Miniature flow cytometer and its application in cancer theranostics-based nanoassays

Panwar, Nishtha

2018

Panwar, N. (2018). Miniature flow cytometer and its application in cancer theranostics-based nanoassays. Doctoral thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/73484>

<https://doi.org/10.32657/10356/73484>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**MINIATURE FLOW CYTOMETER
AND ITS APPLICATION IN
CANCER THERANOSTICS-BASED NANOASSAYS**

NISHTHA PANWAR

**SCHOOL OF
ELECTRICAL AND ELECTRONIC ENGINEERING**

2018

**MINIATURE FLOW CYTOMETER
AND ITS APPLICATION IN
CANCER THERANOSTICS-BASED NANOASSAYS**

NISHTHA PANWAR

SCHOOL OF ELECTRICAL AND ELECTRONIC ENGINEERING

A thesis Submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2018

Dedicated to
My loving parents, Vandana & Anil
and
My adorable husband, Manish

ACKNOWLEDGEMENTS

First of all, I would like to pay my regards to The Almighty who has led me to the path of science and explore the exciting opportunities that come in the offering.

My sincere thanks goes to my supervisor and mentor, Prof. Tjin Swee Chuan, who is always supportive of new ideas and proposals, and has been overly encouraging with his insightful inputs and useful advice for taking my work forward step-by-step.

I want to sincerely thank Prof. Yong Ken-Tye, who always motivated me to explore novel methods for my research, and guided me through challenges that came along the way.

I also want to thank Prof. Murukeshan Vadakke Matham for his constructive feedback which has helped to shape up my work in a big way. I want to give a big thanks to Ms. Toh Han Wei, you had solutions to all my administrative problems right from my first day in NTU.

I wish to acknowledge the unparalleled support of Dr. Song Peiyi for his untiring assistance on performing experiments. It is hard imagine to the amount of work done in all this time without your support and encouragement. I also want thank Mr. Yang Chengbin, Ms. Stephanie Yap Hui Kit, and Dr. Yin Feng, who assisted me in performing experiments with their expertise.

My lab experience would not have been as relaxed and conducive without the presence of Ms. Yee Yang Boey, Mr. Yong Kim Lam, Ms. Low Poh Chee, Mr. Tsay Chi Huang, and Mr. Yeoh Tiow Koon. You all have immensely helped me to ensure flawless flow of work.

Life at NTU will always be full of happy memories, all the more with friends like Dinesh and Muneesh by my side. It's been a treat sharing both light and heavy moments with you, especially during teatime.

This journey would not have been possible without the constant support, encouragement, and blessings of my beloved parents, Mrs. Vandana Panwar and Mr. Anil Panwar, you have always been there. I am indeed blessed to have my siblings, Anoosha and Tanishq –you both never cease to bring me smiles, no matter how each day goes by.

My heartfelt thanks and gratitude to my parents-in-law, Mrs. Fula Devi and Mr. Radha Mohan Singh, for your unconditional love and cooperation. Your belief in me always drives me to strive ahead.

My PhD journey will mark an important chapter of my life, both professionally and personally. Personally because I started the journey of my married life during this phase, and I can't thank enough my loving husband, Manish, for sharing this journey with me. You have stood by me in all ups and downs, and we have nurtured our relationship with trust and friendship all through this while, which I will always be proud of. Thank you so much.

Last but not the least, I wish my lovely grandparents, who have showered uncountable blessings every moment, were alive to see me graduate. I miss you!

ABSTRACT

Flow cytometry is an indispensable tool for identification, classification, and enumeration of single cells/particles in biomedical applications like pathology, immunology, gene sequencing, chromosome sorting, biodefence, and disease diagnosis. Flow cytometers represent a classic example of multidisciplinary instrumentation involving integrated optical, mechanical, and electronic modules in synchronous operation, and are indeed a gold standard for chronic disease diagnosis. However, the bulkiness and high cost limit their use to state-of-the-art research laboratories and high-end clinical settings by skilled personnel. Recent advances in solid-state optics and durable micro-fabrication techniques have inspired scientists and engineers to design micro-flow cytometers that are portable, easy-to-operate, and cost-effective. A conventional flow cytometer comprises of a nozzle system that aligns the input sample cells/particles into a focused stream of single-cells/particles surrounded by a stream of sheath fluid. Sample focusing by this method occurs as a result of differential pressure between the sample and the sheath stream. This pressure-driven particle focusing technique is termed as sheath flow-assisted hydrodynamic focusing. Most miniature flow cytometers also incorporate the same technique for focusing of cells/particles in a microfluidic channel wherein the sample is directed through the main channel and the surrounding sheath fluids are directed into the main channel through inlets on either side of the main channel. This method narrows down the sample stream width, but it requires additional modifications such as changes in the microfluidic channel geometry or active sources, for achieving a single-line focused stream of cells in the outlet channel. Thus, multiple inlet and outlet ports add complexity to the microfluidic set-up and limits reproducibility. Moreover, the presence of sheath fluid results in sample dilution and reduction of the overall throughput of the lab-on-a-chip flow cytometer. The second

hydrodynamic focusing technique used for particle focusing in microfluidic channels is based on inertial forces. This sheathless particle focusing technique usually employs a single inlet and a single outlet microfluidic channel design. Nevertheless, it requires hybrid channel geometries to reduce multiple equilibrium positions to a single position in three dimensions. Thus, the first objective of the work presented in this thesis is to design a novel microfluidic channel that is able to focus randomly distributed cells/particles at the inlet, to a single equilibrium position at the outlet using the sheathless, inertial forces-based hydrodynamic focusing technique. The proposed microfluidic channel geometry is a uniform square cross-section, single inlet and single outlet microchannel, comprising a series of radially increasing semi-arcs interleaved by linear sections, which is used to focus randomly fed cells/particles at the inlet to a single equilibrium position at the outlet. The entire geometry is symmetric from the midline of the microchannel. To our knowledge, this is the first sheathless microfluidic chip with a uniform cross-section throughout the microchannel length to achieve stable, single position focusing for spherical particles/cells with diameters from 10 μm to 30 μm at fast flow rates (up to 700 $\mu\text{l/min}$). Single position focusing for spherical particles/cells that satisfy the condition $0.1875 < a/w_{ch} < 0.9$ (where, a – diameter of the spherical particle/cell and w_{ch} – width of the proposed microchannel) for $10 < Re < 150$ (where, Re represents the channel Reynolds number and is a measure of the ratio of inertial to viscous forces acting on particles flowing in a channel) is achieved. We present a lab-on-a-chip-based miniature flow cytometer that incorporates our proposed microfluidic chip integrated with tapered optical fibers for interrogating the focused cells/particles and compare its performance with a commercial flow cytometer for a beads-based assay.

Nanoassays are imperative to extract any morphological or biological information from flow cytometers. In the context of disease diagnosis, nanoassays are

extensively used for providing and enhancing the signals from biomolecules. Drug delivery, disease progression, and prognosis are studied using nanoassays based on fluorescent beads, organic dyes, quantum dots (QDs), variable sized-colored beads, etc. However, these assays cannot be used as simultaneous carrier (biomolecules/drug/gene delivery) and signal providers (output/fluorescence signal). Thus, the second objective of this work is to develop alternative non-toxic, stable, and biocompatible nanoassays that can be used for both biomolecule carriers and signal analysis for cancer diagnosis and therapy. We have investigated the feasibility of using gold nanorods (AuNRs) and biodegradable charged polyester-based vectors (BCPVs) as gene delivery nanovehicles and signal providers in pancreatic cancer cells and chronic myeloid leukemia (CML) cells, respectively. Following this, a lab-based flow cytometer has been used to measure the performance indicators- gene transfection efficiency and cell apoptosis, to evaluate the effectiveness of the proposed nanoassay. The excellent gene knockdown performance (over 81%) of the proposed model support *in vivo* trials for RNAi-based cancer theranostics. Post transfection with AuNRs-siRNA nanoprobe, the 14-16 μm pancreatic cancer cells can be readily focused in our engineered portable flow cytometry set-up, and the transfection efficiency can be determined by measuring the output fluorescent signal. The lab-on-a-chip flow cytometer offers distinct advantages when compared with the conventional bulky flow cytometer: smaller sample size is needed, fewer sample preparation steps, cartridge-type one-off use chip, easier handling and operation, and low cost.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	v
ABSTRACT	ix
Chapter 1 Introduction.....	1
1.1 Motivation.....	1
1.2 Research Objectives.....	2
1.3 Scope and organization of thesis	4
Chapter 2 Literature Review	7
2.1 Flow cytometer: A Historical Perspective	7
2.2 Applications of a Flow Cytometer.....	7
2.3 Instrumentation and Operation: Inside the Black Box.....	9
2.4 Challenges of Conventional Flow Cytometers	12
2.5 Particle focusing techniques in Microfluidic Channels: Current state-of-the-art.....	14
2.5.1 Sheath flow-assisted Hydrodynamic Focusing.....	14
2.5.2 Inertial forces-assisted Hydrodynamic Focusing.....	19
2.6 Nanoassays for Cancer Theranostics	28
2.7 RNA interference (RNAi): A gene therapy for Cancer	29
2.7.1 siRNA delivery in Pancreatic Cancer Cells.....	32
2.7.2 siRNA delivery in Chronic Myeloid Leukemia (CML) cells	33
2.8 The Role of Flow Cytometers in Cancer Theranostics.....	35
2.9 Hypotheses.....	36
2.10 Summary.....	37
Chapter 3 Inertial forces-based Sheathless Particle Focusing for Lab-on-a-Chip Flow Cytometry	39
3.1 Introduction.....	39
3.2 Experiments and Methods	41
3.2.1 Fabrication of Microfluidic Chip.....	41
3.2.2 Cell Culture and Staining.....	43
3.2.3 Particle Focusing Experiments	43
3.2.4 Embedding Optical Fibers in the Microfluidic Chip	44
3.2.5 Optical Detection of Flowing Beads/Cells	47
3.3 Results and Discussion	49
3.3.1 Proposed Microfluidic Design for 3D Single-position Inertial Focusing.....	49
3.3.2 Theoretical Model for the Proposed Design	51
3.3.3 Simulation Results	58
3.3.4 Experimental Validation of Particle Focusing in the Proposed Microchannel Design.....	60
3.3.5 Effect of Re on Equilibrium Position.....	64
3.3.6 Focusing Performance of Cells.....	65
3.3.7 Lab-on-a-Chip Optofluidic Flow Cytometer	67
3.4 Summary.....	71

Chapter 4	Sheath-assisted Hydrodynamic Focusing in the Viscous and Inertial Regimes.....	73
4.1	Introduction.....	73
4.2	Experiments and Methods.....	75
4.2.1	Modelling of Sheath flow-assisted Particle Focusing in Microfluidic Channel.....	75
4.2.2	Fabrication of the Microfluidic Focusing Chip using Soft-photolithography.....	76
4.2.3	Particle Focusing Experiments	76
4.3	Results and Discussion	77
4.3.1	Role of Re and Re_p on Particle Focusing	77
4.3.2	Experimental validation of inertial hydrodynamic focusing	81
4.3.3	Single-sheath and Dual-sheath Hydrodynamic Focusing at Higher Re_p ..	82
4.3.4	Inertial Forces lead to Single-particle Hydrodynamic Focusing	83
4.3.5	Inertial Forces-based and Sheath-based Miniature Flow Cytometers for Cancer Theranostics.....	88
4.4	Summary	90
Chapter 5	Gold Nanorods-based Nanoassay for Pancreatic Cancer Theranostics.....	93
5.1	Introduction.....	93
5.2	Materials and Methods.....	95
5.2.1	Synthesis of AuNRs and Characterization of Nanoplexes	95
5.2.2	Cell Culture.....	96
5.2.3	Cytotoxicity.....	96
5.2.4	Transfection of cells with IL-8 siRNA	97
5.2.5	Fluorescence Imaging	97
5.2.6	Flow Cytometry	98
5.2.7	Gene Expression Analysis	98
5.2.8	Cell proliferation, Migration, Invasion, and Apoptosis Assays.....	99
5.2.9	Statistical Analysis.....	100
5.3	Results and Discussion	101
5.4	Summary	116
Chapter 6	A biodegradable charged polyester vector-based theranostic nanoassay for Chronic Myeloid Leukemia.....	119
6.1	Introduction.....	119
6.2	Material and Methods	121
6.2.1	Synthesis of BCPVs and Characterization of BCPV-siRNA nanoplexes 121	
6.2.2	Cell Culture.....	122
6.2.3	Transfection of cells with BCR-ABL siRNA	122
6.2.4	Cytotoxicity.....	123
6.2.5	Fluorescence imaging	123
6.2.6	Flow Cytometry	124
6.2.7	Post-transfection Gene expression analysis	124
6.2.8	Cell proliferation and apoptosis assay	125
6.2.9	Statistical analysis.....	125

6.3	Results and discussion	126
6.4	Summary	138
Chapter 7	Conclusions and Future Directions	141
7.1	Summary of the presented work	141
7.2	Challenges to realize miniaturized portable flow-cytometry for cancer theranostics: Present and Future	143
7.3	Current ongoing projects	145
References		147
List of Figures		161
List of Tables		171
Abbreviations and Symbols		173
List of Publications		179

Chapter 1 Introduction

1.1 Motivation

Flow cytometers are indispensable tools for clinical diagnostics, therapeutics, and biomedical studies. As the cells pass one-by-one in the fluidic nozzle of a flow cytometer, they get interrogated by a directional laser beam, yielding information about their size, morphology, and/or fluorescence characteristics. In most cases, flow cytometers employ lasers with their associated cooling systems, and an inter-related chain of optical components for guiding the illuminating beam and detection of transmitted signals, which translate into large and bulky benchtop instruments. Delicate optical detection/filtering devices and complicated control circuits add to the mechanical complexity and cost of such instruments. Besides these limitations, the conventional bench-top flow cytometers require high volumes of sheath fluids to assist the alignment of the cell sample into a single stream of flowing cells for analysis. Moreover, the sample needs to be subjected to multiple pre-treatment steps before it can be fed to the flow cytometer. Thus, it is a tedious process to analyze cell characteristics (during both diagnostic and therapeutic stages) using current conventional flow cytometry systems.

Flow cytometry is increasingly being employed for monitoring *in vitro* cancer theranostics. Cancer cells, after being subjected to gene therapy, need to be monitored at different stages for the therapeutic effect of the administered modality. Suppression of overexpressed gene mutations in cancer cells through RNA interference (RNAi) technique is a therapeutically-effective modality for oncogene silencing. However, small interfering RNA (siRNA) are unstable and unable to enter the cancer cells by themselves owing to their negative charge and nuclease-mediated degradation. In general, transfection agent is needed for siRNA delivery. In this context, flow cytometers play an important role of measuring the transfection efficiencies of different

siRNA nanocarriers. Moreover, flow cytometers are also used to measure cell characteristics during the therapeutic stages, e.g. apoptosis, necrosis, etc. as the cancer cells undergo gene therapy by various nanoassays.

Therefore, there are two urgent challenges that we need to address for making these flow cytometers easily accessible for point-of-care applications. First, new and portable optofluidic flow cytometry methods need to be engineered to enable fast and affordable cancer theranostics. Second, novel theranostics nanoassays that provide non-toxic, biocompatible, and stable non-viral transfection agents for gene delivery in cancer cells and at the same time, are able to extract meaningful diagnostic information from the cancer cells, need to be developed and investigated so as to enhance the capability of flow cytometers.

1.2 Research Objectives

The research work presented in this thesis is broadly divided into two themes: *Miniaturization* and *Cancer theranostics applications*, as shown in **Figure 1.1**, and is further classified as detailed below:

Figure 1.1: Research scope of this thesis

Miniaturization

- To understand the instrumentation and working of a flow cytometer, and its application in cancer theranostics.
- To update with different versions of lab-on-a-chip-based miniature flow cytometers.
- To learn various particle focusing techniques (both sheath-assisted and sheathless) that are employed for aligning and focusing cells to a single-position in microfluidic channels for performing cytometry measurements.
- To devise novel methods of focusing cells/particles inside microfluidic channels using inertial forces without the use of sheath fluid.
- To explain the inertial cell focusing mechanism in the proposed microfluidic channel design analytically and by simulations.
- To distinguish the proposed sheathless inertial focusing method with the sheath-assisted cell focusing method in microfluidic channels for the same working parameters.
- To design a lab-on-a-chip miniature flow cytometer incorporating on-chip inertial fluid flow and tapered optical fibers for interrogation.
- To characterize the designed miniature flow cytometer with fluorescent spherical beads and present a comparative study vis-à-vis conventional flow cytometer.

Cancer theranostics applications

- To acquaint with the use of flow cytometer for evaluating the diagnostic and therapeutic indicators in cancer cells undergoing RNAi-based gene therapy.
- To develop novel nanoassays for gene transfection into pancreatic cancer and leukemia cells, and estimate the gene knockdown effect on cancer cells by flow cytometry.

