, despite the altered level of platelet adhesion and aggregation, in no case did nanoparticle exposure result in significant loss of platelet viability. This work clearly demonstrates that 1) biophysical aspects such as fluid flow have to be considered in studying cellular behaviors and 2) aspects besides viability, such as cellular adhesion and interaction with other cell types, have to be considered in the context of nanotoxicology.
The simple and highly adaptable microfluidic analytical platform used in this chapter will be useful for further studies involving other nanoparticles, different cell types, and different cell functions.

Besides biophysical aspects such as flow, cells in the human body always interact with numerous chemical signals and other cell types around them - this thesis also investigates these interactions. By using a model of chemotaxis, chemically directed cellular migration, the dynamics of cellular behaviors under multiple chemical signals (Chapter 3) and in the context of cell-cell interactions (Chapter 4) were quantitatively studied using a microfluidic platform. The target cells used in these chapters are neutrophils, the most abundant and motile leukocyte in humans. The microfluidic platform establishes a stable and dynamic gradient of chemicals across a cell culture chamber and enables the quantitative investigation of cellular migration in the presence of multiple chemical signals. This study leads to interesting findings such as a hierarchy of neutrophil response to chemical signals, the involvement of an enzyme in regulating neutrophils to decide which signal to follow, and the involvement of endothelial cells in regulating neutrophil chemotaxis through surface marker expression. In addition, this study also reveals the impact of neutrophil activation in neutrophil chemotaxis, providing insights into the potential of neutrophil chemotaxis regulation for therapeutic purposes. This study brings new knowledge about neutrophil chemotaxis by replicating physiologically relevant environments for neutrophils (e.g. multiple chemical signals in the context of cell-to-cell communications), and yields both fundamental and therapeutically relevant insight.
In parallel to above mentioned biochemical studies with in vivo mimetic microfluidic platforms, this thesis also aims to achieve microfluidics-based analytical platforms with powerful chemical identification capability. Microfluidics facilitates parallel, low volume sample manipulation and provides opportunities for various optical signal transduction mechanisms. Chapter 5 of this thesis provides proof-of-concept microfluidic sensor platforms that fully utilize such advantages of microfluidics. The developed microfluidic sensor platforms are equipped with gold-based SERS substrates that enable highly sensitive chemical fingerprinting, and the microfluidic device enables serial dilution of introduced analyte solution that terminates in five discrete sensing elements. Chapter 5 demonstrates the utility of the developed sensor platforms to create a calibration curve or do a limit of detection study in a single experiment.

Overall, the thesis herein advances our current understanding of cellular behaviors occurring inside the human body, and demonstrates the utility and a potential future direction of microfluidics-based experimental platforms. All findings described in this thesis will facilitate better understanding of the human immune system and lead to new development of medical and pharmacological applications.
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List of Abbreviations and Symbols

ACD: acid-citrate-dextrose
ADP: adenosine diphosphate
APTES: 3-aminopropyltriethoxy silane
$A_{U_{\text{max}}}$: maximum fluorescence intensity in arbitrary unit
BLTR: leukotriene B4 receptor
BPE: trans-1,2-bis(4-pyridyl)ethylene
CD11: cluster of differentiation
CFD: computational fluid dynamics
CI: chemotactic index
CMFDA: 5-chloromethylfluorescein
CTAB: cetyltrimethylammonium bromide
CTAC: cetyltrimethylammonium chloride
CXCL2: chemokine c-x-c motif ligand 2
CXCL8: chemokine c-x-c motif ligand 8
CXCR1: chemokine c-x-c motif receptor 1
DI: de-ionized
DLS: dynamic light scattering
DMEM: Dulbecco’s Modified Eagle Medium
DMSO: dimethyl sulfoxide
FBS: fetal bovine serum
FITC: fluorescein isothiocyanate
fMLP: formyl-methionyl-leucyl-phenylalanine
FMS: fluorescently labeled mesoporous silica
FPR2: formyl peptide receptor 2
EC: endothelial cell
ECI: effective chemotaxis index
EDTA: ethylenediaminetetraacetic acid
ELISA: enzyme-linked immunosorbent assay
FON: film over nanospheres
GHP: GH propylene
HBSS: hank’s Buffered Salt Solution
HSA: human serum albumin
HUVEC: human umbilical vein endothelial cell
ID: inner diameter
IL-2: interleukin 2
IL-6: interleukin 6
ICAM-1: intercellular adhesion molecule-1
LOD: limit of detection
LOQ: limit of quantification
LSPR: localized surface plasmon resonance
LTB4: leukotriene B4
MAPK: mitogen-activated protein kinase
MI: motility index
MWCO: molecular weight cut-off
MS: mesoporous silica
MTT: methylthiazolyldiphenyl-tetrazolium bromide
NCs: nanocubes
NP: nanoparticle
NRs: nanorods
PTFE: polytetrafluoroethylene
PBS: phosphate buffered saline
PCI: polymerase chain reaction
PDMS: polydimethylsiloxane
PEG: polyethylene glycol
PKB: protein kinase B
PK-PD: pharmacokinetics-pharmacodynamics
PI3K: phosphatidylinositol 3-kinase
PPP: platelet poor plasma
PRP: platelet rich plasma
PTEN: phosphatase and tensin homolog
PS: penicillin and streptomycin
RITC: rhodamine B isothiocyanate
R6G: rhodamine 6G
SERS: surface-enhanced Raman scattering
TEM: transmission electron microscopy

TEOS: tetraethyl orthosilicate

3D: 3-dimensional

TMS: trimethylsiloxane

UV: ultraviolet

μCCA: micro cell culture analog

XRD: x-ray diffraction
Chapter 1

Microfluidics-based in vivo mimetic systems for the study of cellular biology

Adapted from:

1-1. INTRODUCTION

While understanding the human immune system is essential for medical advance, measurement limitations obscure critical details. The human immune system is a complex network of biophysical (flow, feature dimensions, and gradients) and biochemical (molecules, matrices, and cells) components, and human pathophysiology is a result of those components acting both individually and collectively. While significant research has been performed on the individual components of the system, understanding the interactions among the immune system components has been largely limited due to technical barriers. Conventional approaches are typically either quick in vitro assays on isolated cells in a simplified environment or slow in vivo assays using animal models to account for the complex nature of the immune system - unfortunately, neither of these approaches provide clear insights into the cell-cell interactions occurring inside the human body. As such, there is a clear need, especially for analytical and bioanalytical chemists, to provide new principles and tools to bridge the gaps in our understanding.

Recent advances in technology provide opportunities to overcome the limitations of traditional methodologies - any new analytical platform for this purpose must (1) facilitate incorporation of human cellular components while (2) resolving the behavior of individual components with (3) quick, practical, affordable, and reliable assays. Microfluidics platforms, miniaturized devices with micron-sized channels and chambers, are good candidates for all of these requirements.\textsuperscript{1-7} This chapter presents recent innovations in microfluidics for fast in vitro assay solutions that mimic in vivo environments.
1-2. MICROFLUIDICS IN THE STUDY OF CELLULAR BIOLOGY

Due to significant advances in micro-fabrication technologies from the semiconductor industry, microfluidic channels and chambers can be generated in nearly any geometry in a variety of materials with opportunities for high-throughput and multiplexed analyses. Even within the limited context of biological applications, microfluidic channel geometries vary from simple straight channels to complex 3-dimensional (3D) structures, and the device material varies from glass and silicon, to other polymers, such as polydimethylsiloxane (PDMS) and hydrogels. Hybrid devices have been created that incorporate nanoscale metal structures (mostly gold and silver in biological applications) into the microfluidic device to facilitate molecular detection. Also, various surface chemistries have been adapted to create cell-friendly surfaces, enabling the study of either short-term (seconds/minutes) or long-term (hours/days) cellular behaviors. As such, microfluidics has been intensively employed in various fields of chemical, biological, pharmaceutical, and medical studies in recent years.

The two main focus areas of microfluidics-based approaches to better understand human biology are 1) providing high-throughput single cell analysis to measure heterogeneity of cellular responses and 2) providing an in vivo mimic to account for the dynamic and complex nature of the immune system. This thesis focuses on the latter - significant breakthroughs in the field have been made by exploiting in vivo-mimicking microfluidic assays and have revealed distinct cellular behaviors that are obvious only in environments that model the true in vivo environment. For example, it has been
reported that neutrophils on a fibronectin-coated surface behave differently than neutrophils cultured on endothelial cells.\textsuperscript{31} Also, cultured epithelial cells show more in vivo-like molecular transport behaviors when they are exposed to flow than when they are kept in a static environment.\textsuperscript{32} Herein, this chapter will describe microfluidic applications developed within the last 5 years that focus on 1) modeling biophysical aspects of the immune system (e.g. flow), 2) modeling biochemical aspects of the immune system (e.g. cell-cell networks), and 3) combining higher level biological networks (e.g. organ on-a-chip & animal on-a-chip). These microfluidic approaches facilitate both fundamental understanding of human pathophysiology and therapeutic development.

1-3. RECONSTITUTION OF DYNAMIC ASPECTS OF THE IN VIVO

Not surprisingly, the biochemical and biophysical microenvironment influences cellular adhesion, morphology, and differentiation, and microfluidic methods make it possible to control such factors. For example, Nakao et al. have recently shown achievement of a hepatic cord-like structure of hepatocytes simply by reconstituting the tissue-tissue interface.\textsuperscript{33} Also, simple exposure of epithelial cells to physiological shear stress in the presence of drugs like vasopresine and aldosterone induced in vivo levels of water/salt transport by the epithelial cells.\textsuperscript{32,34} As such, it is important to consider the influence of the microenvironment on cellular behaviors. Among the various possible
Figure 1-1. (A) Whole blood perfusion enables reproducible development of a thrombus at the interface between collagen and blood.\textsuperscript{38} Platelets and thrombin are shown in red and green, respectively, on either collagen (a–c) or collagen/tissue factor scaffolds (d–f). Time-dependent resulting structures at the clot boundary were simulated in COMSOL (g). Reproduced from Ref. 38 with permission. (B) Three layer microfluidic platform for the study of sickle cell blood flow conductance, including an artificial capillary layer for blood flow, a hydration layer, and a gas reservoir.\textsuperscript{39} A digital pressure regulator and two solenoid valves were utilized for device operation, and oxygen concentration in the gas reservoir was measured using a fiber optic probe (a). Deoxygenated red blood cells show a shift in the hemoglobin absorption peak, and in the inset, representative images of oxygenated (transparent) red blood cells and deoxygenated (dark) red blood cells are presented (b). Reproduced from Ref. 39 with permission.
microenvironmental aspects, this section focuses on replicating in vivo flow characteristics within an in vitro experimental system. Fluid flow is ubiquitous in the human body and facilitates connections among all the components of the human immune system; for example, flow-induced mechanical forces are known to impact the cellular cytoskeleton, morphology, and adhesion. One of the natural strengths of microfluidics is very accurate insights into fluid behaviors and easy adjustment of flow-induced forces across a range of physiologically relevant levels, all with high throughput and small sample volumes. Conventional platforms, such as flow chambers, can explore flow-induced characteristics, but the flow control is spatially and temporally limited. In the following section, in vitro microfluidic studies that focus on flow-induced biochemical aspects of the human body will be concisely described to demonstrate the utility of microfluidics. In the human body, flow forces can either induce a biological response or can be the actual biological response to a stimulus; as will become clear below, microfluidics provides simple but powerful ways to consider both aspects of flow and broaden our understanding of critical cellular processes occurring inside the human body.

1-3-1. Replication of flow-induced cellular responses

Let us begin with a simple example of blood clotting – an important process to prevent fatal damage to blood vessels. What causes blood clotting? Clinical and macroscopic answers for this question can vary, but microscopically, it is a result of biochemical/biophysical interactions between the vessel wall and circulating cells.
(mostly platelets and red blood cells). This process starts with platelets adhering to and aggregating at the site of injury on the blood vessel wall, and while this process remains insufficiently characterized, it has been proposed that flow plays a critical role in platelet adhesion/aggregation.\textsuperscript{2,7,43} As such, one obvious approach to understand platelet adhesion/aggregation within blood vessels is to study the impact of flow in the platelet adhesion/aggregation process. Gutierrez et al. developed a microfluidic platform to test the hypothesis that flow-induced shear stress is the cause of different platelet adhesion levels to the extracellular matrix.\textsuperscript{43} This work investigated platelet adhesion to several physiologically relevant extracellular matrixes: von Willebrand factor, fibrinogen, and collagen, under a wide range of shear stress levels. Their on-chip investigation revealed that intra- and extracellular platelet adhesion receptors had different impacts on platelet adhesion under different shear stress levels. Specifically, this study revealed that the inside-out signaling of integrin $\alpha$IIb$\beta$3 varied based on extracellular shear stress levels, greatly influencing platelet adhesion to the aforementioned extracellular matrices. The high-throughput nature of this study allowed study of platelet interaction with various extracellular matrices under several physiologically-relevant shear stress conditions, and enabled easy monitoring of intracellular signaling on the same device. Similar microfluidic approaches have been used for various related studies.\textsuperscript{45-47} In fact, our lab recently published work where cellular biology relevant to blood clotting events are systematically studied using simple microfluidic platforms with controlled flow.\textsuperscript{46-47} Our studies investigated the impact of platelet membrane phospholipid content or the presence of nanoparticles on platelet adhesion under varying flow conditions, and
revealed flow-dependent platelet adhesion in both cases. From these examples, it is clear that microfluidic in vivo mimetic systems enable evaluation of cellular processes in physiologically relevant environments, and the findings from such studies give critical insight into fundamental biological processes. Similar devices and studies could be easily adapted to other biological models, such as leukocyte rolling, and could also contribute to ex vivo studies by connecting the device to, for example, the artery of an animal.

1-3-2. Evaluation of cellular response-caused changes in flow

The previous examples clearly show how microfluidics can be used to evaluate flow-induced causes of cellular processes within the human body. Similar devices can be used to measure flow changes as an outcome of biological events. For example, when blood clotting becomes abnormal, thickened blood vessel walls increase the blood pressure which leads to further blood clotting; there is a positive feedback loop between flow-induced pressure and blood clotting. Muthard et al. have recently reported a microfluidic platform where they monitored flow-generated shear stress and correlated it to clot size/composition. On-chip visualization of thrombosis facilitated assessment of clot formation, structure, size, composition, and the relationship between platelet adhesion and thrombin generation at various shear stress conditions. Their approach clearly provides mechanistic understanding of the positive feedback loop between flow-induced shear stress and blood clotting. In another example, Wood et al. have exploited microfluidics to study a sickle cell disease-relevant hypothesis that de-oxygenated red blood cells (“sickled” cells) flow slowly in the blood stream, enhancing probability of
cell-cell contact, and thus, creating a positive feedback loop for further blood clotting. To test their hypothesis, they developed a comprehensive microfluidic platform that facilitated control of red blood cell de-oxygenation in blood and monitored the velocity of the blood stream flow. Their methodological innovation was the incorporation of simultaneous manipulation of oxygen gas and blood fluid within the device and realization of simultaneous measurement of both cellular morphology and flow velocity. With this innovative microfluidic approach, they could identify individuals with mild or severe sickle cell disease, and even further, could predict patients’ response to therapies. In these two examples, the former approach exemplifies how microfluidics can broaden our understanding of critical biophysical characteristics while the latter exemplifies the potential of microfluidic diagnostics. Such approaches benefit early detection of disease, monitoring the progression, and even the prediction of patients’ responses to treatments and will broaden our understanding of fundamental biochemistry occurring in the human body.

There are many examples of microfluidic studies of flow effects in biology beyond those presented above, including screening of potential drug candidates. Recently, Korin et al. developed a microfluidic approach mimicking in vivo flow to assess drug delivery from therapeutic nanoparticles to a designated site. They developed nanoparticle aggregates that disassemble at a certain shear stress level and tested delivery of anti-thrombotic drugs, validating a novel therapeutic to justify future pre-clinical studies. As in the work exemplified above, microfluidics provides a simple but powerful approach for cellular
Figure 1-2. (A) Schematic of the microfluidic coculture device (a-c) with a representative image of Hela and HUVEC cells cultured in the microfluidic device (d, scale bars: 300 μm). Reproduced from Ref. 2 with permission. (B) HeLa cell-bacteria coculture system in a microfluidic device. HeLa cell monolayer (a), GFP-expressing *E. coli* BW25113 localized in the bacterial islands (b), coculture of HeLa cells and *E. coli* BW25113 (c), magnified view of the coculture (d), RFP-expressing EHEC and GFP-expressing *E. coli* BW25113 (e), and transmitted, green, and red fluorescence images in the device (f). Scale bar in (a-c) represents 500 μm, and the scale bar in (d-f) represents 200 μm. Reproduced from Ref. 52 with permission.
biology studies to account for the flow-induced dynamic nature of the human immune system. In addition to considerations of flow, microfluidics allows experimentalists to better simulate the in vivo biochemical microenvironment by incorporating various biomolecules or cell populations. The complexity of such biophysical and biochemical features of the in vivo milieu have been hindering our understanding of human biology gleaned from in vitro studies; as such, microfluidic in vivo mimetic platforms possess great potential for facilitating better understanding of human biology.

1-4. RECONSTITUTION OF IN VIVO MOLECULAR AND CELLULAR COMPLEXITY

While flow is a critical biophysical parameter that influences cells, biochemical signals, including neurotransmitters, hormones, and chemokines among others, are presented in dynamic, complex mixtures to influence cellular behavior. Conventional methods to experimentally address such complexities has been largely limited to the use of expensive, slow in vivo assays that are not necessarily relevant to human physiology; however, microfluidics provides a unique gateway where such molecular and cellular interactions can be observed in a temporally, spatially, and chemically resolved manner. During in vivo cell-cell interactions, delivery of chemical mediators can be achieved in vivo by convective motion of the chemical mediator-containing fluid, diffusion, or direct delivery upon physical contact, all of which can be simulated in a microfluidic device. For example, Skelley, et al utilized a novel microfluidic device to observe fusion efficiency in a co-culture of fibroblasts, myeloma cells, and embryonic stem cells. They
created arrays of individual cell traps that allowed controlled cell-cell contacts, that is, controlled chemical mediator delivery by physical contact. Their microfluidic approach significantly improved cell-cell pairing efficacy when compared to a conventional meticulous cell pairing, and the critical factors in the fusion event between targeted cell types, such as size matching or flow-induced forces, were closely analyzed in a high-throughput manner. Like this work by Skelley et al., there have been significant research efforts to realize microfluidic mimetics of in vivo molecular and cellular complexities, and these efforts have identified several critical aspects of human biology that were not easily obtainable by conventional methods. As such, this section provides a concise description of recent achievements in the fields categorized by the biological models that have been considered.

1-4-1. Cell-cell interactions in cancer

In cancer, studies of circulating tumor cells (CTCs) are popular because many believe CTCs are the first sign of impending tumor metastasis.\textsuperscript{2,54,55} There are many open questions about how the CTCs become activated, find a susceptible opening in the endothelium, and traverse the endothelium to induce a subsequent tumor. Accordingly, tumor cell-endothelial cell interactions and chemical mediator exchange between those cells are of significant interest. Microfluidic platforms provide a simple but powerful tool to study these interactions. For example, several microfluidic approaches that incorporated semipermeable membranes or pressure-driven microfluidic valves have realized a co-culture system of tumor cells and endothelial cells.\textsuperscript{2,54,55} In the microfluidic
system by Zheng et al., chemical mediator delivery between tumor cells and endothelial cells is closely monitored without interference from actual physical contact. The microfluidic platform in this study is equipped with pressure-driven microfluidic valves as a physical barrier, and upon valve opening, exchange of chemical mediators between tumor cells and endothelial cells begins, and the device allows real time investigation of cellular migration according to the chemical interactions. In another example, Hsu et al. have recently investigated the paracrine loop between cancer cells and fibroblasts, another connective tissue cell that cancer cells interact with, using a microfluidic device. They tested and affirmed the hypothesis that cancer cell-fibroblast interactions induce fibroblast differentiation into another sub-phenotype known as myofibroblasts; in fact, the device allowed them to identify growth factor TGF-ß1, a chemical mediator, as a critical component of this transformation. A similar microfluidic approach was reported by Ma et al to model the complex microenvironments surrounding fibroblast-cancer cell interactions. By enabling chemical communication, without physical contact, between tumor cells and embryonic lung fibroblast cells or mature epithelial cells, this study revealed differences between epithelial cells and embryonic fibroblasts in responding to tumor cells. All the above microfluidic platforms enable 1) manipulation of precise chemical gradients within the device, 2) controlled co-culture of cancer cells and stromal or connective tissue cells, and 3) most importantly, temporally, spatially, and chemically resolved analysis on individual components of the experimental system. In addition, the device used by Zheng et al. exemplifies the versatility of microfluidics - numerous experiments were performed using the same device design, maintaining uniformity for
result comparison and minimizing the time required for device engineering.² In the device employed by Hsu et al., the valuable multiplexing capability of microfluidics is demonstrated based on their ability to incorporate multiple cell types into a single device.⁵⁵

1-4-2. Cell-cell interactions in bacterial infection

Like cancer, cell-cell interaction in bacterial infection is critical but not well characterized. Microfluidic co-culture of multiple cell types provides opportunities to bridge this gap. Epithelial cells line the major cavities in the body and make up some of the most vital and resilient organ systems. They come in contact with the outside world much more frequently than endothelial cells and therefore, are one of the most relevant model cells to co-culture with bacterial pathogens. In this spirit, Kim et al studied the interaction between \textit{E. coli} and HeLa cells in a microfluidic device controlled by pressure-driven microfluidic valves.⁵⁶ As above, the microfluidic device maintained physical separation between the bacterial and epithelial cells using pneumatic control so that a definite “time zero” could be established and time-resolved infiltration of bacteria into the HeLa cells could be measured while also preventing premature epithelial cell death that otherwise occurs after an exposure time of only six hours. Through the use of biofilm islands, this group demonstrated that bacteria display a spatial bias to colonization, gathering insight on how \textit{E. coli} outcompetes commensal bacteria in the gastrointestinal tract. Another example of a microfluidic device utilized in the study of epithelial co-culture with bacteria by Hong et al.⁵⁷ investigated the use of bacteria in
targeting cancer. A device utilizing a large middle chamber separated from separate side channels by a collagen layer allowed for the coculture of both cancer cells and normal cells. Bacteria were introduced through the middle chamber and the chemotactic behavior was observed. The primary goal was to observe the activity of *Salmonella typhimurium* when exposed to the competing chemokine gradients secreted by normal hepatocytes and hepatic carcinoma. Hong et al demonstrated that *S. typhimurium* shows a clear preference for cancer hepatocytes over normal hepatocytes, introducing them as a possible candidate in bacteria-driven cancer targeting.57

Microfluidics is clearly a simple and powerful way to consider molecular and cellular complexity in vivo. For co-culture studies in particular, microfluidics use pressure-driven valves or semipermeable membranes to enable the critical separation between/among cultured cell types to prevent interferences and evolution of sub-phenotypes in the cultured cells. This capability provides opportunities to examine cellular function at a microenvironmental level, even in the context of cell-cell interactions, that is otherwise unachievable. Another way microfluidics can facilitate microenvironmental control is in the creation of tissue spheroids - sphere-shaped, self-assembled tumor microenvironments closely mimicking avascular tumors and micrometastases. Conventional methodologies are unable to form uniform and stable spheroids in high yield. The innate microfluidic control over fluid flow and placement of cells provides opportunities to address such issues.58 In addition, the accessibility of various on-chip
Figure 1-3. Organ-on-chip systems.\textsuperscript{10,59} (A) A PDMS-based microfluidic kidney model composed of stacked PDMS microfluidic channels and a porous polyester membrane (a), a liver-on-a-chip system mimicking the permeable endothelial barrier between the liver sinusoid and hepatocytes (b, scale bar: 50 μm), a 3-D microfluidic system replicating heterotypic interactions between endothelial cells and tumor cells (c). Reproduced from Ref. 10 with permission. (B) Spleen on-a-chip (a),\textsuperscript{60} lung on-a-chip (b),\textsuperscript{34} neuronal network on-a-chip (c),\textsuperscript{61} microfluidic endothelium (d),\textsuperscript{62} myotube patterns for skeletal muscle-cell-based bioassays (e),\textsuperscript{63} liver on-a-chip (f),\textsuperscript{64} nine-cell cardiomyocyte network on-a-chip (g),\textsuperscript{65} on-chip resistance vessel fixation and myography (h),\textsuperscript{66} encapsulated endothelial cells in branched collagen gel tubes (i),\textsuperscript{67} and human intestinal system on a PDMS microfluidic device (j).\textsuperscript{68} Reproduced from Ref. 59 with permission.
detection schemes facilitates in-depth characterization of biochemical events occurring in spheroids. In fact, one can go beyond co-culture or spheroid creation to more complex organ mimetics to provide methods for drug screening or organ transplant assessment. Monitoring the potential impact of drugs or the efficacy of transplantation adds several additional demands on microfluidic device design, including consideration of 3-D structure, geometries, stiffness, permeability, and density of the included cell aggregates. Microfluidics opens opportunities to consider any aspect of the in vivo environment and achieve new understanding of human biology that cannot be achieved with traditional methods.

1-5. ORGANS-ON-A-CHIP

In vitro cell culture platforms and in vivo animal models are essential screening technologies for understanding human pathophysiology and developing novel pharmaceutical and medical treatments. Unfortunately, despite significant investment in these methodologies, in vitro cell culture and animal models often do not predict drug behavior in humans, and it would be highly beneficial if microfluidics could be exploited to better predict in vivo human drug effects and toxicity. Accomplishing this feat requires insight beyond simple cell-flow and cell-cell interactions probed in the aforementioned devices; thus, a new segment of microfluidic research has emerged to pursue more complex systems known as organs-on-a-chip. The goal of an organ-on-a-chip is reconstitution of 3D human organs on a microfluidic platform with structural and physiological characteristics of the actual organs. For the purpose of recapitulating
physical environments, perfusion culture conditions are applied to generate the mechanical forces that cells experience in living organisms. Microfluidics provides precise control over various extracellular matrices to better mimic cell-matrix interactions and support formation of 3D cell layers in in vivo-relevant structures. The integration of microelectrodes into microfluidic devices, the employment of technologies called micro-electro-mechanical systems, is critical for several organs-on-a-chip examples, such as heart tissues and neuronal systems, as these organ systems frequently require electrochemical stimulation. Computational simulation is also critical in realizing organ on-a-chip systems to prepare appropriate flow- and structure-induced mechanical characteristics. Incorporation of organ functions into microfluidic devices allows a platform shift from conventional cell culture models to organ-specific microenvironments, offering a better model for human body response to drugs or other biological stimuli.