1.3 Scope and organization of thesis

This thesis is divided into two broad sections. The first section gives an overview of the fundamental flow cytometer instrumentation and operation. It highlights the challenges and limitations of the conventional flow cytometer and focuses on the need to miniaturize the same. Furthermore, a novel method of focusing cells/particles inside microfluidic channels using inertial forces without the use of sheath fluid is discussed in detail. Also, experimental and analytical data for sheath-assisted particle focusing in microfluidic channels using the same working parameters as in the proposed design are presented, for a comprehensive comparison of the two pressure-driven particle focusing techniques. The second part of this thesis focuses on the application of flow cytometers in cancer theranostics. It features novel nanoassays for gene transfection into pancreatic cancer and leukemia cells, and elaborates the role of flow cytometry in measuring the performance indicators of these cancer therapeutic modalities.

Chapter 2 gives the background on flow cytometer evolution and its increasing penetration in the biomedical field for myriad applications. It discusses the limitations of using conventional table-top flow cytometers for point-of-care applications in low-end clinical settings. It also gives an overview of the research on the design of lab-on-a-chip flow cytometer models over the previous decade, with particular elaboration on the two passive particle focusing techniques used in microfluidic channels for aligning and focusing cells/particles to a single position: sheath-assisted particle focusing and inertial forces-assisted particle focusing. Furthermore, it explains RNAi-based gene therapy for suppressing oncogene mutations in cancer cells and highlights the role of flow cytometers as performance indicators of gene therapy nanoassays in treating various forms of cancer.

Chapter 3 presents a novel method of focusing cells/particles inside microfluidic channels using inertial forces without the use of sheath fluid. Analytical and experimental results along with the ranges of working parameters are presented in this chapter. Furthermore, it outlines the design of a lab-on-a-chip flow cytometer based on the sheathless particle focusing microfluidic chip with tapered optical fibers. Optical detection and characterization of the device is performed using different sized fluorescently-labeled polystyrene beads. We then present a statistical comparison to quantify the lab-on-a-chip flow cytometer vis-à-vis conventional flow cytometer.

Chapter 4 gives a comparative study between the proposed sheathless inertial focusing design, and the sheath-assisted particle focusing method for the same ranges of working parameters. Comparison of two sheath-assisted particle focusing methods, i.e. the single-sheath and dual-sheath designs, for hydrodynamic particle focusing are also presented.

Chapter 5 discusses a novel theranostic nanoassay for pancreatic cancer cells. It then elaborates the role of flow cytometry in measuring the performance indicators (gene transfection efficiency and cell apoptosis) to evaluate the effectiveness of the proposed nanoassay.

Chapter 6 presents a novel study on a non-adherent or suspension cancer cell line – Chronic Myeloid Leukemia (CML) cell line (K562 cells), and investigates the effect of a biodegradable polymer-based theranostic nanoassay to enable high efficiency gene delivery and induce gene silencing, with a qualitative and quantitative assessment of gene delivery studies by flow cytometry.

Chapter 7 summarizes the observations and conclusions of the stated research objectives of this thesis. It presents an overview of the entire work with a descriptive account of the methodologies applied to address some of the challenges during the

various stages of work. Finally, it outlines a brief layout on recommendations for future work directed towards some critical challenges along with current ongoing projects on the miniature flow cytometer.

Chapter 2 Literature Review

2.1 Flow cytometer: A Historical Perspective

Flow cytometry is the science of measuring physical and chemical properties of live cells or other biological particles as they pass in a single-file fluid stream through a measuring apparatus, called the flow cytometer [1]. Each cell or particle is interrogated by a laser beam directed towards it, yielding information about its size, morphology, and/or fluorescence characteristics. Driven by the highly-motivated efforts of biologists to make qualitative records of visual observations of single cells in early 19th century, practical concurrent developments in microscopy and photography, enabled scientists and engineers to develop instruments for differentiating cell types, study cell division and cell reduction mechanisms [2]. Attempts to record and quantify these observations led to demonstrations of cell counting chambers on a glass slide by Gowers [3] and capillary tube-based model by Moldavan [4]. In 1947, the first working flow cytometer was described, in which the air sample stream was confined to the central portion of an air chamber by a sheath of filtered air, and illuminated by a Ford headlight, that could reportedly detect a 0.6 μm diameter particle with 60% probability [5]. Following this sheath flow principle, variants of blood cell counters were developed [6, 7]. Additionally, these counters are now being incorporated with fluorescent markers for analysing, purifying, and sorting living cells [8-10] that have resulted in commercial fluorescence activated cell sorters (FACS) [11-13]. A host of commercial devices, such as FACScan, FACSCalibur, MoFlo, and Influx, are currently used for diagnostics and therapeutics in high-end clinical settings and state-of-the-art research laboratories.

2.2 Applications of a Flow Cytometer

A flow cytometer, as the name suggests, is an instrument that measures the morphological and light-interaction characteristics of cells/particles as they flow past a

liquid stream in a single-particle file. Over the past century, flow cytometers have evolved from a basic cell counter to high-throughput tools that are extensively used for identification, characterization, quantification, and sorting of sample populations in biomedical and clinical settings. Starting from enumerating the number of cells in a given volume of blood, to sorting different cells in a blood sample, flow cytometers have been on the centerstage for laboratory, clinical, and medicinal research. Flow cytometry is a powerful quantitative analysis tool for a myriad of biological applications like immunology and pathology [14], HIV diagnosis, chromosome staining, and sorting [15], measurement of DNA content [16] and DNA sequencing [17, 18]. Additionally, flow cytometry is increasingly being put to use for measuring protein expressions [19, 20] and study of cancer biology (**Figure 2.1**).

Figure 2.1: Application domains of a flow cytometer.

2.3 Instrumentation and Operation: Inside the Black Box

The basic architecture of a flow cytometer consists of an optical unit, a fluidic unit, and signal processing electronics [1, 21]. **Figure 2.2** shows the schematic arrangement of components that constitute a generalized “four-parameter” flow cytometer. The entire process of measuring and analyzing signals from single cells or particles is classified into four major steps and detailed in the following paragraph with reference to **Figure 2.2**.

a) Optical Excitation: Illumination of the cell or particle stream

A laser beam with a circular, radially symmetrical Gaussian profile is used for illuminating the cells as they flow past the incident beam of light. Different lens combinations are used to focus the laser beam to a small diameter such that each cell of the sample is brightly illuminated. To achieve this, it is essential for the cells to be confined to a well-defined path to ensure that each cell is uniformly illuminated as they pass through the beam. The next building block of the flow cytometer, the fluidic unit, serves the purpose of focusing the sample cells or particles to a single-position for extracting quantitative data from every cell or particle in the sample.

Figure 2.2: Schematic representation of flow cytometer instrumentation depicting the four major architectural sections.

b) Fluidics: Centering of cells or particles in the illuminating beam

Pre-treated cells or particles are fed into the sample inlet of the nozzle system. The function of the nozzle system is to align the input sample cells or particles into a focused stream of single-cells or particles. A high-pressure sheath stream envelops the sample stream and focuses the sample into a single-cell line that one or more lasers interrogate (**Figure 2.3a**). The pressures of both the sheath and sample fluid play a crucial role in aligning the cells into a single-cell stream as the cells move through the cytometer nozzle. Following the principles of fluid mechanics, the suspended cells within the sample remain confined in a central core as they flow within the pressure-driven sheath stream through the flow chamber. This phenomenon of aligning the sample within the center of the sheath flow is termed as hydrodynamic focusing. The diameter of the sample flow is maintained in the range of few tens of microns. This narrow core ensures uniform and focused illumination at the center of the stream. Moreover, the narrow channel ensures the cells to be stretched out at a distance from each other, enabling one cell to be illuminated at a time.

c) Interrogation: Detection of signals from focused cells

A typical flow cytometer consists of two or more photodetectors placed in-line or at an angle with the illuminating beam. Each photodetector is tuned to detect optical signals of interest to obtain specific information from the sample cells or particles. The part of the fluidic nozzle where the fluid stream and illuminating light beam intersect is called the interrogation region. It is essential that the light beam and the sample stream are squarely aligned with each other for proper illumination and generation of non-erroneous optical signals from the flowing cells that are subsequently relayed onto the photodetectors through lens assemblies surrounding the interrogation region.

Figure 2.3: a) Schematic of the hydrodynamic focusing process used to align the cells or particles fed to the flow cytometer into a focused stream of single cells. The fluidic unit consists of a nozzle that has inlets for sample and sheath fluid fluids. The sheath fluid surrounds and flows past the sample at a higher pressure such that the sample cells or particles get focused into a single-cell line as they traverse the length of the nozzle. b) The optical signals are acquired by a signal acquisition unit and processed to give distributions of output intensities in terms of cell or particle count.

The photodetector placed in-line at the interrogation region collects the light scattered by the focused particles within an angle of 5° . This is called the forward scatter signal (FSC) and it extracts information regarding the size and volume of the cell/particle, including other parameters such as refractive index (RI). For instance, a cell having higher RI will bend more light than a cell with a lower RI of same size, thereby generating a stronger FSC. The rest of the photodetectors are placed at different angles (less than 90°) to the illuminating beam. One of them registers light that is scattered on the side from the surface of the cell/particle. The strength of this signal is a measure of surface roughness or irregularities or granularity. This signal is called side-scatter (SSC) signal and is related to a cell's surface texture and internal structure besides its size and shape. The other detectors collect the fluorescence from the labeled-cells or particles, yielding information about specific interactions or cell parameters for further investigations.

2.4 Challenges of Conventional Flow Cytometers

In most cases, flow cytometers employ lasers with their associated cooling systems, and an inter-related chain of optical components for guiding the illuminating beam and detection of transmitted signals, which translate into large and bulky benchtop instruments. Delicate optical detection/filtering devices and complicated control circuits add to the mechanical complexity and cost of such instruments. Therefore, the conventional flow cytometers pose the following limitations which restrict their use on a large scale in low-end clinical settings:

- Bulky
- High cost
- Trained personnel
- Large volumes of sheath fluid for sample focusing
- Multiple sample pre-treatment steps
- Large sample size needed for analysis
- Mechanical and optical complexity

Escalated development in microfluidics and nanotechnology have spurred research on lab-on-a-chip devices for nano-immunoassays, nano-diagnostic assays, micro-scale cell/particle sorting and separation, particle mixing modules, etc. Having proven its mettle in cell/particle counting, characterization, and sorting, flow cytometers have made an irreversible bond with clinical diagnostics and immunophenotyping- both in clinical practice as well as in medical research. Thus, there have been tremendous efforts for the development of portable, accurate, and fast lab-on-a-chip-based models of flow cytometer, integrating fluidics and optical principles at the micro-scale with an important objective of a cost-effective, larger footprint in the medical community.

Figure 2.4 gives an estimate of the research efforts in terms of translating the table-top version into a miniature lab-on-a-chip flow cytometer. Motivated research efforts have resulted in simpler, smaller, and inexpensive flow cytometers. Preliminary designs for on-chip flow channels have been proposed for specific target applications, such as estimation of targeted drug delivery [22], detection of fluorescently-labeled beads [23], differentiation of cells in a sample based on their size, and the detection of virus concentration in a given sample [24].

The principles behind the design and operations of miniature flow cytometers can be categorized into optical fluorescence-based [22], impedance-based [25, 26], both fluorescence and impedance-based [27], and magnetic bead-based [24]. The main idea is to design and develop an on-chip geometry that is conducive for the sample flow in a laminar regime with one particle or cell passing through the interrogation region at a time. These lab-on-a-chip-based designs proposed for flow cytometry applications are perhaps in the proof-of-concept or characterization stages. Nevertheless, the performance of these miniature versions vis-à-vis the conventional counterpart is encouraging enough to explore and investigate novel flow cytometer designs for achieving consistent results outside the laboratory settings.

Figure 2.4: a) Statistical trend depicting the increasing relevance of flow cytometers. b)

Research output for microfluidic flow cytometers during the last two decades.

2.5 Particle focusing techniques in Microfluidic Channels: Current state-of-the-art

Precise focusing of particles to a single position within the microfluidic channel is paramount to the accurate operation of the miniature flow cytometer. Derived from the particle focusing in macroscale systems, various particle focusing techniques are applied for aligning micro-/nano-meter sized particles in microfluidic systems. The most popular method is the hydrodynamic focusing wherein the applied pressure is the primary factor that defines particle alignment to a single-line stream across the length of the microchannel. Based on the forces acting on the particles that result in definite equilibrium positions along the microchannel length, this pressure-driven hydrodynamic focusing technique can be broadly classified into two categories:

- a) Sheath flow-assisted hydrodynamic focusing
- b) Inertial forces-assisted hydrodynamic focusing

Besides the pressure-driven particle focusing technique, some groups also proposed to use active approaches including acoustic [28-30], magnetic [31-33], electrokinetic [26, 34, 35], hydrophoretic [36-38], and dielectrophoretic [39-41] methods for supplementing the hydrodynamic focusing in microfluidic channels. However, these additional external forces add to the complexity of the microfluidic systems and are not a part of discussion in this thesis. In this section, we outline both categories of hydrodynamic focusing- their working mechanisms, the ranges of various operating parameters that define them, and the various types of microchannel geometries used.

2.5.1 Sheath flow-assisted Hydrodynamic Focusing

Based on the working of the conventional flow cytometer, sheath flow-assisted hydrodynamic focusing in microfluidic channels involves movement of cell/particle

stream surrounded by a sheath flow, wherein the pressure difference (differential flow rates) between the sample and sheath fluids regulates the cell stream within a narrow region between the sheath streams (**Figure 2.5**). The width of the focused stream depends on the following factors:

- Particle diameter ' a '
- Width of the main channel ' w_{ch} '
- Aspect ratio (AR) of the rectangular channel (defined as h/w_{ch} ; h is the channel height)
- Flow rate ratio (FRR = sample flow rate ' Q_s '/sheath flow rate ' Q_{sh} ')
- Viscosity ratio of the sample and sheath fluids

Figure 2.5: Parameters of a typical sheath flow-assisted hydrodynamic focusing in a microfluidic channel layout.

Various symmetrical and asymmetrical channel geometries involving single and multiple sheath flows for hydrodynamic particle focusing in microfluidic flow cytometers have been proposed [23, 24, 42-44]. Lin et al. presented a micromachined flow cytometer with two embedded etched optical fibers to carry out on-line detection of particles and cells, as shown in **Figure 2.6a** [22]. Lithographic technique was used to fabricate the cytometer on soda-lime glass substrates. The miniature flow cytometer used single-sheath hydrodynamic focusing to align and focus particles and cells and was

shown to count polystyrene beads and human blood cells as each particle passed through the detection region.

Figure 2.6: a) Embedded etched optical fiber based detection of different-size particles on a lithographically fabricated substrate [22]. b) Groove-generated sheath flow for particle focusing [42].

Three-dimensional (3D) particle focusing was demonstrated in a curved microchannel with a series of repeated symmetric sharp corner structures on both side walls using $7.32\ \mu\text{m}$ particles at a sample flow rate of $66.7\ \mu\text{L min}^{-1}$ and a sheath flow rate of $400\ \mu\text{L min}^{-1}$ [45]. A combination of sheath flow-induced microfluidic drifting and momentum-induced inertial drifting led to particle focusing at the center of the microchannel. Another microflow cytometer was demonstrated wherein particle focusing occurred using groove-generated sheath flow [42]. Chevron-shaped groove features were fabricated on the top and bottom of the channel to aid sheath-assisted hydrodynamic focusing (**Figure 2.6b**). Mao and co-workers demonstrated an on-chip microfluidic flow cytometer system based on a 3D hydrodynamic focusing technique termed as microfluidic drifting [23]. The fluidic module, shown in **Figure 2.7a**, comprised of multiple sheath flows for particle focusing and was integrated with an

input fiber to deliver the excitation light to the focused cell stream, and three detection fibers arranged around the main channel at different angles so as to simultaneously detect the FSC, SSC, and FL signals through photomultiplier tubes (PMTs). An optofluidic flow cytometer was illustrated using dual-sheath hydrodynamic focusing and integrated optical signal detection [44]. Tumor cell apoptosis was studied by focusing HeLa cells within 200 μm wide microchannels with sample and sheath flow rates of 30 $\mu\text{L min}^{-1}$ and 150 $\mu\text{L min}^{-1}$, respectively (**Figure 2.7b**). A cell counting rate of 866 cells per second was recorded. Further, numerical and experimental studies were shown for particle focusing in a two-layer dual-sheath microchannel design at $Re < 5$ [46]. Particle focusing was studied at sample flow rate of 3.3 $\mu\text{L min}^{-1}$ for a range of ARs. An additional lifting port inlet design was introduced in a dual-sheath configuration for focusing and counting different sized-polystyrene beads and Jurkat cells at sample flow rate = 1 $\mu\text{L min}^{-1}$, sheath flow rate = 50 $\mu\text{L min}^{-1}$, flow rate of sideports = 5 $\mu\text{L min}^{-1}$ and flow rate of lifting inlet = 20 $\mu\text{L min}^{-1}$ [47].

Figure 2.7: a) 3D hydrodynamic focusing and detection of particles using microfluidic drifting technique [48]. b) Dual-sheath hydrodynamic focusing and integrated optical signal detection [44].

The existing works concerning sheath-assisted hydrodynamic focusing models predict the focused sample stream width in the low Reynolds number regime ($Re \ll 1$) of the Navier-Stokes (NS) equations, wherein the viscous forces dominate the inertial forces. In Chapter 4, we will discuss particle focusing behavior in single-sheath and dual-sheath flows using simulation and experiments, and outline the effect of particle focusing at higher Re ($60 < Re < 130$) laminar flows. Moreover, we will explain sheath-based particle focusing at lower and higher Re_p , and also differentiate the performance of sheath-assisted focusing from the sheathless focusing techniques. Inasmuch, the range of operating conditions of sheath-assisted hydrodynamic focusing imposes inherent challenges, listed below, that limit the scalability of this model for lab-on-a-chip flow cytometry applications.