1-5-1. Lung on-a-chip

One area of representative pioneering work in organ-on-a-chip is human lung biomimetic microsystems. For instance, Huh et al. created a microdevice to model human alveolar-capillary interfaces, simulating a fundamental unit of the human lung, by bonding two microchannels separated by a thin flexible porous PDMS membrane. This sandwich design enabled air flow into the upper compartment, with epithelial cells coating the membrane, and medium circulation in the lower compartment with endothelial cells coating the other side of the membrane. Vacuum application on both microchannels
during cell culture facilitated growth of both cell layers at the air-liquid interface while simulating pulmonary breathing movements. Direct, real-time visualization of individual cells demonstrated that the medium containing cytokine molecules up-regulated the expression of adhesion molecules produced by endothelial cells and induced transmigration of leukocytes through confluent cell layers, whereas cyclic mechanical strain had no impact on apparent inflammatory response markers.

1-5-2. Liver on-a-chip
Hepatotoxicity is recognized as the one of the major issues that causes side effects in drug development. Domansky et al. reported a reliable and efficient liver-on-a-chip platform equipped with electronic controlled pneumatic micropumps that sustained parallel 3D liver cell culture. All included tissues were maintained with constant fluidic perfusion and kept functionally viable for at least one week, facilitating high throughput observation of drug candidate hepatotoxicity. Furthermore, oxygen distribution in each liver culture was modeled by a computational tool to predict gas transfer and consumption, and this model was consistent with the on-chip measurement of luminescence-based analyte concentrations.

1-5-3. Heart on-a-chip
Replicating relevant cardiac tissue is another urgent need for drug testing due to the high risk of heart failure resulting from unforeseen drug toxicities. A novel “muscular thin film” (MTF) assay has been proposed to explore the structure/function relationship and
drug dose effects on anisotropic cardiac myocytes, overcoming the shortcomings of traditional single cell studies and isotropic cardiac assays. The MTF device is composed of a metallic temperature controller, embedded microelectrodes, an elastomeric thin film array of cultured cardiac cells, and a transparent top to allow optical screening. PDMS thin film cantilevers and cardiac myocytes were utilized to replicate the laminar structure of the heart ventricle, and deflection of each myocyte-incorporated cantilever in the array was recorded after electrophysiological stimulation to calculate diastolic and systolic stresses. The utility of this MTF device was further explored by exposing the MTF-based heart-on-a-chip system to varying doses of isoproterenol, a non-selective beta adrenergic agonist and monitoring cardiac contractibility of the engineered tissue system. The micro-engineered heart-on-a-chip system showed dose-dependent changes in the cantilever deformation due to cardiac contractibility and proved its utility as a drug screening platform.

1-5-4. Immune system on-a-chip

Rapid progress in developing organ-on-a-chip platforms has aroused interest in building pharmacokinetics-pharmacodynamics (PK-PD) models to study drug behavior in multi-organ systems. One precursor study employed a compact three-chamber “micro cell culture analog” (μCCA) device based on a PK-PD model. Sung et al. assembled this μCCA system for hydrogel cell culture of liver, tumor, and bone marrow cell lines. Gravity-generated fluid flow eliminated the need for an external pump and prevented bubble formation, and this unique flow recirculation system allowed the cells to retain
viability for three days. A brief comparison of drug cytotoxicity in the theoretical PK-PD model and dynamic μCCA conditions proved that experimental results fit computational estimates satisfactorily, providing opportunities for such combined approaches to predict the drug effects in complex biological systems.

Like the innovations detailed in the previous sections, the proper adaptation or use of these on-chip mimetics of living organs will lead to better understanding of human biology. The main challenge of this newly emerging technology is that, like all other cutting-edge technologies, these devices are currently only accessible to those of who with the expertise and equipment to design/fabricate the devices. Significant interdisciplinary expertise and effort are required to design, fabricate, and apply these devices. Hopefully, the great efforts, like those detailed in the above examples, will bring organ-on-a-chip technology from cutting-edge to practical use in areas like drug metabolism studies, which are typically time-consuming, expensive, and often fail to translate to clinical drug tests.

1-5. CONCLUSIONS
As described above, microfluidic mimetics of in vivo environments provide powerful platforms to study human pathophysiology. These efforts to replicate in vivo-relevant environments on an in vitro platform have identified critical cell-flow, cell-chemical mediator, and cell-cell interactions that influence cellular behavior. In the future, human immune systems, bacterial systems, eco-systems, and even interactions among these will
be closely replicated on a microfluidic system and provide opportunities to significantly improve our understanding of human biology, and thus, public health.

Of course, despite the recent achievements described above, the field is still in need of further innovation. One critical need is easy incorporation of more detection/analysis methods. Nearly all microfluidic devices employ optical microscopy techniques (e.g. phase contrast or immunofluorescence microscopy); however, realization of drug screening or transplant assessment will require simultaneous measurement of various cellular mediators and markers that all need to be quantitatively analyzed in a spatially and temporally resolved manner. There have been significant research efforts to enable state-of-the-art detection on microfluidic platforms: surface-enhanced Raman scattering, mass spectrometry, NMR, electrophoresis, and electrochemistry have all been recently reported.\textsuperscript{17,71-74} There has also been intensive research effort to incorporate conventional biochemical assays, such as the polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA), onto microfluidic platforms.\textsuperscript{75,76}

Another challenge that on-chip in vivo mimetic systems, as well as the entire field of human biology, faces is the lack of available human cells. Even the available human cells are not sufficiently stable for long-term in vitro cultures, making multiple organ-combined systems like the immune system on-a-chip very difficult. One promising alternative is the use of stem cells; however, further development of stem cells is deterred by ethical concerns.

Lastly, microfluidic devices will require more user-friendly interfaces to be employed as point-of-care diagnostics systems or analytical platforms for fundamental sciences. This
requires, in addition to reproducibility and reliability as an analytical platform, easy
operation by unskilled users. Clearly, this requires simplification of fabrication protocols,
achieving mass production of such in vivo-mimetic systems, and automation of device
operation.

The above innovations will require significant input from materials chemists, analytical
chemists, cellular biologists, pharmacologists, and engineers. Such developments in the
field will significantly benefit our fundamental understanding of human pathophysiology
and improve current pharmaceutical and medical applications.
Chapter 2

On-chip Evaluation of Shear Stress Effect on

Cellular Behaviors and Interactions

Adapted from:


2-1. INTRODUCTION

Nanoparticles (NPs) have drawn significant attention in biological and medical sciences due to their promising characteristics for targeting, diagnosis, and drug delivery applications.1-4 Toward to the realization of these NP applications, there have been intense in vitro and in vivo studies on the effects of NPs on biological systems. Unfortunately, despite the great achievements through in vitro approaches, it is always questioned whether in vitro approaches provides in vivo-relevant information.5-6 Also, despite the strength of in vivo approaches, there are issues of in vivo approaches such as cost, labor-intensiveness, speed of assays, ethical issues of killing animals, and most importantly, its relevancy to human. As such, there has been a great need for a new in vitro platform that better models relevant in vivo systems. With no doubt, realization of such in vitro systems for a human model will facilitate a more accurate and deeper understanding of cell-NP interactions and the implications for resulting toxicity.

Fluid flow is a critical feature in our body and known to be related to a variety of cellular behaviors.14-16 Flow may be especially important in considerations of NP toxicity as, in addition to the generated forces on the cells (i.e. shear stress on the endothelium) by flow, it will likely influence cell-NP contact time. To date, a couple studies have investigated particle interaction with cells under flow conditions as an indicator of leukocyte adhesion to the endothelium17-23 but the direct effect of flow conditions on cell-NP interaction, and cell-NP-cell interaction, have largely gone unconsidered.

Toward achieving physiological relevance in the study of cell-NP interaction under flow conditions, this research realizes an on-chip assay platform that mimics the human blood
vessel environment. The cell type employed herein is human endothelial cells, the type of cells that line blood vessels (Figure 2-1). They are known to have flow-dependent behavior, such as morphology changes and activation,\textsuperscript{14-15} and will come in direct contact with injected NPs in the case of intravenous NP injection. The microfluidic system described herein enables human endothelial cell culture inside the device and investigation of NP-endothelium interactions under more in vivo-like, dynamic conditions. In addition, to further develop this on-chip mimetic of blood vessel for cellular function studies, this work introduces a platelet stream over the endothelial cell layer for platelet adhesion/aggregation studies under physiologically relevant, vascular mimetic conditions. The platelet stream is subjected to various doses of mesoporous silica (MS) nanoparticles to examine the nanoparticle impact on platelet-endothelial cell interactions.

To note, while many different nanoscale materials are being used or considered for use in consumer and/or therapeutic products, the NPs chosen for consideration herein are MS NPs. These materials are a promising candidate for controlled and stealth drug delivery based on their materials characteristics, including size control, large internal surface area and easy surface modification.\textsuperscript{24-25} Prior to practical use in clinics with intravenous injection as a delivery method, however, the biocompatibility of MS NPs under dynamic flow conditions should be carefully examined and well characterized. To date, researchers have found that the MS NPs are nontoxic to mammalian cells at exposure concentrations less than 200 μg/mL,\textsuperscript{24,26-28} but all of these studies were performed under static conditions.
Figure 2-1. Schematic of on-chip blood vessel mimetic nanotoxicity assay and platelet adhesion/aggregation assay platform.
Here, MS NPs, at the concentration of 20 μg/mL, 200 μg/mL, and 1000 μg/mL, are used as a model to study the shear stress effect on the cytotoxicity of NPs. In this chapter, the MS NPs are fluorescently labeled (denoted as FMS) have diameters less than 50 nm. For stability and biocompatibility screening, the FMS NPs were either with or without surface modification, and the surface modification performed was a co-modification with polyethylene glycol (PEG) and chlorotrimethylsiloxane (TMS) (surface-modified MS NPs denoted as FMS@PEG/TMS). Fluorescent probes were either fluorescein isothiocyanate (FITC, FITC-labeled NPs denoted as FITC-FMS) or Rhodamine isothiocyanate (RITC, RITC-labeled NPs denoted as RITC-FMS). In summary, there are FITC-FMS, FITC-FMS@PEG/TMS, and RITC-FMS@PEG/TMS, and all NPs were synthesized using related procedures by colleagues in the Haynes group (Yu-Shen Lin, Katie Hurley and Joseph Buchmann).¹

2-2. EXPERIMENTAL DETAILS

2-2-1. Device fabrication

The microfluidic channels were fabricated using standard photolithography techniques. Mask device designs were printed on transparent film (Cad/Art Service Inc., Bandon, OR), transferred onto a 5 x 5 chrome mask plate (Nanofilm, Westlake Village, CA), and then developed in 351 developer solution (Rohm and Hass Electronic Materials LLC, Marlborough, MA). For completion of the mask, the exposed chrome layer was etched down in a chrome etchant solution (Cyantek Corporation, Fremont, CA), and then, the
plate was washed and dried. To fabricate a device master, a 4-inch silicon wafer was cleaned using 1:10 hydrofluoric acid (Avantor Performance Materials,Phillipsburg, NJ):DI water solution for 1 minute to remove potential oxides on the surface, dried on a 115°C hotplate, and spin-coated with a negative photoresist, either SU-8 50 or SU-8 2100, depending on the channel height (Microchem, Newton, MA). For endothelial cell-NP interaction assessment under fluidic conditions, the channel width and height used in this research are described in Table 1, and the channel length is 20 mm for all devices. For platelet adhesion/aggregation studies, the channel dimensions are 2000 μm (width) x 100 μm (height) x 25000 μm (length). While devices could be designed in a way that one inlet leads to multiple identical channels for high-throughput, individual channels were separate for simplification purposes as the goal of this research is method development. After a baking step, the photoresist layer was exposed to UV light through the previously prepared mask to transfer the channel images onto the photoresist. After another baking step, the wafer was developed in SU-8 developer. Upon completion of the master, Sylgard 184 elastomer and curing agent (Ellsworth Adhesives, Germantown, WI) were mixed in 10:1 weight ratio, de-gassed, cast onto the master, and cured overnight at 95°C. The PDMS layer was then cut and punched for inlet and outlet holes. After cleaning, the PDMS layer was permanently bonded onto a glass coverslip by oxygen plasma treatment at 100 W for 10 seconds. Glass coverslips were prepared before this bonding process by cleaning them in 1:1 volume ratio of 30% hydrogen peroxide and 99.9% sulfuric acid (Avantor Performance Materials, Phillipsburg, NJ) for 5 min followed by acetone, methanol, isopropyl alcohol, and DI water rinsing.
The complete device was then brought into a biosafety cabinet and 20 µL syringe tips (Thermo Scientific, Rockford, IL) were inserted in both the inlet and outlet holes. The device was exposed to UV light for an hour and kept in the biosafety cabinet overnight to promote sterility. Then, the channels were washed using a 70 volume percent ethanol solution in sterilized Milli-Q water (Millipore, Billerica, MA) followed by sterile Milli-Q water rinsing three times. The device was dried and kept in the biosafety cabinet until use. This use of micropipette tips is trivial but worth noting as the tips addressed several practical issues. They facilitated exchange of the syringe without damaging the inlet and outlet holes as well as cell loading and feeding using standard culture procedures with minimal inducement of shear stress in the channel.

2-2-2. Endothelial cell culture

Human endothelial cells (Hy926) were purchased from American Type Culture Collection (ATCC, Manassas, VA) and kept frozen in a liquid nitrogen tank. Upon thawing, endothelial cells were expanded into Dulbecco’s Modified Eagle Medium (DMEM) with high glucose (formula: 4mM L-glutamine, 4.5g/L L-glucose, and 1.5g/L sodium pyruvate (Gibco®, Carlsbad, CA)), supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin (Sigma Aldrich, Milwaukee, WI), and cultured in a T-flask in an incubator with 5% CO$_2$ at 37° C (New Brunswick Scientific, Edison, NJ). Cells were fed every other day and, if needed, split once per week. Endothelial cells were used only between the third and tenth passages.
2-2-3. Platelet isolation

Human platelets were isolated from freshly-drawn human blood. Human blood samples were obtained from trained professionals at the Memorial Blood Center (IRB protocol E&I ID#07809) where the blood was drawn directly into tubes containing EDTA as an anti-coagulant. Briefly, once blood samples were obtained, the entire contents of the vial (5 mL) was transferred into a 15 mL centrifuge tube, and centrifuged at 270xg for 20 minutes with no brake. Then, the top two-thirds of the platelet rich plasma (PRP) layer were transferred into a new centrifuge tube. Hirudin was added to at a concentration of 10 U/mL and then the PRP was allowed to rest at 37°C for 25 minutes. Following the rest, one-third volume of acid-citrate-dextrose (ACD) was added and the PRP was again centrifuged at 800xg for 10 minutes with low brake. The platelet poor plasma (PPP) was removed and the pellet was resuspended into Tyrode’s buffer. The PRP was placed in the incubator for 30 minutes for rest, and then centrifuged again at 800xg for 10 minutes with low brake after adding ACD. The formed pellet was again resuspended in Tyrode’s buffer and the platelets were kept in the incubator again for 30 minutes for rest prior to being incubated with 2 μM 5-chloromethylfluorescein (CMFDA) dye for 30 minutes so that platelets adhered onto endothelial cells could be fluorescently monitored. All results reported in this work derive from 6 biological replicates (6 donors) unless otherwise specified.

2-2-4. Endothelial cell coating of a microfluidic channel
First, 250 µg/mL of human fibronectin (Invitrogen, Carlsbad, CA) in sterilized Milli-Q water was injected through the channel, and the device was incubated under 5% CO₂ at 37 °C for an hour. Meanwhile, endothelial cells in a T-flask were washed with phosphate buffered saline (PBS) (Invitrogen, Carlsbad, CA) solution 3 times, trypsinized (Invitrogen, Carlsbad, CA), and kept in the incubator for 4 min. The trypsin solution containing endothelial cells was collected and re-suspended into two-fold volume of DMEM with high glucose, 10% fetal bovine serum and 1% penicillin and streptomycin in a centrifuge tube and centrifuged at 150xg for 5 min. During the centrifugation, fibronectin solution was removed from each channel. The obtained endothelial cell pellet was then re-suspended into the same DMEM media again with the desired cell density (5 - 8 x 10⁶ cells/mL), injected through the channel, and the device was kept in the incubator. After 2 hours, the media was exchanged to remove non-adherent cells, and the endothelial cells in the device were fed every twelve hours. Cells were cultured in the device for a minimum of 3 days and maximum of 5 days to ensure that endothelial cells cover the entire channel surface as different cell number would significantly affect the cytotoxicity results.

2-2-5. Mesoporous silica NP synthesis materials

All chemicals were used as received. n-Cetyltrimethylammonium bromide (CTAB), tetraethyl orthosilicate (TEOS), chlorotrimethylsilane (TMS) and fluorescein isothiocyanate (FITC) were purchased from Sigma-Aldrich (Milwaukee, WI). 2-[Methoxy(polyethyleneoxy)propyl]-trimethoxysilane, (PEG-silane, MW 596-725 g/mol,
9-12 EO) was obtained from Gelest (Morrisville, PA). Absolute anhydrous 99.5% ethanol and 95% ethanol were purchased from Pharmco-Aaper (Brookfield, CT). The de-ionized (DI) water was generated using a Millipore Milli-Q system (Billerica, MA). Ammonium hydroxide (NH₄OH, 28-30 wt% as NH₃) was obtained from Mallinckrodt (Phillipsburg, NJ). Acetic acid was obtained from BDH (West Chester, PA).

2-2-6. Synthesis of unmodified fluorescent mesoporous silica NPs (FMS NPs)

The preparation of bare fluorescent MS NPs was accomplished using our published procedure. First, 1.9 mg of FITC was first dissolved in 1 mL of 99.5% ethanol. Two μL of APTES was then added to the FITC ethanolic solution. The solution was stirred under dark condition for 18 hours at room temperature. Next, 0.29 g of CTAB was dissolved in 150 mL of 0.256 M NH₄OH solution at 50 °C. After one hour, 1 mL of FITC-APTES ethanolic solution and 2.5 mL of 0.88 M ethanolic TEOS solution were added simultaneously under vigorous stirring. After one hour, the stirring was stopped and the colloidal solution was aged for 20 hours at 50 °C. After aging, the as-synthesized colloidal solution was passed through a 0.45 μm GH propylene (GHP) filter and diluted to 40 mL with DI water. The surfactant was removed from the as-synthesized FMS NPs using a dialysis process described by Urata et al. The as-synthesized sample was transferred to regenerated cellulose dialysis tubing (with a molecular weight cut off, MWCO, of 12,000-14,000, Fisherbrand) and placed into a 250 mL acid solution composed of 95% ethanol and 2 M acetic acid. The acid solution was replaced every 24 hours and repeated two times. The particles were then dialyzed against 500 mL of D.I.
water three more times. Finally, the dialyzed FITC-FMS NPs were filtered through a 0.8 μm filter and stored at 4 °C until use. To prepare rhodamine B isothiocyanate (RITC) for incorporation into the silica network, it was first functionalized with 3-aminopropyltriethoxy silane (APTES) by incubating 2.6 mg RITC and 2 μL APTES in 1 mL 99% ethanol with stirring overnight. The next day, 0.29 g cetyltrimethylammonium bromide (CTAB) surfactant was dissolved in 150 g of 0.256 M NH₄OH by stirring at 300 rpm at 50 °C. After 1 h, 2.5 mL of 0.88 M ethanolic tetraethylorthosilicate (TEOS) were added, followed quickly by 1 mL of the ethanolic RITC/APTES reaction resulting in sub-50 nm diameter RITC-FMS NPs.

2-2-7 Synthesis of highly organo-modified fluorescent mesoporous silica NPs (FMS@PEG/TMS NPs)

The synthesis of FITC-FMS@PEG/TMS NPs was based on a newly developed method by our group.³ First, 0.29 g of CTAB was added to 150 mL of 0.256 M NH₄OH solution at 50 °C. Then, 1 mL of ethanolic FITC-APTES solution and 2.5 mL of 0.88 M ethanolic TEOS solution were added simultaneously to solution under continuously stirring. After one hour, 450 μL of PEG-silane was added to the as-synthesized colloidal solution. The mixture solution was stirred for 30 minutes and then 68 μL of TMS was added. After another 30 minutes, stirring was stopped and the obtained solution was aged at 50 °C for 20 hours. The as-synthesized modified MS colloidal solution was filtered with a 0.45 μm GHP filter and diluted to 50 mL with DI water. The filtered colloidal solution was then heated at 90 °C for 24 hours in a sealed vessel. The surfactant removal steps followed the
centrifugation method. Finally, the surfactant-free FITC-FMS@PEG/TMS NPs suspended in 99.5% ethanol were filtered using a 0.2 \( \mu \)m polytetrafluoroethylene (PTFE) filter. The products were dried and stored at room temperature until use. For surface-modified RITC-FMS NPs, the mixture prepared in the above section 2-2-6 was stirred at 600 rpm and heated at 50°C for 1 h, followed by the addition of 450 \( \mu \)L PEG-silane (500-700 MW). After 30 minutes, 68 \( \mu \)L of chlorotrimethyl silane (TMS) were added. Stirring was stopped after 30 minutes, and the sample was aged at 50 °C for 20 h, allowing excess water to evaporate from the solution (final volume ~50 mL), co-modified with PEG and TMS (RITC-FMS@PEG/TMS). All nanoparticle batches were passed through a 0.45 \( \mu \)m filter to exclude large aggregates and placed in an oven at 90 °C for 24 h. Treated particles were then purified by dialysis and centrifugation and stored in MQ DI water prior to use. All reactions and post-synthetic steps were performed under dark conditions to prevent photobleaching.

2-2-8. NP characterization

Transmission electron microscopy (TEM) micrographs were taken on a JEOL 1200 EXII with a 100 kV voltage. Powder X-ray diffraction (XRD) patterns were measured on a Siemens Bruker-AXS D-5005 X-ray diffractometer using filtered Cu K\( \alpha \) radiation (\( \lambda = 1.5406 \) Å) at 45 kV and 20 mA. Data were recorded by step scan with a step size of 0.040° and a step time of 1.0 second. Hydrodynamic diameter data were measured at particle concentration of 200 \( \mu \)g/mL using dynamic light scattering (DLS) with a Brookhaven 90Plus/BIMAS particle analyzer (Holtsville, NY) equipped a 655 nm laser.
Three runs and one minute run duration were set for each measurement. The DLS size distribution was plotted using a lognormal analysis method.

Similar procedures were followed to characterize RITC-FMS@PEG/TMS NPs. Dynamic light scattering measurements were performed on a Brookhaven Instruments 90Plus particle analyzer (Holtsville, NY) with 35 mW red diode laser at 600 nm. Samples were diluted to 1 mg/mL in water and measured at room temperature. A FEI Tecnai T12 transmission electron microscope with 120 kV operating potential was used to collect TEM images for particle morphology and sizing. Diameters were calculated from an average of 500 NPs. Nitrogen adsorption/desorption isotherms were acquired on a Micromeritics ASAP 2020 instrument after being degassed at 120 °C for 8 h.

2-2-9. Shear stress control in microfluidic channels

Shear stress levels applied to the endothelial cells were controlled by adjusting channel dimensions and flow rate. Shear stress levels at given flow rate and channel dimensions were calculated using equation (1) and confirmed by computational fluid dynamics (CFD) modeling using COMSOL Multiphysics v4.1.

2-2-10. On-chip fluorescence imaging for NPs

The FMS and FMS@PEG/TMS NPs used in this research were synthesized with incorporated FITC and RITC to facilitate easy characterization of cell/NP interaction. Following cell exposure to NPs, the cells were washed with PBS for 3 min and then
fluorescence imaging was performed on an inverted Nikon TE3000 microscope (Melville, NY).

2-2-11. On-chip fluorescence imaging for platelet adhesion/aggregates

Once NPs, platelets and endothelial cell-coated microfluidic devices were ready, the population of platelets was divided and treated as desired. The total number of conditions was eight, and the first four conditions involved platelet exposure to no NPs, 20 µg/mL, 200 µg/mL, or 1000 µg/mL NPs without intentional platelet activation, and the other four were the exact same NP conditions except that platelets were activated with adenosine diphosphate (ADP). For ADP-activated conditions, platelets were exposed to the same four NP conditions and then exposed to 5 µM ADP. Two of the most important platelet functions are adhesion/aggregation and secretion, and ADP is a strong platelet aggregation agonist that results in no significant secretion;\textsuperscript{38,39} by using ADP activation, this study specifically focused on the impact of NPs on the adhesion/aggregation process of platelets. Following exposure, the platelet suspension was introduced through the microfluidic channel using a syringe pump set at a flow rate of 30 µL/min. Given the dimensions of the microfluidic channel, the shear generated by this flow rate, \( \sim 0.1 \text{ N/m}^2 \), cause minimal impact on the cells inside channel.\textsuperscript{40} The stream of platelets was maintained for 25 minutes and the channels were washed with fresh Tyrode’s buffer to remove non-adherent platelets within the channel. The platelet suspension coming through the channels was collected at the outlet for further analysis, and the devices were brought to the microscope equipped with fluorescence imaging setup. Metamorph V.7.7.5
was used as the imaging software. For adhesion studies, 3 replicates of 400 µm x 400 µm images (20x magnification) were obtained from 3 random locations and for aggregation study, 3 replicates of 200 µm x 200 µm images (40x magnification) were obtained from 5 random locations. Platelet aggregation is further analyzed using fluorescence imaging using a higher magnification objective that is described below. For the purposes of this study, platelet aggregates were defined as more than one platelet. Due to day-by-day differences in the absolute number of platelets adhered onto the endothelial cell layer, reported adhesion levels are in ratio to the control (conditions without NPs in both not-activated and ADP-activated platelets).

2-2-12. On-chip in vitro MTT assay on endothelial cells

After visually insuring appropriate cell coverage of a microfluidic channel, the inlet and outlet tips were connected to syringes via 0.0038” ID Teflon tubes. The presence of any bubbles compromises shear stress control in the device, so extra care was taken to avoid and identify bubbles in the tips and device. Then, each channel was washed with DMEM with high glucose and 1% penicillin and streptomycin (henceforth, DMEM without serum) for 3 minutes, and the syringe was exchanged to that with either DMEM without serum (control) or DMEM without serum but containing 200 µg/mL silica NPs (experimental). After 2 hours of flow, the syringe was changed to that with DMEM without serum to remove non-adherent NPs, and a 0.5 mg/mL MTT solution was injected through the channel for 1 minute. After 2 hours incubation, the tube was connected to a syringe filled with dimethyl sulfoxide (DMSO), and the channel contents were collected.
into an eppendorf tube. If needed, the total volume of DMSO in each eppendorf tube was balanced so that all tubes contained the same final volume. Absorbance of the collected DMSO solutions was measured at 570nm with 655nm measurement for background using a plate reader (Bio-Rad, Hercules, CA) in triplicate. Toxicity was assessed by taking absorbance of the collected DMSO from experimental condition channel (with NPs) and dividing that by absorbance of the collected DMSO from control condition channel (without NPs).

2-2-13. Off-chip in vitro trypan blue assay on platelets

20 μL of each condition of the collected platelets was mixed with an equal volume of 0.4% Trypan Blue. Then, 20 μL of the resulting solution was placed on a hemocytometer. Both the number of live and dead (stained) cells was counted, and percent viability was calculated.

2-2-14. NP dose calculations for the flow conditions

2-2-14-1. Number of NPs per gram:

\[
\text{Density of amorphous silica} = 2.2 \ \left( \frac{g}{cm^3} \right) \\
\text{Primary pore volume} = 0.81 \ \left( \frac{cm^3}{g} \right)
\]

Assumption: The NPs is a hexagonal prism.
The total volume of a single NP:

$$\text{Volume} = 6 \times \frac{\sqrt{3}}{4} \times L^2 \times H$$

where L is the length of a side of the hexagon and H is the height of the entire structure.

Herein, the values for L and H are determined based on TEM imaging.

$$= 6 \times \frac{\sqrt{3}}{4} \times (21 \times 10^{-7})^2 \times (42 \times 10^{-7}) = 4.812 \times 10^{-27} \left(\frac{\text{cm}^3}{\text{NP}}\right)$$

Defining \(X_{42}\) as the number of NPs per gram,

$$\left[\left(X_{42} \times 4.812 \times 10^{-27} \left(\frac{\text{cm}^3}{\text{NP}}\right)\right) - \left(1 \times 0.81 \left(\frac{\text{cm}^3}{\text{g}}\right)\right)\right] \times 2.2 \left(\frac{\text{g}}{\text{cm}^3}\right) = 1$$

$$\Rightarrow X_{42} = 2.6 \times 10^{22} \left(\frac{\text{No. NPs}}{\text{g}}\right)$$

2-2-14-2. Potential volume of NP-containing fluid interacting with endothelial cell layer:

Diffusion length in \(z\)-axis (height) \(\times\) Channel width \(\times\) Total traveled length

$$= 1 \times 10^{-6} \text{m} \times 150 \times 10^{-6} \text{m} \times 0.2 \text{ m/sec} \times 7200 \text{sec} \times \frac{1000 \text{ L}}{\text{m}^3}$$

$$= 2.16 \times 10^{-4} \text{L}$$

2-2-14-3. Number of NPs exposed to endothelial cells:
2-3. RESULTS AND DISCUSSION

2-3-1. NP characterization

The unmodified FMS and organically modified FMS NPs were synthesized using our published and recently developed methods. The transmission electron microscopy (TEM) images of FMS NPs and polyethylene glycol (PEG)/trimethyl silane (TMS)-modified fluorescent MS NPs (denoted as FMS@PEG/TMS) show that both FMS and FMS@PEG/TMS have similar diameter (~40 nm) (Figure 2-2). In addition, the TEM images and XRD patterns reveal that the pore structure inside FMS@PEG/TMS NPs is not as ordered as FMS, confirming a high amount of organic silane modification on either the outer or interior surface of FMS NPs. The detailed pore structure of FMS and FMS@PEG/TMS is shown in the figure 2-2. Compared to the hydrodynamic diameter of FMS NPs (~200 nm) in serum-free cell culture medium (Dulbecco’s modified Eagle’s...
Figure 2-2. (a, b) TEM images of FITC-FMS and FITC-FMS@PEG/TMS, (c) TEM image of RITC-FMS@PEG/TMS, and (d, e) Hydrodynamic size distribution of FITC-FMS and RITC-FMS NPs measured by dynamic light scattering (DLS).
medium, DMEM), the FMS@PEG/TMS NPs have smaller hydrodynamic diameter (~60 nm), showing that unmodified FMS NPs agglomerate in a highly salted environment while the PEG/TMS modification prevents agglomeration of FMS NPs (Figure 2-2). The incorporated fluorophore, fluorescein isothiocyanate (FITC), facilitated fluorescence imaging to assess NP association with the endothelial cells under controlled shear stress conditions.

FMS@PEG/TMS nanoparticles were obtained through a procedure based on our previous work. These MS nanoparticles are surface functionalized with both PEG and TMS, a co-modification procedure that has been shown to produce long-term colloidal stability in biological environments such as protein-containing or high ionic strength media.41, 42 TEM imaging of these nanoparticles revealed relatively monodisperse particles with a diameter of 47.9 ± 7.1 nm (Figure 2-2). DLS hydrodynamic diameter in water was slightly higher, as expected, at 66.8 ± 0.3 nm (Figure 2-2). Photos of bulk nanoparticle solution under ambient and ultraviolet light (Figure 2-2) show the bright and homogeneous fluorescence achieved with these materials. This high surface area will be critical in future studies where these nanoparticles will be used to deliver drug cargo to platelets or other cell types.

2-3-2. Shear stress control within a microfluidic device
Figure 2-3. (a) Complete device (14 single channels on a cover slip) (b) device design, (c) cross-section operation schematic, and (d) an example COMSOL simulation with channel dimensions of 300µm, 80µm, and 500µm for width, height, and length, respectively. Flow rate is set 75 µL/min.
The microfluidic system consists of a simple channel, but is an integrated platform on which cell culture, exposure to experimental conditions, imaging, and a cell viability assay are performed. Figure 2-3 shows the complete device, the design, and a schematic of the platform. The microfluidic channel is fabricated in polydimethylsiloxane (PDMS), and the uncomplicated design simplifies device production, fluid modeling and control, and device operation with the accurate control of flow. The shear stress levels targeted in this work range from 0 to 6.6 N/m², based on physiologically relevant shear stress levels in various vessels of the human body. The shear stress levels at 75 μL/min flow rate with varying channel dimensions were determined using the equation,

\[ \tau = \frac{6 \mu Q}{wh^2} \quad (1) \]

in which \( \mu \), \( Q \), \( w \) and \( h \) represent dynamic viscosity of the media (0.00084 Pa·s), volumetric flow rate (75 μL/min), channel width, and channel height, respectively. The calculated values for various devices used herein can be found in Table 2-1. While the equation above is generally accepted and used to estimate shear stress, this equation is obtained with an assumption that the channel width is infinite (i.e., that there are no side wall effects). Accordingly, the numbers obtained using equation (1) were confirmed using computational fluid dynamics (CFD) (Figure 2-3, d). CFD provides steady state modeling of fluid rheology with given channel dimensions, allowing quantitative analysis of fluid rheology parameters on all four sides of the channel. COMSOL Multiphysics 4.1 software was used to produce a single phase laminar flow model with given channel
<table>
<thead>
<tr>
<th>Channel dimensions$^a$</th>
<th>150W80H</th>
<th>150W65H</th>
<th>300W45H</th>
<th>300W80H</th>
<th>300W200H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate (µL/min)</td>
<td>75</td>
<td>50</td>
<td>45</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>Calculated shear stress (N/m²)</td>
<td>6.6</td>
<td>6.6</td>
<td>6.2</td>
<td>3.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Modeled shear stress (N/m²)</td>
<td>7.3</td>
<td>7.1</td>
<td>5.6</td>
<td>3.2</td>
<td>0.6</td>
</tr>
</tbody>
</table>

$^a$W: Width (µm); H: Height (µm)

**Table 2-1. Summary of shear stress levels calculated and simulated in the given channel dimensions.**
dimensions and fluid properties at 37 °C. The model was simplified in length (set at 500 µm rather than the actual channel length of 20 mm) to reduce the amount of time for computing, and the volumetric flow rate at the inlet was set as described in Table 2-1. The shear stress patterns in all channel geometries were the same as shown in Figure 2-3; at the bottom channel surface, the effective zone (the zone where the shear stress level changes less than 1 unit from the reported value) of shear stress across all channel geometries was ~10 µm thick at minimum, indicating that the endothelial cells, which are sitting on the bottom surface of the channel, experience the intended shear stress level. All the modeled values reported herein are the maximum shear stress values from each model geometry. According to Table 2-1, the COMSOL modeling confirmed that accurate control of shear stress in a physiologically relevant range could be achieved in endothelial cell-coated device channels. The channels with 150 µm width showed slightly higher shear stress level in modeled results than in the calculated ones (e.g. 6.6 N/m² calculated versus 7.3 N/m² modeled for the 150W80H case), and the geometries with 300 µm width (higher aspect ratio than 150 µm cases) showed more accurate shear stress control, indicating that high aspect ratio devices may be advantageous in future work.

2-3-3. Cytotoxicity of FITC-FMS under dynamic conditions

Figures 2-4 a and e, and figure 2-4 b and f show bright field and fluorescence micrographs, respectively, obtained after 2 hour endothelial cell exposure to FMS NPs at different shear stress levels following a PBS wash to remove non- or weakly adherent NPs. The fluorescence imaging confirms that a portion of the presented FMS NPs was
Figure 2-4. Bright field and fluorescence images of endothelial cells exposed to FMS (a, b, e, and f) and FMS@PEG/TMS (c, d, g, and h) NPs for 2 hours at 200 µg/mL concentration in 2 different shear stress conditions: a, c, e, and g are under a shear stress level of 3.3 N/m² and b, d, f, and h are under a shear stress level of 6.6 N/m². Compared to FMS NPs (e and f), FMS@PEG/TMS NPs associate significantly less with endothelial cells than unmodified NPs under the same conditions (g and h).
strongly associated with endothelial cells under both conditions, while no significant amount of FMS@PEG/TMS NPs was observed under the same flow conditions (Figures 2-4, g and h).

To investigate the cytotoxicity of these associated FMS NPs to endothelial cells under various shear stress conditions, an in vitro viability assay (methylthiazolyldiphenyltetrazolium bromide or MTT assay) was performed. The MTT assay is a well-established method used to assess cytotoxicity of both molecular toxins and NPs in many studies. While the MTT assay has been shown to produce false positive results in the presence of some nanoscale materials, previous work has clearly established that it is an appropriate choice for toxicity assessment of silica NPs. A NP concentration of 200 µg/mL in serum-free cell culture media was chosen for comparison with previous work where significant toxicity was apparent in endothelial cells after 24 hour incubation in static conditions. A direct comparison of cytotoxicity was performed under static conditions and shear stress conditions, with shear stress ranging from 0.5 to 6.6 N/m². Within the healthy human body, arterioles and capillaries experience an average of ~ 5 - 6 N/m² shear stress, but these values can fluctuate depending on the person and condition. Thus, the shear stress values used here represent NP exposure conditions upon introduction into normal or abnormal arterioles or capillaries. Figure 2-5 shows the toxicity results obtained from both static and shear stress conditions. Endothelial cells were exposed to the unmodified FMS NP stream for two hours, and as shown in the figure, unmodified FMS NPs were significantly more toxic to endothelial cells under the two highest shear stress conditions (88.9 ± 0.9 % and 70.4 ± 3.3 % viability at 3.3 and 6.6
Figure 2-5. MTT assay results after endothelial cell exposure to NPs for 2hrs. (a) Cytotoxicity results for FMS NPs in different shear stress conditions. Concentration of FMS NPs is 200 µg/mL (b) Dose effect in the cytotoxicity of FMS NPs under static conditions (c) Dose effect on the cytotoxicity of FMS NPs in the same (similar) shear stress conditions (d) Effect of PEG/TMS modification on the cytotoxicity of MS NPs. (*, **, and *** represent p < 0.1, p < 0.01, and p < 0.0001, respectively.)
N/m², respectively) while no apparent toxicity was found under either the static (96.5 ± 1.5 %) or 0.5 N/m² (103.4 ± 0.7 %) shear stress conditions with the same exposure time and concentration. These results indicate that shear stress influences cytotoxicity of FMS NPs to endothelial cells, and FMS NPs do not show significant toxicity to endothelial cells under static or very low shear stress conditions. These results indicate that the dynamic nature of the environment (flow conditions) is one of the likely causes of the discrepancy between in vivo and in vitro nanotoxicity assays. It is a generally accepted concept that NP toxicity is dependent on both NP dose (amount) and exposure (time). Under the flow conditions, however, concentration cannot represent the dose because, at least in this work, the absolute number of NPs passing through the microfluidic channel is larger under flow conditions than static conditions, and therefore, the shear stress-dependency in the toxicity result might not be from the effect of shear stress. To investigate this possibility, the NP dose under flow conditions was estimated and varied by tuning NP concentration under static conditions and channel dimension and flow rate under flow conditions (Figure 2-5, b and c). Taking both flow velocity and NP diffusion length into account, NPs within 1 µm above the endothelial cell layer are estimated to have the potential to interact with the endothelial cells. Using this limit, the number of NPs that interact with endothelial cells under the 6.6 N/m² shear stress condition was calculated to be 1.1 x 10¹² NPs, while the value for the static condition was calculated to be 5.2 x 10¹¹ NPs (Table 2-2); so, the original experimental conditions led to endothelial cells under the highest shear stress condition interacting with approximately double the number of NPs as in the static condition. To account for this dose difference, three
Table 2-2. Dose comparison between static and the highest shear stress conditions.

<table>
<thead>
<tr>
<th></th>
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<th>6.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shear Stress (N/m²)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flow Rate (μL/min)</td>
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<td>75</td>
</tr>
<tr>
<td>Concentration</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td># of NPs</td>
<td>$5.2 \times 10^{11}$</td>
<td>$1.1 \times 10^{12}$</td>
</tr>
</tbody>
</table>

Table 2-3. Dose variation done in static conditions.

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<th></th>
<th>200</th>
<th>475</th>
<th>950</th>
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<tr>
<td>Shear Stress (N/m²)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Concentrations (μg/mL)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td># of NPs</td>
<td>$5.2 \times 10^{11}$</td>
<td>$1.2 \times 10^{12}$</td>
<td>$2.4 \times 10^{12}$</td>
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</tbody>
</table>

Table 2-4. Dose variation done in shear stress conditions.

<table>
<thead>
<tr>
<th></th>
<th>150W80H</th>
<th>150W65H</th>
<th>300W45H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Channel Dimensions (μm)</td>
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<td></td>
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</tr>
<tr>
<td>Flow Rate (μL/min)</td>
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<td>45</td>
</tr>
<tr>
<td>Shear Stress (N/m²)</td>
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<td>6.6</td>
<td>6.2</td>
</tr>
<tr>
<td>Concentrations (μg/mL)</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td># of NPs</td>
<td>$5.2 \times 10^{11}$</td>
<td>$4.5 \times 10^{11}$</td>
<td>$5.6 \times 10^{11}$</td>
</tr>
</tbody>
</table>

* W: Width, H: Height
additional experiments were performed; two under static conditions but with ~ two- (475 µg/mL, 1.2 x 10^{12} NPs) and four-fold (950 µg/mL, 2.3 x 10^{12} NPs) higher concentrations of NPs to achieve the same and higher dose as the 200 µg/mL exposure under the flow conditions (1.1 x 10^{12} NPs), and one where flow rates are varied to achieve the same dose as the 200 µg/mL (5.2 x 10^{11} NPs) exposure under static conditions (Table 2-3 and 2-4). In the latter case, channel dimensions were altered to achieve the same 6.6 N/m² shear stress under the lower flow rates used; this shear stress was verified by COMSOL modeling (Table 2-1). Figures 2-5 b and c summarize the cytotoxicity results as the number density of NPs is maintained as described; the results confirm that the increased toxicity under shear stress was clearly the result of the flow. Under static conditions, two hour exposure of endothelial cells to 5.2 x 10^{11}, 1.2 x 10^{12}, and 2.3 x 10^{12} NPs results in 96.5 ± 1.5, 88.9 ± 4.1, and 88.8 ± 5.5 % endothelial cell viabilities, respectively. Under flow conditions, on the other hand, 70.4 ± 3.3, 73.1 ± 3.0, and 75.9 ± 4.0 % endothelial cell viabilities were obtained from 1.1 x 10^{12}, 4.5 x 10^{11}, and 5.6 x 10^{11} NPs, respectively. While there is a possible, slight dose effect in cytotoxicity results under static conditions, this assessment clearly confirms that the difference in the toxicity results in the original assessments (static vs. flow) came from the shear stress rather than dose.

One potential explanation for this phenomenon is that increased shear stress induces activation of the endothelial cells. Several studies have reported changes in endothelial cell behavior under various shear stress conditions. In fact, clear morphological changes were observed in this work in endothelial cells under shear conditions of 6.6 N/m² compared to static conditions (data not shown). While the specific changes to the
endothelial cell population is the subject of future work, it is already clear that flow plays a critical role in the interaction between NPs and cells, making in vivo cells vulnerable to silica NP exposure.

With the intention to realize MS NP applications in the human body, the toxicity of FMS NPs must be controlled. One strategy known to ameliorate silica NP toxicity is based on blocking the interaction between surface silanol groups and the cellular membrane. Specifically, modification with low molecular weight poly(ethylene glycol) successfully masks the surface silanol groups in static conditions; herein, we consider the role of this surface modification under flow conditions. The NP cytotoxicity for FMS@PEG/TMS NPs under 3.3 and 6.6 N/m\(^2\) shear stress conditions were obtained and compared to the previously obtained unmodified FMS NP toxicity results under the same shear stress conditions. In each shear stress condition, the cytotoxicity results indicate that FMS@PEG/TMS NPs do not have significant toxicity (99.11 ± 0.3 and 97.22 ± 1.6 % viability at 3.3 and 6.6 N/m\(^2\), respectively) to the endothelial cells, revealing that NP surface chemistry plays an important role in flow-mediated cell-NP interactions (Figure 2-5, d). One possible explanation for this is that, while these endothelial cells were activated by shear stress, the PEG surface modification prevented non-specific association of the NPs, resulting in decreased interaction between endothelial cells and NPs, and thus, decreased toxicity.

2-3-4. RITC-FMS@PEG/TMS and platelet viability

Trypan Blue staining of platelets collected from the microfluidic device showed no
Figure 2-6. % Viability of platelets by trypan blue assay.
significant loss in platelet viability for any of the conditions (Figure 2-6). This benign nanoparticle interaction was expected for FMS@PEG/TMS nanoparticles as they have been shown to be non-toxic to endothelial cells. The main interest of our investigation, then, is in identifying any non-lethal effects of FMS@PEG/TMS nanoparticle exposure on platelet adhesion and aggregation.

2-3-5. RITC-FMS@PEG/TMS and platelet adhesion/aggregation

To investigate the impact of nanoparticle presence on platelet adhesion, varying concentrations of nanoparticles were introduced into the platelet stream on endothelial cells. The chosen concentrations of nanoparticles are 20 μg/mL, 200 μg/mL, and 1000 μg/mL. Assuming 3-5% loading by weight of doxorubicin, a typical cancer therapeutic, a single nanoparticle dose via intravenous injection would be in the range of 3000-4000 mg in a total volume of 10s to 100s of mL. Thus, the doses used in this study are relevant, particularly for platelets near the site of injection. As platelets are sensitive to flow conditions, the device operation was kept at a flow rate (30 μl/min in a channel that is 100 μm thick and 2000 μm wide) that minimizes flow-induced activation of platelets. The device used herein had multiple straight channels to perform simple parallel analysis of replicate samples, but it could easily be adapted to include multiple branches to increase the throughput or various channel dimensions to simulate more complex vasculature. The number of platelets adhering to endothelial cells had a slight variation from day to day (or donor-by-donor); as such, the adhesion values reported in this chapter are presented as the ratio compared to the control cells from that donor. Thus,
Figure 2-7. Adhesion of (a) non-activated and (b) ADP-activated platelets onto endothelial cells.
control cells from both unactivated and ADP-activated cells always show 1 as the adhesion value. Without ADP activation, FMS@PEG/TMS nanoparticle exposure had a significant impact on platelet adhesion to endothelial cells. Both 20 µg/mL and 200 µg/mL nanoparticles resulted in reduced adhesion of platelets to endothelial cells (p < 0.0001 for both compared to control, Figure 2-7), and the trend was concentration-dependent. In our experimental system, ~ 3.5 x 10^6 platelets are introduced to the confluent endothelial cell layer and, assuming an average platelet diameter of 2.5 µm, an average nanoparticle diameter of 48 nm, the shape of each nanoparticle as a hexagonal prism, a pore volume of 0.81 cm^3/g, and a diffusion length of 1 µm for a nanoparticle, ~ 2000 nanoparticles surround a single platelet during an experiment. Intuitively, the presence of nanoparticles within the platelet suspension may interfere with platelets interacting with one another as well as with the layer of endothelial cells, and this might be a cause of the reduction in adhesion. We know that the FMS@PEG/TMS nanoparticles have shown no significant impact on the viability of endothelial cells, so the change in adhesion behavior can be attributed to nanoparticle effects on the platelets directly. The same trend was found in ADP-activated platelets. Compared to the control cells (no nanoparticles) activated by ADP, ADP-activated platelets exposed to both 20 µg/mL and 200 µg/mL FMS@PEG/TMS nanoparticles also showed reduced adhesion to endothelial cells (p < 0.0001 for both, Figure 2-7). Thus, it can be concluded that the presence of nanoparticles interferes with the platelet-endothelial cell interaction and reduces physical contact between them. This preliminary conclusion requires further studies into platelet and endothelial cell biology to characterize why this interaction is
Figure 2-8. Representative images from fixed-cell fluorescence imaging. (a) ADP-activated, 0 µg/mL nanoparticle condition showing the major population of platelets in single or small aggregates, and (b) ADP-activated, 1000 µg/mL nanoparticle condition showing major population of platelets in big aggregates. Images processed for overlaying by ImageJ (green: platelets, purple: nanoparticles).
impeded – possible causes include changes in surface receptor expression, outer bilayer phospholipid composition, or secretion of soluble mediators. These experiments will be the topic of future research. While ADP-activated platelets still had nanoparticle concentration dependence in their adhesion behavior, the trend was opposite to that found in unactivated platelets. ADP-activated platelets exposed to 200 µg/mL nanoparticles show slightly higher adhesion to endothelial cells when compared to the ADP-activated platelets exposed to 20 µg/mL nanoparticles (p = 0.025). The previous results with endothelial cells showed that activation of cells may result in different trends in cellular responses against nanoparticles compared to their unactivated counterparts. This difference, depending on whether or not platelets are ADP activated, implicates cell-nanoparticle interaction beyond a physical interaction level in platelet adhesion to endothelial cells; at a certain concentration, the presence of nanoparticles may cause cellular activation that changes adhesion behavior. This reasoning is supported by the trend in platelet adhesion exposed to the highest concentration of nanoparticles. Both unactivated and ADP-activated platelets exposed to 1000 µg/mL nanoparticles showed a significant increase in their adhesion to endothelial cells (Figure 2-7). Several data support the hypothesis of a potential signaling cascade triggered by this large dose of nanoparticles. First, fluorescence analysis confirmed nanoparticle association to endothelial cells, and while it was not very clear from on-chip assessment due to the interference from nanoparticles on endothelial cells, our fixed-cell fluorescence imaging (vide infra) confirmed nanoparticle association with platelets (Figure 2-8). Second, our preliminary assessment of platelet adhesion to fibronectin-coated (i.e.
Figure 2-9. Aggregation of (a) non-activated and (b) ADP-activated platelets.
without endothelial cells) surfaces has shown that both unactivated and ADP-activated platelets adhered less to fibronectin when they were exposed to 1000 µg/mL than 200 µg/mL (data not shown). Together, these imply an additional signaling cascade between platelets and endothelial cells that is triggered by the high concentration of nanoparticles presented. As adhesion of platelets is a critical step for their in vivo function, future work will probe platelet secretion of chemical messengers and adhesion studies under the same nanoparticle exposure conditions but with different biological stimuli (e.g. thrombin that induces direct secretion from platelets).

In addition to adhesion, the presence of nanoparticles also influenced platelet aggregation behavior (Figure 2-9). Unlike the adhesion assay, the aggregate value obtained in these on-chip experiments is the absolute number of aggregates (normalized to the total area of the collected image). In general, compared to the control cells, the number of platelet aggregates did not significantly change except at the highest nanoparticle dose. Only the 20 µg/mL nanoparticle condition of ADP-activated platelets showed a slightly lower number of aggregates when compared to that in 200 µg/mL condition of ADP-activated platelets (p = 0.01). Both unactivated and ADP-activated platelets exposed to 1000 µg/mL nanoparticles show significantly increased aggregation. These results support the conclusion from the adhesion assay above that the impact of nanoparticles on platelet adherence is mostly physical interference until they reach a certain dose. While on-chip investigation provides an initial assessment of platelet aggregation, the majority of the platelet suspension will pass over the layer of endothelial cells and flow through the device outlet. To assess aggregate formation in non-adherent platelets, we performed
conventional fluorescence imaging on fixed platelets delivered to the outlet to complement our on-chip assay results.

Once collected at the outlet, platelets were fixed on a cover slip and then imaged using a 100x/1.40 oil immersion objective. The imaging studies of the collected platelet suspensions exhibited similar trends to those found in the on-chip assay (Figure 2-10). First, in both the unactivated and ADP-activated platelets, the majority of the platelets were not part of an aggregate. The isolated state of the majority of platelets is consistent with the fact that activated platelets are likely to adhere to the endothelial cells and form aggregates within the microfluidic device. For unactivated conditions, platelets exposed to 200 and 1000 µg/mL nanoparticle concentrations showed a significantly increased number of aggregates (p = 0.06 and p = 0.03, respectively). For ADP-activated conditions, only the 1000 µg/mL nanoparticle condition showed a statistically significant increase in aggregation (p = 0.02). The small population of aggregates was analyzed further, focusing specifically on the larger aggregates, namely those with more than 4 platelets. These were counted, and the ratio of the number of aggregates to the total number of platelets in the population was calculated. The unactivated platelet aggregate ratios were the same under all control and nanoparticle exposure conditions. On the other hand, the ADP-activated platelet ratios were similar to those from the unactivated platelets except for the 1000 µg/mL condition. These ADP-activated platelets showed an increased number of large aggregates at this nanoparticle dose (p = 0.02 Figure 2-10), which is consistent with the on-chip aggregation results. To be noted, as shown in Figure 2-8, the fluorescence imaging also revealed that the nanoparticles are co-localized with the
Figure 2-10. Aggregation assay of (a) non-activated and (b) ADP-activated platelets by fixed-cell fluorescence imaging. (c, d) show aggregation of more than 4 platelets in (c) non-activated platelets and (b) ADP-activated platelets.
platelet aggregates. Isolated platelets show weak nanoparticle fluorescence when the platelet and nanoparticle fluorescence signals are overlaid (Figure 2-8, a) while the platelet aggregates appear to be highly associated with the fluorescent nanoparticles (Figure 2-8, b). All of these results support the conclusion from the on-chip adhesion/aggregation assay, showing high levels of activation and aggregation of platelets at the high nanoparticle dose.