- Multiple inlet and outlet ports in the microfluidic set-up adds to the complexity of successful functioning and limits reproducibility of results.
- Presence of sheath fluid results in sample dilution.
- As the sample flow focusing is governed by sheath flow pressure, the sample throughput is limited to very low flow rates such the microfluidic chip is able to withstand the overall pressure, thereby limiting the overall throughput of the cytometry device.
- The flow parameters (speed, direction, and pump on/off timing) for the main channel and the sheath channels need to be carefully set and controlled by syringe pumps.
- Many researchers have investigated complicated structures, including the effect of multiple ARs and introduction of grooved 3D patterns in a single chip for accurate sheath assisted-particle focusing in microchannels. These features make the microfluidic fabrication process a bit cumbersome and complex.

2.5.2 Inertial forces-assisted Hydrodynamic Focusing

Inertial forces are also used for nano-/micron-sized particle separation, sorting, and filtration in microfluidics devices. Before citing examples of inertial forces-assisted hydrodynamic focusing for microfluidic applications, I will give a brief introduction about the origin and fundamentals of inertial forces in fluid dynamics. The motion of particles in a flow is governed by the N-S equation which accounts for both viscous and inertial effects. More often, theoretical research on particle motions in viscous fluids is predicted on three approximations:

1. Reynolds number is zero and inertial effects are completely neglected. Reynolds number Re is a measure of the ratio of inertial to viscous forces acting on particles flowing in a channel, and is expressed in **Equation 2.1** as

$$Re = \frac{\rho U_m D_h}{\mu} \quad (2.1)$$

where ρ is the fluid density, μ is the coefficient of viscosity of the fluid, U_m is the maximum velocity of the fluid, and D_h is the hydraulic diameter of the channel, defined as $D_h = 2hw_{ch}/(h + w_{ch})$, where h and w_{ch} denote the height and width, respectively, of the channel cross-section.

2. Particles have a fixed shape.
3. The suspending fluid is Newtonian. For a Newtonian fluid subjected to shear, the resulting strain rate is linearly related to the applied stress. It is numerically expressed as $\tau_{xy} = 2\mu\epsilon_{xy}$, where τ_{xy} represents the applied shear stress and ϵ_{xy} denotes the resulting strain rate [49]. Water, oils, and gases are common examples of Newtonian fluids. On the other hand, fluids which yield non-linear functional relationship between applied stress and strain rate are termed as Non-Newtonian.

Under weak departures from these approximations, the instantaneous velocity or pressure fields more or less follow the theoretically-predicted values. However, small

contributions of inertia, nature of flow, and/or changes in particle shape have a dominant effect on particle position and orientation as demonstrated by the lateral migration of spherical particles in a Poiseuille flow by Segre and Silberberg [50]. In their experiment with ~ 1 mm particles flowing in a ~ 1 cm diameter cylindrical pipe, they found particles migrating to an annulus at a distance of 0.6 times the pipe radius, from the center of the pipe. Such lateral cross-migration between streamlines in a laminar flow has been explained analytically to be a consequence of the inertial force component of the N-S equation in independent works of Ho & Leal [51] and Vasseur & Cox [52]. A shear gradient-induced lift force ' F_{IS} ' contributes to significant changes in particle velocity to allow lateral drift of rigid particle from its initial position/orientation. This force interacts with the wall-induced lift force ' F_{IW} ' which by nature repels particles from the channel walls, and results in equilibrium positions across the channel cross-section. A general framework based on asymptotically small departures from these approximations has been developed for the prediction of steady-state configuration of particles wherein $Re \ll 1$ [53-55]. Implicit in this framework is the fact that the particle is negligibly small as compared to the scale of flow boundaries (bounded as well as unbounded flows), and is referred to as point-particle interaction with the flow. Within bounded flows, non-linear inertial lift effects ' F_I ' act on rigid particles to induce lateral migration across streamlines of an undisturbed flow with a particle Reynolds number (Re_p) dependent-translational velocity [53]. Following the theoretical and experimental evidences of the effect of inertial forces in driving particles trajectories in macroscale fluid dynamics, inertial forces-based focusing has attracted talented research groups to explore particle behavior at the microscale, i.e. inertial forces in microfluidics.

Typically, inertial forces dominate in microchannels where the Re_p , defined in **Equation 2.2**, is of the order of 1.

$$Re_p = Re \frac{a^2}{D_h^2} \quad (2.2)$$

Inertial lift forces driven trajectories of neutrally buoyant particles in a square duct over an Re range of 100 through 1000 were numerically investigated by Chun and Ladd using the lattice-Boltzmann method [56]. They described the evolution of equilibrium position of particles from a stable ring in a cylinder [50] to discrete equilibrium positions in a duct. The first experimental evidence of particle focusing, ordering, and separation in microfluidic channels using inertial forces was presented by Di Carlo and co-workers [57]. They experimentally demonstrated the effect of inertial lift forces ' F_I ' on particle migration at high Re (15, 30 and 90) and Re_p (0.48, 0.97 and 2.9) in three microfluidic structures: a) rectangular microchannels, b) symmetric curving geometry, and c) asymmetric curving geometry (**Figure 2.8**). They demonstrated four symmetric equilibrium positions in rectangular microchannels, whereas two and one equilibrium position in the latter two cases. Meanwhile, Papautsky's group [58, 59] demonstrated the effect of channel AR on the stability of equilibrium positions in a rectangular straight channel. The number of equilibrium positions in low AR rectangular channels favored on the center of the wider channel face, as there was negligible shear on the narrow face. The reduced number of equilibrium positions in the curved microchannels is attributed to the presence of secondary forces called the Dean Drag forces ' F_D '. Dean forces are a function of Dean Number ' De ' (**Equation 2.3**) and curvature ratio ' δ ' (**Equation 2.4**) of the microchannel, which cause particle rotations along the two counterrotating vortices above and below the channel midline.

$$De = Re \sqrt{D_h/2r} \quad (2.3)$$

$$\delta = D_h/2r \quad (2.4)$$

The secondary rotation causes outward and inward drag along the channel midline and channel edges, respectively. Thus, this sheathless approach of ordering and focusing particles inside microchannels is a breakthrough for designing simpler miniature flow cytometers. The effect of flow parameters and channel geometry on inertial forces-assisted particle focusing have been explored by various research groups since then. The interaction between F_I and F_D is detailed in Chapter 3, where we introduce a novel microfluidic design for 3D particle focusing without the use of sheath fluid or any active method. The following part of this section will introduce the reader to different categories of sheathless particle focusing layouts based on inertial lift forces or so to say, sheathless particle manipulation layouts, which have been proposed after Di Carlo's pioneering work. A brief outline about the working principles that define particle behavior in each of these categories is also underlined.

Figure 2.8: a) Schematic representation of particle ordering in curved channels. b) Images of 9 μm diameter particles flowing in a 50 μm square channel, showing distinct (four) number of equilibrium position at different Re_p . c) Two equilibrium positions in a symmetric curving channel the symmetry. d) A single equilibrium position in an asymmetric curving system [57].

More experimental results for particle equilibrium positions in straight high AR rectangular microchannels were soon reported [58]. The role of Dean flows in curved microchannels was analyzed [60, 61] and the effect of the dimensions of curved geometries in inertial focusing was presented [62]. In 2010, an extreme throughput imaging flow cytometer with a sampling rate of 1 million focused blood cells per second was proposed [63]. Based on inertial focusing, the microfluidic device consisting of 256 parallel high AR ($w_{ch} = 16 \mu\text{m}$, $h = 37 \mu\text{m}$) microchannels in each of which two streams of focused cells assembled. This lab-on-a-chip particle focusing device shows extremely high-throughput, but would perhaps be an expensive overall product when combined with state-of-the-art wide-field optical imaging techniques for flow cytometry.

Bhagat and group proposed a spiral microchannel design, shown in **Figure 2.9**, for sheathless focusing of particles under the influence of F_I coupled with Dean Drag [64]. Their device was a 10-loop, $100 \mu\text{m} \times 50 \mu\text{m}$ ($w_{ch} \times h$) spiral microchannel and could achieve particle focusing towards the channel side, and produce a particle counting throughput of $2100 \text{ particles sec}^{-1}$ for $6 \mu\text{m}$ particles.

A more comprehensive study of inertial focusing dynamics and particle behavior in low AR ($\sim 1/1$ to $1/8$) spiral microchannels was reported by Martel and Toner [65]. Oakey and co-workers disclosed a staged channel design consisting of both curved and straight sections that combine to order particles into a single streamline with longitudinal spacing using inertial and secondary forces [66]. The straight part of the channel has a rectangular cross-section ($30 \mu\text{m} \times 50 \mu\text{m}$), with varying channel width in the asymmetrical curved regions. The focusing performance for $10.2 \mu\text{m}$ beads was demonstrated for a maximum flow rate of $100 \mu\text{l/min}$. However, there was no comment on the number of the asymmetrical curves or the overall length of the microchannel.

Figure 2.9: Schematic of a spiral microfluidic design illustrating sheathless 3D particle focusing under the action of inertial lift forces and Dean Drag forces for flow cytometry applications [64].

Chung and co-workers demonstrated single-stream particle focusing platform in wider microchannels ($\geq 100 \mu\text{m}$) with sequential obstacles by the combined effect of inertial lateral migration and locally-tuned secondary flows [67]. A particle focusing throughput of $13000 \text{ particles s}^{-1}$ was achieved for $19 \mu\text{m}$ polystyrene microspheres at $Re = 66.7$. Following this work, a 1:2 AR, straight channel interspersed with stepped parts was shown to focus different sized particles and cells ($8 - 16 \mu\text{m}$) to a single position at $Re = 83.33$ (**Figure 2.10**) with an even higher throughput of $36000 \text{ particles s}^{-1}$ [68] .

Figure 2.10: Schematic of a single-stream particle focusing microfluidic channel design consisting of a low AR straight channel with stepped, orthogonally-arranged constrictions [68].

The effect of inertial lift forces and secondary flow drag on the sheathless focusing of 9.9 μm particles in a simple straight channel with asymmetrically patterned triangular expansion-contraction cavity arrays were studied up to flow rates of 700 $\mu\text{l}/\text{min}$ [69]. A microchannel design consisting of a low AR channel bifurcated into high AR channels was shown to achieve particle focusing using inertial migration and asymmetric flow separation [70]. The sheathless focusing device illustrated focusing of 15 μm microbeads at $Re = 40$ and was also integrated with a laser counting system to build a micro flow cytometer. The device yielded a throughput of 2200 particles s^{-1} . A four-stage inertial focusing device was suggested by Reece et al. [71] comprising of i) a straight wide rectangular channel, ii) asymmetrically curved channel, iii) straight tall

rectangular channel, and iv) an expansion part at the outlet in sequence, with promising single and multiple pass concentration efficiency. Jiang and co-workers described a symmetrical serpentine microchannel with a rectangular cross-section ($80\ \mu\text{m} \times 40\ \mu\text{m}$) [72]. Simulation results, based on IB-LBM model, governed the sorting of $5\ \mu\text{m}$ and $10\ \mu\text{m}$ particles under the action of F_I and F_D . The numerical study illustrated the effect of Re and Re_p on the particle focusing positions across the curved channels. The symmetrical sinusoidal channel demonstrated two vertical equilibrium positions for $4.8\ \mu\text{m}$ and $9.9\ \mu\text{m}$ particles up to flow rates of $220\ \mu\text{l/min}$. However, they do not define conditions for achieving a single equilibrium focusing position for any particular particle size or flow parameter.

Figure 2.11: Sheathless particle focusing in a microfluidic channel with arc-shaped grooves on the top surface [73].

Very recently, a sheathless and 3D particle focusing device consisting of a low AR straight channel ($AR = 0.2$) and an arc-shaped groove array pattern on the surface was presented (**Figure 2.11**). Inertial and secondary forces were shown to focus $10 - 24\ \mu\text{m}$ particles and cells at flow rates reaching $1100\ \mu\text{l/min}$ [73]. Few review reports are

also available which provide a comprehensive picture of the present theoretical understanding and experimental evidences of inertial focusing of particles/cells in microchannels for various applications [74-76]. **Table 2.1** presents an overview of various microfluidic channel geometries utilized for inertial focusing of microparticles and cells.

Table 2.1: Overview of microfluidic channel geometries on inertial focusing of microparticles

Type of geometry	Equilibrium positions	Flow parameters ($Re/Re_p/De$)	Channel Aspect Ratio	Particle size (μm)	Throughput
Straight (square) [57, 77]	Four	$Re = 90$ $Re_p = 2.9$	1	9	-
Straight (rectangular) [58, 59]	Two	$Re = 30$	0.27	20	-
Curved (symmetric) [72]	Two	$Re = 75$	0.5	5 - 10	~300 $\mu\text{l/min}$
Curved (asymmetric) [66]	One	$Re_p = 5.9$ $De = 25.6$	0.5 (curved) 1.67 (straight)	10.2	700 $\mu\text{l/min}$
Spiral [64]	One	$Re = 20$	0.2	6	2100 sec^{-1} particles
Stepped [68]	One	$Re = 83.33$	0.5 (linear) 0.25 (stepped)	9.9	6400 sec^{-1} particles
Pillars [67]	One	$Re = 66.7$	0.3125	15 - 19	13000 sec^{-1} particles
Expansion-contraction arrays [69]	One, two	$Re = 41.63 - 270.57$	0.25	3.2 - 9.9	700 $\mu\text{l/min}$
Grooved [73]	One	$Re = 29.2 - 160.4$	0.2	10 - 24	1100 $\mu\text{l/min}$

Thus, inertial focusing provides a sheathless way of focusing particles in a single-line stream without the need of additional active sources. Hence, inertial focusing methods can be utilized for developing lab-on-a-chip flow cytometers for high-throughput applications. **Figure 2.12** is a statistical representation of the growing

research efforts to design and develop inertial forces-based lab-on-a-chip flow cytometers.

Figure 2.12: a) Publication trend for sheathless inertial focusing in microfluidic devices. b) Publication trend for sheathless inertial forces-based lab-on-a-chip flow cytometers.

2.6 Nanoassays for Cancer Theranostics

Nanoassays play a vital role in extracting any morphological or biological information from flow cytometers. In the context of cancer diagnosis, nanoassays are extensively used for providing and enhancing the signals from biomolecules. Drug delivery, disease progression, and prognosis are studied using nanoassays designed using fluorescent dyes [78, 79], beads [80], QDs [81], and organic dyes [82]. However, these traditional nanoassays cannot be used as simultaneous carrier (biomolecules/drug/gene delivery) and signal providers (output/fluorescence signal). From the perspective of point-of-care healthcare, it is desirable to develop novel nanoassays that are feasible for cancer “theranostics” i.e. assays designed to yield diagnostic as well as therapeutic assessment of the targeted tumor. As depicted in **Figure 2.13**, by the term theranostics, we mean nanossays which serve as carriers (“therapeutics”) and are signal providers as well (“diagnostics”). Nanomaterials are

increasingly used for nanoassay applications because of their unique and remarkable properties such as a) small size, b) tunable charge-size ratio, c) size-dependent optical properties, d) biocompatibility, e) low toxicity, and most importantly, f) surface functionalization ability. Examples of such nanomaterials are gold nanoparticles, Molybdenum disulfide (MoS_2), QDs, graphene, etc. In our work, we focus on developing theranostic nanoassays using these nanomaterials that initiate gene therapy and at the same time, yield output signals to measure the effect of gene therapy on cancer cells. RNAi-based gene therapy is one of the potential therapeutic techniques which catalyzes the suppression of overexpressed gene mutations in cancer cells. In the next section, we will describe the process of RNAi and elaborate on the pressing need for developing theranostic nanoassays for RNAi-based gene therapy and their importance in portable flow cytometry.

Figure 2.13: The constituents of the term theranostics.

2.7 RNA interference (RNAi): A gene therapy for Cancer

The discovery and role of RNAi in regulating gene expression [83] has propelled research in addressing oncogene mutations in tumor cells where conventional cancer therapies fail to succeed [84-86]. RNAi is a natural cellular process that regulates gene expression and provides innate defense mechanism against invading viruses [83].

RNAi-based gene therapy can be realized through small-interfering RNAs (siRNAs), which are short double-stranded RNAs, 20-24 nucleotides in length [87]. siRNAs can be applied directly or engineered, to target specific gene of interest. When siRNAs are applied to cells, they induce degradation of targeted cellular compartment upon complimentary mRNA encounter. RNAi-based therapies are being extensively explored by the research community for their therapeutic role in treating fatal disorders like cancers [84-86, 88].

Transfection of siRNA into cells is required for initiating RNAi process for targeted gene silencing. Naked siRNAs are unstable and vulnerable to nuclease-mediated degradation [89]. Their small size and negative charge prevent them from crossing the cellular membrane to reach the intra-cellular translation processes [90]. Moreover, free siRNA molecules have a very short half-life to sustain themselves from endogenous nucleases under physiological conditions [91]. Therefore, for effective RNAi initiation for the gene suppression of the targeted mRNA, it is imperative that siRNAs be directed to the targeted cells and tissues by conjugating them with safe and robust carriers that ensure proper release of siRNAs to the treatment site and prevent them from triggering an immune response in the blood stream [92].