2-4. CONCLUSIONS

This study exploits the use of microfluidics to explore the impact of mesoporous silica nanoparticles on individual components of a simulated blood vessel system, an important portion of the human immune system. As the use of nanoparticles in commercial and pharmacological/medical products increases, in particular those with a proposed delivery method of injection into the blood stream, it is important to understand what potential interactions will occur between nanoparticles and the various blood components. In addition to conventional assessment of nanoparticle impact in cell death, it is critical to go beyond considerations of live/dead viability and assess changes in critical cellular functions upon nanoparticle exposure, and the simple microfluidic platform proposed herein provides a simple but powerful way to investigate the impact of nanoparticles on blood vessel components under physiological conditions. The initial investigation tests the cytotoxicity of FMS and FMS@PEG/TMS NPs under flow and static conditions revealing flow-dependent cytotoxicity of MS NPs (enhancement of cytotoxicity with flow) and providing a potential solution to remove such enhanced cytotoxicity (surface
modification with biocompatible molecules). Then, this study considers cell-NP interactions beyond live/dead type viability by proving its usefulness in studying important cellular functions that occur in blood vessels – platelet adhesion and aggregation. In addition to the variations in the platelet adhesion/aggregation on endothelial cells with different doses of NPs (especially at high dose of NPs) under flow conditions, this follow-up study indicated platelet responses to nanoparticles under flow conditions varied depending on the presence of endothelial cells. This also indicates the importance of considering physiological environments, the flow and cell-cell interactions, when evaluating the impact of nanoparticles on the immune system. To date, a couple studies have investigated microparticle (particles in the scale of microns) interaction with cells under flow conditions as an indicator of leukocyte adhesion to the endothelium\textsuperscript{17-23} but the direct effect of flow conditions on cell-NP interaction has largely gone unconsidered. Accordingly, this research provides very important and interesting insights about nanoparticle-immune system interactions. It will be of significant interest in future studies to study the impact of different shear stress levels on such cell-nanoparticle interactions using a similar approach as well as drug-release profiles under controlled flow conditions. In addition, this platform can be used with cell types beyond platelets and endothelial cells – thus, assessment of other critical cellular functions of other cell types can be easily incorporated. Further development of such approaches will facilitate better understanding of the human immune system and exploit innovative applications for pharmacological and medical purposes.
Chapter 3

Neutrophil Chemotaxis in the Presence of Competing Signals

Adapted from:

3-1. INTRODUCTION

Chemotaxis is an important cellular process in immune response by which cells migrate toward a site of interest, such as the site of infection\(^1,2\), inflammation\(^3,4\), injury\(^5,6\), or even cancer metastasis\(^7-9\). In chemotaxis, cells are guided by signaling molecules called chemoattractants. In unusual circumstances, such as inflammation or infection, specific chemoattractants are up/down-regulated or newly introduced. Fifty years of studies on chemotaxis has identified various chemoattractants and revealed that each cell type within the immune system responds to a unique set of chemoattractant signals\(^10-16\), however, conventional assay techniques have limited ability to fully delineate the biophysical and biochemical mechanisms of chemotaxis. Conventional chamber-based assay platforms, such as the Boyden chamber\(^17-19\) or the Dunn chamber\(^20,21\) rely on diffusion of asymmetrically introduced molecules to establish a chemical gradient; as such, the platforms do not allow precise control over the gradient, the resulting gradient degrades with time, and provide limited ability for quantitative analyses or to delineate relative strength of each chemoattractant for a given cell type. More importantly, experiments using conventional assays are commonly performed in an over-simplified environment, such as static buffer conditions or a single chemoattractant gradient\(^18-25\), which is insufficient to reflect the real chemotactic environment of the cells in vivo as the chemical signaling events in the in vivo milieu occur under complex and dynamic environments. Only a few research efforts have endeavored to take such environmental aspects into account, and these studies have revealed several characteristics not obvious using classical methods, such as the effect of dynamic gradients on chemotaxis or cellular
integration of multiple chemotactic signals. To address the challenge of introducing complexity into chemotaxis studies, this research utilizes microfluidics to form competing gradients of two chemoattractants while monitoring the resulting behavior of individual human neutrophils. Microfluidic platforms enable creation and control of dynamic microenvironments while providing other advantages, such as small volume sample requirements, device optical transparency, and biocompatibility, based on the small device dimensions and the device media (polydimethylsiloxane or PDMS in this research). Using a microfluidic platform, this work investigates chemotaxis of neutrophils, the most abundant leukocytes in human, against all pairwise combinations of four potent chemoattractants. This research reveals relative strength of chemoattractants, adjustment of surface marker expression of neutrophils responding to these molecular signals, and involvement of p38 mitogen-activated protein kinase (MAPK) in regulating chemotaxis.

Neutrophils are chosen as the model cell type to study chemotaxis as they are the most abundant and motile cell type in human with various critical roles in the human immune system. When abnormal events such as infection occur, neutrophils are the first cells that migrate to the event site through chemotaxis, and in several diseases, including lung cancer and asthma, patients have shown abnormalities in their neutrophil populations or the levels of neutrophil chemoattractants. A variety of chemoattractants have been shown to induce neutrophil chemotaxis, including chemokine C-X-C motif ligands 2 and 8 (CXCL2 and CXCL8), leukotriene B4 (LTB4), and formyl-met-leu-phe (fMLP), the four chemoattractants considered in this study. CXCL2 and CXCL8 are part of a
chemokine sub-family produced by many cell types (including neutrophils, T-cells, endothelial cells, epithelial cells, and others)\textsuperscript{36,42} with known roles in several inflammatory diseases. LTB4 is a major lipid product of several cell types and is reported as an indicator of neutrophil activation.\textsuperscript{39, 41, 43} LTB4 is known to play a role in cell adhesion, oxygen metabolite production, and degranulation of immune cells.\textsuperscript{39} Lastly, fMLP is believed to originate from bacterial protein degradation and is known to activate several cell types, including neutrophils, to produce tissue-destructive oxygen-derived free radicals in phagocytic cells as well as induce neutrophil chemotaxis.\textsuperscript{44} The first three are used in this study to mimic a situation in which only host-derived signals exist, and fMLP is employed to mimic a situation in which host- and bacterially derived signals conflict. Neutrophils responding to these molecular signals coordinate a variety of signaling cascades to interpret the input signals and regulate their chemotaxis toward a particular signal. Among those, stimulation of chemotaxis requires phosphorylation of protein kinase B (PKB). Many previous studies have demonstrated that phosphatidylinositol 3-kinase (PI3K)- and p38 mitogen-activated protein kinase (MAPK)-involved signaling cascades are critical to achieve this phosphorylation.\textsuperscript{45,46} p38 MAPK is a protein kinase that governs a wide array of cell functions such as survival, differentiation and proliferation,\textsuperscript{36,47-51} and diverse cytokines, including chemoattractants, have been shown to phosphorylate p38 MAPK in neutrophils.\textsuperscript{48,49} In general, it is clear that inhibition of p38 MAPK impairs neutrophil chemotaxis, but the mechanism of this impairment has not been clearly established. Literature precedent suggests the potential involvement of p38 MAPK in providing directional guidance to the cells.\textsuperscript{48-52} For
example, Heit et al. showed the role of phosphatase and tensin homolog (PTEN) in prioritizing a certain chemical signal soon after Shen et al. demonstrated the role of p38 MAPK in the regulation of PTEN. It is critical for neutrophils to navigate through complex signals in pursuit of bacteria or to the site of injurious events; thus, deeper understanding of the role played by p38 MAPK in chemotaxis will facilitate both fundamental understanding of chemotaxis and the development of potential therapeutic treatments for the diseases mentioned above.

Thus, in this study, neutrophil chemotaxis against all pairwise combinations of the four CXCL2, CXCL8, LTB4, and fMLP chemoattractant signals and the role of p38 MAPK-dependent signaling in neutrophil chemotaxis against such signals was investigated using a microfluidic platform (Figure 3-1). A microfluidic platform is employed in effort to present the neutrophils with a complex and controlled chemoattractant environment. The microfluidic platform allows creation of stable gradients of multiple chemoattractants while facilitating individual neutrophil trajectory analysis and optical assessment of receptor expression. In the consideration of the four chemoattractants, CXCL2, CXCL8, LTB4, and fMLP, used in this study, expression of their respective surface receptors, CXC-motif chemokine receptor 1 (CXCR1), the LTB4-receptor (BLTR), and formyl peptide receptor 2 (FPR2) is fluorescently monitored upon exposure of neutrophils to a chemoattractant gradient. In addition, the surface adhesion molecules CD66b and CD11b are considered based on their purported critical role in neutrophil chemotaxis. CD66b is a cell surface receptor that regulates neutrophil adhesion to fibronectin in the extracellular matrix or E-selectin on endothelial cells. CD11b is an
integrin family receptor that also plays a key role in neutrophil adhesion to endothelial cells by binding to intercellular adhesion molecule-1 (ICAM-1). As such, investigation of these surface markers on p38 MAPK-blocked neutrophils, in parallel to their chemotaxis assessment, can provide insight into how p38 MAPK regulates those surface markers in neutrophil chemotaxis. The commercially available p38 MAPK inhibitor, SB203580, is used in this work; it is a pyridinylimidazole compound that binds selectively to p38 MAPK to inhibit the p38 MAPK signaling cascade.\textsuperscript{56,57} Literature precedent has demonstrated that SB203580 is an effective inhibitor for the p38 MAPK pathway-relevant cellular functions by monitoring oxidative burst activity, stress-induced apoptosis, or downstream substrates of p38 MAPK, such as transcription factors.\textsuperscript{32,58-60} While a couple of recent studies have investigated the chemotaxis of neutrophils in a complex environment,\textsuperscript{41,48,49} situational chemotaxis studies on hierarchical preference of neutrophils, its correlation to surface marker expressions and intramolecular mechanism (involvement of p38 MAPK) has not yet been studied. Therefore, accomplishment of this study improves fundamental understanding chemotaxis as well as progression of pathophysiology of neutrophil-involved diseases.

3-2. EXPERIMENTAL DETAILS

3-2-1. Neutrophil preparation

Ethylenediaminetetraacetic acid (EDTA)-anticoagulated whole human blood samples were obtained from Memorial Blood Center (St. Paul, MN) according to approved IRB
Figure 3-1. Schematic of the microfluidic chemotaxis assay with representative trajectories from 20 neutrophils
protocol E&I ID#07809. All blood samples were collected from healthy donors by skilled professionals at the Memorial Blood Center on the day that experiments were performed and were used immediately after the samples were obtained. Neutrophils were isolated from 5 mL of blood by density gradient centrifugation (Solon, OH) following a previously reported protocol. Neutrophils were resuspended in Hank’s Buffered Salt Solution (HBSS, Sigma-Aldrich, St. Louis, MO) containing 2% human serum albumin (HSA, Sigma-Aldrich, St. Louis, MO) that was kept at 37 °C.

3-2-2. Device fabrication

The microfluidic devices were fabricated using photolithography techniques. Mask designs were printed on transparent film (Cad/Art Service Inc., Bandon, OR) and transferred onto a chrome mask plate with a positive photoresist (Nanofilm, Westlake Village, CA). The transferred designs were developed using a developer solution (351 developer, Rohm and Hass Electronic Materials LLC, Marlborough, MA) to expose the chrome layer underneath the photoresist. Finally, the exposed chrome was etched down in a chrome etchant solution (Cyantek Corporation, Fremont, CA); the remaining photoresist was removed, and the plate was washed and dried. To fabricate a device master, a silicon wafer was cleaned using 1:10 hydrofluoric acid (Avantor Performance Materials, Phillipsburg, NJ):DI water solution and spin-coated with a negative photoresist, SU-8 50 (Microchem, Newton, MA). The mixing channel dimensions were 50 μm (W) x 100 μm (D) x 2330 μm (L) while the cell culture chamber was 400 μm (W) x 100 μm (D) x 2500 μm (L). After a baking step, the device design was transferred onto the photoresist...
layer on a silicon wafer through the previously prepared mask. Following a second baking step, the device design on the silicon wafer was developed in SU-8 developer (Microchem, Newton, MA). Upon completion of the master, a Sylgard 184 elastomer and curing agent mixture (10:1 ratio, Ellsworth Adhesives, Germantown, WI) was cast onto the master and cured overnight at 95 °C. After punching inlet and outlet holes, the PDMS layer was bonded onto a glass coverslip via oxygen plasma treatment at 100 W for 10 seconds. The complete device was then brought into a biosafety cabinet where the device was exposed to UV light for an hour and kept in the biosafety cabinet to promote sterility until use.

3-2-3. Device preparation for experiments

Before use, the channels were washed using a 70% v/v ethanol solution in sterilized Milli-Q water (Millipore, Billerica, MA). After rinsing the channels with sterilized Milli-Q water, a 250 µg/mL solution of human fibronectin (Invitrogen, Carlsbad, CA) in sterilized Milli-Q water was introduced through the channels, and the device was incubated under 5 % CO₂ at 37 °C for an hour. Following fibronectin incubation, 50 μL of neutrophil suspension (2 - 8 x 10⁶ cells/mL) in HBSS containing 2% HSA were injected into the cell culture chamber inlet, and the device was kept in the incubator for another hour to promote neutrophil adhesion. The device was then connected to a syringe pump, and HBSS containing 2% HSA was injected through the channel for 2 minutes to remove non-adherent neutrophils. Then, the desired combination of chemoattractants (e.g. HBSS containing 2% HSA vs. CXCL2 in HBSS containing 2% HSA) was introduced
through the two chemical inlets. CXCL2, CXCL8, and fMLP were purchased from Sigma Aldrich (St. Louis, MO) and LTB4 was purchased from Cayman Chemical (Ann Arbor, MI). All chemicals were used as received.

3-2-4. Gradient confirmation
To ensure that the device was appropriate to generate the expected chemoattractant gradients, computational fluid dynamics (CFD) modeling was performed using COMSOL Multiphysics ver. 4.1. To reduce the time necessary for modeling, stationary analysis was performed on a single mixing channel geometry in 3-D. Flow rates used for modeling were 50, 75, and 100 μL/h, and diffusion coefficients for molecules introduced into the channels ranged from 1.5 x 10^{-5} to 1.0 x 10^{-6} cm²/s, values simulating both small molecule and small protein diffusion.

3-2-5. Time-lapse microscopy
Time-lapse images of neutrophils migrating inside the microfluidic cell culture chamber were obtained using Metamorph Ver. 7.7.5 imaging software on an inverted microscope equipped with a 10 x objective (Nikon, Melville, NY) and a CCD camera (QuantEM, Photometrics, Tucson, AZ). Images of neutrophils in the chamber were acquired every 10 seconds for 30 minutes.

3-2-6. Chemicals
Chemoattractants solutions, 1, 10, and 100 ng/mL of fMLP, CXCL2, CXCL8, and LTB4,
whether or not containing 5.3 μM SB203580, were prepared in HBSS. CXCL2, CXCL8 and fMLP were purchased from Sigma-Aldrich (St. Louis, MO), LTB4 was purchased from Cayman Chemical (Ann Arbor, MI), and SB203580 from Sigma-Aldrich (St. Louis, MO). Anti-CD11b conjugated with AlexaFluor 700 (Life Technologies, Grand Island, NY), anti-CD66b conjugated with AlexaFluor 647 (BD Pharmingen, San Diego, CA), anti-FPR2 conjugated with AlexaFluor350 (Bioss Inc., Woburn, MA), anti-BLTR conjugated with AlexaFluor 647 (AbD Serotec, Raleigh, NC), and anti-CXCR1 conjugated with fluorescein isothiocyanate (Novus Biologicals, Littleton, CO) for human were used as instructed by the manufacturer.

3-2-7. Analysis of data
For experiments with a single chemoattractant gradient of CXCL2, CXCL8, LTB4, or fMLP, 15 or more neutrophils were randomly chosen from each device and analyzed. For experiments with competing gradients of chemoattractants, 10 or more neutrophils were randomly chosen from both the left and right sides of a channel (total of 20 or more neutrophils per device), and separately analyzed. For each condition, neutrophils from minimum 6 different donors were used. MetaMorph, the imaging software, tracked the position of each cell in each frame, and the tracked data was further analyzed using Microsoft Excel. From the obtained data, the migration patterns of neutrophils were described using 3 numeric parameters: motility index (MI), chemotactic index (CI), and effective chemotactic index (ECI). Similar interpretation has been previously done to describe neutrophil chemotaxis. MI indicates how much of total possible neutrophil
movement was in the overall dominant xy direction and is defined as the ratio of final
displacement (d) and maximum displacement (d_{max}) of a neutrophil:

\[ MI = \frac{d}{d_{max}} \]

where \( d_{max} \) is defined as the product of the average migration speed of the tracked
neutrophil and the total migration time.

The orientation of neutrophils during migration is quantified by CI which is defined as:

\[ CI = \frac{x}{d_{total}} \]

where \( x \) is the final displacement along the x-axis (the direction of the gradient) and \( d_{total} \)
is the total migration distance.

ECI (the product of MI and CI) is used to quantify how effective chemotaxis was in the
neutrophil migration. In this report, movement toward the right side of the channel results
in positive CI and ECI values. These three parameters for individual cells are plotted as
histograms, and averaged values of these parameters are plotted per condition. The
migration rate of individual cells was given in pixel/second by the MetaMorph software,
and the % population represents the number of cells in % that moved AWAY from the
fMLP signal. Lastly, in each frame, angular displacement of individual cells was given
either a score of “+1” or “-1” depending on which direction the cell moved (+1 when
toward a higher concentration of fMLP). This data was then averaged for the entire
collection time to indicate how much a cell was distracted on the way toward the fMLP
signal. This angle distribution parameter was measured for the first 1, 4, and 7 minutes,
and for the entire measurement to analyze temporal response of neutrophils to set the
direction of their migration.
T-test and analysis of variance (ANOVA) without assumption of a Gaussian distribution (also called Kruskal-Wallis test) are used for statistical comparison, and the error in all figures in this work are represented as the standard error of the mean.

3-3. RESULTS AND DISCUSSION

3-3-1. Building a gradient using microfluidic mixing channels

The microfluidic platform consists of many serpentine channels and an observation channel (the cell culture chamber) (Figure 3-1). Chemoattractant-containing solutions introduced into the series of serpentine channels for mixing establish a stable gradient of chemoattractant in the observation channel. Simply speaking, when 10 ng/mL fMLP solution to the right inlet and 10 ng/mL CXCL8 solution to the left inlet are introduced, a lateral 0 – 10 ng/mL concentration gradient of fMLP and a 10 – 0 ng/mL concentration gradient of CXCL8 are generated within the observation channel. The key to establishing such a gradient in the observation channel is a complete mixing process in each serpentine channel; therefore, a CFD module in COMSOL Multiphysics 4.1 was used to verify that the device operated as desired (Figure 3-2). In the simulations, 1.5 x 10^{-5} cm^2/s and 1.0 x 10^{-6} cm^2/s diffusion coefficients were used to model small molecules and small proteins, respectively, diffusing through the channels. Figure 3-2 shows an example result for 1.5 x 10^{-5} cm^2/s diffusion coefficient and 100 μL/h flow rate, and all other model results showed a complete mixing process like that depicted in Figure 3-2, confirming that neutrophils will be exposed to the desired concentration gradient of
chemoattractants as specified below. Final gradients can be further evaluated using fluorescence imaging, and there is a study in the literature with intense assessment of gradient establishment in the same type of device. For the purpose of this study, the investigation of neutrophil chemotaxis in a complex environment, the effect of flow itself was also evaluated. The shear stress in the main observation channel generated by the flow conditions was below 0.03 N/m² at the maximum (calculated using COMSOL Multiphysics simulation, data not shown), which is significantly smaller than the shear stress levels in in vivo environments (~1 N/m² - 6 N/m²). Therefore, it is likely that the effect of the flow itself on neutrophil chemotaxis is minimized in this research.

3-3-2. Neutrophil migration under single gradients

In order to understand neutrophil chemotaxis under competing gradients of chemoattractants, neutrophil chemotaxis under single gradients of individual chemoattractants has to first be understood. One reason behind this is because neutrophil chemotaxis is dependent on the type of chemoattractants. The receptors for each chemoattractant are different (CXCR1 and CXCR2 for CXCL2 and CXCL8, BLTR for LTB4, and FPR2 for fMLP) so the expectation from this investigation was that neutrophils would respond differently to different gradients. Another reason behind this investigation is because neutrophil chemotaxis is also dependent on the gradient itself. Even though the same chemoattractant is used, for example, neutrophils under 0 – 10 ng/mL fMLP behave differently to those under 0 – 100 ng/mL fMLP. Thus, using the
Figure 3-2. (a) Schematic of the device used in this work (not to scale), and (b) an example result for the mixing process modeled by COMSOL multiphysics (flow rate of 100 \( \mu \text{L/h} \) and a diffusion coefficient of \( 1.5 \times 10^{-6} \, \text{cm}^2 \text{s}^{-1} \), molecule introduced to the right side inlet with the concentration of 50 ng/mL).
microfluidic platform shown in Figure 3-1, neutrophil chemotaxis under single gradients of CXCL2, CXCL8, LTB4, and fMLP was studied using histogram analysis (data not shown) and the above-mentioned three numerical parameters (MI, CI, and ECI) to quantify how effective chemotaxis is in the neutrophil migration. First, as a control, neutrophil chemotaxis under media containing no chemoattractant was investigated and, as shown in Figure 3-3, cells under the stream of no chemoattractants showed the smallest effectiveness of chemotaxis (the smallest ECI) in their movement. This supports our hypothesis that ~0.03 N/m² shear stress generated by the 100 μL/h flow condition has minimum impact on neutrophil chemotaxis. Then, several gradients of individual chemoattractants were tested to find a gradient that resulted in indistinguishable ECI values. Again, the goal of this study was to reveal preference of neutrophils among the considered chemoattractants; therefore, use of the gradient resulting in similar ECI values among chemoattractants eliminates the possible interference on neutrophil chemotaxis from the gradient itself. First, the results confirmed that neutrophil responses are dependent both on the type and the concentration of chemoattractants, which is in agreement with previously reported results and proves the relevance of the microfluidic approach. Also, the results showed that the gradient with 10 ng/mL maximum concentration of all four chemoattractants gave indistinguishable ECI values (Figure 3-3) (LTB4: 0.19 ± 0.04, fMLP: 0.14 ± 0.04, CXCL8: 0.16 ± 0.02, CXCL2: 0.17 ± 0.03, p = 0.7193). To be noted, MI values for the 10 ng/mL gradient of chemoattractants are different (Figure 3-3). Previous studies have reported that the
Figure 3-3. (a) MIs, (b) CIs, and (c) ECIs under the control condition and single gradient conditions (N = 50 for control, 58 for CXCL8, 55 for CXCL2, 57 for LTB4, and 60 for fMLP). Results were statistically tested using Kruskal-Wallis test (*: p < 0.1, **: p < 0.01, ***: p < 0.0001)
phosphatidylinositol 3-kinase (PI3K) signaling pathway is a critical pathway in neutrophil chemotaxis against host-derived chemoattractant, while p38 mitogen-activated protein (MAP) kinase is used in fMLP-induced neutrophil chemotaxis.\textsuperscript{48,49,52} Considering this previous finding and specific receptors for each chemoattractant, our results confirm that, whether or not the chemical mediators (signal) share receptors or intracellular signaling cascade (PI3K), the resulting migration is not necessarily the same.

3-3-3. Neutrophil migration under fMLP-containing competing gradients

As we found a gradient for all the four chemoattractants that results in similar level of chemotaxis, the investigation of neutrophil chemotaxis proceeded to consideration of competing gradients (CXCL8-fMLP: Figure 3-4, CXCL2-fMLP: Figure 3-5, and LTB4-fMLP: Figure 3-6, and Table 3-1 for summarized results). As mentioned in the experimental section, in each device, 10 or more cells from left side of the culture chamber were chosen and analyzed separately from 10 or more cells randomly chosen from the right side of the culture chamber. In all cases, overall migration of the cells exposed to the three pairwise competing gradients was toward fMLP, indicating that fMLP is the strongest chemoattractant among the four chemoattractants (Table 3-1). For example, under the CXCL8-fMLP competing gradient condition in which 10 ng/mL CXCL8 solution was introduced from the left side inlet while 10 ng/mL fMLP solution was introduced from the right side inlet, the overall trend for migration from both sides of
Figure 3-4. Results from CXCL8-fMLP-mediated chemotaxis (**: p < 0.0001 using unpaired T-test). (a) ECIs from each sides of the channel with $N_{left} = 31$ and $N_{right} = 36$ (positive sign indicates that the chemotaxis was toward fMLP while negative sign indicates that the chemotaxis was toward CXCL8), (b) Populations of the cells moved toward a chemoattractant, (c) MI, CI, and ECI comparison to those from fMLP-mediated chemotaxis (positive sign indicates that the chemotaxis was toward fMLP while negative sign indicates that the chemotaxis was toward CXCL8), and (d) MI, CI, and ECI comparison to those from CXCL8-mediated chemotaxis (positive sign indicates that the chemotaxis was toward CXCL8 while negative sign indicates that the chemotaxis was toward fMLP).
the channel was toward fMLP (Figure 3-4). Precisely, 77% of cells from the left side of the channel and 68% of cells from the right side of the channel moved toward a higher concentration of fMLP (Figure 3-4). The population of cells moving away from fMLP was 27%, which was different from 16% observed during single gradient fMLP-mediated chemotaxis, indicating that the cells sensed and responded to both CXCL8 and fMLP simultaneously. Ultimately, however, fMLP-induced chemotaxis overwhelmed the CXCL8, and 73% of cells in the entire channel moved towards fMLP. Similar trends were observed in all fMLP-containing competing gradient conditions (Figure 3-4 through 3-6). Considering that the effectiveness of chemotaxis under the single gradient of individual chemoattractants was kept the same (Figure 3-3), these data reveal that there is a hierarchy among the four chemoattractants, with the strongest neutrophil response to fMLP. This is in agreement with previous reports that showed that activation of the p38 MAPK pathway overwhelms the PI3K pathway (activated by CXCL8, CXCL2, or LTB4 in this research), and the chemotaxis of neutrophils in the system is dominated by p38 MAPK pathway (activated by fMLP in this research).48,52