Both viral and non-viral vectors can be used as siRNA/gene delivery vehicles to cells, depending upon the target tissue and specificity. Viral vectors have shown prolonged gene silencing both *in vitro* and *in vivo* with excellent tissue-specific tropism and high transfection efficiency [93]. However, safety concerns (e.g. insertion mutagenesis and immunogenicity) and difficulties with large-scale manufacture limit the use of viral vectors for siRNA delivery in clinical setting [94, 95]. Hence, non-viral vectors are increasingly being probed as effective transfection agents owing to their

advantages such as ease of synthesis, low immune response, biosafety, and unrestricted gene material size.

Non-viral vectors can be mainly categorized into cationic liposomes and polymeric nanoparticles. Many commercially available cationic liposomes, for e.g. Lipofectamine2000 and Oligofectamine, are commonly used as transfection reagents due to their high transfection efficiencies, nonetheless they are highly cytotoxic [96]. Polymer nanoparticles that have been reported for siRNA delivery include polyethylenimine (PEI), chitosan, poly-L-lysine (PLL), and PEG-based polymers [92, 97]. These polymer nanoparticles and inorganic aggregates like calcium phosphate have been successfully employed for gene delivery with high transfection efficiencies but their use comes with trade-offs such as non-tunable interdependence of charge and size ratio, and increased cytotoxicity at high concentration of polymers [98-100]. Therefore, we need to engineer novel nanoassays that a) function as non-toxic, tunable, biocompatible, and stable non-viral siRNA transfection agents for safe gene delivery in cancer cells, b) that can simultaneously yield diagnostic or output signals, and c) that are easy to prepare, and can be used outside clinical settings in microfluidic flow cytometers.

In Chapters 5 and 6 of this thesis, we investigate the efficacy of RNAi-based gene silencing by developing novel nanoassays for two different types of cancer cells: a) adherent pancreatic cancer cells (Panc-1 and MiaPaCa-2) and b) suspension chronic myeloid leukemia (CML) cells (K562). Secondly, we demonstrate the essential applications of flow cytometers in measuring the efficiencies of these theranostic nanoassays. The following parts of this section present an overview of siRNA delivery in pancreatic and CML cells.

2.7.1 siRNA delivery in Pancreatic Cancer Cells

Many forms of cancer have poor prognosis, due to the fact that they are diagnosed at advanced stages of their progression, leading to ineffective treatment strategies that eventually end up in death. Pancreatic cancer is one such type of cancer that is usually diagnosed at very advanced stages, thus being the fourth-largest cause of cancer-related deaths in the US [101]. Research studies have shown the over-expression of a pro-inflammatory CXC chemokine, IL-8 [102], in pancreatic cancer cells and its role in promoting angiogenesis, tumorigenicity, and metastasis therein [103, 104]. Among a host of human pancreatic cancer cell lines including, L3.3, MiaPaCa-2, Panc-1, CaPan-1, CaPan-2, and HPAFII cells, the highest IL-8 mRNA expression has been detected in L3.3, followed by MiaPaCa-2 and Panc-1 cells [105]. Therefore, targeting the IL-8 signaling pathway within the tumor microenvironment by siRNA strategy may sensitize pancreatic cancer cells to undergo apoptosis for systemic treatment of metastatic disease [106].

For the first theranostic nanoassay, we investigate the feasibility of using gold nanorods (AuNRs) as non-viral transfection agents for the delivery of IL-8 siRNA to MiPaCa-2 and Panc-1 pancreatic cancer cell lines, and characterize their cytotoxicity, loading capacity, and transfection efficiency. Gold nanoparticles in the form of AuNRs have been used as a gene delivery agent, both *in vitro* in dopaminergic neuronal cells [107] and *in vivo*, by stereotaxic injection of AuNR-siRNA into the brain of Sprague-Dawley rats [108]. Easy to synthesize, AuNRs offer high biocompatibility in loading with siRNA. Moreover, they can be engineered to different sizes and ARs according to the necessity of the bio-molecule under consideration. They facilitate functionalization with simple conjugation techniques, and offer high transfection efficiency due to their shape and surface area. The surface of AuNRs can be modified to become cationically

charged to enable them to form electrostatic complexes with negatively-charged siRNA [107]. They offer a non-viral gene delivery platform with a lower cytotoxicity, ease of conjugation, and tunable properties. To summarize, gold nanoparticles manifest themselves with tunable physical and chemical properties such as surface charge, size, and shape that bestow them with physical and chemical stability and low toxicity to be used as effective non-viral gene delivery vehicles with high transfection efficiencies [109, 110]. **Figure 2.14** is an illustrative schematic of the proposed nanostrategy to transfect pancreatic cancer cells with AuNRs-siRNA nanoplex to bring about IL-8 gene knockdown by RNAi.

Figure 2.14: Illustration of the RNAi phenomenon for IL-8 mRNA suppression in MiaPaCa-2 pancreatic cancer cells. AuNRs act as nanocarriers for transfecting IL-8 siRNA inside the cell. Upon successful delivery into the cytoplasm, siRNA forms an assembly with RNA-induced silencing complex (RISC) and gets dissociated with its antisense strand. Thus, degradation of IL-8 mRNA results when it encounters the sense strand of siRNA.

2.7.2 siRNA delivery in Chronic Myeloid Leukemia (CML) cells

CML is one of the most aggressive cancer diseases which is mostly caused by chromosomal translocations between chromosomes 9 and 22 that results in the fusion

of BCR and ABL genes to form an oncogenic hybrid gene, BCR-ABL [111]. The BCR-ABL chimeric protein is known for its increased active tyrosine kinase activity [112] [113] which leads to oncogenic growth and reduction of apoptosis [114], thus promoting CML. As reported in literature, activated tyrosine kinase activity plays an important role in abnormal proliferation of CML progenitors including increased proliferation in progenitor culture, reduced adhesion to fibronectin, and reduced chemotaxis to stroma-derived factor-1 α [115]. Several critical phenotypic abnormalities observed in primary CML cells, including larger myeloid colony-forming units and delayed apoptosis are largely caused by the overexpression of BCR-ABL oncoprotein [116]. A BCR-ABL inhibitor, imatinib mesylate (or STI 571), an orally administered drug, has been widely used for suppression of BCR-ABL expressing cells and induction of apoptotic cell death. However, various clinical reports have highlighted the induced resistance of CML cells to STI 571 [117-121].

Albeit various viral and non-viral siRNA vectors have been developed for transfection, no carrier can however contribute high transfection efficiency on the leukemia cells owing to the non-adherent nature of CML cells. In general, commercially available transfection agents and synthetic polymers, such as Lipofectamine2000TM, TransMessenger, PEI, PLL, and poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) result in a high transfection efficiency of about 70 % in non-adherent leukemia cells [97, 122-124]. Though a fairly acceptable transfection efficiency, the long-term cytotoxicity, non-degradable properties, and lack of reactive sites for further chemical modifications in these vectors remain the persistent concerns for further investigations for clinical setting.

Based on these reasons, several polymeric-based carriers are being investigated for siRNA delivery in suspension cells. The triblock copolymer polybutadiene-block-

poly(methacrylic acid)-blockpoly(2-(dimethylamino)ethyl methacrylate) (BMAAD) can form well-defined micelle structures and has been reported to display superior transfection capabilities for adherent cells and human leukemia cells [125]. Recently, SNALP-like lipids with different compositions have been explored for siRNA transfection in suspension cells, and the optimal composition of SNALP-like lipid was determined based on its transfection results [126]. However, the long-term physiological toxicity and transfection efficiency need to be further assessed *in vivo*.

Degradable cationic polymers with insignificant cytotoxicity have presented far-reaching applications as gene carriers, and are synthesized using step polymerization and living polymerization techniques. In Chapter 6, we propose another theranostic nanoassay that makes use of a biodegradable charged polyester-based vector (BCPV), shown in **Figure 2.15**, as a nanocarrier for the delivery of BCR-ABL-specific siRNA to the suspension culture of K562 CML cell line.

Figure 2.15: The chemical structure of BCPV.

We demonstrate the siRNA transfection into K562 cells through this nanoassay and study the transfection efficiency of BCR-ABL siRNA-loaded BCPVs, and cytotoxicity of BCPVs in non-adherent K562 cells. Moreover, we determine the gene silencing effect of BCPVs, both in FBS-present and FBS-absent cell culture media and compare the performance of the proposed nanoassay with Lipofectamine2000TM.

2.8 The Role of Flow Cytometers in Cancer Theranostics

In this thesis, we propose and study novel nanoassays to induce specific siRNA oncogene silencing in two types of cancer cell lines: adherent pancreatic cancer cells

(MiaPaCa-2 and Panc-1) and suspension CML cells (K562). The gene delivery and gene silencing effects of these nanoassays are evaluated by flow cytometry. The role of a flow cytometer in quantitatively measuring disease diagnosis and therapy (collectively termed as “theranostics”) has high potential for future clinical applications. As a multi-parameter, bulky, and expensive instrument, conventional flow cytometers are restricted to highly specialized laboratories and high-end clinical settings. While a course of diagnostic or therapeutic analysis may seem to be done within a couple of hours in developed countries, the same might actually take weeks to be completed in poorer and developing nations, as they do not have easy access to such sophisticated clinical infrastructure in their close vicinity. Hence, such locations become more vulnerable to epidemics given the inability to curb or treat diseases at an early stage.

Flow cytometers hold immense potential in cancer theranostics-based nanoassays. Through our investigations on these nanoassays, we aim towards a theme of “one-shot” nanoassays which are compatible with miniature flow cytometers and can be used in field applications (mass screening or therapeutics in rural settings). Hence, the development of microfluidic flow cytometry is a major contribution towards point-of-care disease theranostics.

2.9 Hypotheses

Based on the pressing need of miniature and portable flow cytometers, and their predominant role in cancer theranostics-based nanoassays, we hypothesize that:

- A careful analysis of inertial forces acting on spherical particles/cells at $Re_p > 1$ is necessary for the design of a sheathless microfluidic channel geometry to achieve accurate, 3D single-position particle focusing in microfluidic chips over a wide range of channel Reynolds number (Re).

- Inertial forces also have a significant effect on determining hydrodynamic focusing of particles in sheath-assisted flows at higher Re .
- An integrated optofluidic flow cytometry approach with a single-input, single-output, sheathless particle focusing mechanism can lead to high-throughput lab-on-a-chip miniature flow cytometers.
- Designing one-shot theranostics nanoassays compatible with microfluidic flow cytometers can contribute towards easy accessibility to point-of-care disease theranostics.

2.10 Summary

In this chapter, we first introduced the reader to what a conventional flow cytometer is and why it is so useful, and gave a brief summary of its historical evolution. Given the limited accessibility of conventional flow cytometers for the vast applications, we highlighted the need for developing miniature flow cytometers. Being a necessary requirement for flow cytometry, we elaborated the current state-of-the-art on single-position particle focusing techniques using sheath-assisted and inertial forces-assisted particle focusing mechanisms in microfluidic channels, and outlined their present limitations. Furthermore, we underlined the pressing need for developing theranostic nanoassays for extracting meaningful information from flow cytometers for effective disease management. In this regard, we presented a nanoassay development procedure based on RNAi phenomenon for gene therapy of pancreatic and leukemia cancer cells. Finally, we outlined the indispensable role of a flow cytometer in quantitatively evaluating the theranostic effect of the proposed nanoassays in cancer cells.

Chapter 3 Inertial forces-based Sheathless Particle Focusing for Lab-on-a-Chip Flow Cytometry

Being apprised of the fact that flow cytometers occupy a pre-eminent position in disease diagnostics, cancer therapy, immunophenotyping, etc., we have pressingly elicited the need for miniature, lab-on-a-chip flow cytometers for widespread and affordable point-of-care biomedical theranostics in the previous chapter. Engineering miniature flow cytometers calls for integrating multiple-disciplines: microfabrication, microfluidics, optical design, nanotechnology, and electronics, to operate closely. In this chapter, we propose a novel microfluidic method to achieve 3D single-position focusing of flowing particles/cells inside microchannels based on inertial microfluidic effects. The proposed method does not involve the use of active sources or sheath fluids, and is thus a self-containing technique that utilizes the interaction of induced inertial forces throughout the length of the microchannel to manipulate particle migration behavior in the microchannel cross-section. Furthermore, we propose a novel method to embed tapered microfibers during microfluidic chip fabrication process for signal interrogation. Finally, we demonstrate the operation of the fabricated optofluidic chip-based flow cytometer and compare its performance with a commercial flow cytometer for a beads-based assay. The proof-of-concept results are a milestone for developing sheathless, optofluidic flow cytometers, which would greatly benefit point-of-care diagnostic and therapeutic disease management.

3.1 Introduction

The first and foremost requirement for developing a portable, lab-on-a-chip flow cytometer is the design of microfluidic channel layout such that the randomly fed cells/particles at the inlet port of the microfluidic chip get focused to a single-position

train of moving particles at the outlet port. In other words, we need to construct a microchannel geometry such that the superposition of all forces acting on a particle result in a single equilibrium position across the channel cross-section in the interrogation region of the channel (which is the outlet region of the microfluidic channel). Detailed in Chapter 2, a few microchannel geometries including spiral [64, 65], staged [66, 70, 71], pillared [67], stepped [68], arrayed [69], grooved [73], and serpentine [72], have been proposed in recent years for studying the particle migration behavior and number of equilibrium positions under the influence of inertial lift forces coupled with Dean Drag.

However, several factors limit the applications of these microfluidic geometries for lab-on-a-chip flow cytometry applications. First, complicated design geometries (multiple AR, bifurcations, asymmetry) result in larger footprint and hinder the fast fabrication of microfluidic chips. Second, most studies concern a single particle size ' a ' for a particular flow condition (Re) and do not highlight the ranges of operating parameters (flow conditions, particle size) which would support 3D single-position inertial focusing. Third, some of the reported schemes do not describe the entire geometrical parameters, for e.g., number of the asymmetrical curves or the overall length of the microchannel. Most studies are experimental demonstrations and lack the theoretical analysis for defining the focusing conditions for a range of operating parameters. There is no report however, describing a unity AR, single-layer, symmetric microchannel design that precisely focuses particles to a single equilibrium position for a wide Re range.

In this chapter, we present a simple and symmetrical microfluidic channel design, intended for 3D single-position particle focusing of microparticles and cells at the channel outlet, as they traverse the microchannel length under the influence of

inertial lift forces and Dean Drag forces. The microfluidic chip comprises a square microchannel with a series of radially increasing uniform semi-arcs interleaved by linear sections - which is symmetric from the midline of the microchannel. Inertial microfluidics-based 3D particle focusing allows the microfluidic chip to withstand higher pressures to achieve higher throughput. We demonstrate single-position 3D focusing for a range of relative particle sizes $0.175 < a/w_{ch} < 0.9$ under Re ranging between 20 and 153. We perform fluorescence and confocal microscopic imaging to verify the single-position 3D focusing of cells or particles at the microchannel outlet. Furthermore, we present an empirical model relating the ratio of inertial lift forces and Dean Drag forces, and the geometrical parameters of the design to determine the rule of thumb for the precise single-position focusing of particles in the proposed design. As a proof-of-concept, we present a novel optofluidic flow cytometer using the proposed particle-/cell-focusing technique in a microfluidic chip for point-of-care healthcare applications in resource-limited conditions.

3.2 Experiments and Methods

3.2.1 Fabrication of Microfluidic Chip

We fabricated the optimized microchannel design using standard SU-8 photolithography, PDMS soft-lithography and molding techniques (**Figure 3.1**) for experimental validation of the proposed focusing mechanism. First, we printed a *.gds drawing of the microchannel design on a photomask (Chromium on soda-lime glass). Then, we spun SU-8 photoresist on a Silicon (Si) wafer at 2000 rpm for 80 seconds twice to get the desired thickness of 80 μm . Following each recipe, the SU-8-coated Si wafer was relaxed at 60°C for 10 minutes followed by a 20 minute pre-baking at 120°C. For photolithography, we subjected the SU-8-coated Si wafer to a 25 second UV exposure (40 mW) under the photomask. Thereafter, the wafer was baked (post-baking)

on a hotplate at 110°C for 20 minutes and thoroughly rinsed in SU-8 developer. Post development, only the channel layout part remained on the Si wafer while the blank parts got dissolved. The wafer is referred to as the ‘master’ post this stage. We then baked the master (hard-baking) at 200°C for 2 hours for strengthening the crosslinking between the SU-8 and the wafer.

Figure 3.1: Schematic of the stepwise soft-photolithography process for fabrication of the microchannel chip. a) Printing of microchannel design on glass wafer. b) Wafer view of the fabrication processes: cleaning and spin coating SU-8 photoresist on a Si wafer (1, 2), UV exposure on the SU-8 coated-Si-wafer (3), post-baking, developing and hard-baking the wafer- ‘master’ wafer (4), curing of the PDMS-based molding suspension poured over the master wafer at 70°C (5), Removal of patterned PDMS chip from the master wafer (6), irreversible bonding of the chip to a clean glass slide by plasma treatment for performing flow experiments (7).

Once the master wafer was ready for molding, we prepared a PDMS-based molding suspension by mixing the PDMS elastomer base and curing agent together in a 10:1 ratio. The suspension was degassed, and then poured over the master wafer, degassed again, and allowed to cure for about 4 hours at 70°C. We then gently peeled off the cured PDMS from the master wafer to obtain a PDMS chip patterned with the designed microchannel. We refer to this chip as the “microfluidic chip” in the entire thesis. Thereafter, we punctured holes at the inlet and outlet reservoirs of the microfluidic chip using a standard puncher (Harris Uni-Core) for microtubing connections, cleaned the microfluidic chip with acetone and nitrogen-dried it before finally irreversibly bonding it to a clean glass slide by oxygen plasma treatment (40 W, 40 seconds, 200 mTorr) for performing flow experiments.

3.2.2 Cell Culture and Staining

We cultured human pancreatic cancer cell line, MiaPaCa-2 (CRL-1420) (American Type Culture Collection (ATCC)) and human macrophage (RAW cell, ATCC) in Dulbecco’s modified Eagle’s medium (DMEM, Hyclone), 10% fetal bovine serum (FBS, Hyclone), penicillin (Gibco, 100 µg/mL) and streptomycin (Gibco, 100 µg/mL) in an incubator at 37°C in a humidified atmosphere with 5% CO₂. Prior to flow in the microfluidic chip, we stained MiaPaCa-2 cells with Fluorescein (FITC kit, Thermo Fisher) and macrophages with Cadmium Selenide (CdSe) QDs (10 µg/ml). Cells were made into a singular cell suspension in deionized (DI) water before flowing.

3.2.3 Particle Focusing Experiments

We purchased fluorescent polystyrene particles from Polysciences Asia Pacific, Inc. (10 µm, CV 15% and 20 µm, CV 4%) and Phosphorex, Inc (15µm, CV 7%). For flow experiments, we separately mixed each of the particles in DI water in concentration ratio between 0.1 to 1% wt/vol. The mixture was shaken vigorously for 15 minutes and

allowed to settle for 5 minutes before withdrawing into syringes. We then fed the cell or particle sample through the inlet port of the microfluidic chip at flow rates controlled by a syringe pump (NE-1000, Newera). The waste flowing out from the outlet port was collected in a separate dish. We used fluorescently-labeled cells or particles so as to track their focusing positions as they travelled through the microchannel length. As the samples were fed at the set flow rate, we acquired fluorescent images of the outlet part of the channels with a fluorescence microscope (Eclipse-Ti, Nikon). To observe precise particle movement inside the channels, we recorded high-speed videos of the moving particles at 50000 frames per second (fps) at a fixed focal plane with a FASTCAM Mini UX50 (Photron, Japan) to observe particle focusing and determine the final equilibrium position across the channel width.

3.2.4 Embedding Optical Fibers in the Microfluidic Chip

For performing flow cytometry measurements, we integrated the microfluidic chip with optical fibers at the outlet part. First, we tapered down optical fibers to 80 μm diameter to match the size of microchannel in order to achieve maximum illumination of the flowing particles and collect maximum output photo-signals for analysis of particle information. We performed adiabatic-tapering of optical fibers using a fiber drawing machine (Vytran, GPX-3000) which is equipped with a graphite filament and motorized fiber holding stages. We maintained a normalized power of the filament to 50W and the power degradation rate of -15% during the waist drawing period. The fabricated microfiber had a waist diameter of 80 μm with minimal change in the core, as shown in **Figure 3.2**. **Table 3.1** shows the taper profile mode parameters used for the tapering. The taper profile of the microfiber can nonetheless be tailored to conform to the microchannel cross-sectional dimensions provided the fiber mode profile characteristics are maintained within the tolerable limits [127].

We then gently placed the tapered optical fibers (one single mode fiber and one multimode fiber) at 30° with each other with their cleaved tapered ends facing the interrogation region at the outlet region of the microfluidic channel on the master wafer and glued the fibers near the edge of the wafer with a small pinch of stick-on. Following this, we also placed the 3D-printed wall (**Figure 3.3a**), which was essentially an extruded version of a rectangle, with dimensions of a normal glass slide, enclosing the microfluidic channel. We fixed the fiber positions with the aid of a microscope, and once the set-up was stable, very small quantity of PDMS was poured over the fiber part and also on the edges on the 3D-printed wall and left to cure overnight. Thereafter, when the fiber positions were affixed with the cured PDMS, more PDMS was now poured and was left to cure at high temperature as detailed in Section 3.2.1. Hence, we obtained the optical fiber-integrated PDMS microfluidic chip, shown in **Figure 3.3b**.

Table 3.1: Taper profile mode parameters

Down-taper length Waist length Up-taper length Waist diameter Cladding diameter	10 mm 20 mm 10 mm 40 mm 125 μm	Gas flow rate	0.50 l/min
		Background Argon	0.15 l/min
		Taper pull velocity	0.50 mm/sec
		Initial furnace move	0.0
		Filament start	50 W
		Post-purge time	5 sec
		Pre-purge time	5 sec
		Taper pull delay	0.20 sec
		Filament delta	0.0%

Figure 3.2: Snapshots of single mode optical fiber during the tapering process. a) Diameter of the cladding region at the taper waist, b) diameter of the core region at the taper waist, c) diameter of the cladding region between the taper waist and untapered part, d) diameter of the cladding region at the untapered part, and e) diameter of the core region at the untapered part.

Figure 3.3: a) Schematic drawing of the 3D-printed wall. b) The microfluidic chip integrated with optical fibers.

3.2.5 Optical Detection of Flowing Beads/Cells

We used the optical fibers-integrated microfluidic chip to perform flow cytometry measurements. **Figure 3.4** is a detailed schematic representation of the complete lab-on-a-chip flow cytometry instrumentation. The light from the 488 nm fiber-coupled laser source was coupled to the tapered single-mode excitation fiber and

the PMT module (Hamamatsu, H10722-01) was connected to the multi-mode detection fiber. The PMT head was fitted with an interchangeable filter adaptor, so that the filters can be changed according to the wavelength of fluorescent signal to be detected. In our experiments, we used 532 nm notch filter and 633 nm bandpass filter to detect the side-scattered florescent signals from the flowing FITC-labeled polystyrene beads and CdSe QD-labeled macrophages, respectively.

Figure 3.4: Lab-on-a-chip flow cytometry instrumentation. a) Schematic representation of the microchannel chip with auxiliary instrumentation to detect output photo-signals. b) Amplified views of the fiber interrogation part, showing the tapered single mode fiber (Fiber IN) and tapered multimode fiber (Fiber OUT). c) The complete experimental set-up. d) Amplified photo of the microfluidic channel. e) Microscope image of the optical fibers integrated inside the PDMS microfluidic chip.

The output photo-signals from flowing cells or particles were converted to output voltage signals voltage by the PMT module. The PMT module was connected to a data acquisition and multifunctional I/O device (DAQ PCI-6221, National Instruments) from where the output data was extracted at a sampling rate of 100 KS/s. The data was processed using a customized Labview program. The number of voltage signals is a measure of cell or particle count and the signal intensity is a measure of

various morphological or biological properties of the cells, for e.g., cell size, biomarker tag, etc.

3.3 Results and Discussion

3.3.1 Proposed Microfluidic Design for 3D Single-position Inertial Focusing

Our proposed microfluidic focusing device consists of a square cross-section microchannel ($AR = 1$) with a single inlet and single outlet. The meandering portion between the inlet and the outlet comprises of four series of semi-arcs interleaved by a linear path between two adjacent series of semi-arcs (**Figure 3.5**). The series of semi-arcs arranged closest to the inlet, referred to as primary arc, has the smallest radius ' r_s ', and the series of semi-arcs arranged furthest from the inlet (i.e. closest to the outlet) has the largest radius. The semi arcs belonging to the same series have the same radius. The semi-arcs belonging to different series have different radius. Thus, the entire microchannel design is a symmetrical, periodically increasing, curvilinear square duct.

We consider three versions for analysis and experiments in this thesis: (i) Design 1 (D1): $r_s = 150 \mu\text{m}$, (ii) Design 2 (D2): $r_s = 200 \mu\text{m}$ and (iii) Design 3 (D3): $r_s = 250 \mu\text{m}$. The schematic of these three designs are shown in **Figure 3.6**.

Figure 3.5: a) The complete dimensional drawing of the microchannel geometry for 3D focusing of cells or particles. b) Pictorial schematic of the functionality of the microchannel design. Randomly-ordered cells or particles are fed at the inlet at pre-determined flow rates. As they traverse through the linear and curved parts of the channel, interplay of transverse inertial lift forces ' F_l ' and Dean Drag forces ' F_D ' leads to 3D single-position focusing of cells or particles at the outlet.

Figure 3.6: The dimensional representation of the three designs D1, D2 and D3 used to demonstrate 3D single-position inertial focusing. The dimensions in each design represent the diameter of each series of the semi-arcs.

3.3.2 Theoretical Model for the Proposed Design

Inertial forces in microfluidic devices have been previously employed for micron-sized particle separation [128], sorting [129], and filtration applications [57, 62, 130-132]. High ARs straight rectangular microchannels lead to modulation of shear rate that cause size-dependent lateral migration of particles [132]. For a given particle size, a continuous increase in hydraulic diameter D_h along the microchannel height h results in shifting of equilibrium positions towards the channel walls. This observation suggests a direct correlation of the ' F_{IS} ' and the inverse dependence of the ' F_{IW} ', with the channel AR (h/w_{ch}) for a given particle size, a . Moreover, the ratio of minimum particle size ($a_{\min}$) to channel width (w_{ch}) that is capable of undergoing inertial focusing under allowable flow rates is $a_{\min}/w_{ch} = 0.07$ [57]. As the channel Reynolds number increases ($Re \rightarrow 500$), the equilibrium position at the center region of the channel becomes unstable with only the corner regions remaining stable and therefore, the particle equilibrium positions shift towards the channel walls [50, 55, 133]. Results of the numerical computations of the 3D steady state N-S equations yielding the transverse lift

forces acting on isolated non-point particles ($0.1 \leq a/w \leq 0.9$) describe the four-point equilibrium positions in a straight square microchannel duct as illustrated in **Figure 3.7**.

Figure 3.7: (a-c) Behavior of lift coefficient (f_L) in a square channel. Poiseuille flow of randomly distributed particles of diameter, a in the square channel of width, w_{ch} with maximum velocity at the center, U_{max} . Pictorial representation of a straight square channel with four particle equilibrium positions, one along each of the face center. (d-e) Variation of f_L as a function of flow rate and particle size near the channel center and near the walls [77].

For $a_{min}/D_h \geq 0.1$, the particle Reynolds number, Re_p , becomes important ($1 \leq Re_p \leq 10$), and defines the lateral trajectories of particles as they traverse the length of the microchannel under viscous and inertial effects. The larger particles tend to shift towards channel center following the four-point equilibrium position symmetry. Herein, the following two cases may be delineated with respect to the relative particle size:

(i) Point-particle assumption ($a/w_{ch} \ll 1$)

Computed by the matched asymptotic assumptions method, the transverse inertial lift force ' F_I ' scales identically throughout the channel cross-section as $f_L(G, Re)\rho U_{max}a^4/w_{ch}^2$, where f_L is a nondimensional lift coefficient that bears empirical

dependence on the shear rate, G and Re [55, 133]. However, this force behavior is valid only on spheres with sufficiently small relative size, in which case the transverse velocity of the sphere that leads to lateral migration across streamlines does not cause disturbance to the main channel flow [4,5]. Also, since $Re_p \ll Re$ ($0.02 < Re_p < 0.5$), the ' F_{WI} ' is negligible across major part of channel width, except for the very small $w_{ch}Re^{-1/2}$ distance from the channel walls [55]. The region where the ' F_{WI} ' is dominant is referred to as the extent of the wall-induced inertial lift ' d_{WI} '.

(ii) Non-point-particle assumption ($0.1 < a/w_{ch} < 0.9$)

The lateral forces acting on particles in bounded flows bear functional dependence on the relative size of particles. With increasing Re , the transverse inertial lift force increases at the channel centerline, while it decreases near the walls. This explains the shift of equilibrium positions towards the walls at higher Re , and the shift of equilibrium positions towards the channel centerline for increasing particle size, at a particular Re [77, 134]. In this case, the inertial lift due to wall-induced shear effect is also significant as the equilibrium position lies within comparable order of d_{WI} (**Figure 3.8**). Thus, both, linear shear and the wall-induced inertia acting on the particle interplay towards lift contributions in a confined flow.

Figure 3.8: Graphical representation of the extent of wall induced-inertial lift force (d_{wl}) measured from the wall as a function of Re , highlighting the significant of wall-induced shift in particle focusing in confined flows.

The distinguishing feature that delineates the two cases is the nature of the lift coefficient, f_L . For case (i) that corresponds to the transverse migration of a rigid spherical particle in an unbounded flow, f_L which monotonically decreases with increasing Re over the complete domain i.e. $0 < 2d_{eq}/w_{ch} < 1$ [55]. This phenomenon is a direct consequence of the diminishing effect of the wall-induced lift contributions for higher Re , as shown in **Figure 3.8**. For case (ii), the particle size dominates the lateral migration behavior as $Re_p > 1$. While in the near-wall regions, the size-dependent- f_L decreases with increasing Re , it increases near the channel-center region [77] as shown in **Figure 3.7d-e**.

The proposed microfluidic device demonstrates 3D single-position focusing in the range, $0.175 < a/w_{ch} < 0.9$ for Re between 20 and 153. For focusing of cells or particles to a single equilibrium position along the microchannel width, Dean Drag

forces ' F_D ' of appropriate magnitude are utilized. The square cross-section microchannel ($AR = 1$) starts with a straight section with sufficient entrance length where the randomly distributed cells or particles get ordered in four equilibrium positions along the centers of each wall-face. The cells or particles then enter the first semicircular arc termed as primary arc, where the generated asymmetrical vortices due to ' F_D ' bias one/more of the four previous equilibrium positions. The cell or particle travels the full primary semi-arc followed by a symmetrical semi-arc in the opposite direction, and start to align at the equilibrium position across the channel width where both the ' F_I ' and the ' F_D ' balance each other. As the cells or particles progress through the microchannel length, they travel through secondary semi-arcs of increasing curvature and finally enter the outlet linear region where a single-line of focused cells or particles are obtained (**Figure 3.9a-d**).

Typically, three parameters play an important role in defining the precise focusing of particles in the microchannels to a single stream. They are i) geometrical parameters of the microchannel (namely the hydraulic diameter D_h of the channel, and the minimum radius of curvature of the primary arc r_s), ii) the flow parameters (Re), and iii) the size of particle a (particle Reynolds number Re_p).

Figure 3.9: (a-b) Schematic illustration of particle focusing as they traverse the linear and curved regions of the channel; (c-d) Pictorial representation of the interplay of inertial lift forces and Dean Drag force for cell or particle focusing at the channel outlet.

The symmetrical semi-arcs play a central role in balancing the ' F_I ' by introducing the ' F_D ' with comparable order of magnitudes. The approximate ratio of F_I and F_D is given as

$$F_I/F_D \approx \frac{2r_s}{D_h} \left(\frac{a}{D_h}\right)^3 Re^n, \quad (n < 0) \quad (3.1a)$$

The dependence of f_L on the size of particle and channel Reynolds number imposes a lower limit on the ratio of F_D and F_I . For 3D single-position focusing of particles, it is essential that $F_D > F_I$ such that one of the four multiple equilibrium positions in the square microchannel gets biased. However, excess F_D leads to random motion of particles in the flow. Hence, we put an upper limit on the magnitude of F_D .

For the proposed design scheme, the first condition defines a limit on the magnitude of F_D relative to F_I , as shown in **Equation 3.1b**, below which the F_D causes undesired particle mixing in the channels.

$$F_I/F_D \approx > 0.016 \quad (3.1b)$$

Second, since F_I/F_D is a function of the curvature ratio, δ , the curvature ratio of the primary arc, $D_h/2r_s$ must obey the inequality criterion for a smaller focusing length:

$$1.2D_h \sim < \sqrt{2}ar_s \quad (3.2)$$

The primary arc is mainly responsible for focusing of particles to a single equilibrium position, and the secondary arcs have increasing radii that regulate particle-particle spacing and prevent particle disorientation as the generated F_D subsequently scales down due to larger curvatures. Therefore, the geometrical parameters of the primary semi-arc in microchannels that introduce Dean Drag forces for focusing of microparticles should follow the empirical boundary limit, as described by **Equation 3.2** for inertial focusing of cells or particles in a symmetrical, periodically increasing, curvilinear square microchannel. **Figure 3.10** is a graphical interpretation of **Equations 3.1** and **3.2** for size-dependent focusing of cells or particles at $Re = 65.6$.

Figure 3.10: Theoretical condition for inertial focusing of cells or particles in a symmetrical, periodically increasing, curvilinear square microchannel. The shaded area in the curve represents the range of geometrical and flow parameters that will lead to 3D focusing of cells or particles in the microchannel design.