More details about chemotaxis can be gained using the measure of MI to indicate motility of neutrophils’ migration, CI to quantify the orientation of neutrophils during migration, and ECI to quantify how effective chemotaxis is in the neutrophil migration. MI, CI, and ECI values from competing gradient conditions were statistically compared to those from the single gradient experiments. In the CXCL8-fMLP competing gradient condition, for
Figure 3-5. Results from CXCL2-fMLP-mediated chemotaxis. (a) ECIs from each side of the channel with $N_{\text{left}} = 30$ and $N_{\text{right}} = 31$ (positive sign indicates that the chemotaxis was toward fMLP while negative sign indicates that the chemotaxis was toward CXCL2), (b) Populations of the cells moving toward a chemoattractant, (c) MI, CI, and ECI comparison to those from fMLP-mediated chemotaxis (positive sign indicates that the chemotaxis was toward fMLP while negative sign indicates that the chemotaxis was toward CXCL2), and (d) MI, CI, and ECI comparison to those from CXCL2-mediated chemotaxis (positive sign indicates that the chemotaxis was toward CXCL2 while negative sign indicates that the chemotaxis was toward fMLP).
example, MI, CI, and ECI values obtained from CXCL8-fMLP competing gradient experiments (0.45 ± 0.02, 0.13 ± 0.03, and 0.06 ± 0.01, respectively) were compared to the values from both the fMLP single gradient condition (0.42 ± 0.04, 0.20 ± 0.04, and 0.14 ± 0.04, respectively, Figure 3-4), and the CXCL8 single gradient condition (0.43 ± 0.03, 0.30 ± 0.03, and 0.16 ± 0.02, respectively, Figure 3-4). A general trend was found from the three fMLP-containing competing gradient conditions; the numerical indices from the competing gradient experiments were comparable to those from the stronger chemoattractant (fMLP in this case) single gradient experiments (Figure 3-5). MI, CI, and ECI values from all fMLP-containing competing gradient conditions, except the MI's under the CXCL2-fMLP condition and LTB4-fMLP condition, were statistically indistinguishable from those of the fMLP single gradient conditions (Figure 3-4 through 3-6, c and d). These results reveal that chemoataxis under the fMLP-containing competing gradient conditions are dominated by the fMLP-induced chemotaxis behavior. The existence of a dominant signal can also be found based on overall population of cells moving away from the stronger signal source. 27%, 20%, and 12% of cells moved away from fMLP under CXCL8-fMLP, CXCL2-fMLP, and LTB4-fMLP competing gradients, respectively. Evidence showing that neutrophils use an hierarchy to prioritize certain chemical signals are accumulating in the literature,49 and the results in this study also suggest that the cells in a complex environment use a hierarchy to prioritize particular signals from the surrounding environment, with an order of fMLP > CXCL8 > CXCL2 >
Figure 3-6. Results from LTB4-fMLP-mediated chemotaxis. (a) ECIs from each side of the channel with $N_{left} = 30$ and $N_{right} = 30$ (positive sign indicates that the chemotaxis was toward LTB4 while negative sign indicates that the chemotaxis was toward fMLP), (b) Populations of the cells moving toward a chemoattractant, (c) MI, CI, and ECI comparison to those from fMLP-mediated chemotaxis (positive sign indicates that the chemotaxis was toward fMLP while negative sign indicates that the chemotaxis was toward LTB4), and (d) MI, CI, and ECI comparison to those from LTB4-mediated chemotaxis (positive sign indicates that the chemotaxis was toward LTB4 while negative sign indicates that the chemotaxis was toward fMLP).
LTB4 when the fMLP signal was superimposed to host-derived chemical signals. Interestingly, the results also indicate that, while there is a dominant signal, all present signals still influence neutrophil chemotaxis. While cells orient toward fMLP in complex conditions (based on CI and % population), the MIs, motilities under the varied competing gradients, are not dominated by fMLP (Figure 3-7). The MIs under CXCL2-fMLP and LTB4-fMLP conditions are 0.71 ± 0.07 and 0.61 ± 0.04 and these are significantly different from the MI for fMLP-mediated chemotaxis (0.42 ± 0.04). More importantly, the analyses show that the competing gradient MI values are actually comparable to those measured from single CXCL2-, LTB4-, and CXCL8-mediated chemotaxis, 0.60 ± 0.04 (p = 0.1792), 0.60 ± 0.06 (p = 0.9285), and 0.45 ± 0.02 (p = 0.7875), respectively (Figure 3-7). The MIs under fMLP-containing competing gradient conditions seem to originate from the secondary chemoattractant, suggesting that, while overall chemotaxis is dominated by one signal, the minor chemoattractant contributes to the character of chemotaxis, as well (Figure 3-7). Combined, these data suggest that neutrophils prioritize a certain signal, and both the dominant and the secondary chemoattractants contribute to the cellular migration behavior (orientation and motility). The results demonstrate a hierarchy among the employed chemoattractants in which neutrophils prioritize fMLP > CXCL8 > CXCL2 > LTB4 (Table 3-1 and Figure 3-7) while every signal present in the environment contributes to their chemotaxis. Again, this order reflects the fact that the p38 MAPK-dependent signaling pathway (for the fMLP signal) overwhelms PI3K-dependent signaling pathway (for the host-derived
Figure 3-7. MI, CI, and ECI comparison summary (a) Comparison of MIs, CIs, and ECIs from fMLP-containing conditions, (b) Comparison of MIs, CIs, and ECIs from CXCL8-containing conditions, (c) Comparison of MIs, CIs, and ECIs from LTB4 containing conditions, and (d) Comparison of MIs, CIs and ECIs from CXCL2-containing conditions. MIs, CIs, and ECIs from competing gradient conditions were statistically tested using unpaired T-test against MI, CI, and ECI from single gradient conditions (*: p < 0.1, **: p < 0.01, ***: p < 0.0001).
chemoattractants). An in vivo study reported that the PI3K-dependent pathway is not required for fMLP-induced chemotaxis but that it accelerates fMLP-induced chemotaxis. Comparison to this in vivo study reveals whether or not the results obtained using this in vitro platform mimic the in vivo milieu, while also providing some biophysical insight into neutrophil behavior.

3-3-4. Neutrophil migration under a competing gradient of host-derived chemoattractants

Neutrophils were also exposed to competing gradients that were combinations of only host-derived chemoattractants (LTB4-CXCL8, CXCL2-CXCL8, and CXCL2-LTB4). This investigates the hierarchy of chemoattractants that, whether they share receptors or not, trigger the same chemical signaling cascades (PI3K-dependent). In LTB4-CXCL8 and CXCL2-CXCL8 conditions, it was common for the cells to migrate preferentially toward CXCL8 (Figure 3-8 and 3-9). For example, under the LTB4-CXCL8 competing gradient condition (10 ng/mL LTB4 solution on the left side and 10 ng/mL CXCL8 solution on the right side), overall neutrophil chemotaxis was toward CXCL8 (Figure 3-8), with a total of 66% of cells in the entire channel moved toward CXCL8 (Figure 3-8). The three numerical parameters, MI, CI, and ECI, were used to quantitatively analyze the neutrophil migration patterns (Table 3-1). MI, CI, and ECI values from competing gradient conditions were statistically compared to those from single gradient experiments (Figure 3-8 and 3-9). In the LTB4-CXCL8 competing gradient condition (Figure 3-9), the obtained MI, CI, and ECI values were compared to
Figure 3-8. Results from LTB4-CXCL8-mediated chemotaxis (**: \( p < 0.01 \) and ***: \( p < 0.0001 \) using unpaired T-test). (a) ECIs from each side of the channel with \( N_{\text{left}} = 32 \) and \( N_{\text{right}} = 33 \) (positive sign indicates that the chemotaxis was toward CXCL8 while negative sign indicates that the chemotaxis was toward LTB4), (b) Populations of the cells moving toward a chemoattractant, (c) MI, CI, and ECI comparison to those from CXCL8-mediated chemotaxis (positive sign indicates that the chemotaxis was toward CXCL8 while negative sign indicates that the chemotaxis was toward LTB4), and (d) MI, CI, and ECI comparison to those from LTB4-mediated chemotaxis (positive sign indicates that the chemotaxis was toward LTB4 while negative sign indicates that the chemotaxis was toward CXCL8).
Figure 3-9. Results from CXCL2-CXCL8-mediated chemotaxis. (a) ECIs from each side of the channel with $N_{\text{left}} = 35$ and $N_{\text{right}} = 33$ (positive sign indicates that the chemotaxis was toward CXCL8 while negative sign indicates that the chemotaxis was toward CXCL2), (b) Populations of the cells moving toward a chemoattractant, (c) MI, CI, and ECI comparison to those from CXCL8-mediated chemotaxis (positive sign indicates that the chemotaxis was toward CXCL8 while negative sign indicates that the chemotaxis was toward CXCL2), and (d) MI, CI, and ECI comparison to those from CXCL2-mediated chemotaxis (positive sign indicates that the chemotaxis was toward CXCL2 while negative sign indicates that the chemotaxis was toward CXCL8).
Figure 3-10. Results from CXCL2-LTB4-mediated chemotaxis. (a) ECIs from each side of the channel with $N_{\text{left}} = 30$ and $N_{\text{right}} = 31$ (positive sign indicates that the chemotaxis was toward LTB4 while negative sign indicates that the chemotaxis was toward CXCL2), (b) Populations of the cells moving toward a chemoattractant, (c) MI, CI, and ECI comparison to those from LTB4-mediated chemotaxis (positive sign indicates that the chemotaxis was toward LTB4 while negative sign indicates that the chemotaxis was toward CXCL2), and (d) MI, CI, and ECI comparison to those from CXCL2-mediated chemotaxis (positive sign indicates that the chemotaxis was toward CXCL2 while negative sign indicates that the chemotaxis was toward LTB4).
the values from both the CXCL8 single gradient condition and the LTB4 single gradient condition (Figure 3-8, c and d). While CIs, ECIs, and % population data indicate chemotaxis in favor of CXCL8 under LTB4-CXCL8 and CXCL2-CXCL8 gradients, CIs and ECIs under the two competing gradient conditions were significantly lower than the CIs and ECIs obtained under single gradient conditions, and the MI of neutrophils under the LTB4-CXCL8 gradient was higher than that of neutrophils under CXCL8 gradient (Figure 3-8 and 3-9). These indicate that, similarly to neutrophil chemotaxis under fMLP-containing competing gradients, neutrophil chemotaxis under host-derived chemoattractant competing gradient conditions cannot be represented as the stronger chemoattractant (CXCL8 in this case)-mediated chemotaxis. In the CXCL2-LTB4 condition (Figure 3-10), the results suggested that LTB4 was more potent than CXCL2, and it was observed that the MI, CI, and ECI values were significantly reduced compared to those from either single CXCL2 or LTB4 single gradient conditions. This indicates that neutrophil chemotaxis under the CXCL2-LTB4 competing gradient condition cannot be represented by neutrophil chemotaxis under the more potent chemoattractant (LTB4); however, as stated, it was found that there was still a hierarchy that neutrophils use to prioritize the LTB4 signal. In summary of all above results under host-derived chemoattractant competing gradients, it is found that neutrophils have a hierarchy of CXCL8 > LTB4 > CXCL2 among the three chemoattractants.

Experiments under the CXCL2-CXCL8, CXCL2-LTB4, and LTB4-CXCL8 investigate how neutrophils respond to signals that trigger the same signaling cascade (in this case, PI3K-dependent pathway). As a brief reminder, stimulation of neutrophil chemotaxis
requires phosphorylation of PKB, and PI3K- and p38 MAPK-involved signaling cascades are two main signaling routes to achieve this phosphorylation.\textsuperscript{45,46} CXCL2 and CXCL8 interact with neutrophils via CXCR1 and CXCR2 receptors while LTB4 interacts with neutrophils via BLTR receptors,\textsuperscript{36,47-51} and in general, competing gradients of chemoattractants that initiate signaling cascades via either CXCR receptors or BLTR receptors suppressed chemotaxis toward the competing signal. For example, a LTB4 signal (via BLTR receptor) suppressed CXCL2-induced chemotaxis (via CXCR receptors), resulting in significantly lower MI, CI, and ECI values than those from CXCL2-mediated chemotaxis (Figure 3-10). CXCL2 (via CXCR receptors) also suppressed the effectiveness of CXCL8-induced chemotaxis (Figure 3-9). These results suggest that, even when competing chemoattractants trigger the same signaling cascade, there is still a dominant signal and a minor signal, both of which mediate and contribute to the outcome neutrophil chemotaxis.

Interestingly, depending on whether or not the p38 MAPK-dependent pathway is triggered with the PI3K pathway, a different hierarchy among the three host-derived chemoattractants is obtained. When only the PI3K pathway is triggered (host-derived chemoattractants only), CXCL8 is the strongest among the three host-derived chemoattractants, and LTB4 showed stronger effects on neutrophil chemotaxis when it is introduced in competition with CXCL2. The overall population of cells that moved toward LTB4 under the LTB4-CXCL8 gradient was more than those that moved toward CXCL2 under the CXCL2-CXCL8 condition (Figure 3-8 and 3-9). Experiments under the CXCL2-LTB4 condition confirmed the hierarchy of CXCL8 > LTB4 > CXCL2
Table 3-1. Result summary for all single and competing gradient conditions.

<table>
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<tr>
<th>Conditions</th>
<th>N_{total}</th>
<th>% of cells moved toward right * (%)</th>
<th>MI</th>
<th>CI</th>
<th>ECI</th>
<th>ECI (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N_{left}</td>
<td>N_{right}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCL8-fMLP</td>
<td>67</td>
<td>77</td>
<td>68</td>
<td>73</td>
<td>0.447 ± 0.0224</td>
<td>0.135 ± 0.0251</td>
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<tr>
<td>CXCL2-fMLP</td>
<td>61</td>
<td>91</td>
<td>69</td>
<td>80</td>
<td>0.713 ± 0.0705</td>
<td>0.176 ± 0.0355</td>
</tr>
<tr>
<td>LTB4-fMLP</td>
<td>60</td>
<td>81</td>
<td>98</td>
<td>88</td>
<td>0.606 ± 0.0444</td>
<td>0.813 ± 0.0402</td>
</tr>
<tr>
<td>LTB4-CXCL8</td>
<td>65</td>
<td>89</td>
<td>38</td>
<td>66</td>
<td>0.631 ± 0.0558</td>
<td>0.130 ± 0.0415</td>
</tr>
<tr>
<td>CXCL2-CXCL8</td>
<td>68</td>
<td>66</td>
<td>78</td>
<td>73</td>
<td>0.455 ± 0.0350</td>
<td>0.116 ± 0.0282</td>
</tr>
<tr>
<td>CXCL2-LTB4</td>
<td>61</td>
<td>74</td>
<td>83</td>
<td>69</td>
<td>0.278 ± 0.0314</td>
<td>0.106 ± 0.0208</td>
</tr>
<tr>
<td>fMLP</td>
<td>60</td>
<td>N/A</td>
<td>N/A</td>
<td>84</td>
<td>0.423 ± 0.0413</td>
<td>0.204 ± 0.0406</td>
</tr>
<tr>
<td>CXCL8</td>
<td>58</td>
<td>N/A</td>
<td>N/A</td>
<td>89</td>
<td>0.430 ± 0.0274</td>
<td>0.301 ± 0.0277</td>
</tr>
<tr>
<td>LTB4</td>
<td>57</td>
<td>N/A</td>
<td>N/A</td>
<td>79</td>
<td>0.599 ± 0.0603</td>
<td>0.257 ± 0.0418</td>
</tr>
<tr>
<td>CXCL2</td>
<td>55</td>
<td>N/A</td>
<td>N/A</td>
<td>82</td>
<td>0.596 ± 0.0375</td>
<td>0.234 ± 0.0347</td>
</tr>
</tbody>
</table>

1 * indicates the chemoattractants in bold.
Figure 3-11. Neutrophil chemotaxis within an fMLP gradient (a) Motility index (MI) and chemotaxis index (CI) of untreated (green) and p38 MAPK-blocked (blue) neutrophils. (b) Average migration rate of untreated (green) and p38 MAPK-blocked (blue) neutrophils. (c) Angle distribution of untreated (circle) and p38 MAPK-blocked (triangle) neutrophils with a value of 1 indicating no deviation from the direction toward fMLP (each point represents angular distribution of a cell and the solid line indicates the average).
(Figure 3-10). However, under fMLP-containing competing gradient conditions where the PI3K pathway is triggered along with the p38 MAPK-dependent pathway, the hierarchy was CXCL8 > CXCL2 > LTB4, showing stronger effects of CXCL2 than LTB4. This alteration in the hierarchy might be caused by the extra p38 MAPK-dependent pathway. Alternatively, the alteration might be caused by saturation/desensitization of surface receptors for a certain chemoattractant under the conditions used in this study (10 ng/mL concentration), which likely result in suppressed chemotaxis toward the overdosed chemoattractant. Thus, this study further investigated the role of p38 MAPK in regulating neutrophil chemotaxis.

3-3-5. Chemotaxis and p38 MAPK
As the initial investigation provided a haunch for the involvement of p38 MAPK in prioritizing a certain signal, the role of p38 MAPK in neutrophil chemotaxis was studied more in detail. As briefly mentioned above, a commercial inhibitor of p38 MAPK, SB203580, was used at the final concentration of 5.3 μM SB203580. This concentration was chosen based on precedent reports demonstrating the effectiveness of SB203580 at the concentration range of 1 – 10 μM in p38 MAPK inhibition by monitoring secretion of superoxide,74 cytokines such as interleukin 6,59 tumor necrosis factor α,58 or downstream proteins of p38 MAPK such as transcription factor 2 or MAPK-activated protein kinase 2.60 To be noted, high concentrations of SB203580 (above 10 μM) can activate extracellular signal-regulated kinase (ERK), which is also suggested to have a role in neutrophil chemotaxis59,60,75-78 but is not a focus of this work. This investigation started
with monitoring chemotaxis of neutrophils, whether or not exposed to SB203580, against fMLP signal. Again, the numerical parameters, MI as an indicator of how active cells are, CI as an indicator of the directionality in cellular migration, % population as an indicator of how potent a chemoattractant is, and average migration rate (pixel/second) and angular distribution were used to quantitatively analyze neutrophil migration. As a brief reminder, angular distribution is a measure of “physical” deviation of cellular migration from the direction toward signal. As shown in Figure 3-11, pre-incubation with SB203580 resulted in slightly hastened migration rate of neutrophils when compared to untreated neutrophils (0.34 ± 0.01 compared to 0.29 ± 0.01, p = 0.004). However, this enhanced speed was not reflected in MI and CI (Figure 3-11). This seems to be because SB203580-treated neutrophils, neutrophils lost p38 MAPK activity, lost their ability to set their direction to the fMLP signal correctly, and wasted their extra mobility by moving back and forth in the parallel direction of the fMLP gradient (0.27 ± 0.05 compared to 0.42 ± 0.04, p = 0.0217).

The results demonstrate that neutrophils are still capable of recognizing and migrating toward a strong fMLP signal without p38 MAPK activity - during 30 minutes of exposure to the fMLP gradient, a majority population of cells migrated toward the fMLP signal (only 10% of untreated cells and 17% p38 MAPK-blocked cells moved away from the fMLP signal). In addition, the results indicate that more cells deviate from the direction of the gradient when treated with SB203580. Previous studies reported disruption of neutrophil chemotaxis when treated with SB203580, and the physical deviation observed in our results might be the cause of the disruption – as the previous studies used
conventional chemotaxis assays, they were not able to provide any further information beside the reduced number of migrated neutrophils as the indicator of “disruption”. Also, the enhanced migration rate in p38 MAPK-blocked cells could be explained by the known effect of p38 MAPK in controlling actin polymerization.\textsuperscript{79,80} Again, however, despite the single, straightforward chemoattractant signal, the cells did not translate this extra mobility into directed motion when they lost p38 MAPK activity.

On the other hand, as this deviation from the gradient direction seems more significant in the initial 4 minutes (Figure 3-11, c), another hypothesis was made: while pre-expressed p38 MAPK was blocked by the inhibitor, the 30 minute period on the device without inhibitor might be long enough for de novo expression of enzymes (p38 MAPK in this case).\textsuperscript{51,53} To test such capability of neutrophils, the chemotaxis within the fMLP gradient was compared for neutrophils exposed to inhibitors for enzymes before and throughout assessments. In these experiments, the p38 MAPK inhibitor, SB203580 (5.3 \textgreek{M}) and LY294002, a commercially available inhibitor for PI3K (6.5 \textgreek{M}, Sigma-Aldrich, St. Louis, MO) were used as enzyme inhibitors. 1 – 10 \textgreek{M} LY294002 has shown its effectiveness in PI3K inhibition with no influence on ERK.\textsuperscript{81-84} In one set of experiments (denoted as “Temporal”), neutrophils were treated with either SB203580 or LY294002, plated onto a device, and then exposed to a 0 – 10 ng/mL fMLP gradient. In the other set of experiments (denoted as “Continuous”), neutrophils were treated with either SB203580 or LY294002, plated onto a device, and then exposed to a 0 – 10 ng/mL fMLP gradient that contained a constant concentration of either 5.3 \textgreek{M} SB203580 or 6.5 \textgreek{M}
Figure 3-12. Chemotaxis against the fMLP gradient. (a) Neutrophils temporally (green) and continuously (blue) exposed to p38 MAPK inhibitor, SB203580. (b) Neutrophils temporally (green) and continuously (blue) exposed to PI3K inhibitor, LY294002. p > 0.05 in all cases.
LY294002. There were no significant differences in the chemotactic behavior among these two exposure conditions (Figure 3-12). This suggests that 30 minutes is not long enough for the cells to newly express sufficient enzyme to influence chemotactic behavior, and thus, our investigation is not biased by potential resurrection of p38 MAPK upon neutrophil activation.

To further investigate the role of the p38 MAPK-dependent pathway in neutrophil chemotaxis, p38 MAPK-blocked neutrophils were then exposed to competing gradients of fMLP, CXCL8, CXCL2, and LTB4. Pairwise comparison of chemotaxis of neutrophils, whether or not pre-incubated with SB203580, in these gradients reveals the response hierarchy and the potential role of p38 MAPK in providing directional cues to neutrophils.

Again, for p38 MAPK inhibition, neutrophils were exposed to 5.3 µM SB203580 before being plated onto the device. The schematic to establish competing gradients of chemoattractants was the same as is the initial investigation. As an example, the introduction of 10 ng/mL fMLP to the right inlet and 10 ng/mL CXCL8 to the left inlet establishes a 10 – 0 ng/mL concentration gradient of CXCL8 superimposed a 0 – 10 ng/mL concentration gradient of fMLP in the observation channel (Figure 3-1). First, the results demonstrate that the majority of neutrophils are still able to prioritize the fMLP signal under all competing gradients (Figure 3-13 through 3-15). As shown in Figure 3-13, the p38 MAPK-blocked cells responding to the CXCL8-fMLP gradient did not result in altered MI and CI when compared to untreated cells. This trend of unaltered MI and CI was observed from all competing gradient conditions (Figure 3-13 through 3-15). As shown in Figure 3-13, the migration speed was slightly slower in p38 MAPK-blocked
Figure 3-13. Neutrophil chemotaxis within a CXCL8-fMLP competing gradient (a) Motility index (MI) and chemotaxis index (CI) of untreated (green) and p38 MAPK-blocked (blue) neutrophils. (b) Average migration rate of untreated (green) and p38 MAPK-blocked (blue) neutrophils. (c) Angle distribution of untreated (circle) and p38 MAPK-blocked (triangle) neutrophils with a value of 1 indicating no deviation from the direction of fMLP (each point represents angular distribution of a cell and the solid line indicates the average).
Figure 3-14. Chemotaxis against a CXCL2-fMLP competing gradient (a) Motility index (MI) and chemotaxis index (CI) of untreated (green) and p38 MAPK-blocked (blue) neutrophils. (b) Average migration rate of untreated (green) and p38 MAPK-blocked (blue) neutrophils. (c) Angle distribution of untreated (circle) and p38 MAPK-blocked (triangle) neutrophils with a value of 1 indicating no deviation from the direction toward fMLP (each point represents angular distribution of a cell and the solid line indicates the average).
Figure 3-15. Chemotaxis against LTB4-fMLP competing gradient (a) Motility index (MI) and chemotaxis index (CI) of untreated (green) and p38 MAPK-blocked (blue) neutrophils. (b) Average migration rate of untreated (green) and p38 MAPK-blocked (blue) neutrophils. (c) Angle distribution of untreated (circle) and p38 MAPK-blocked (triangle) neutrophils with a value of 1 indicating no deviation from the direction toward fMLP (each point represents angular distribution of a cell and the solid line indicates the average).
Figure 3-16. % population of untreated (green) and p38 MAPK-blocked (blue) cells that moved away from the fMLP signal within competing gradients. (a) CXCL8-fMLP gradient. (b) CXCL2-fMLP gradient. (c) LTB4-fMLP gradient. (d) Summary. CXCL8-fMLP, CXCL2-fMLP, LTB4-fMLP gradients are denoted as 8-fMLP, 2-fMLP, and 4-fMLP, respectively.
neutrophils, compared to untreated cells, under the CXCL8-fMLP gradient (0.26 ± 0.01 for p38 MAPK-blocked cells compared to 0.33 ± 0.02 for untreated neutrophils, p = 0.0011). Also, p38 MAPK-blocked neutrophils showed more directional deviation in general. Under the CXCL8-fMLP competing gradient, significantly more p38 MAPK-blocked neutrophils hesitated in deciding which signal to follow (0.00 ± 0.05 for p38 MAPK-blocked cells compared to 0.26 ± 0.04 for untreated cells, p < 0.0001). This trend was consistent among all competing gradient conditions, and was more obvious in the first 4 minutes of each trial than the entire 30 minutes (Figure 3-13 through 3-15). A more important trend is shown in Figure 3-16. As mentioned, the % population data shows how many cells move AWAY from the fMLP signal; thus, under competing gradient conditions, this % population data represents how potent the non-fMLP chemoattractant is against fMLP. Untreated neutrophils showed their preference for chemoattractants in the order of CXCL8 > CXCL2 > LTB4 (47.8, 32.4, and 29.7% cells moved away from fMLP) while p38 MAPK-blocked neutrophils had them in the order of CXCL8 > LTB4 > CXCL2 (45.6, 39.5, and 32.6% population of cells moved away from fMLP), indicating a role for p38 MAPK in prioritizing a certain signal. In the previous study of this research, it was found that fMLP was the most potent chemoattractant among those considered. Here, it is clear that, despite the difference in extent, neutrophils without p38 MAPK activity are still capable of prioritizing fMLP over CXCL8, CXCL2, and LTB4. In addition, the previous study showed that fMLP was a signaling cue that altered the hierarchical response of neutrophils to CXCL2 and LTB4; CXCL2 was more potent when either of those competes with fMLP while LTB4 was
more potent when they compete with each other (where no fMLP is present). Such alternating preference from the previous study does not hold in p38-blocked neutrophils; even though the chemical signals were competing with fMLP, LBT4 was always more potent than CXCL2 in p38 MAPK-blocked neutrophils. The results suggest that the role of p38 MAPK falls in sensing and prioritizing a certain signal. p38 MAPK-blocked neutrophils under competing gradient conditions had a slower migration rate than untreated cells, likely because neutrophils lost their ability, in part, to interpret surrounding signals.

3-3-6. Surface receptors and p38 MAPK

Expanding the investigation of this research to the role of p38 MAPK in neutrophil chemotaxis, this research added another analytical strength into the microfluidic platform. Considering the fact that cells communicate with surrounding chemical signals through surface receptors, this study monitored surface receptor expressions, in parallel to monitoring chemotaxis, by fluorescence imaging. As it was revealed that p38 MAPK has a role in directing neutrophils to a designated site, this investigation provides insight into whether the p38 MAPK regulation is through controlling surface receptors. Herein, surface expression of FPR2, BLTR, and CXCR1, the specific receptors for the chemoattractants used in this research, on p38 MAPK-blocked cells was assessed from individual cells using fluorescently-labeled antibodies and compared to those from untreated cells. Again, control cells indicate cells exposed to no chemoattractant, and all reported values here are maximum fluorescence intensity in arbitrary units (AU\textsubscript{max}). In
Figure 3-17. Expression of surface receptors after exposure to no gradient, fMLP gradient, CXCL8 gradient, and a CXCL8-fMLP competing gradient (denoted as 8-fMLP). (a) FPR2 expression in untreated (green) and p38 MAPK-blocked (blue) neutrophils. (b) BLTR expression in untreated (green) and p38 MAPK-blocked (blue) neutrophils. (c) CXCR1 expression in untreated (green) and p38 MAPK-blocked neutrophils.
Table 3-2. Fluorescence assessment of surface markers.