3.3.3 Simulation Results

We performed simulations for optimization of the geometrical parameters of the proposed microfluidic design on COMSOL Multiphysics 5.1 (COMSOL, MA, USA) platform for 3D modelling of the velocity profiles of flowing polystyrene particles (Density $\rho = 1055 \text{ kg m}^{-3}$) of a given size. DI water was modelled as the medium of flow ($\rho = 10^3 \text{ kg m}^{-3}$). Modeling was performed for an incompressible, Stokes flow with input flow rates and entrance lengths obeying the boundary conditions of laminar inflow. The mesh size comprised of 4742 tetrahedral elements and 4548 triangular elements. The total mesh volume was $2.421 \times 10^8 \mu\text{m}^3$ with the average element quality being 0.5619. The average time taken for a single run for velocity estimation was 14 seconds.

As shown in **Figure 3.11a**, the simulated velocity profiles reflect the presence of counter-rotating vortices (shown in black lines) caused by F_D in the symmetrical arcs. This “drag” is believed to be responsible for biasing one of the four equilibrium positions in confined flow-square microchannels and bring about ordering of flowing particles towards the channel side. Finally, these ordered particles then laterally migrate to their final equilibrium position as they travel in the linear outlet. Thus, the final equilibrium position is governed by particle size, the flow rate and interplay of shear and wall induced-inertial lift forces. Velocity profile in the outlet region of 20 μm polystyrene particles flowing at 300 $\mu\text{l}/\text{min}$ ($Re = 65.6$), shown in **Figure 3.11b**, indicates that the particles will migrate laterally and get focused around the center of the microchannel.

Figure 3.11: a) Simulation result for particle velocity profile and particle trajectory under the influence F_I and F_D in the curved and b) linear regions. (The color bar indicates increasing magnitude of velocity, red color being the highest).

3.3.4 Experimental Validation of Particle Focusing in the Proposed Microchannel Design

We studied the focusing performance of the three designs by using spherical fluorescent polystyrene particles of different diameters, a (10 μm , 15 μm , 20 μm). We conducted experiments for $20 < Re < 153$ for each particle size in all three designs. First, we mixed the particles in DI water before they were fed to microchannel inlet. Next, we performed imaging of the flowing sample at the channel outlet to determine the particle focusing outcomes. Long exposure imaging (≥ 5 seconds) was used to observe the fluorescent trace from the flowing beads.

Figure 3.12a shows the snapshots of the outlet sections showing focusing of 15 μm beads flowing in D3 over a range of Re . We also observed particle drifting towards the edges in the curved parts of the microchannel, which confirm the effect of F_D (**Figure 3.12b**). To validate single-position focusing, we recorded the intensity distribution of the fluorescent signal across the channel width at the outlet. A measure of full width at half maximum (FWHM) from the Gaussian fit of the intensity distribution plots gave an estimate of single-position focusing of particles aligned at the channel outlet (**Figure 3.12c**).

Figure 3.12: a) Flow rate-dependent lateral shift in equilibrium position. The data shown are fluorescent streaks of 15 μm spherical polystyrene beads at the outlet part of the microfluidic channel (Design 3) for Re from 21 through 153 (Scale bar, 80 μm). b) The effect of F_D is responsible for the biasing of equilibrium position to the channel side and thus, establish single-position inertial focusing. Inset shows the fluorescent signal from flowing beads at 300 μl/min in the arcs (Scale bar, 80 μm). c) Normalized intensity distribution of fluorescent 20 μm polystyrene beads across the micro-channel outlet showing single-position focusing.

In addition, we captured high-speed images of flowing beads at frame rates of 25000 fps to monitor the particle trajectories of individual beads at different flow rates. **Figure 3.13** confirms that the equilibrium position at a given Re remains uniform through the entire length of the microchannel outlet.

Figure 3.13: High speed images of the outlet section taken at 25000 fps showing a single 20 μm bead flowing in D3 at 300 μl/min. The series of images (i)-(ix) capture particle trajectory for a time interval of 0.36 msec. The equilibrium position remains uniform through the entire outlet (Scale bar, 50 μm).

Figure 3.14: Snapshots of the fluorescent images of the microfluidic channel outlet demonstrating the focusing of polystyrene particle from concentrations of $10^3 \sim 10^6$ particles/mL. For each sample concentration, the flow rate was set to 300 μl/min. (Scale bar, 80 μm)

We also examined the focusing ability of the proposed microfluidic design for samples of varying bead concentrations (from 10^3 to 10^6 particles/mL). As shown in **Figure 3.14**, we observed single-position particle focusing even for higher concentrations ($10^5 \sim 10^6$ particles/mL), and this interesting finding supports the feasibility of the proposed microfluidic design for flow cytometry applications, wherein higher sample concentrations are usually encountered. Furthermore, we conducted confocal microscopy imaging of polystyrene particles flowing in the microchannel to validate the 3D single-position focusing of particles inside the microchannel outlet (**Figure 3.15**).

Figure 3.15: a) Confocal microscopy images for verifying the single-position 3D focusing of spherical polystyrene beads at the microchannel outlet. 3D fluorescent imaging of flowing polystyrene beads (300 $\mu\text{L}/\text{min}$) in the microchannel (Scale bar, 50 μm). b) Cross-section view (y-z plane) of the microchannel with flowing beads, the dotted circle indicates the measured focusing position (Scale bar, 20 μm). c) Side-view (x-z plane) of the microchannel with flowing beads (Scale bar, 50 μm). d) Schematic representation of the microfluidic chip configuration. The red box indicates the section that was imaged.

3.3.5 Effect of Re on Equilibrium Position

Figure 3.16 is a plot of intensity distributions of fluorescent signals of 15 μm beads flowing in D3 for a range of Re . The graph conveys two important results: first, the microchannel design parameters support single-position focusing over $20 < Re < 153$, and second, there is a lateral shift in equilibrium position at higher Re . A careful analysis of the equilibrium position relative to channel center in all three designs shows a distinct pattern of flow rate-dependent lateral shift (**Figure 3.17**). The equilibrium position shifts towards the channel wall as Re increases. Moreover, we observed a steep increase in shift in the flow rate regime of $55 < Re < 120$. The observed trend of lateral shift toward the channel wall is in resonance with the scale-down pattern of wall induced-shift, as shown in **Figure 3.8**.

Figure 3.16: a) Normalized intensity profiles of 15 μm fluorescent polystyrene beads flowing at different flow rates in the Design 3 microfluidic channel. The intensity profiles depict flow rate-dependent particle focusing at different positions relative to channel center. b) Schematic representation of the lateral displacement of the particles at equilibrium from the channel centerline.

Moreover, the magnitude of lateral shift in the three designs also varies as: $D1 > D2 > D3$. This observation is also evident from the theoretical model (**Figure 3.10**),

wherein $(F_D/F_I)_{D1} > (F_D/F_I)_{D2} > (F_D/F_I)_{D3}$. Thus, F_D dominates over the overall weak inertial forces (F_{IS} and F_{IW}) to push the equilibrium position closer to channel walls in D1 as compared to D2 and D3.

Figure 3.17: Experimental results on the effect of Re on the lateral migration of equilibrium position of 15 μm spherical polystyrene particles relative to channel center in the three designs. Design 1: $r_s = 300 \mu\text{m}$; Design 2: $r_s = 400 \mu\text{m}$; Design 3: $r_s = 500 \mu\text{m}$.

3.3.6 Focusing Performance of Cells

Besides validating the sheathless focusing performance of the proposed microfluidic device with polystyrene beads, we checked the compatibility of the PDMS microfluidic chip and the focusing performance with pancreatic cancer cells (14-16 μm) and macrophages (20-22 μm) in different experiments for further lab-on-a-chip applications. **Figure 3.18a** shows precisely focused dye-stained pancreatic cancer cells, MiaPaCa-2 (14 μm) and **Figure 3.18b** shows QDs-stained macrophages (20 μm) at the microchannel outlet at a flow rate of 300 $\mu\text{l}/\text{min}$. Likewise the beads, the cells also follow a similar trend in the shift in equilibrium position as the Re increases. **Figure**

3.19a compares the relative shift in position of cells and 15 μm beads for the same design (D3) over the range of Re . The difference in the relative equilibrium position between beads and cells may be possibly due to the fact that the cells being denser, experience a higher shear with respect to the flow with increasing Re , and thus a correspondingly lesser relative lateral shift occurs. **Figure 3.19b** shows the actual experimental data points along with errors computed from the fitted curve (**Figure 3.19a**). We captured high-speed images of macrophages flowing inside the microchannel at frame rates of 50000 fps to demonstrate randomly flowing single cells at the inlet (**Figure 3.18c**) and getting focused at outlet region (**Figure 3.18d**).

Figure 3.18: a) Dye-stained MiaPaca-2 cells (14 μm , scale bar 50 μm) and b) QDs-stained macrophages (20 μm , scale bar 80 μm) are precisely focused at the channel outlet. c) High-speed CMOS camera images of the inlet and d) outlet region at 50000 fps. Inlet: Randomly flowing macrophages fed at a flow rate of 300 $\mu\text{l}/\text{min}$. (Scale bar, 80 μm .) Outlet: Cells getting focused at the outlet. The blue circles represent the trajectory of one cell. Images were taken continuously (Scale bar, 80 μm).

Figure 3.19: a) Experimental comparison of the lateral migration of equilibrium position of 15 μ m spherical polystyrene beads and 14 μ m pancreatic cancer cells (MiaPaCa-2) relative to channel center in the D3. b) Residual errors for 15 μ m beads (top) and 14 μ m cells from the fitted curve.

3.3.7 Lab-on-a-Chip Optofluidic Flow Cytometer

Exploiting the unique behavior of the transverse inertial lift forces and Dean Drag forces in confined particle flows, the present microfluidic design totally alleviates the need of using any sheath flow or external force for 3D single-position focusing of micron-sized cells or particles.

With its novel and sheathless hydrodynamic design, the microfluidic chip can be engineered to a greatly simplified, lab-on-a-chip flow cytometry system with the following advantages: (i) very short channel length (~ 38 mm) and compact geometry; (ii) no additional multiple sheath flow channels required for focusing of sample cells or particles; (iii) no complicated cell/particle controlling technique (e.g. additional geometries in the microchannel, multi-layer microfluidic structure, etc.); (iv) channel geometry supports cell/particle focusing at higher flow rates (up to 700 μ l/min); (v) demonstrates successful focusing of dye- or nanoparticle-conjugated cells at high flow

rates; (vi) demonstrates 3D single-position focusing even at higher concentrations; (vii) easy, fast, and robust fabrication process of the single-layer PDMS-molded microfluidic chip; (viii) single equilibrium position is a desirable feature for accurate flow cytometry; (ix) can readily be integrated with optical fibers for excitation and detection of output photo-signals from cells or particles.

Figure 3.20: Output signals recorded for 1 second interval for 18.6 μm beads flowing at a $Re = 65.6$. Inset: Amplified 100 ms interval snapshot of the output voltage signal. The system gives a throughput of the system was 445 particles/sec for 18.6 μm particle size at 100KS/s.

We performed an experimental demonstration for proof-of-concept of the working efficiency of the miniature flow cytometer using fluorescent 18.6 μm and 26 μm polystyrene beads. We used the experimental set-up, illustrated in **Figure 3.4**, which comprised of the optofluidic flow cytometer chip integrated with tapered optical fibers for interrogation of fluorescent signals from flowing cells/particles. The optical fibers (excitation fiber: single-mode and detection fiber: multi-mode) were adiabatically tapered to a diameter of 80 μm to conform with the microchannel dimensions for maximum illumination of the flowing particles and collect maximum output photo-signals for analysis of particle information. The adiabatic taper ratio of 0.64 and other taper parameters listed in **Table 3.1**, allow the single-mode fiber profile characteristics to be maintained within the tolerable limits [127]. The fiber coupled PMT was interfaced to a data acquisition instrument for extracting the output signals at a data sampling rate

of 100 KS/s. The arrangement of optical fibers in the present optofluidic system enabled to obtain side-scattered fluorescent signals from cells/particles as they passed through the excitation beam. As depicted in **Figure 3.20**, the miniature optofluidic flow cytometer was able to achieve a throughput of 445 particles/sec for 20 μm beads at $Re = 65.6$ for a beads concentration of 10^6 beads/ml.

Figure 3.21: The count distributions for 18.6 μm and 26 μm beads, indicating a CV of 24% and 15% respectively for 1 sec duration of the entire output signal.

Moreover, we determined the coefficient of variation (CV) for 18.6 μm and 26 μm beads to be 24.37% and 15.91%, respectively for 1 second duration (**Figure 3.21**), which underlined the ability of the miniature optofluidic flow cytometer system to accurately detect the side-scattered signals vis-à-vis conventional flow cytometer. Furthermore, the pulse width ' t_{pw} ' for the fluorescent signal from 18.6 μm and 26 μm beads was measured to be 22 μs and 30 μs respectively, which means that our system was capable of resolving even higher concentration samples, and therefore, a maximum

throughput as high as 15000 particles/sec (18.6 μm) and 11000 particles/sec (26 μm) ($1\text{s}/(3 \times t_{pw} \mu\text{s})$) could be achieved by the current configuration. We used an in-house MATLAB program to extract useful quantitative and statistical information from the obtained LABVIEW data.

Figure 3.22: Comparison of the performance of the FL signal of the 26 μm beads from the (a) miniature and (b) commercial flow cytometer in terms of CV

Figure 3.22 shows the statistical side-scattered fluorescent signal count for a beads-based size assay obtained from our miniature and commercial flow cytometer (FACSCalibur, Becton Dickinson). **Table 3.2** presents a comparison of the performance of our miniature flow cytometer with the commercial flow cytometer in terms of CV. The current CV values for our present system are relatively higher than that of the commercial flow cytometer. Nonetheless, the CV of our system can be significantly lowered by optimizing the flow parameters for a particular size or sample type.

Table 3.2: Comparison of the miniature flow cytometer with the commercial flow cytometer

Particle size	Coefficient of variation	
	Miniature flow cytometer	Commercial flow cytometer
18.6 μm	22.16	12.2
26 μm	16.4	11.5

Thus, based on the current performance of the proposed miniature inertial focusing based-optofluidic flow cytometer, we predict a significant improvement in the device throughput by i) optimizing the flow parameters depending on the size or morphology of the sample, ii) increasing the sampling rate of the detection instrumentation and iii) optimizing the optical fiber-coupled laser beam shaping. The optofluidic cytometer system is thus, greatly simplified with a much smaller footprint, and is an easy-to-operate device in resource-limited conditions.

3.4 Summary

In this chapter, we proposed and demonstrated a sheathless and hydrodynamic method of ordering and focusing cells or particles based on transverse inertial lift forces and Dean Drag forces in confined-particle flows. We identified the range of relative particle sizes ($0.175 < a/w_{ch} < 0.9$) that can be three-dimensionally focused to a single-position by the proposed design over a given flow regime ($20 < Re < 153$). We also outlined the effect of Re on defining different equilibrium positions across the channel width. Further, we presented an empirical model relating the ratio of inertial lift forces and Dean Drag forces, and the geometrical parameters of the design to determine the rule of thumb for the precise focusing of particles in the proposed microchannel design. We demonstrated the focusing performance of the microfluidic device by experimental characterization with high repeatability for fluorescent polystyrene microparticles (15

μm and $20\ \mu\text{m}$), pancreatic cancer cells MiaPaCa-2 ($14\text{-}16\ \mu\text{m}$) and macrophages ($20\text{-}22\ \mu\text{m}$) at flow rates up to $700\ \mu\text{l}/\text{min}$. The proposed design offers a greatly simplified single-layer microfluidic version for sheathless ordering and focusing of particles or cells in 3D, as confirmed by confocal microscopy imaging. Furthermore, we integrated the inertial focusing-based microfluidic chip with tapered optical fibers at the outlet region and engineered it to a portable, easy-to-use lab-on-a-chip optofluidic flow cytometer. We presented a comparison on the performance of the miniature flow cytometer vis-à-vis commercial flow cytometer for a beads-based size assay. The encouraging results of the miniature flow cytometer underline its potential towards clinical applications in resource-limited conditions with more precise instrumentation techniques. We envision the microfluidic platform as a cartridge-type, one-off use chip for management of infectious diseases, and also for studying drug-delivery for disease theranostics.

Chapter 4 Sheath-assisted Hydrodynamic Focusing in the Viscous and Inertial Regimes

In Chapter 3, we introduced and discussed sheathless particle-focusing utilizing inertial forces in microchannels. We demonstrated 3D single-position focusing for a range of relative particle sizes $0.175 < a/w_{ch} < 0.9$ under Re ranging between 20 and 153. At such conditions, the $Re_p > 1$, and laminar flow occurs in the confined-particle regimes and hence, inertial forces dominate. We discussed the underlying phenomena explaining the effect of the inertial lift forces and secondary Dean Drag forces to cause lateral migration of particles as they flow past the microchannel length in the confined-particle flows. Herein, we introduce sheath-assisted hydrodynamic focusing and elaborate the phenomena in both point-particle ($a/w_{ch} \ll 0.1$) and confined-particle flows ($a/w_{ch} > 0.1$). Thus, this chapter will give an insight into the sheath-based particle focusing at lower and higher Re_p , and also differentiate the performance of sheath-assisted focusing from the sheathless focusing techniques.