<table>
<thead>
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<th></th>
<th>Untreated</th>
<th></th>
<th>p38 MAPK-blocked</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>FMLP</td>
<td>CXCL8</td>
<td>8-MLP</td>
</tr>
<tr>
<td>CD11b</td>
<td>1498 ± 28.5</td>
<td>1078 ± 5.3</td>
<td>1056 ± 4.2</td>
<td>1056 ± 4.7</td>
</tr>
<tr>
<td></td>
<td>(p &lt; 0.0001)</td>
<td>(p &lt; 0.0001)</td>
<td>(p &lt; 0.0001)</td>
<td>(p &lt; 0.0001)</td>
</tr>
<tr>
<td>CD66b</td>
<td>2786 ± 60.3</td>
<td>2007 ± 45.1</td>
<td>1785 ± 35.0</td>
<td>2206 ± 94.3</td>
</tr>
<tr>
<td></td>
<td>(p &lt; 0.0001)</td>
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</tr>
<tr>
<td>FPR2</td>
<td>2046 ± 25.7</td>
<td>1816 ± 10.1</td>
<td>2095 ± 19.3</td>
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<tr>
<td></td>
<td>(p &lt; 0.0001)</td>
<td>(p &lt; 0.0001)</td>
<td>(p &lt; 0.0001)</td>
<td>(p &lt; 0.0001)</td>
</tr>
<tr>
<td>BLTR</td>
<td>1216 ± 6.4</td>
<td>1183 ± 7.8</td>
<td>1328 ± 29.5</td>
<td>1766 ± 100.9</td>
</tr>
<tr>
<td></td>
<td>(p &lt; 0.01)</td>
<td>(p &lt; 0.0001)</td>
<td>(p &lt; 0.0001)</td>
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</tr>
<tr>
<td>CXCR1</td>
<td>1501 ± 9.1</td>
<td>1472 ± 8.6</td>
<td>1638 ± 24.5</td>
<td>1682 ± 31.2</td>
</tr>
<tr>
<td></td>
<td>(p &lt; 0.1)</td>
<td>(p &lt; 0.0001)</td>
<td>(p &lt; 0.0001)</td>
<td>(p &lt; 0.0001)</td>
</tr>
</tbody>
</table>

* Maximum fluorescence intensity in arbitrary units (p value from t-test comparing to the control)
untreated cells, the surface receptors, FPR2, BLTR, and CXCR1, showed decreased expression upon stimulation. Compared to the untreated cells exposed to no chemoattractant signals, cells exposed to the fMLP gradient showed lower expression of all three receptors (Figure 3-17, Table 3-2). In the case of FPR2 expression, this may not mean actual down-regulation of FPR2 after stimulation as fMLP can bind to this receptor, making the site unavailable for labeling. fMLP exposure of p38 MAPK-blocked cells showed a similar trend in surface receptor expression. FPR2, BLTR, and CXCR1 expressions were generally lower in fMLP-exposed cells than those in control cells (Figure 3-17, Table 3-2). On the other hand, exposure to the CXCL8 gradient or the CXCL8-fMLP gradient resulted in different trends. First, up-regulation of FPR2, BLTR, and CXCR1 surface expression was observed in untreated cells exposed to CXCL8 (Figure 3-17, Table 3-2). Exposure of untreated cells to CXCL8-fMLP gradient resulted in, again, up-regulated FPR2, BLTR, and CXCR1 level (Figure 3-17, Table 3-2). However, exposure of p38 MAPK-blocked cells to the CXCL8 or the CXCL8-fMLP gradient resulted in down-regulation of those surface markers. In p38 MAPK-blocked cells, compared to no chemoattractant condition, surface receptors FPR2, BLTR, and CXCR1 were down-regulated when cells were exposed to the CXCL8 (Figure 3-17, Table 3-2). Similarly, FPR2, BLTR, and CXCR1 expression of p38 MAPK-blocked cells exposed to CXCL8-fMLP were lower than those in control cells (Figure 3-17, Table 3-2). These results indicate that p38 MAPK also participates in the regulation of these specific surface receptors upon stimulation.

In untreated cells, exposure to an fMLP gradient resulted in down-regulation of
chemoattractant receptors, with the largest effect on FPR2. This may be due to fMLP binding to the receptor. On the other hand, exposure to CXCL8 or the CXCL8-fMLP competing gradient resulted in up-regulation of FPR2, BLTR and CXCR1 in general. To be noted, BLTR expression in p38-blocked cells did not show as significant change as that seen in CXCR1 level. The chemotaxis data show that the inhibition of p38 MAPK resulted in stronger favor of neutrophils for LTB4 than CXCL2, and the fluorescence data suggest that this may be in part through diminishing the preference on CXCL2 rather than enhancing the preference on LTB4. Comparison of untreated vs. p38 MAPK-blocked neutrophils responding to fMLP shows higher levels of FPR2, BLTR, and CXCR1 in p38 MAPK-blocked cells (p < 0.01 for FPR2 and BLTR, and p = 0.0185 for CXCR1). On the other hand, p38 MAPK-blocked cells responding to CXCL8 or CXCL8-fMLP resulted in lower levels of FPR2, BLTR, and CXCR1 expression compared to untreated cells (p < 0.01 for all receptors in CXCL8 condition and p < 0.0001 for all receptors in CXCL8-fMLP condition). These indicate that p38 MAPK is involved in the responding mechanism of neutrophils to fMLP and CXCL8 to a different extent. Also, in untreated cells, FPR2, BLTR, and CXCR1 expression was particularly high in the CXCL8-fMLP condition compared to either the CXCL8 or fMLP conditions, indicating a synergistic impact of CXCL8 and fMLP in the regulation of those surface receptors. However, this synergistic impact of CXCL8 and fMLP was unclear in p38 MAPK-blocked cells, indicating potential involvement of p38 MAPK in intracellular signal enhancement. All results demonstrate again that the role of p38 MAPK is more important in sensing/interpreting surrounding signals, obviously in part by regulating surface receptor
Figure 3-18. Expression of adhesion molecules after exposure to no gradient, fMLP gradient, CXCL8 gradient, and CXCL8-fMLP competing gradient (denoted as 8-fMLP). (a) CD11b expression in untreated (green) and p38 MAPK-blocked (blue) neutrophils. (b) CD66b expression in untreated (green) and p38 MAPK-blocked (blue) neutrophils.
expression, rather than actual movement.

3-3-7. Adhesion molecules and p38 MAPK

In addition to surface receptor – chemoattractant interaction, adhesion of neutrophils to a surface/cell layer is critical in chemotaxis; thus, surface expression of adhesion molecule CD11b and CD66 was assessed in both untreated and p38 MAPK-blocked neutrophils. As it was revealed that p38 MAPK has a role in neutrophil chemotaxis, this investigation provides insight into whether the p38 MAPK regulation is, at least in part, through controlling cellular adhesion. In this study, surface markers on individual cells were assessed using fluorescently labeled antibodies specific for either CD11b or CD66b, and all reported values in this chapter are maximum fluorescence intensity from individual cells in arbitrary units (denoted as AU_max). For clarification purpose, throughout this chapter, untreated cells indicate cells that are not treated to SB203580, and control cells indicate cells, whether or not treated with SB203580, exposed to no chemoattractant. In untreated cells, exposure to the fMLP gradient resulted in down-regulation of CD11b and CD66b expression when compared to control cells (Figure 3-18, Table 3-2). However, SB203580-treated cells exposed to the fMLP gradient showed unchanged expression of CD11b (p = 0.1804) and up-regulation of CD66b (Figure 3-18, Table 3-2) when compared to control cells. Similarly, despite the difference in extent, both CD11b and CD66b were down-regulated in untreated cells after exposure to CXCL8 (Figure 3-18, Table 3-2). In p38 MAPK-blocked cells, on the other hand, exposure to CXCL8 resulted in unchanged CD11b levels (p = 0.1623) and up-regulated CD66b levels (Figure 3-18, Table 3-2). The same trend was found in the CXCL8-fMLP conditions. In untreated cells,
both CD11b and CD66b (Figure 3-18, Table 3-2) were down-regulated upon exposure to the CXCL8-fMLP gradient. On the other hand, in p38 MAPK-blocked cells, the CXCL8-fMLP gradient resulted in unchanged CD11b levels ($p = 0.6673$) and up-regulated CD66b levels (Figure 3-18, Table 3-2). To be noted, treating neutrophils with SB203580 resulted in significant down-regulation of both CD11b and CD66b expression ($p < 0.0001$ for both CD11b and CD66b), and CD66b expression of p38 MAPK-blocked cells exposed to a chemoattractant gradient is always higher than that of untreated cells ($p < 0.0001$ for all comparison). These results clearly demonstrate the involvement of p38 MAPK in the regulation of CD11b and CD66b.

Down-regulation of adhesion molecules upon p38 MAPK inhibition may lead to less adhesion of neutrophils, but once they are adhered, they may be better equipped to respond faster. Our observations were indicative of this as more cells washed out of the device at the beginning of experiments in p38 MAPK-blocked cells, but the adherent cells moved faster within a gradient of fMLP. In untreated cells, CD11b levels were highest in control cells and similar between a gradient of fMLP, CXCL8 and a competing gradient of CXCL8-fMLP conditions. Interestingly, on the other hand, CD66b expression levels were in the order of CXCL8-fMLP > fMLP > CXCL8 in untreated cells. This indicates that CXCL8 and fMLP have different impacts on CD66b expression, and that CXCL8 and fMLP have a synergistic impact in regulating CD66b expression. This helps to explain our previous finding that all presenting signals cooperatively contribute to the outcome chemotaxis.\textsuperscript{54} A similar cooperative effect was found in the p38 MAPK-blocked cells even though the synergistic effect was not as clear as that in untreated cells. The
CD66b expression was highest in p38-blocked cells exposed to CXCL8-fMLP. Again, this indicates that CXCL8 and fMLP cooperate to affect neutrophils. The general trend of CD66b expression upon stimulation is down-regulation in untreated cells; however, p38-blocked cells showed significantly up-regulated CD66b expression upon stimulation. Also, upon stimulation, CD11b levels were down-regulated in untreated cells but unchanged in p38 MAPK-blocked cells. This may have allowed the cells to have more time to interpret the surrounding signal, thus, increasing the opportunity for the cells to deviate from its route to a signal, especially when there is more than a single chemoattractant signal present. The migration rate data in which p38 MAPK-blocked cells moved slower than untreated cells under competing gradient conditions support this conclusion. All above demonstrate that p38 MAPK is involved in regulating surface expression of CD11b and CD66b.

It has been reported that stimulation of neutrophils induces release of granules filled with adhesion molecules, including CD11b and CD66b;\textsuperscript{44,84} however, in our study, untreated neutrophils challenged with chemoattractant gradients showed lower or similar level of those adhesion molecules on their surface. This might be due to 1) CD11b and CD66b being stored in different granules with different threshold for degranulation,\textsuperscript{44,84-87} 2) our assessments were on neutrophils (both untreated and p38 MAPK-blocked), already adhered to a fibronectin-coated surface,\textsuperscript{88-90} or 3) our separation of neutrophils from other blood components. In previous studies that have shown up-regulation of these adhesion molecules, stimulation was frequently applied in the presence of other cell type.\textsuperscript{85-87} It will be of significant interest in future studies to investigate the potential existence of
separate signaling pathways for degranulation and distribution of granular contents into the plasma membrane, kinetics of surface expression of those adhesion molecules, or potential communication mechanism of neutrophils with other parties in the immune system.

3-4. CONCLUSIONS

This work provides detailed information about neutrophil chemotaxis under single and competing gradients of multiple chemoattractants (fMLP, CXCL8, CXCL2, and LTB4), and the role of p38 MAPK in chemotaxis and surface marker expressions in neutrophils responding to these chemical signals. The microfluidic platform successfully incorporates two chemoattractants simultaneously to which the cells sense and respond. The single gradient experiments confirmed that the type of chemoattractants, mean concentration, and gradient itself are important aspects to be considered when studying neutrophil chemotaxis, and provided the proper gradient for each chemoattractant for competing gradient studies. Through competing gradient experiments, it was found that neutrophils respond to multiple chemoattractants by integrating and prioritizing the signals. A hierarchy of chemoattractants is found, and the hierarchy among the chemoattractants (fMLP > CXCL8 > CXCL2 > LTB4) is dynamic depending on which signaling cascades are triggered. Importantly, this research reveals that the in vivo mimetic in vitro platform can provide results that correspond well with in vivo studies; specifically herein, each chemoattractant signal contributes to the cellular migration (motility from the weaker chemoattractant and orientation from the dominant chemoattractant). The distinct
behavior of each chemoattractant within the heterogeneous population of primary culture neutrophils is confirmed by analysis of the MI, CI, and ECI parameters.

With regard of the role of p38 MAPK in regulating neutrophil chemotaxis, using a p38 MAPK inhibitor SB203580, on-chip evaluation of neutrophil chemotaxis revealed that neutrophils were still capable of migrating toward fMLP but not as effective as untreated neutrophils. Assessment of surface markers (adhesion molecules CD11b and CD66b and chemoattractant receptors FPR2, BLTR, and CXCR1) suggests the involvement of p38 MAPK in surface expression of those markers, and thus, the critical role of p38 MAPK in interpreting surrounding signals and prioritizing particular signals. All of the results suggest that p38 MAPK is more important in neutrophil chemotaxis signal prioritization than actual movement and this is accomplished in part by regulating surface markers. This research illuminates fundamentals of neutrophil chemotaxis by revealing the important role of p38 MAPK-dependent pathway in neutrophil chemotaxis. This tool for screening neutrophil response to a variety of chemoattractants will lend significant insight into clinical research on neutrophil-related diseases such as asthma, chronic obstructive pulmonary disease or chronic granulocytic leukaemia.
Chapter 4

On-Chip Evaluation of Neutrophil Activation and

Neutrophil-Endothelial Cell Interaction during Neutrophil Chemotaxis

Adapted from:

4-1. INTRODUCTION

Because they are the most abundant white blood cell type in the human circulatory system, abnormal behavior of neutrophils has significant impact on human immune response. Neutrophils originate from bone marrow and circulate in search of foreign invaders or dead/dying host cells, playing active roles in both innate and adaptive immunity in humans. When abnormal events such as infection occur, neutrophils are the first cells that migrate to the event site through a process called chemotaxis.\textsuperscript{1-3} Chemotaxis is regulated by concentration gradients of chemotaxis-inducing chemical mediators; however, in the body, neutrophils co-exist with a variety of non-chemotaxis inducing chemical mediators and other cell types.\textsuperscript{4-9} As such, it is highly likely that such interactions between neutrophils and other immune system components will have an influence on neutrophil chemotaxis. Unfortunately, however, conventional experimental methods are frequently incapable of adapting critical extracellular environmental aspects while monitoring neutrophil chemotaxis; thus, our understanding of neutrophil chemotaxis is limited to measurements in oversimplified environments. Animal experiments are another genre of studies frequently used to investigate chemotaxis- or neutrophil-related pathophysiology; however, these studies are expensive, slow, labor-intensive, hard to control, and frequently not representative of human physiological response.

Microfluidics is a powerful approach to overcome such limitations.\textsuperscript{10-12} Microfluidic platforms offer advantages for human cell biology studies by enabling creation of stable but dynamic environments with precise control and small volume sample
requirements. Thus, increased experimental complexity, such as multiple chemical signals and/or cell types, can be easily incorporated using a microfluidic platform. Interestingly, the microfluidics-supported microenvironment is simply an altered culture condition; as such, analysis on individual target cells can be done with minimal complication from the added biological complexity. This is a big analytical strength as many studies have pointed out heterogeneity in cellular behaviors and the importance of understanding such heterogeneity in addition to the collective behavior of cells. Chapter 3 showed disrupted chemotaxis in neutrophils with decreased p38 mitogen-activated protein kinase activity, which was apparent based on the strengths of single cell analysis techniques, and as will become clear below, this microfluidic platform keeps the single cell analysis capability despite the addition of an endothelial cell component. Thus, in this study, neutrophil chemotaxis is studied in the context of “interaction” (Figure 4-1). Neutrophils always interact with endothelial cells, the cells lining blood vessel walls, and molecules for which they express receptors. Endothelial cells were chosen because they are ubiquitous in the body and actively interact with neutrophils during neutrophil migration (Interaction C in Figure 4-1). In addition to the impact of neutrophil-endothelial cell interaction in neutrophil chemotaxis, the effect of chemical mediators in neutrophil chemotaxis is also considered herein, since chemical mediators are a major cell-cell signaling method. Previous work, including Chapter 3, has focused on comprehensive analyses of various chemotaxis-inducing mediators, known as chemoattractants, in neutrophil chemotaxis (Interaction B in Figure 4-1). Those studies revealed that neutrophils respond to all present chemoattractants while prioritizing
Figure 4-1. Neutrophil-involved interactions in vivo - neutrophils interacting with chemical mediators that do not directly induce chemotaxis (A), neutrophils interacting with chemoattractants (B), neutrophils interacting with other cell types (C).
a particular chemoattractant. In this study, the impact of non-chemotaxis inducing mediators is considered (Interaction A in Figure 4-1). Literature precedent indicates active roles for interleukin-2 and interleukin-6 (IL-2 and IL-6) in neutrophil biology.\textsuperscript{5,6,8,9,21} Known impacts of IL-2-induced neutrophil activation include enhanced chemical mediator production,\textsuperscript{5} reduced apoptosis,\textsuperscript{5} enhanced surface Fcγ expression,\textsuperscript{4} and altered gene expression.\textsuperscript{6} On the other hand, IL-6 challenge of neutrophils is known to alter neutrophil production of mediators such as platelet-activating factor (PAF)\textsuperscript{22} and reactive oxygen species.\textsuperscript{23} Also, challenging IL-6-activated neutrophils with fMLP is known to enhance the elastase release from neutrophils.\textsuperscript{6,24} Such IL-2- and IL-6-induced alterations of neutrophil biology likely have an impact on the entire immune system, and as such, abnormal IL-2 and IL-6 levels are frequently reported in the context of infections.\textsuperscript{25-33} Based on the known IL-2- and IL-6-induced impacts on neutrophil biology, this work explores interleukin-exposed neutrophil behavior in a concentration gradient of the bacterial chemoattractant fMLP. While the impact of IL-2, IL-6, or endothelial cells on neutrophil function has been described by a few studies, there are no comprehensive studies on their contributors to chemotaxis and surface CD11b and CD66b expression, especially in the context of cell-cell interactions. Despite the additional complexities (e.g. endothelial cells and interleukins) in the experimental platform, neutrophils in each condition were individually monitored, and the impact of the additional complexities was comprehensively analyzed in this study.

\textbf{4-2. EXPERIMENTAL DETAILS}
4-2-1. Neutrophil preparation

Human neutrophils were prepared as previously described in the section 3-2-1. Briefly, whole human blood, anti-coagulated with ethylenediaminetetraacetic acid (EDTA), was obtained from Memorial Blood Center (St. Paul, MN) according to approved IRB protocol E&I ID#07809. All blood samples were collected on the day of experiments from healthy donors and were used immediately after the samples were obtained. Each condition has 6 biological replicates (6 different donors). Polymorphprep (Accurate Chemical & Scientific Corp., Westbury, NY) was used to isolate neutrophils by density gradient centrifugation as recommended by the manufacturer. Hank’s buffered salt solution (HBSS, Sigma-Aldrich, St. Louis, MO) was used while washing and resuspending neutrophils during isolation, and finally, ~$5 \times 10^6$ isolated neutrophils were resuspended in HBSS containing 2% human serum albumin (HSA, Sigma-Aldrich, St. Louis, MO). The final cell suspension was kept at 37 °C until use.

4-2-2. Device fabrication

Device fabrication and endothelial cell culture in the device were done using standard soft lithography techniques and is described in detail in previous sections 2-2-1 and 3-2-2. The channel dimensions were 50 µm (W) x 100 µm (D) x 2330 µm (L) for the mixing channel and 400 µm (W) x 100 µm (D) x 2500 µm (L) for the cell culture channel.

4-2-3. Endothelial cell culture in a microfluidic channel
A detailed description of how endothelial cells (hy926 human endothelial cell line) were cultured is given in the previous section 2-2-2. To note, the endothelial cell culture media, denoted as DMEM w/ FBS and PS throughout this article, was Dulbecco’s modified eagle medium (DMEM) with high glucose (formula: 4mM L-glutamine, 4.5g/L L-glucose, and 1.5g/L sodium pyruvate (Gibco®, Carlsbad, CA)), supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin (Sigma Aldrich, Milwaukee, WI).

4-2-4. Chemicals

Before introducing isolated human neutrophils into a microfluidic device, they were pretreated with either IL-2 or IL-6 (Sigma Aldrich, St. Louis, MO) in HBSS at the final concentration of 10 ng/mL. The gradient of chemoattractant concentrations, fMLP and LTB4, was 0 – 10 ng/mL. 10 ng/mL fMLP, IL2, IL-6, and LTB4 are 22.9 nM, 29.7 nM, 0.6 nM, and 0.4 nM, respectively.

4-2-5. Device preparation for experiments without endothelial cells

For experiments without endothelial cells, the channels were washed using a 70% v/v ethanol solution in sterilized Milli-Q water (Millipore, Billerica, MA) and then treated with a 250 µg/mL solution of human fibronectin (Invitrogen, Carlsbad, CA) in sterilized Milli-Q water followed by an hour long incubation under 5 % CO₂ at 37 °C. Following fibronectin incubation, the media in the channels was exchanged for fresh HBSS with 2% human serum albumin (HSA); then, 20 µL of neutrophil suspension (~5 x 10⁶ cells/mL),
Figure 4-2. (a) Microfluidic device schematic. Solutions introduced into the two inlets are mixed while traversing the serpentine channels to establish a stable concentration gradient across the observation channel. While a gradient of fMLP is established by introducing media into inlet 1 and fMLP solution into inlet 2, a competing gradient of LTB4-fMLP is established by introducing LTB4 solution into inlet 1 and fMLP solution into inlet 2. (b) Neutrophils on a fibronectin-coated surface in the observation channel. (c) Trajectories of 20 representative control neutrophils (unactivated) within the fMLP gradient. (d) Neutrophils on an endothelial cell-coated surface in the observation channel. (e) Trajectories of 20 representative control neutrophils (unactivated) within the fMLP gradient.
either treated or not treated with interleukins, was injected into the cell inlet on the microfluidic device. After another hour of incubation to promote neutrophil adhesion, the device was connected to a syringe pump to introduce the chemoattractant gradient (0 – 10 ng/mL fMLP) into the observation channel. This 0 – 10 ng/mL concentration gradient was established by introducing HBSS containing no fMLP into the left inlet (0 ng/mL fMLP) and 10 ng/mL fMLP into the right inlet (shown in Figure 4-2).

4-2-6. Device preparation for experiments with endothelial cells

For experiments on endothelial cells, the endothelial cells cultured in the device were visually inspected to confirm uniformity of the endothelial cell layer. The endothelial cell layer within the cell culture channel was achieved by the same procedure described in the previous section 2-2-4. Once confirmed, 20 \( \mu \text{L} \) of neutrophil suspension (~5 x 10^6 cells/mL), either un-activated or activated with interleukins, was injected into the microfluidic cell culture chamber inlet. After an hour of incubation to promote neutrophil adhesion, the device was connected to a syringe pump to introduce the chemoattractant gradient (0 to 10 ng/mL fMLP) over the cells in the observation channel.

4-2-7. Sample preparation for fluorescence imaging

For fluorescence imaging, 100 \( \mu \text{L} \) of neutrophil suspension (~5 x 10^6 cells/mL) was injected into each well of a 96-well plate, incubated for 1 hour to promote adhesion to the surface, washed three times with HBSS, and subject to interleukin exposure, endothelial cell-conditioned media, and/or fMLP. Endothelial cell-conditioned medium was prepared
from the medium in an endothelial cell culture. Rather than a regular media change, the medium in a culture was collected and centrifuged at 150xg for 10 minutes to remove non-adherent cells. Then, the supernatant was collected and kept frozen until use; this supernatant was used to examine the effect of soluble species produced by endothelial cells. For single stimulation conditions, cells were challenged with IL-2, IL-6, or fMLP at the final concentration of 10 ng/mL or endothelial-cell conditioned medium for an hour. For co-stimulation conditions with both interleukins and fMLP, cells were subject to IL-2 or IL-6 for an hour first and then challenged with fMLP for another hour. For co-stimulation conditions with endothelial cell-conditioned medium and fMLP, cells were subject to endothelial cell-conditioned medium for an hour first and then challenged with fMLP for another hour. For co-stimulation conditions with interleukins, endothelial cell-conditioned medium, and fMLP, cells were subject to interleukins with endothelial cell-conditioned medium, to be consistent with on-chip chemotaxis investigation, for an hour and then challenged with fMLP for another hour. After stimulation, cells were washed with HBSS three times and then subject to anti-CD11b conjugated with AlexaFluor 700 (Life Technologies, Grand Island, NY) and anti-CD66b conjugated with AlexaFluor 647 (BD Pharmingen, San Diego, CA) following instructions from manufacturers, incubated for 1 hour, washed with HBSS three times, and then surface expression of those adhesion molecules on individual cells were fluorescently monitored.

4-2-8. Analysis of migration data
Migration data analysis was performed as described in the previous section 3-2-7. Briefly, two unitless numeric parameters: motility index (MI) and chemotactic index (CI), were used as previously described.\textsuperscript{1,10} MI is an indicator of how active/mobile individual cells are, while CI is an indicator of how directional individual cells’ migration is. In this report, movement toward the right side of the observation channel, toward a higher concentration of fMLP, results in positive CI. In addition to MI and CI, % population represents cells with overall migration opposite to the direction of the fMLP gradient. Also, the angular displacement of individual cells were digitized by giving a score of “+1” to cells in each frame that moved with $-90^\circ < \text{angle} < 90^\circ$, and giving a score of “-1” to cells in each frame moved with angle $> 90^\circ$ or with angle $< -90^\circ$. The angle value was obtained with respect to the x-axis (parallel to the gradient direction); thus, this parameter is descriptive of how much deviation from the parallel to the fMLP gradient occurred during migration. An angle distribution value of “+1” would indicate that the cell moved straight toward the fMLP signal in every collected image. Again, to assess individual neutrophils’ response in a time-resolved manner, this angle parameter was calculated for the first 1, 4, and 7 minutes, and for the entire duration of the experiment. All angle distribution plots provided in this thesis contain data for the first 4 minutes and for the entire duration. T-tests were used for statistical comparison, and errors are reported as +/- standard error of mean, SEM, throughout the chapter.