4.1 Introduction

Pressure-driven hydrodynamic focusing is the commonly adopted approach for focusing of cell or particle samples to a singular-particle stream in microfluidic channels for designing lab-on-a-chip flow cytometers. Typical hydrodynamic focusing-based microfluidic configurations comprise of single-sheath or dual-sheath rectangular channels wherein the cell or particle sample flowing in the main channel is confined to a narrow stream, for single-cell interrogation by the surrounding sheath fluids which join the main channel through inlets on either side of the main channel. Hydrodynamic focusing models predict the focused sample stream width in the low Reynolds number regime ($Re \ll 1$) of the N-S equations, wherein the viscous forces dominate the inertial

forces. Nonetheless, operating in the viscous regime of the laminar microfluidic flow results in high relative focused stream width, and also limits the efficiency of microfluidic flow cytometers as sample throughput is low due to extremely low flow rates. Various sheath-assisted hydrodynamic focusing models have been reported to predict the focused sample stream width in the low Reynolds regime ($Re \ll 1$) of the N-S equations, wherein $Re_p \ll 1$ and the viscous forces dominate the inertial forces [135-137]. Yet, existing works do not outline the effect of focused stream width at high Re_p in confined-particle flows ($0.1 < a/w_{ch} < 0.9$; a , particle diameter; w_{ch} , width of the microchannel). The low Re assumption, does not take into account the inertial component in sheath-assisted flows. Thus, to enhance the power of microfluidic cell focusing, study of the hydrodynamic focusing mechanism at high Re is required.

In this work, we investigate hydrodynamic particle focusing in the inertial regime and present a comparative analysis of particle focusing performance in both the viscous and inertial regimes. We present experimental results for pressure-driven hydrodynamic focusing inside microchannels in the higher Re ($60 < Re < 130$) laminar flows in both the viscous and inertial regimes and compare them with particle trajectory simulations. We analytically explain the deviations of the results from the theoretical predictions of hydrodynamic focusing at $Re \ll 1$. Furthermore, we discuss the conditions and ranges of all parameters that define these focusing techniques. These studies underline the conditions for single-position focusing for a range of flow parameters and relative particle sizes in sheath-assisted hydrodynamic flows, particularly intended for lab-on-a-chip flow cytometry applications.

4.2 Experiments and Methods

4.2.1 Modelling of Sheath flow-assisted Particle Focusing in Microfluidic Channel

We performed the analysis of the 3D N-S equations for pressure-driven fluid flow inside microchannels in the laminar regime using the Finite element method (COMSOL Multiphysics 5.1, MA, USA). The channel height was fixed at $80\text{ }\mu\text{m}$ and various microchannels with $AR = 0.4, 0.8$ and 1.6 corresponding to microchannel widths ' w_{ch} ' of $200\text{ }\mu\text{m}$, $100\text{ }\mu\text{m}$ and $50\text{ }\mu\text{m}$, respectively, were studied for analysis. For the single-sheath design, we designed a pair of sheath inlets joining the main channel from either side at an angle of 30° (**Figure 4.1a**). For the dual-sheath design, two pairs of sheath inlets joined the main channel from either side at an angle of 30° (**Figure 4.1b**).

Figure 4.1: Schematic representation of the a) single-sheath and b) dual-sheath hydrodynamic particle focusing microfluidic geometries.

The flow was modeled in the laminar flow regime at a sheath flow rate of 150 $\mu\text{l}/\text{min}$. The sample flow rates were modulated at different values relative to the sheath flow rate to study the particle focusing process at various FFRs. Sample parameters corresponded to polystyrene beads ($\rho = 1055 \text{ kg}/\text{m}^3$) dispersed in DI water and the sheath fluid was DI water ($\rho = 10^3 \text{ kg}/\text{m}^3$). The flow was modeled as incompressible with shallow channel approximation. Simulations were performed at various FFRs from 1:5 through 1:1. Besides modelling the focusing effect as a consequence of sheath flows in the single-sheath and dual-sheath configurations, we also simulated the particle trajectories for particle diameters of 5 μm and 20 μm in the presence and absence of sheath flow. This is indeed a very useful modeling approach as it provides significant results to predict equilibrium particle positions across the microchannel width as a function of particle size and FFR, which is critical for flow cytometry studies.

4.2.2 Fabrication of the Microfluidic Focusing Chip using Soft-photolithography

For conducting experiments, we fabricated single-sheath and dual sheath microfluidic layouts with channel width, $w_{ch} = 80 \mu\text{m}$. We followed the same recipe for fabricating these microfluidic chips, as mentioned before in Section 3.2.1.

4.2.3 Particle Focusing Experiments

We purchased fluorescent polystyrene particles from Polysciences Asia Pacific, Inc. (5.7 μm , CV 7 %; 9.8 μm , CV 6 %; and 18.7 μm , CV 4 %). For each flow experiment, particles were mixed in DI water in a concentration ratio of 0.5 % wt/vol. The mixture was shaken vigorously for 15 minutes and allowed to settle for 5 minutes before extraction into syringes. The particle sample was fed through the inlet port of the chip at flow rates controlled by a syringe pump (NE-1000, Newera). We utilized the fluorescence from flowing particles to track their focusing positions across the microchannel width. As the samples were fed at the set flow rate, fluorescent images of

the outlet part of the channels were acquired with an inverted fluorescence microscope (Eclipse-Ti, Nikon).

4.3 Results and Discussion

4.3.1 Role of Re and Re_p on Particle Focusing

For FEM-based computations of the 3D steady state N-S equations, the entire microchannel structure (**Figure 4.2a**) is subdivided into microdomains (**Figure 4.2a** inset), commonly known as meshing of domains, where the velocity profile, concentration profile and particle tracing calculations are performed. Shown in **Figure 4.2b** is the velocity distribution for a fully-developed, laminar and incompressible flow, governed by N-S equation (Equation 4.1) and the continuity equation (Equation 4.2) across the microchannel width, w_{ch} . As expected, a parabolic velocity profile with a FRR-dependent magnitude across w_{ch} was obtained. Following this, we modeled the width of the focused particle stream in the main channel ' w_f ' using the diffusion-convection equation (Equation 4.3) in COMSOL Multiphysics 5.1 (COMSOL, MA, USA) with concentration as the dependent variable (dependent on velocity) and thus obtained the concentration profile of the sample across w_{ch} as shown in **Figure 4.2c**.

$$\rho(u \cdot \nabla)u = \nabla \cdot [-pI + \mu(\nabla u + (\nabla u)^T)] - 12 \frac{\mu u}{d_z^2} + F \quad (4.1)$$

$$\rho \nabla \cdot u = 0 \quad (4.2)$$

$$\nabla \cdot (-D_i \nabla c_i) + u \cdot \nabla c_i = R_i \quad (4.3a)$$

$$N_i = -D_i \nabla c_i + u c_i \quad (4.3b)$$

Here, where μ represents the viscosity, u is the velocity, T is the operating temperature, ρ is the density, p is the pressure, F represents the sum of the body forces, D represents the diffusion coefficient ($D_i = 10^{-10} \text{ m}^2/\text{s}$), and c_i is the initial concentration, R_i represents the sources or sinks in the reaction and N_i is the molar flux. The velocity of the channel walls was set to zero to simulate the wall-bounded flow.

Figure 4.2: a) COMSOL simulation model of single-sheath hydrodynamic focusing. Inset: meshing domains used for calculation of velocity and concentration of flowing sample. b) Velocity profile obtained for an $80 \mu m$ (w_{ch}) wide channel and c) Concentration profile showing the focusing of the samples surrounded by sheath fluid. d) Velocity profiles for single-sheath and dual-sheath configurations across the microchannel width for $FRR = 2$. Inset: The hydrodynamic focusing effect due to single-sheath and dual-sheath flows as a function of sample concentration across the w_{ch} ($50 \mu m$). A sample could be fluorescent-labeled particles/cells or a dye solution.

Furthermore, we investigated the effect of AR on the relative width of the focused particle stream ' w_f/w_{ch} ' in single-sheath (**Figure 4.3a**) hydrodynamic flows as a function of FRR is shown for $w_{ch} = 50 \mu\text{m}$ (AR 1.6), $100 \mu\text{m}$ (AR 0.8), and $200 \mu\text{m}$ (AR 0.4). We compared the relative focused stream widths (w_f/w_{ch}) in different AR rectangular cross-section microchannels obtained using FEM analysis with the theoretical focused stream width obtained for a Poiseuille velocity profile for $Re \ll 1$ computed by [135] using Equation 4.4 and Equation 4.5,

$$\frac{w_f}{w_{ch}} = \frac{1}{\gamma(1+2/FRR)} = \varphi \quad (4.4)$$

$$\gamma = \frac{v_f}{v_{ch}} = \frac{1 - \frac{192}{\pi^5} AR \sum_{n=0}^{\infty} \frac{1}{(2n+1)^5} \frac{\sinh\left[\frac{(2n+1)\pi(\varphi)}{2AR}\right]}{\cosh\left[\frac{(2n+1)\pi}{2AR}\right]}}{1 - \frac{192}{\pi^5} AR \sum_{n=0}^{\infty} \frac{1}{(2n+1)^5} \tanh\left[\frac{(2n+1)\pi}{2AR}\right]} \quad (4.5)$$

where, γ is the ratio of the average velocity of the focused sample v_f and main channel v_{ch} .

It was interesting to observe that simulation trend largely deviated from the solution of the Poiseuille velocity equation for the relative focused stream width, plotted in **Figure 4.3b**. This was primarily because the microchannel dimensions in their studies correspond to $Re < 1$ and $Re_p \ll 1$ whereas the present analysis (**Figure 4.3a**) concerns hydrodynamic focusing in laminar flows in the range $60 < Re < 130$ where Re_p is of the order of unity. It is thus, interesting to investigate the effect of sheath-assisted hydrodynamic focusing at higher Re_p , the regime where the inertial effects start to dominate.

Figure 4.3: a) Simulation results of the relative focused particle stream width in single-sheath configuration as a function of FRR at $Re \gg 1$ for $Re_p > 1$; Q_{sh} is fixed at 150 $\mu\text{l}/\text{min}$. The focused stream width obtained in this case is 8 μm for $Q_{sh}/Q_s = 5$. b) Simulation results of the relative focused particle stream width in single-sheath configuration as a function of FRR at $Re < 1$ for $Re_p \ll 1$; Q_{sh} is fixed at 12 $\mu\text{l}/\text{min}$. The focused stream width obtained in this case is 35 μm for $Q_{sh}/Q_s = 5$.

4.3.2 Experimental validation of inertial hydrodynamic focusing

We fabricated a unity AR ($w_{ch} = 80 \mu\text{m}$) PDMS microfluidic chip to study inertial particle focusing in sheath-assisted hydrodynamic flows. Two sets of fluorescent polystyrene beads, $5.7 \mu\text{m}$ ($a/w_{ch} = 0.7$, $0.4 < Re_p < 0.54$) and $9.8 \mu\text{m}$ ($a/w_{ch} = 0.12$, $1.2 < Re_p < 1.58$), were chosen to study the effect of relative particle size on the width of the focused stream at higher Re . Due to the larger Re_p , inertial forces become more significant in the hydrodynamic focusing of $9.8 \mu\text{m}$ beads than $5.7 \mu\text{m}$ beads.

Figure 4.4a shows the snapshots of the microscope images depicting the fluorescent streaks of the flowing particles. With all parameters remaining same, it was observed that the larger $9.8 \mu\text{m}$ beads focused to a narrower stream width as compared with the smaller $5.7 \mu\text{m}$ beads (**Figure 4.4b**). This is a possible result of the additional inertial lift forces that come into play at the dimensional level of the larger beads. The particle Reynolds number, Re_p , is typically of the order of 1 for the larger $9.8 \mu\text{m}$ beads, and hence the inertial lift forces dominate. It was interesting to note that while the lift forces act to move the particles away from channel center [57] in sheathless flows, the particle stream remain confined to the channel center because of the higher surrounding lateral pressure in sheath-assisted flows. The smaller relative particle diameter ($a/w_{ch} < 0.1$) and therefore the Re_p ($\ll 1$) of the $5.7 \mu\text{m}$ beads, however, resulted in a much wider focused sample width ($\sim 5 \times a$) suggesting an inverse dependence of the pressure-driven hydrodynamic focusing on the particle diameter.

Figure 4.4: a) Inverted fluorescent microscope images of the main channel of single-sheath microfluidic chip showing the hydrodynamic focusing of 5.7 μm and 9.8 μm beads for $77 < Re < 105$ (Scale bar, 80 μm). b) Relative focused stream width plots for 5.7 μm and 9.8 μm beads as function of FRR. The fluorescent intensity distribution across the microchannel width is fitted to a Gaussian profile and the FWHM is calculated to determine the relative focused stream width.

4.3.3 Single-sheath and Dual-sheath Hydrodynamic Focusing at Higher Re_p

We plotted the experimentally obtained relative focused stream width as a function of FRR of flowing 9.8 μm beads in single-sheath and dual-sheath configurations, shown in **Figure 4.5a-b**, for $77 < Re < 105$. The fluorescent intensity profiles for $FRR = 0.5$ across w_{ch} in both cases are shown in the inset of **Figure 4.5b**. Unexpectedly, it was the single-sheath configuration that yielded single-particle focusing at the channel center ($\sim 1.6 \times a$). This resulting observation was possibly due to the huge cumulative sheath flow pressure in the dual-sheath configuration, as defined in **Equation 4.6a,b** (provided both sheath fluids are pumped at the same pressure),

$$Q_{sh(dual)} = 2 \times Q_{sh(single)} \quad (4.6a)$$

$$Q_{sh(cumulative)} = 2 \times Q_{sh(single)} = 4 \times Q_{sh(dual)} \quad (4.6b)$$

In our experiment, $Q_{sh(\text{single})} = 150 \mu\text{l/min}$. The particles flowing in dual-sheath flows experience more shear force relative to particles flowing in single-sheath flows. In this situation, shear-induced inertial forces tend to exert a lift force away from the channel center and thus, a notable difference was observed ($\sim 1.4 \times a$) between the relative focused stream width of dual- and single-sheath configurations at the same operating parameters.

Figure 4.5: Effect of single-sheath and dual-sheath hydrodynamic focusing at higher Re_p . a) Schematic illustration of single-sheath and dual-sheath configurations for the studying the hydrodynamic focusing effect in confined-particle flows ($a/w_{ch} = 0.12$) at $Re \gg 1$. b) The experimentally determined relative focused stream width profile for 9.8 μm fluorescent polystyrene beads in single-sheath and dual-sheath configurations. Inset: Fluorescent intensity distribution showing the FWHM of the focused stream width in single-sheath (red) and dual-sheath (blue) configurations at $Re = 88$.

4.3.4 Inertial Forces lead to Single-particle Hydrodynamic Focusing

We further investigated the effect of inertial forces on the efficiency of sheath-assisted hydrodynamic focusing. We carried out FEM simulations for tracing particle trajectories with and without sheath flows at $FRR = 0.5$. While no focusing was observed for 5 μm diameter particles without sheath flow (**Figure 4.6a**), a much constricted

particle trajectory was obtained for 5 μm and 20 μm diameter particles in the presence of sheath flow (**Figure 4.6b,c**). The magnified images, however revealed that the smaller 5 μm particles are not well-ordered to a single-particle level, a necessary feature required for accurate flow cytometry applications.

Figure 4.6: Role of sheath fluid in achieving hydrodynamic focusing in single-sheath configuration. The simulation images show particle trajectories recorded 0.2 sec after the onset of flow. In the absence of (a) sheath flow, negligible lateral migration of particles is observed. In the presence of sheath flow, (b) 5 μm and (c) 20 μm polystyrene particles laterally migrate to a narrow width at the channel center. The enlarged images of the main channel indicate that the focused stream of the smaller 5.7 μm particles consist of the bunch of individual particles and not a single-particle train.

Following this, we compared these simulation findings of 5 μm and 20 μm diameter particle sizes with the experimental investigation of the focused particle stream widths using 5.7 μm and 18.7 μm fluorescent polystyrene beads. The focused stream widths were determined by calculating the FWHM of the intensity profiles obtained from the fluorescent images of the flowing 5.7 μm and 18.7 μm beads (**Figure 4.7b-i,ii**). The FWHM values, shown in **Figure 4.7a**, for both the particle sizes are comparable: 30.78 μm (for 5.7 μm beads) and 28.07 μm (for 18.7 μm beads).

Figure 4.7: a) Experimentally determined FWHM of the focused stream width of 5.7 μm and 18.7 μm polystyrene beads to demonstrate the effect of Re_p at higher $Re = 88$. The FWHM of both sizes of beads is approximately the same, indicating the role of inertial forces at higher Re_p . b) The microscope images of the fluorescent streaks of (i) 18.7 μm and (ii) 5.7 μm polystyrene beads agree with the particle trajectory simulation results (Scale bar, 80 μm).

These results also support the fact that inertial focusing aids viscous hydrodynamic effects to obtain single-particle focusing in sheath-assisted hydrodynamic focusing at higher Re_p (>1). The inertial lift forces actively participate to order the 18.7 μm diameter beads to a narrower focused stream as $0.1 < a/w_{ch} < 0.9$ for $a = 18.7 \mu\text{m}$, whereas only viscous forces act in the case of smaller 5.7 μm particles as $a/w_{ch} < 0.1$ and therefore, Re_p is also < 1 . **Figure 4.8** presents the difference in the scaling of Re as we progress towards the inertial regime in laminar, confined-particle flows ($0.1 < a/w_{ch} < 0.9$) from the viscous regime in point-particle flows ($a/w_{ch} \ll 0.1$).