**4-3. RESULTS AND DISCUSSION**
Figure 4-3. Neutrophil chemotaxis within the fMLP gradient on fibronectin and on endothelial cells. (a) Motility index (MI) and chemotactic index (CI) of neutrophils on fibronectin (green) and on endothelial cells (blue). (b) Angular distribution of neutrophil movement on fibronectin (circles) and on endothelial cells (triangles) with a value of 1 indicating migration of a cell straight toward fMLP. Each point represents the angular displacement of an individual neutrophil, and the solid bar indicates the average. (c) Average rate of neutrophil migration on fibronectin (green) and on endothelial cells (blue).
4-3-1. Neutrophil migration against the fMLP gradient – the impact of the presence of endothelial cells

To investigate the impact of cell-cell interactions between neutrophils and endothelial cells, chemotaxis of neutrophils was assessed when the human cells were introduced onto a layer of endothelial cells (B&C in Figure 4-1). Figure 4-2 shows representative trajectories of 20 neutrophils on fibronectin- and endothelial cell-coated devices. The representative trajectories indicate that neutrophils on endothelial cells are not as directed toward the fMLP signal as neutrophils on a simplified fibronectin-coated surface, and their migration patterns are analyzed in more detail using numerical parameters defined in the experimental section of Chapter 3. As shown in Figure 4-3, mobility and directionality in neutrophil migration toward the fMLP signal (MI and CI) were significantly lower in neutrophils on endothelial cells (0.44 ± 0.04 and 0.16 ± 0.06 compared to 0.64 ± 0.03 (p < 0.0001) and 0.40 ± 0.03 (p < 0.0001), respectively). Interestingly, rather than diminishing migration speed, the reduced MI and CI seemed to be caused by deviation in migration direction. The average velocity of individual cells on endothelial cells was similar to that on fibronectin (0.26 ± 0.03 compared to 0.25 ± 0.01, p = 0.67, Figure 4-3, c) while the angular distribution data showed more deviated migration on endothelial cells (0.23 ± 0.07 compared to 0.61 ± 0.06 for the first 4 minutes (p < 0.0001), and 0.25 ± 0.08 compared to 0.47 ± 0.06 for the entire 30 minute duration of the experiment (p = 0.03), Figure 4-3, b). Again, this angle distribution represents the level of deviation from the gradient direction for individual cells during migration, and a value close to “+1” indicates that the neutrophil moved straight toward the fMLP signal.
Figures 4-4. % Population of neutrophils on fibronectin (green) and on endothelial cells (blue) that moved away from the fMLP signal.
in every collected image. The deviation in migration angle for neutrophils on endothelial cells is also linked to the higher % population data; 34.4% neutrophils moved away from the fMLP signal on endothelial cells while only 10.9% cells moved away from the fMLP signal on a fibronectin-coated surface (Figure 4-4).

4-3-2. Neutrophil migration against fMLP gradient – the impact of cytokines

To explore the effect of non-chemotaxis-inducing cytokines on neutrophil chemotaxis, neutrophils were pre-incubated with either 0.6 nM interleukin-2 (IL-2) or 0.4 nM interleukin-6 (IL-6), and then exposed to an fMLP gradient (A&B in Figure 4-1). Challenging neutrophils with 0.6 nM IL-2 or 0.4 nM IL-6 has been shown to be effective in activating neutrophils, but also, few nM level elevation of these interleukins are frequently found in individuals with abnormal immune response. In this section, comparison is performed between neutrophils that were not activated (denoted as “control”) and that were pre-activated by IL-2 or IL-6 (denoted as “IL-2-activated” or “IL-6-activated”). The results indicate that neutrophils move faster when they are pre-incubated with interleukins (0.29 ± 0.02 for IL-2 (p = 0.06) and 0.34 ± 0.02 for IL-6 (p < 0.0001) compared to 0.25 ± 0.01 for control, Figure 4-5, c) even though MI and CI were the largest in control neutrophils. Remembering that MI and CI have a speed term (multiplied by the time) in the denominator by definition, this indicates that interleukin-exposed cells do not reflect their increased migration speed into the actual motility and directionality. Specifically, IL-2-activated neutrophils had decreased MI (0.55 ± 0.03 for IL-2-activated compared to 0.64 ± 0.03 for control, p = 0.04, Figure 4-5, a), and IL-6 pre-
Figure 4-5. Neutrophil chemotaxis within the fMLP gradient: unactivated (control), IL-2-activated, and IL-6-activated neutrophils on fibronectin. (a) Motility index (MI) and chemotactic index (CI) of neutrophils. (b) Angle distribution of neutrophils, with a value of 1 indicating migration of a cell straight toward fMLP. Each point represents the angular displacement of a cell, and the solid bar indicates the average. (c) Average migration rate of neutrophils.
activation led to the down-regulation of both motility and directionality (0.51 ± 0.05 and 0.18 ± 0.05 for IL-6-activated compared to 0.64 ± 0.03 (p = 0.012) and 0.40 ± 0.03 (p = 0.01) for control, Figure 4-5, a). The angle distribution shows that, in both pre-activated conditions, more cells deviated from the fMLP signal during migration (0.23 ± 0.06 (p = 0.01) for IL-2-activated and 0.32 ± 0.07 (p = 0.09) for IL-6-activated compared to 0.47 ± 0.06 for control). % population data also supported this deviated migration pattern: 18.2% IL-2-activated cells and 21.1% IL-6-activated cells moved away from the fMLP signal while only 11% of control cells moved away from the fMLP signal (Figure 4-4). The first 4 minutes of movement was especially influenced, with significantly more pre-activated neutrophils moving away from the fMLP signal (0.21 ± 0.07 (p < 0.0001) for IL-2-activated and 0.30 ± 0.07 (p < 0.0001) for IL-6-activated compared to 0.61 ± 0.06 for control, Figure 4-5, b). Consistent with the neutrophils in contact with endothelial cells, these data indicate that increased neutrophil migration speed was compensated by deviated migration from the direction of fMLP signal.

4-3-3. Neutrophil migration – the synergistic impact of cytokines and endothelial cells

As the microfluidic platform allowed separate study of cytokine impact (A&B in Figure 4-1) and endothelial cell impact (B&C in Figure 4-1) on neutrophil chemotaxis, this work also considered the simultaneous impact of both cytokines and endothelial cells on neutrophil chemotaxis (A&B&C in Figure 4-1). Comparison in this section was between neutrophils on endothelial cells, and the neutrophils were either unactivated (control),
Figure 4-6. Neutrophil chemotaxis within the fMLP gradient: unactivated (control), IL-2-activated, and IL-6-activated neutrophils on endothelial cells, (a) Motility index (MI) and chemotactic index (CI) of neutrophils. (b) Angle distribution of neutrophils, with a value of 1 indicating migration of a cell straight toward fMLP. Each point represents the angular displacement of a cell, and the solid bar indicates the average. (c) Average migration rate of neutrophils.
pre-activated with IL-2 (IL-2-activated), or pre-activated with IL-6 (IL-6-activated). Only
the MI in IL-2-activated neutrophils on endothelial cells was altered when compared to
control neutrophils on endothelial cells (0.59 ± 0.03 compared to 0.44 ± 0.04, p = 0.01,
Figure 4-6, a). The average migration rates for IL-2- and IL-6-activated conditions were
not significantly different from the control condition on endothelial cells, although IL-6
pre-incubation resulted in a slightly faster migration rate than IL-2 pre-incubation (0.30 ±
0.04 for IL-6-activated compared to 0.24 ± 0.02 for IL-2-activated, p = 0.1, Figure 4-6, c).
While angle distribution data showed no significant differences among conditions by
mean ± SEM comparison, the distribution patterns were noticeably different. The
distribution was most focused toward the fMLP signal in IL-2-activated neutrophils on
endothelial cells (Figure 4-6, b). The % population data also supported this deviated
migration: on endothelial cells, 15% IL-2-activated cells and 22% IL-6-activated cells
moved away from the fMLP signal while 34% control cells moved away from the fMLP
signal (Figure 4-4).
Unlike pre-activated neutrophils on a fibronectin-coated surface (Figure 4), directionality
of pre-activated neutrophils migrating on endothelial cells seems to be independent of
pre-activation (no significant differences in CIs or angle distributions, Figure 4-6). As
such, we further studied chemotaxis of pre-activated neutrophils on endothelial cells by
incorporating a competing gradient of chemoattractants. In Chapter 3, we showed that
the microfluidic platform allows incorporation of multiple chemoattractant gradients and
that neutrophils prioritize a fMLP signal over a LTB4 signal. Herein, we adapted the
same competing gradient approach from the previous study into the
Figure 4-7. Neutrophil chemotaxis within the competing gradient of LTB₄-fMLP: unactivated (control), IL-2-activated, and IL-6-activated neutrophils on endothelial cells. (a) Motility index (MI) and chemotactic index (CI) of neutrophils. (b) Average migration rate of neutrophils. (c) % population of neutrophils moved away from the fMLP signal.
microfluidic platform and exposed neutrophils on endothelial cells to a competing gradient of LTB4 and fMLP (LTB4-fMLP). A LTB4-fMLP gradient is established by introducing 10 ng/mL LTB4 and 10 ng/mL fMLP into the left and right inlet, respectively (Figure 4-2). This achieves a 10 – 0 ng/mL LTB4 gradient superimposed on a 0 – 10 ng/mL fMLP gradient within the observation channel. The gradient of each chemoattractant was chosen based on previous study; in this concentration gradient, neutrophils exposed to the LTB4 gradient performed a similar chemotaxis level when compared to neutrophils exposed to the fMLP gradient.¹⁹ Thus, chemotaxis investigation within such a competing gradient reveals the hierarchy of neutrophil response among the molecular signals. In previous work, it was demonstrated that neutrophils prioritize fMLP over leukotriene B4 (LTB4) on a fibronectin-coated surface; however, we were concerned that other cells or the activation state of neutrophils will influence the neutrophil behavior. Herein, neutrophils were exposed to an LTB4-fMLP competing gradient on endothelial cells. Neutrophils were either unactivated (control), pre-activated by IL-2 (IL-2-activated), or pre-activated by IL-6 (IL-6-activated). Most importantly, the results demonstrate that the major population of neutrophils moved toward fMLP in all conditions (Figure 4-7). This indicates that neutrophils still prioritized fMLP signal over LTB4 signal, and that the neutrophil interaction with non-chemotaxis inducing contributors (IL-2, IL-6, and endothelial cells) did not change the hierarchy of neutrophil response to these molecular signals. Also, while no noticeable variation was observed from the angle distribution data, the results indicate that IL-6 is the most potent contributor in these conditions: MI, average migration rate, and % population were
noticeably different from all other conditions in IL-6 pre-incubated neutrophils on endothelial cells (Figure 4-7).

4-3-4. Neutrophil chemotaxis: cytokines and endothelial cells

Neutrophils circulate in the human body seeking abnormalities on the endothelial cells lining the inner wall of blood vessels. They make a contact with endothelium repeatedly, and once they confront an abnormality, they stay in contact with endothelium and seek the exact site of the abnormality. As such, neutrophils inevitably interact with endothelial cells while performing their critical roles in the immune system. Also, neutrophils express surface receptors for IL-2 and IL-6 which are frequently up-regulated in the human body in various abnormal circumstances. Thus, it is highly likely that neutrophils utilize IL-2 and IL-6 before/during migration as a way to communicate with other components of the immune system; however, there have been no comprehensive studies of such interactions due to technical difficulties. Herein, we exploit a simple microfluidic platform to introduce such biological complexity into experimental investigations. The interactions among non-chemotaxis inducing mediators (A in Figure 4-1), chemoattractants (B in Figure 4-1), and other cell types (C in Figure 4-1), that are the critical in generating immune response, are accounted for in our microfluidic platform. While maintaining high fidelity analysis of individual neutrophil behavior, our platform provides mechanistic insight in a resolved manner for each of contributing complexities (endothelial cells and chemical mediators). In this discussion, Table 4-1 will be used to summarize the bar chart results shown above. Again, pre-incubation with IL-2 or IL-6
represents interaction A in Figure 4-1, fMLP, or LTB4-fMLP gradient exposure represents interaction B (chemotaxis) in Figure 4-1, and added endothelial cell represents interaction C in Figure 4-1. The neutrophil chemotaxis was significantly disturbed on endothelial cells compared to that on a simple fibronectin coating, which proves that neutrophils behave differently when they interact with endothelial cells (B&C in Figure 4-1). The interaction of neutrophils with endothelial cells does not seem to significantly impact migration speed. However, neutrophil migration on endothelial cells was not as directed as that on fibronectin. It may be, in part, because in vivo neutrophils migrate through the junctions between endothelial cells rather than over the endothelial cells, which will cause a slight disturbance in the directional movements. Directional deviation from the chemoattractant gradient direction was also observed from IL-2-activated and IL-6-activated neutrophils (A&B in Figure 4-1, Table 4-1). All of these demonstrate that, while IL-2, IL-6, and endothelial cells do not directly induce chemotaxis, they play a role in neutrophil chemotaxis by adjusting both direction and movement of neutrophils.

To be noted, neutrophils on endothelial cells respond to the interleukins differently than those on fibronectin (All interactions in Figure 4-1 together, Table 4-1). As mentioned, IL-2- and IL-6-activated neutrophils on a fibronectin-coated surface had decreased motility and chemotactic indices (MI and CI) and altered migration patterns (angle distribution and % population) despite increased migration rate when compared to control, un-activated, neutrophils. On the other hand, when compared to control neutrophils on endothelial cells, IL-2-activated neutrophils on endothelial cells had an increased MI and
more directed migration toward the fMLP signal (focused angle distribution and lower % population). The migration rate of IL-2-activated neutrophils was not significantly different from control neutrophils when they were on endothelial cells. IL-6-activated neutrophils on endothelial cells had similar MI and CI and showed more directed migration toward the fMLP signal (similar angle distribution but lower % population). The migration rate of IL-6-activated neutrophils on endothelial cells was higher than that of control neutrophils on endothelial cells. From these results, it is obvious that IL-2 or IL-6 cooperatively contribute with endothelial cells to neutrophil chemotaxis. It was previously found that neutrophils simultaneously respond to all present chemoattractants, and this study demonstrates that non-chemotaxis inducing mediators also make their own individual/synergistic contributions. In this study, neutrophils, whether pre-activated by interleukins or not, exposed to the competing chemoattractant gradients (LTB4-fMLP) prioritize fMLP over LTB4 on endothelial cells, which is consistent with our previous finding from neutrophils on a fibronectin surface. Our results demonstrate that indirect contributors to chemotaxis alter the capability of neutrophils to follow a signal rather than their prioritization of a particular signal.

4-3-5. Surface expression of adhesion molecules – the individual and synergistic impact of cytokines and endothelial cells

As shown above, the microfluidic platform employed herein allowed detailed investigation of neutrophil chemotaxis and its relation to surrounding cytokines and different cell types that do not directly induce neutrophil chemotaxis, and revealed the
Table 4-1. Summary of neutrophil chemotaxis under the fMLP gradient – Control: unactivated, IL-2: IL-2-activated, and IL-6: IL-6-activated.

<table>
<thead>
<tr>
<th></th>
<th>On fibronectin-coated surface</th>
<th>On endothelial cell-coated surface</th>
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<tbody>
<tr>
<td>MI</td>
<td>Control &gt; IL-2 &gt; IL-6</td>
<td>IL-2 &gt; Control = IL-6</td>
</tr>
<tr>
<td>CI</td>
<td>Control = IL-2 &gt; IL-6</td>
<td>Control = IL-2 = IL-6</td>
</tr>
<tr>
<td>Angle distribution</td>
<td>Control &gt; IL-2 = IL-6</td>
<td>Control = IL-2 = IL-6</td>
</tr>
<tr>
<td>Migration rate</td>
<td>IL-6 &gt; IL-2 &gt; Control</td>
<td>Control = IL-2 = IL-6</td>
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impact of each of these contributors on the outcome chemotaxis. To broaden the mechanistic understanding of the IL-2, IL-6, and endothelial cell impacts on neutrophil chemotaxis, surface expression of the adhesion molecules CD11b and CD66b were fluorescently monitored. Adhesion of neutrophils is the initial step for chemotaxis and CD66b and CD11b are well-known key surface markers for neutrophil adhesion to endothelial cells. To mimic neutrophils migrating on endothelial cells, neutrophils were treated with endothelial cell-conditioned medium (denoted as “EC-treated”). As such, comparison in this section is between two populations of cells, untreated vs. EC-treated, either with no stimulation (control), activation with IL-2 (IL-2-activated), activation with IL-6 (IL-6-activated), stimulation with fMLP (denoted as “fMLP-stimulated”), or activation with IL-2 or IL-6 followed by fMLP stimulation (denoted as “IL-2-fMLP” and “IL-6-fMLP”); all statistical comparisons are shown in Table 4-2. The CD11b levels in untreated neutrophils had a general trend of down-regulation upon stimulation (Figure 4-8, a and c). Interestingly, co-stimulation resulted in higher expression of CD11b than individual stimulation. Compared to activation with IL-2 or IL-6 and fMLP stimulation, the CD11b expression was higher in IL-2-fMLP and IL-6-fMLP conditions (Figure 4-8, Table 4-2). CD66b levels in untreated cells had a similar trend; cells expressed less CD66b when stimulated (Figure 4-8, Table 4-2). Unlike CD11b expression, the synergistic impact of interleukins and the chemoattractant fMLP on the CD66b expression was only clear in the IL-6-fMLP condition (Figure 4-8, Table 4-2). On the other hand, both surface CD11b and CD66b expression was significantly different in EC-treated cells (Figure 4-8). While stimulation of untreated cells resulted in down-
Figure 4-8. Individual and cooperative impact of IL-2 (a and b) and IL-6 (c and d) with endothelial cells on surface CD11b and CD66b expression by neutrophils: un-activated (control), IL-2-activated, IL-6-activated, fMLP-stimulated, IL-2-fMLP co-stimulated, and IL-6-fMLP co-stimulated neutrophils. (a and c) CD11b expression of untreated (green) and EC-treated (blue) neutrophils. (b and d) CD66b expression of untreated (green) and EC-treated (blue) neutrophils.
Table 4-2. Fluorescence results summary for all activation/stimulation conditions.

All statistical comparisons are between the experimental condition and the indicated control.
regulation of CD11b, stimulation of EC-treated cells resulted in up-regulation of CD11b (Figure 4-8, Table 4-2). Also, while stimulation of untreated cells resulted in a decreasing trend in surface CD66b expression, stimulation of EC-treated cells resulted in an increasing trend in surface CD66b expression. Only IL-2-activated EC-treated cells resulted in down-regulation of CD11b and CD66b (Figure 4-8, Table 4-2).

4-3-6. Neutrophil surface marker expression: cytokines and endothelial cells

Once neutrophils sense a chemical signal, they adhere to the endothelium and start migrating; thus, adhesion is an important component of chemotaxis. To correlate this phenomenon with the measured chemotaxis, individual human neutrophils were analyzed for expression of the surface adhesion molecules CD11b and CD66b via fluorescence imaging. Again, two populations of cells, untreated or EC-treated, were activated/stimulated by IL-2 (IL-2-activated), IL-6 (IL-6-activated), fMLP (fMLP-stimulated), IL-2 followed by fMLP (IL-2-fMLP), or IL-6 followed by fMLP (IL-6-fMLP), and surface expression of CD11b and CD66b of those cells were compared to those from un-stimulated cells (control). All measured results demonstrate that IL-2, IL-6, and endothelial cells influence the expression of neutrophil CD11b and CD66b (Figure 4-8, Table 4-3).

The most noteworthy trend in CD11b and CD66b expression was a down-regulation in untreated cells but an up-regulation in EC-treated cells. In untreated neutrophils, exposure to IL-2, IL-6, and fMLP resulted in down-regulation of CD11b and CD66b and this down-regulation may have contributed to the faster migration rate in neutrophils
Table 4-3. Summary of surface CD11b and CD66b expressions – Control: unactivated, IL-2: IL-2-activated, IL-6: IL-6-activated, fMLP: fMLP-stimulated, IL-2-fMLP: IL-2-activated and fMLP-stimulated, IL-6-fMLP: IL-6-activated and fMLP-stimulated, EC-treated: endothelial cell culture medium-treated.

<table>
<thead>
<tr>
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<th>CD11b</th>
<th>CD66b</th>
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<tr>
<td>Untreated</td>
<td>Control &gt; IL-2-fMLP &gt; IL-2 &gt; fMLP</td>
<td>fMLP &gt; Control &gt; IL-2-fMLP &gt; IL-2</td>
</tr>
<tr>
<td>Control</td>
<td>Control &gt; IL-6-fMLP &gt; IL-6 &gt; fMLP</td>
<td>fMLP &gt; IL-6 &gt; Control = IL-6-fMLP</td>
</tr>
<tr>
<td>EC-treated</td>
<td>fMLP &gt; Control &gt; IL-2-fMLP &gt; IL-2</td>
<td>fMLP &gt; Control = IL-2-fMLP &gt; IL-2</td>
</tr>
<tr>
<td>fMLP</td>
<td>fMLP &gt; IL-6 &gt; Control = IL-6-fMLP</td>
<td>fMLP &gt; IL-6 &gt; IL-6-fMLP &gt; Control</td>
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upon stimulation. On the other hand, in EC-treated cells, IL-6 activation and fMLP stimulation resulted in an up-regulation of CD11b and CD66b. To be noted, both untreated and EC-treated neutrophils show a decreased CD11b and CD66b expression when activated by IL-2. This indicates that IL-2 down-regulates surface CD11b and CD66b expression in neutrophils, and its impact is more potent than the neutrophil-endothelial cell interaction. In addition, in general, the impact of individual IL-2, IL-6, or fMLP on CD11b and CD66b expression was different than the cooperative impact of IL-2-fMLP or IL-6-fMLP, indicating a synergistic effect for the interleukins and fMLP. An interesting aspect in this research is the CD11b and CD66b down-regulation upon neutrophil stimulation. The same trend, down-regulation of these adhesion molecules, was found in our previous study where neutrophils were stimulated by CXC-motif chemokine 8 (CXCL8); however, as multiple literature precedents also reported up-regulation of those molecules from neutrophils after stimulation, this unexpected down-regulation was unexplained in the previous study. Upon close examination of precedent work done in co-culture, we see that CD11b and CD66b expressions were up-regulated in EC-treated cells. As such, it was hypothesized that the neutrophil-endothelial cell interaction initiates a signaling cascade that converts a down-regulation of CD11b and CD66b upon stimulation to an up-regulation. As the EC-treated condition contained a step that exposes neutrophils to a different medium, the impact of the medium in CD11b and CD66b expression was analyzed to assess this hypothesis. The endothelial cell culture medium was, as described in experimental section, DMEM w/FBS and PS (DMEM with high glucose supplemented with 10% fetal bovine serum and
Figure 4-9. Surface adhesion molecule expression of untreated, DMEM-treated, and DMEM 10%FBS 1%PS-treated neutrophils. Expression of (a) CD11b and (b) CD66b on untreated (green), DMEM-treated (blue), and DMEM with FBS and PS-treated (purple) neutrophils.
1 % penicillin and streptomycin), while the base medium for neutrophil handling was Hank’s balanced salt solution (HBSS). Thus, while keeping all other conditions the same, neutrophils were treated with DMEM with high glucose, and DMEM w/ FBS and PS and then respective stimuli, and the CD11b and CD66b expression was assessed. (Figure 4-9) Again, the control condition in each cell population was the un-activated cells. As shown in Figure 4-9, both IL-2 and IL-6 stimulation of neutrophils with DMEM with high glucose or DMEM w/ FBS and PS resulted in down-regulated CD11b and CD66b expression when compared to controls. Only the fMLP showed a possible interaction with DMEM with high glucose or DMEM w/ FBS and PS. This demonstrates that our results of CD11b and CD66b up-regulation in EC-treated cells are not attributed to the medium, and thus, neutrophil-endothelial cell interaction plays a role in regulating surface CD11b and CD66b in neutrophils. It will be of particular interest in the near future to profile neutrophil- and endothelial cell-expressed mediators and find out what signaling cascade is responsible for this process.

4-4. CONCLUSIONS

This work provides detailed information about neutrophil chemotaxis upon interaction with non-chemotaxis inducing components of the immune system. The microfluidic platform successfully incorporates neutrophils and endothelial cells, enabling the investigation of neutrophil chemotaxis on a layer of endothelial cells. All of various parameters above that describe neutrophil migration and surface marker expressions, each critical in defining neutrophil biology, are obtained at significantly reduced amount
of cost, labor, time, and consumption of samples and chemicals, when compared to conventional assays. Moreover, the insight gleaned from the single cell analysis approach is not easily available with conventional assays, proving that this microfluidic approach will provide opportunities to broaden our understanding beyond the traditional level. Our findings also demonstrate the strength of the microfluidic approach by revealing the contribution of chemical and cellular mediators in neutrophil chemotaxis. Specifically, our results show that indirect chemotaxis-inducing mediators (interleukins and endothelial cells) have an active role in the regulation of neutrophil chemotaxis, and the role is, in part, mediated through adjustment of surface adhesion molecule expression. IL-2 and IL-6 have their own individual and synergistic impact on neutrophil chemotaxis, but this impact does not alter the neutrophils’ ability to respond to an fMLP chemoattractant signal. The most noteworthy finding is that, in all conditions, the presence of endothelial cells or endothelial cell-expressed mediators altered neutrophil response to both cytokines and chemoattractants. Every single immune response is managed by cell-to-cell communications; as such, our finding may form the basis for chemotaxis studies in the context of cell-cell communications. It will be of particular interest in the future to profile neutrophil/endothelial cell-expressed mediators to delineate their interaction in more detail, leading to a more complete understanding of pathophysiology of neutrophil-mediated diseases.
Chapter 5

Simple Approaches for On-Chip SERS Sensors:

Realization of One Shot Limit-of-Detection Assessment

Adapted from:

5-1. INTRODUCTION

Microfluidics has become an often-used technique for sample manipulation in bioanalytical/biomedical sciences because the devices are largely biocompatible, require small (viz., nanoliter) sample volumes, create less biohazard waste, and provide shorter analysis time and higher throughput than many bench-top techniques. These advantages make microfluidics especially promising for biological applications and bioanalytical sensor developments, particularly when interrogated with optical detection. Among the various optical detection methods that can be used in conjunction with microfluidic platforms, Raman spectroscopy is particularly interesting for bioanalytical applications because it yields a vibrational fingerprint for detected analytes and its performance is not compromised in aqueous samples. Normal Raman spectroscopy has inherently small scattering cross-sections, yielding poor sensitivities; however, surface-enhanced Raman scattering (SERS) provides opportunities to enhance detection sensitivity significantly.1-3 This enhancement is attributable to the large molecular dipole induced when a Raman-active molecule experiences electromagnetic fields generated at the surface of nanoroughened plasmonic noble metal surfaces. Practically speaking, the SERS limit of detection can reach the single-molecule detection realm, but nM and pM analyte concentration detection is routinely observed with high quality plasmonic substrates.4 Accordingly, SERS has been employed on microfluidic platforms to identify chemical moieties in bioanalyte systems such as cells, viruses, bacteria, DNA, proteins, drugs, cellular mediators, and even organelles and sub-organelles.5-6
Colloidal noble-metal nanoparticles are the most commonly used SERS-active substrates within microfluidic devices.\textsuperscript{1,7-11} In general, the SERS-active nanoparticles are injected through a microfluidic channel where they encounter analyte species at a specified physical location within the device. The primary challenge with this type of approaches is that it is difficult to achieve large signal enhancement without colloidal nanoparticle aggregation.\textsuperscript{4,11} It has been reported that the best SERS signal enhancement for gold nanoparticles can be achieved when nanoparticles are in contact, or at least separated by not more than 1-2 nm;\textsuperscript{4-11} however, controlling nanoparticle aggregation is challenging, presenting persistent experimental roadblocks related to stability and sensitivity of the obtained signal. Some work also reports unexpected loss of nanoparticles or defects in their aggregation/arrangement due to nanoparticle interactions with channel surfaces or flow profile.\textsuperscript{12} To avoid these difficulties, there have been several reported systems where a SERS-active substrate is incorporated into a microfluidic device.\textsuperscript{13-16} In these reports, SERS-active substrates were largely fabricated either on glass or silicon using high-end lithography techniques, aligned, and bound to a layer containing the microfluidic channel geometries. These finely-tuned SERS-active substrates, such as ‘nanoholes’ or ‘nanogaps’, have very high signal enhancements compared to simple nanoparticle colloids; however, fabrication can be prohibitively expensive and requires access to specialized instrumentation for time- and labor-intensive fabrication protocols.

Herein, we present two types of proof-of-concept microfluidic-SERS sensing platforms with potential to address the aforementioned challenges: a microfluidic-gold film-over-nanospheres (AuFON) platform and a flexible SERS sensor platform with nanoparticles
incorporated directly into the microfluidic polymer layer. Gold was chosen over silver for the plasmonic material despite its lower signal enhancement compared to silver, because of its stability against dissolution and oxidation, lower toxicity, and ease of surface functionalization for future bioanalytical application purposes.\textsuperscript{5-6}

The microfluidic-AuFON platform, as the name implies, employs a AuFON as the plasmonic component. We have previously demonstrated, using silver film-over-nanospheres (AgFON), that noble metal FON substrates can provide strong, reproducible SERS enhancement factors. Similar capabilities have been reported for AuFONs as well.\textsuperscript{17} The most appealing aspect of FONs as SERS substrates is that, despite relatively simple and inexpensive fabrication techniques, they achieve extraordinary signal enhancements ($10^6 \sim 10^8$) with uniform and reproducible analyte response over cm$^2$ substrate areas. As such, the noble metal FONs have become the gold standard of SERS substrates. In addition to aforementioned advantages of AuFONs over AgFONs, gold also provides better compatibility than silver with the microfabrication techniques that are used to combine the SERS-active substrates with a polydimethylsiloxane (PDMS)-based microfluidic platform.

For the flexible SERS sensor platform, gold nanorods (AuNRs) and gold nanocubes (AuNCs) are used as nanoparticle SERS substrates. These nanoparticles are directly dispersed into the PDMS device and enable SERS detection of analytes flowing through the device. This approach allows ease of device fabrication, adaptability to any desired channel geometries, optical transparency and flexibility of the device, and low consumption of nanoparticles. To our knowledge, there are only a few reported research
efforts where nanoparticles were embedded directly into the device.\textsuperscript{18,19} In these studies, gold nanocomposites are created by vapor deposition or in situ synthesis onto microfluidic channels or by stamping nanocomposites onto the side of a microfluidic device. Despite the potential benefits of these methods, the in situ synthesis approach and vapor deposition frequently consume an unnecessary amount of gold and complicate further device fabrication steps. In addition, the potential of these approaches to achieve the optimally enhancing nanoparticle aggregates is dubious.

The employment of PDMS-based microfluidics herein enables the realization of a portable, durable SERS sensing platform with added analytical strengths. In the proof-of-concept microfluidic-SERS sensor platforms presented herein, the microfluidic channels are designed to enable easy limit of detection (LOD) or limit of quantification (LOQ) studies. The fundamental principle of the device is based on previously reported microfluidic devices for creation of chemical gradients\textsuperscript{20} with a slight modification to achieve simultaneous detection of different concentrations of an analyte. Another advantage of the PDMS-based SERS sensor is that PDMS provides strong Raman-active bands in a 490-700 cm\textsuperscript{-1} region, acting as an internal standard. As our results show, the presented microfluidic-SERS platforms demonstrate capability of quantitative, single experiment LOD/LOQ assessment and their potential for multiplexed high-throughput analysis. Further development of these proof-of-concept platforms will facilitate the development of several diagnostic devices for early detection of diseases and continuous monitoring of patients.
5-2. EXPERIMENTAL DETAILS

5-2-1. AuFON fabrication

After a base bath cleaning step in 10:1:1 water:ammonium hydroxide:hydrogen peroxide for an hour, 13 μL of 600-nm-diameter silica nanospheres (Bangs Laboratories) were spread onto a clean silicon wafer. The silica nanospheres were distributed on the silicon wafer until there were no visible mixing lines. The wafer was allowed to air dry. After self-assembly and drying, 200 nm of Au (99.999 % Au Kurt J Lesker, Clairton, PA) was deposited onto the nanosphere template, as measured by a quartz crystal microbalance (Denton Vacuum, Moorestown, NJ). The localized surface plasmon resonance (LSPR) of the substrate was measured to be $\lambda_{\text{max}} = 790$ nm using a fiber optic probe (Ocean Optics, Dunedin, Florida) with a flat gold film as the reflective standard, and complete AuFONs were stored in a closed petri dish.

5-2-2. Nanoparticle characterization

The AuNRs (48 ± 11 x 13 ± 2 nm, 5 nM) and AuNCs (53 ± 10 nm in edge length, 4 nM) were graciously provided by the Murphy group at Northwestern University and by the Mirkin group at University of Illinois, Urbana Champaign, respectively. These nanoparticles were handled in the synthesis solution, probably leaving some cetyltrimethylammonium bromide or cetyltrimethylammonium chloride moieties on the surface, and characterization of these nanoparticles was performed by transmission
electron microscopy (TEM) and UV-vis extinction (Ocean Optics, Dunedin, Florida). The LSPR $\lambda_{\text{max}}$ values were 526 nm and 760 nm for AuNRs and 618 nm for AuNCs.

5-2-3. Microfluidic device fabrication for microfluidic-AuFON device

Standard photolithography procedures described in Chapter 3 were followed. Briefly, device design (Figure 5-1), imaged onto a transparent film (CAD/Art Services Inc., Bandon), was transferred to a blank chrome mask plate (Nanofilms, Westlake Village, CA for the blank masks). Then, using the mask, the design was transferred to a SU-8 photoresist layer (Microchem, Newton, MA) on a silicon wafer. Then, 10:1 weight ratio of Sylgard 184 resin and curing agent mixture (Ellsworth Adhesives, Germantown, WI) was cast onto the master and cured at 65 °C overnight to complete the PDMS channel layer. Channel dimensions were 100 μm wide and 100 μm high. For practical purposes, the terminal channels where measurements were performed had 1000 μm width. Bonding of the PDMS layer to AuFON was done via oxygen plasma treatment after inlet and outlets were punched. The entire fabrication was done in the Minnesota Nano Center (MNC) at the University of Minnesota.

5-2-4. Flexible SERS sensor fabrication

Flexible SERS sensors were fabricated using the same SU-8 mold used for microfluidic-AuFON devices. Nanoparticle solutions (1 mL of 5 nM AuNRs and 4 nM AuNCs in water) were centrifuged at 10,000 xg for 10 minutes, and the supernatants were removed
Figure 5-1. Device design (left) and photographs of microfluidic-AuFON (middle) and flexible SERS sensor (right).
using a micropipette. Then, nanoparticles were re-suspended into 100 μL of the curing agent component of Sylgard 184, and then mixed with 900 μL Sylgard 184 resin, degassed, and then poured onto the terminal channel array area of the SU-8 mold. After 30 minutes of curing on a 65 °C hot plate, 10:1 resin:curing agent mixture of Sylgard 184 was poured onto the mold, covering the entire device design, and cured overnight. Then, the device layer was peeled, cut, and punched for inlets and outlets. At this stage in device fabrication, SERS measurement on the microfluidic platform was possible but often not successful, likely because a large majority of the nanoparticles were completely incased in PDMS (i.e. not exposed to the in-channel stream of analyte). To enhance nanoparticle/analyte interaction, the PDMS layer was dry etched via reactive ion etching (75% CF₄ and 25% O₂, 100W, one hour) and then this layer was bound to another, clean and flat PDMS layer via oxygen plasma treatment. Again, channel dimensions were 100 μm wide (1000 μm for the observation channels) and 100 μm high.

5-2-5. Solution preparation

10 mM trans-1,2-bis(4-pyridyl)ethylene (BPE, Sigma Aldrich) was made fresh in ethanol, followed by dilution to 10 nM with MilliQ water (Millipore, Billerica, MA). 50 μM Rhodamine 6G (R6G, Sigma-Aldrich, St. Louis, MO) stock solution was also made fresh in ethanol, and final 1 μM R6G solution was made in water by diluting the stock with MilliQ water.
5-2-6. Fluorescence microscopy

An inverted microscope with a 20x objective (Nikon, Melville, NY) and a CCD camera (QuantEM, Photometrics, Tucson, AZ) was used to collect on-chip fluorescence images in three replicates. Metamorph Ver. 7.7.5 was used as the imaging software. Images of each channel were acquired with 10 ms exposure times. Once collected, the maximum fluorescence intensity, in arbitrary unit (AU\text{max}), in the region was recorded from each channel.

5-2-7. SERS measurement

The prepared microfluidic device-AuFON substrate was attached to an xyz stage (Thor Labs), and SERS spectra were measured with a SnRI ORS system (λ\text{ex}=785 nm, Snowy Range Instruments, Laramie, Wyoming). The LSPR λ\text{max} values of the microfluidic SERS substrates were 790 nm for the AuFON, 760 nm for AuNR, and 618 nm for AuNC; thus, the SnRI ORS system excitation wavelength is most appropriate for the AuFON and AuNR substrates. An xyz micromanipulator stage was used to achieve fine control over the measurement spots and focus throughout the experiment. Measurements were made across all five channels at five different positions within each channel. Each spectrum was collected for 1 second in three replicates (averaged) at a laser power of 3 mW. SERS measurements were conducted after 10 minutes flow through the device to ensure that the gold surface was equilibrated to the solution-phase analyte concentration so that the measurement was not biased with time. To note, as shown in Figure 5-8, a sharp peak was frequently obtained at ~520 cm\(^{-1}\) in the flexible SERS sensors. In the literature,
several studies have observed this peak during SERS measurements in a PDMS device, but the most convincing identity of this peak that we find is mono- or poly-crystalline silicon.\textsuperscript{23,24} For SERS measurements in a flexible SERS sensor, a silicon wafer was always present underneath the flexible SERS devices during measurements to better visualize channels, which may have led to the rise of the sharp peak. Another possibility is that the reactive ion etching chamber is frequently used for silicon etching, and even during etching the flexible SERS PDMS layer, there was a silicon wafer present in the chamber; as such, nanoparticles might be exposed to silicon during the etching process.

5-3. RESULTS AND DISCUSSION

5-3-1. Device operation/simulation

The microfluidic device used herein was designed to establish a concentration gradient of injected solutions so that a range of discrete concentrations are presented at each detection element,\textsuperscript{20} once solution 1 and 2 are injected through inlets 1 and 2, respectively, the solutions repeatedly mix and split while passing through serpentine channels (denoted as mixing channels, Figure 5-2). The original design combines all branches at the end so that linear concentration gradient of the solutions is established in one wide channel downstream of the mixing channels,\textsuperscript{20} but herein, all outcome branches are separated so that the analyte coming out of each channel can be monitored. To achieve the desired results, complete mixing is required within each mixing channel; similarly to the simulation in Chapter 3-3-1, computational fluid dynamics (CFD)
calculations assuming a 3-dimensional single laminar flow model were performed using COMSOL Multiphysics 4.3 to assess the mixing process for the given channel geometry (Figure 5-2). Channel dimensions were modeled as fabricated except that the channel height was set as 50 \( \mu \text{m} \) with symmetry to shorten computing time. As such, in this simulation, incompressible flow and the no slip boundary condition were used with user-defined diffusion coefficients, ranging from \( 0.5 \times 10^{-5} \text{ cm}^2/\text{s} \) to \( 1.0 \times 10^{-5} \text{ cm}^2/\text{s} \), and flow rates of 5, 10, 20, and 30 \( \mu \text{L/minute} \). Figure 5-2 shows the microfluidic device design with a representative simulation data plot. As shown in the figure, the simulation results confirm that, in all given conditions, each mixing channel achieves complete mixing, that is, the device operates as desired. In the experiments described below, a flow rate 10 \( \mu \text{L/minute} \) was used.

### 5-3-2. Fluorescence imaging

Before performing SERS measurements, device performance was experimentally evaluated by fluorescence imaging. Figure 5-3 shows the actual device bound to a clean glass substrate and fluorescence images from each location marked using red boxes. By visual inspection, the achieved fluorescence images showed the expected behavior, and thus, the obtained data were further analyzed quantitatively. Figure 5-3 summarizes fluorescence intensity data (maximum fluorescence intensity in arbitrary unit, \( \text{AU}_{\text{max}} \)) from 3 representative devices. In total, seven devices were tested for linearity of fluorescence response from the array of Channels, and the average \( R^2 \) value was 0.94 ±
Figure 5-2. Microfluidic platform design and COMSOL modeling for mixing process (diffusion coefficient of $1.0 \times 10^{-5}$ cm$^2$/s and flow rate of 10 μL/min).
0.02 (mean ± standard error of mean). This linear response was further evaluated by obtaining measurements from 3 vertical locations in each of the five terminal channels of a device (data not shown). For this assessment, fluorescence intensity from the array of five channels was obtained from 3 fixed y-axis positions of the stage, and all collected data demonstrated linear response from the array of channels.

5-3-3. SERS assessment of the microfluidic-AuFON device

Having achieved the desired performance of the microfluidic portion of our sensing platform, we next evaluated SERS performance of our microfluidic-AuFON platform. As PDMS does not bind well to gold surfaces, the AuFON was intentionally removed at preferred device binding locations to expose the silicon surface, which does bind to PDMS. Then, oxygen plasma treatment was used to induce permanent bonding between the PDMS device and AuFON substrate. Once assembled as shown in Figure 5-1, a BPE solution was introduced into the device following the same procedure used for fluorescence imaging: media without BPE into one inlet and BPE solution into the other inlet using a 10 μL/minute flow rate. We started with a high concentration of BPE (10 mM). BPE adsorb onto gold surfaces, so the channels were rinsed before SERS measurements. The LSPR $\lambda_{\text{max}}$ of the in-device AuFON was 790 nm, a good plasmonic match for the SERS excitation wavelength of 785 nm. SERS spectra of BPE have characteristic bands at 1021, 1210, 1610, and 1640 cm$^{-1}$ shift, and all of these bands
Figure 5-3. (a) Complete microfluidic device without SERS substrate - water into the left inlet and 50 μM R6G solution into the right inlet for demonstration purposes, device design, and fluorescence images from each point marked by red boxes in the design. (b) Fluorescence response analysis.
were observed in the measurement terminals (data not shown). All attempts confirmed that the characteristic BPE bands were a result of SERS, rather than normal Raman from the solution, because the SERS bands were still present after rinsing and either with water in the channels or after the device was allowed to dry (data not shown). As such, a much lower concentration of BPE (10 nM) was used to further evaluate the utility of the microfluidic-AuFON platform; from the left to the right in Figure 5-4, the respective/expected concentrations from each outlet channel 1 through 5 are 0, 1.25, 5, 8.75, and 10 nM. As shown in Figure 5-4, all BPE-characteristic SERS bands are obtained from individual channels with varying intensities, and a complete calibration curve with the assessment of LOD/LOQ was successfully obtained. It should be noted that we observed device-to-device variations, based on differences in AuFON quality. However, irrespective of the overall quality of the AuFON, the device showed linear response to linear concentration gradients of BPE. Even though lower than 1 nM BPE was not successful every time, the difference between 0 nM channel and ~ 1 nM channel was always clear. LOD evaluation by extrapolating data shown in Figure 5-4 yields an LOD of 1.01 ± 0.06 nM for this microfluidic-AuFON device. It should also be noted that on few occasions, the first channel, which should present no analyte, showed a slight BPE signal; this is due to an imperfection at the beginning of the device operation and can be easily fixed. Interestingly, as shown in Figure 5-5, different BPE Raman bands result in differing slopes in linear regression.
Figure 5-4. (a) Device design, (b) representative SERS spectra from the microfluidic-AuFON operated with a 10 nM BPE solution (*: BPE peaks), (c) SERS response analysis.
Figure 5-5. SERS response analysis a microfluidic-AuFON platform with $10 \text{ mM BPE}$. 
This result is interesting because the peak heights are the product of the enhancement factor (EF), Raman scattering cross section, and concentration of the analyte - assuming a constant EF with a known concentration of analyte in the channel, the slopes from all bands would be similar if the vibrational cross-sections were similar. A potential explanation for this variation in slope could be due to the data analysis routine where the presented data is obtained after baseline subtraction using a published algorithm. The algorithm is imperfect at choosing good baseline values especially when the spectrum is crowded, and this could contribute to the variation in slopes observed in our linear regression analysis. Further analysis will be required to delineate whether this is an artifact of analysis or a true reflection of scattering cross-section differences among vibrational modes.

5-3-4. SERS measurement on the flexible SERS microfluidic device

The performance of the flexible SERS device was assessed using the same experimental system detailed above. The device performance was assessed in a step-by-step fashion to characterize the performance of the Au nanoparticles embedded within the PDMS layer. As a preliminary experiment, a prototype straight channel microfluidic device was fabricated (Figure 5-6) with channel dimensions of 2000 μm (width) x 50 μm (height) x 25000 μm (length) with incorporated citrate-capped gold nanospheres (AuNSs). Without dry etching, small SERS signal was recorded, indicating that at least some of the AuNSs were extruding out of PDMS surface to make contact with the analyte. However, the
Figure 5-6. (a) Photograph of the prototype device. (b) SEM image showing nanoparticles extruding out of the PDMS surface. SERS spectra of (c) 1 mM BPE (*: BPE peaks) and (d) 1 mM benzenethiol (BT, *: BT peaks) obtained from the prototype device ($\lambda_{ex}=785$ nm, 10 second exposure, 3 mW power, and average of 3 replicates by the Snowy Range Instruments).
success rate of obtaining high quality SERS spectra was significantly lower than that achieved with devices that underwent reactive ion etching. SEM assessment of nanoparticle-embedded PDMS, after reactive ion etching, clearly demonstrated nanoparticles protruding from the PDMS surface, and SERS spectra from both 1 mM BPE and 1 mM benzenethiol, both well-known SERS probes, were successfully obtained using the preliminary flexible AuNS SERS platform (Figure 5-6). With these preliminary data, we proceeded with well-characterized, high enhancement factor-producing gold nanoparticles provided by the Mirkin group at Northwestern (gold nanocubes, AuNCs) and the Murphy group at University of Illinois, Urbana Champaign (gold nanorods, AuNRs), both shown in Figure 5-7. All attempts to obtain LSPR measurements from the completed device were only marginally successful, likely due to the small number density of nanoparticles available for these experiments, but we could observe a slight LSPR $\lambda_{\text{max}}$ peak shift compared to the colloidal nanoparticles before incorporation into PDMS. The shift is probably due to uncontrolled nanoparticle aggregation within the PDMS media. Using the same microfluidic platform described for the microfluidic-AuFON sensor, and AuNRs and AuNCs were directly embedded into the terminal channel array portion of the PDMS. 1 mM BPE was used as the analyte for this assessment; as such, expected concentrations for the five terminals were 0, 0.125, 0.5, 0.875, and 1 mM BPE. The linearity assessment was performed comparing the intensity of BPE’s 1640 cm$^{-1}$ shift band to the 490 cm$^{-1}$ shift PDMS band. While the LOD was higher than the microfluidic-AuFON platform, the flexible SERS sensor with both AuNRs (Figure 5-8) and AuNCs (Figure 5-8) also showed the expected linear response to
Figure 5-7. Nanoparticle characterization. LSPR measurement (a) and TEM images (b) of AuNRs and LSPR measurement (c) and TEM images (d) of AuNCs.
BPE analyte. The $R^2$ value for AuNC devices significantly improved with the use of a PDMS standard peak (0.89 ± 0.03 mean ± standard error of the mean, compared to 0.69 ± 0.05, Figure 5-8). Spatial variation in SERS responses within each terminal channel was also evaluated, and SERS responses of BPE at 3 discrete locations were similar. Together, these results demonstrate the utility of the embedded AuNR- and AuNC-based microfluidic SERS sensors.

There are many opportunities for further optimization of these flexible devices during the fabrication process. For example, the exact amount of gold nanoparticles added to and protruding from the PDMS layer has not been optimized. It is already clear, as observed under dark-field microscopy (data not shown), that a thinner nanoparticle-containing PDMS layer will lead to more uniform presentation of nanoparticles on the surface of PDMS while also requiring a smaller concentration or volume of colloidal nanoparticle solution. This will present an opportunity for more reproducible, and potentially more sensitive, SERS measurements. Furthermore, many groups are working to optimize SERS enhancement factors by controlling gaps between nanoparticles (e.g. creation of nanoparticle dimers or trimers). In the flexible SERS sensor platform, nanoparticles are embedded and fixed with, presumably, only a portion of the nanoparticle protruding into the microfluidic channel. This may allow, for example, nanoparticle dimer creation with a reduced degree of freedom and enable extraordinarily sensitive SERS sensing.

5-4. CONCLUSIONS
Figure 5-8. (a) Representative SERS spectra and (b) SERS response analysis from the flexible SERS sensor with embedded AuNRs, and (c) representative SERS spectra and (d) SERS response analysis from the flexible SERS sensor with embedded AuNCs. Solvent vs. 1 mM BPE solution was used as the analyte system.
Herein, we explore the use of two microfluidic SERS sensor platforms, a microfluidic-AuFON and a flexible SERS sensor, for high enhancement factor, low volume SERS measurements. The microfluidics portion of the sensor platforms provides desired control over the sample fluids as simulated theoretically and verified experimentally, and monitoring varying concentrations of analyte and assessment of LOD/LOQ are simultaneously achieved by SERS measurements. While the dimension of the entire devices presented herein is relatively large (2.5 cm x 7 cm), the size can be easily adjusted as required by applications. The material used to realize SERS detection is gold, a noble metal presenting minimum reactivity and toxicity to biological systems. Furthermore, the ease of surface functionalization of gold make the microfluidics-incorporated SERS approaches well-suited for applications where it is necessary to introduce biological functionalities, e.g. antibodies, aptamers, enzymes, nucleic acids, proteins, or other small molecules. Once incorporated with such surface-functionalities, simultaneous detection of multiple analytes with high throughput should be easily achieved. Live cell imaging is another potential application for the presented sensor platforms as the flexible SERS sensor-like approach presented herein may provide an opportunity to allow membrane-specific SERS imaging. Further developments on our proof-of-concept microfluidic SERS sensor platforms will provide unique advantages to researchers in bioanalytical sciences and bring significant impact in biomedical applications by providing opportunities for wearable sensors or on-site (portable) point-of-care devices.
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Appendix

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Hanyang Study Abroad Awards, Hanyang University (Translated)  2008
Fellowship for outstanding students, Hanyang University (Translated)  2003

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University of Minnesota, Minneapolis, MN, U.S.A.   2008 – Present
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On-chip Evaluation of immune cell response mechanisms against inflammatory cytokines
- Neutrophil Chemotaxis in Complex Environment
- Neutrophil Chemotaxis and p38 MAPK
- Neutrophil Chemotaxis: The Role of Interaction to Surrounding Cytokines and Endothelial Cells
- The Effect of Shear Conditions on Toxicity of Mesoporous Silica Nanoparticles
- Platelet Adhesion to Endothelium: The Role of Phospholipids in the Plasma Membrane
- Platelet Adhesion: The Interaction with Drug-carrying Nanoparticles
- On-Chip Target Specific Sensor Platform using AuFÖN
- Beyond the Limit: Realization of Extremely High Throughput Single Cell Amperometry using Segmented-Flow Microfluidics
- The Effect of Serotonin Contents in Nerve Cords on the Locomotion of Leeches
Hanyang University, Seoul, South Korea

2005 – 2007

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On-line Applications of Quantitative Raman Spectroscopy using the Wide Area Illumination Scheme

Peer-reviewed Publication List

“Microfluidics-based in vivo mimetic systems for the study of cellular biology”


“Stereochemistry- and concentration-dependent effects of phosphatidylserine enrichment on platelet function”


“Simple Approaches for On-Chip SERS Sensors: Realization of One Shot Limit-of-Detection Assessment”


“Exploring the Role of Phospholipids in Platelet Adhesion and Secretion”


“On-chip Evaluation of Platelet Adhesion and Aggregation upon Exposure to Mesoporous Silica Nanoparticles”


“On-Chip Evaluation of Neutrophil Activation and Neutrophil-Endothelial cell Interaction during Neutrophil Chemotaxis”


“The Role of p38 MAPK in Neutrophil Functions: Single Cell Chemotaxis and Surface Marker Expression”


“Neutrophil Chemotaxis within a Competing Gradient of Chemoattractants”


“On-Chip Evaluation of Shear Stress Effect on Cytotoxicity of Mesoporous Silica Nanoparticles”

“Electroanalytical Eavesdropping on Single Cell Communication”

“Simple and Reliable Raman Measurement of an Etchant Solution Directly Through Teflon Tubing”

“A New Quantitative Raman Measurement Scheme using Teflon as a Novel Intensity Correction Standard as well as the Sample Container”

Presentations
40th FACCS Conference, SciX 2013@Milwaukee 2013
  Simple Approaches for On-Chip SERS Sensors: Realization of One Shot Limit-of-Detection Assessment

  Neutrophil Chemotaxis under Competing Gradient of Chemoattractants
  * Outstanding Poster Award by JACS
  The Effect of Shear Stress on Cytotoxicity of Silica Nanoparticles

American Institute of Chemical Engineers (AICHE), Minneapolis, MN, US 2011
  The Effect of Shear Stress on Cytotoxicity of Silica Nanoparticles

  On-Chip Evaluation of Neutrophil Chemotaxis against Competing Gradient of Chemoattractants

  A Droplet-based Microfluidic Device for Single Immune Cell Characterization

The Korean Society of Analytical Sciences, Jeju, South Korea 2007
  Simple and Direct Raman Measurement of Etchant Solution using Teflon Tube as an External Standard as well as Sample Container (Translated)

International Conference on Raman Spectroscopy, Yokohama, Japan 2006
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Co-author on a grant proposal to National Institutes of Health (NIH)
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Participation in workshop: Innovations in Chromatography and Sample Preparation Certified by Fluka & Supelco, Sigma Aldrich

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