Figure 4.8: a) Comparison of variation of Re with FRR in confined-particle flows ($a/w_{ch} > 0.1$; $AR = 1$) and point-particle flows ($a/w_{ch} \ll 0.1$; $AR = 0.016$). Increase in FRR leads to much significant changes in the flow profile for confined-particle flows (red) than in point-particle flows (blue).

Figure 4.9 shows the prediction trend for relative focused stream width in low and high Re regimes with respect to relative particle sizes. These studies are an integral part of the essential tool-kit for designing microfluidic flow cytometers that underline the effect of relative particle sizes, as different channel dimensions lead to single-position focusing for different particle sizes at the given flow parameters.

These observations indicate the contribution of inertial forces on hydrodynamic focusing behavior in sheath-assisted flows. Therefore, the cumulative effects of channel dimensions, relative particle diameters, flow parameters and number of sheath channels (single/dual) on the relative focused stream width must be taken into consideration while designing microfluidic flow cytometers where single-position particle focusing is essential.

Figure 4.9: Theoretically obtained focused stream width profile at low Re according to Equation 4.5 (blue). Plot of relative focused stream width obtained by simulation of single-sheath hydrodynamic focusing at higher Re (red).

Working in the inertial regime is advantageous from the point of view of lab-on-a-chip flow cytometry applications as it can yield enormously high throughputs as compared with low-throughput obtained in hydrodynamic focusing in the viscous regimes. Moreover, the PDMS-fabricated microfluidic chips could easily withstand total fluid pressures upto 450 $\mu\text{l}/\text{min}$ during the experiments. Thus, these experiments demonstrate the ability of the sheath-assisted hydrodynamic focusing in attaining single equilibrium position across the microchannel width in straight channels as opposed to multiple equilibrium positions in straight channels in the case of sheathless inertial flows at higher Re . The presented results can well be optimized for rare cell counting, tumor cell analysis and white blood cells characterizations. These studies underline the development of commercial miniature lab-on-a-chip flow cytometers to improvise upon existing disease screening methods in low-resource settings.

4.3.5 Inertial Forces-based and Sheath-based Miniature Flow Cytometers for Cancer Theranostics

In Chapter 3 and the preceding sections, we have illustrated the in-depth studies for particle focusing using a) a sheathless inertial forces-based microfluidic chip and b) a sheath-based microfluidic chip. We have also investigated the effects of higher Re and higher Re_p on sheath-based particle focusing. **Table 4.1** outlines the distinct ranges of parameters that define particle focusing in both types of hydrodynamic focusing techniques. It is clear from the experimental results that inertial forces-based hydrodynamic focusing can lead to single-position focusing even at higher flow rates (up to 700 $\mu\text{l}/\text{min}$). It is also possible to achieve high-throughput (particles/sec) output in sheath-based microfluidic particle focusing methods by operating at higher Re – either by tailoring the channel dimensions (such that $a/w_{ch} < 1$) and/or by changing the flow parameters ($Q_{s(max)} = 150 \mu\text{l}/\text{min}$). Since viability of cells is critical in many biomedical studies [138-140], a careful evaluation and optimization of the particle focusing technique thus needs to be done with regards to a particular application, type of cell under study, concentration of cell sample, and permissible flow parameters.

Table 4.1: Hydrodynamic Particle Focusing Techniques

<i>Parameters</i>	<i>Sheath flow-assisted</i>	<i>Inertial forces-assisted</i>
<i>Channel width ‘w_{ch}’</i>	100 – 500 μm	0.5 - 100 μm
<i>Relative particle diameter ‘a/w_{ch}’</i>	$a/w_{ch} \ll 0.1$	$0.1 < a/w_{ch} < 0.9$
<i>Sample flow rate ‘Q_s’</i>	0.05 - 75 $\mu\text{l}/\text{min}$	50 – 700 $\mu\text{l}/\text{min}$
<i>Sheath flow rate ‘Q_{sh}’</i>	<150 $\mu\text{l}/\text{min}$	N.A.
<i>Reynolds number ‘Re’</i>	< 1	$10 < Re < 150$
<i>Throughput</i>	low	high

The role of a flow cytometer for quantitative disease diagnosis and therapy (collectively termed as “theranostics”) has high potential for future clinical applications. As a multi-parameter, bulky, and expensive instrument, conventional flow cytometers

are restricted to highly specialized laboratories and high-end clinical settings. While a course of diagnosis or therapeutic analysis may be completed within a couple of hours in developed countries, the same might actually take weeks to be achieved in poorer and developing nations, as they do not have easy access to such sophisticated clinical infrastructure in close vicinity. Hence, such locations become more vulnerable to epidemics given the inability to curb or treat diseases at an early stage.

The following Chapters 5 and 6 give an in-depth account of the exceptional relevance of flow cytometers in cancer theranostics. Flow cytometers hold immense utility in cancer theranostics-based nanoassays. Therefore, we need to work on low-cost and easy-to-operate flow cytometers to address the urgent need for disease theranostics using an affordable platform. Optofluidic flow cytometers show promising potential as portable, easy-to-use, miniaturized on-chip flow cytometers designed as cartridge-type one-off use chip for affordable diagnostic and therapeutic healthcare in low-end clinical settings. Furthermore, we need efficient nanoassays in order to be able to extract quantitative as well as qualitative disease theranostic information from flow cytometers. As discussed in Section 2.6 of Chapter 2, the current state-of-the-art largely comprises of fluorescence-based, size-based, bead-based nanoassays, which are essentially used for signal analysis i.e. they encompass the “diagnostics” domain of the “theranostics”. Therefore, we need to develop novel nanoassays which enable us to drive simultaneous theranostics. Moreover, we need to focus on preparing “one-shot” theranostics nanoassays such that they are compatible with our miniature flow cytometers and can be used in field applications (mass screening or therapeutics in rural settings).

Figure 4.10a is a schematic pictorial representation of what a theranostics nanoassay would look like, and how it would be useful in miniature flow cytometers (**Figure 4.10b,c**). In Chapters 5 and 6, we will mainly try to address these issues by

proposing and investigating two novel nanoassays which can be readily used in “small” and “big” flow cytometers.

Figure 4.10: a) Pictorial representation of a theranostics nanoassay, consisting of a drug/gene carrier - the gold nanoparticle and the output signal provider- the fluorescent tag on the drug/gene. b) Illustration of a lab-on-a-chip flow cytometry device for theranostics applications, with the associated fluidic (inlet and outlet) and optical instrumentation (laser diode, optical fibers, PMTs and DSO). The designed nanoassay can be fed into the inlet, and the desired drug/gene delivery information can be deduced from the two PMTs. b) Microscopic image of the optical fibers integrated with the PDMS microfluidic chip near the outlet region (Scale bar, 80 μ m). (Size of cells/particles not to scale).

4.4 Summary

In this chapter, we presented a simulation-based and experimental study to determine the effect of inertial forces ($Re > 1$) on particle focusing in sheath-assisted hydrodynamic flows. The role of flow parameters (Re) and relative particle size (Re_p) on hydrodynamic particle focusing was outlined. The focused stream width deviates from the theoretical predictions (done for lower Re) as inertial forces come into play at

higher Re . Thus, the focused stream width predictions for focusing spherical particles in microchannels with sheath-assisted flows at higher Re should be inclusive of inertial forces. Moreover, we experimentally demonstrated the effect of multiple sheath flows on the focused stream width in the inertial regime. We studied the influence of inertial focusing in pressure-driven confined-particle flows ($a/w_{ch} < 0.1$) at the single-particle level. These studies provide crucial information for designing lab-on-a-chip miniature flow cytometers for high-throughput applications. Furthermore, the results also demonstrate the difference in focused stream width using single- and dual-sheath flows at higher flow rates compared to those obtained at lower flow rates. Finally, we introduced the need for designing novel cancer theranostics-based nanoassays and highlighted their relevance in miniature flow cytometry.

We outline the main conclusions of this chapter as follows:

- The focused stream width predictions for focusing spherical particles in microchannels with sheath-assisted flows at higher Re should be inclusive of inertial forces.
- In case of lower Re , only the viscous forces lead to narrowing of the sample stream and thus, it is not possible to achieve single-position particle focusing for smaller size particles ($< 10 \mu\text{m}$).
- At higher Re , both viscous and inertial forces act to make single-position particle focusing possible even for smaller size particles ($< 10 \mu\text{m}$).
- However, the allowable flow rates in the sheath-assisted method ($50 \mu\text{l/min}$) are far low as compared to sheathless focusing methods ($700 \mu\text{l/min}$). Hence, sheath-assisted focusing method still remains a low-throughput method as the microfluidic chip requires to withstand the additional pressures due to sheath flows also.

Chapter 5 Gold Nanorods-based Nanoassay for

Pancreatic Cancer Theranostics

In Chapter 2, we outlined the predominant role of flow cytometers in RNAi-based gene therapy for cancer theranostics. We also highlighted the relevance of formulating novel nanoassays that are “robust and one-shot” and can be suited to be used with portable optofluidic flow cytometers for determining the transfection efficiency of the nanoassays in real time. Thus, we demonstrate a novel *theranostic* nanoassay which is capable of inducing gene therapy of pancreatic cancer cells (*therapeutic*) and at the same time, also capable of providing output signals to measure the effectiveness of the nanoassay (*diagnostic*) through the powerful technique of flow cytometry. Illustrated in Chapter 3, our microfluidic particle focusing chip is able to achieve 3D single-position focusing for pancreatic cancer cells. Hence, the proposed nanoassay is compatible with our miniature flow cytometer and therefore, is suitably designed for use in both “big” and “small” flow cytometers.

5.1 Introduction

Pancreatic cancer is one of the deadliest types of cancer with a survival rate of 6 % at all stages of diagnosis, leading to over 35,000 deaths in the United States every year [141]. Poor prognosis and high mortality can be attributed to both intrinsic (*de novo*) and extrinsic (acquired) drug resistance [142] . To overcome the high mortality rate, new therapeutic modalities such as adjuvant therapy [143], targeted therapy [144-146], and stromal therapy [147], which envision effective treatment to the pancreatic cancer have been proposed. RNAi-based gene silencing possesses great ability for therapeutic interventions in pancreatic cancer. RNAi-assisted therapeutic interventions enable the screening of patients for over-expression of different genes, thereby

facilitating personalized cancer therapy targeting specific mutant genes [148]. The phenotypic detail provided by genomic technologies can be an effective guide for targeted therapeutics for defining prognosis and treatment at the individual level [149, 150]. Among various prognostic indicators, the Interleukin-8 (IL-8) gene mutations are found to be overexpressed in many pancreatic cell lines. In general, transfection agent is needed for siRNA delivery because naked siRNAs are unstable and too small to permeate the cell membrane. Also, they are negatively charged and possess very short half-lives to survive nuclease mediated degradation. Therefore, non-toxic, biocompatible and stable non-viral transfection agents need to be developed and investigated for gene delivery in cancer cells. Perhaps transfection agents could be both viral and non-viral, however, non-viral agents are a preferred choice due to their simple synthesis techniques, biodegradability, low toxicity and low immune response.

In this chapter, we demonstrate IL-8 gene silencing by employing RNAi-based gene therapy approach and this is achieved by using gold nanorods (AuNRs) for efficient delivery of IL-8 siRNA to the pancreatic cell lines of MiaPaCa-2 and Panc-1. **Figure 5.1** gives a pictorial overview of the AuNRs-siRNA nanoplex formation and the IL-8 gene knockdown mechanism in pancreatic cancer cells as a consequence of siRNA transfection.

Hence, we investigate the suitability of using AuNRs as a novel non-viral nanocarrier for the efficient uptake and delivery of IL-8 siRNA in realizing gene knockdown of both MiaPaCa-2 and Panc-1 cells. Flow cytometry and fluorescence imaging techniques are applied to confirm transfection and release of IL-8 siRNA. Upon successful delivery of IL-8 siRNA into cancer cells, we also determine the effects of IL-8 gene knockdown in terms of gene expression, cell invasion, cell migration and cell

apoptosis assays. In addition, we compare our results with a commercial transfection reagent, Oligofectamine, serving as positive control.

Figure 5.1: An overview of the AuNRs-siRNA nanoplex formation and the IL-8 gene knockdown process followed by siRNA transfection.

5.2 Materials and Methods

5.2.1 Synthesis of AuNRs and Characterization of Nanoplexes

We prepared AuNRs by the seed-mediated growth method in Cetyl trimethylammonium bromide (CTAB) surfactant solution as detailed in [151]. CTAB shapes into rod-like micelles above its critical micelle concentration and therefore forms the template for the subsequent synthesis of AuNRs. Perhaps the CTAB-coated AuNRs exhibit positive charge, but cytotoxicity of CTAB limits the use of AuNRs in this form. To address this issue, we first used negatively-charged polymer PSS to coat on the surface of AuNRs (AuNR/PSS), followed by PAH to bind with PSS, thus obtaining a positively charged AuNR/PSS/PAH nanocarrier. For the sake of simplicity, we address this modified AuNR/PSS/PAH nanocarrier as “AuNRs” in the following text. Then, we characterized the synthesized AuNRs by transmission electron microscopy (TEM).

Thereafter, we prepared AuNRs-siRNA nanoplexes with different weight ratios by gently mixing siRNA with AuNRs, and allowing the mixture to settle for 30 minutes. To determine the effective binding of the siRNA with the AuNRs, we performed agarose gel electrophoresis on a UV transilluminator (Bio-Rad). Binding efficiency was determined in terms of electrophoretic mobility and fluorescent emission signals of different AuNRs-siRNA nanoplexes. The formation of stable complexation was confirmed by measurement of hydrodynamic size and surface charge (zeta potential) of AuNRs-siRNA nanoplexes at 25°C using a 90-Plus particle size analyzer (Brookhaven Instrument Corp.).

5.2.2 Cell Culture

We cultured human pancreatic cancer cells, MiaPaCa-2 (CRL-1420), and Panc-1 (CRL-1469) (ATCC) in DMEM (Hyclone), supplemented with 10% FBS (Hyclone), 100 µg/mL penicillin (Gibco) and 100 µg/mL streptomycin (Gibco). Cells were cultured in the UV incubator at 37 °C in a humidified atmosphere with 5% CO₂, and maintained continuously [89].

5.2.3 Cytotoxicity

We measured the cell viability by the MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma) assay kit following the procedure described in [152]. MiaPaCa-2 and Panc-1 cells were seeded at 5×10^3 per well into 96-well plates and allowed to adhere for 24 hours. Cells were treated with different concentrations of AuNRs-siRNA nanoplex for 24 and 48 hours. Prior to this treatment, the medium of the 96-well plate was changed and incubated for 20 minutes. MTT (5 mg/mL, 20 µL) in phosphate-buffered saline (PBS) was added and the cells were incubated for 4 hours at 37°C with 5% CO₂. Dimethylsulfoxide (DMSO, 150 µL, Sigma) was then added to solubilize the precipitate with 5 min gentle shaking. We then measured absorbance with

a microplate reader (Bio-Rad) at a wavelength of 490 nm. The cell viability was obtained by normalizing the absorbance of the sample well against that from the control well and expressed as a percentage, assigning the viability of non-treated cells (Blank) as 100%.

5.2.4 Transfection of cells with IL-8 siRNA

The MiaPaCa-2 and Panc-1 cells (1×10^4) were seeded into 6-well plates in DMEM medium without antibiotics to give 30-50% confluence at the time of transfection. AuNRs (1 mg/mL) dispersion was respectively mixed gently with IL-8 siRNA^{FAM} (20 μ L, 10 μ M) (Sense: 5' -AMGUUGGAGCUUGUGGCGUAGUU-3'; Antisense: 5'-CUACGCCACAAGCUCCAACUU-3') or NC-siRNA^{FAM} (20 μ L, 10 μ M) (negative control siRNA without targeting any gene, Sense: 5'-UUCUCCGAACGUGUCACGUTT-3'; Antisense: 5'-TTAAGAGGCUUGCACAGUGCA-3') and incubated for 30 minutes. Before transfection, the medium of the cultured cells was changed with OPTI-MEM (Invitrogen), and incubated for about 20 minutes. Then, we added different concentrations of the above mentioned AuNRs-siRNA^{FAM} mixture to the 6-well plate wells and the cells were continuously cultured for four hours. Commercial transfection reagent OligofectamineTM (Invitrogen) coupled siRNA^{FAM} (Oligo-siRNA) was used as positive control. Following this, we separated the different groups of transfected cells into two parts each, one for imaging under inverted fluorescence microscope and the other for flow cytometry for detection of transfection efficiency. Transfection efficiency examination and fluorescence imaging were performed 4 hours post-treatment. The gene expression was monitored at 48 hours post-transfection.

5.2.5 Fluorescence Imaging

We performed *in vitro* fluorescence microscopy (Eclipse-Ti, Nikon) to visually inspect the proportion of cells that may have undergone siRNA transfection. After 4

hours of incubation, the transfected cells were washed twice with PBS, and fixed with 4% formaldehyde. Following that, the cells were mixed with DAPI (Sigma) for nuclear counterstaining prior to imaging. To image the cells, filter sets for DAPI (excitation at 405 nm and emission collection with a 450/50 nm band pass filter) and FITC (excited with 488 nm laser and emission collection with a 525/50 nm band pass filter) were applied for DAPI (with excitation/emission maximum at 359/461 nm) and FAM (with excitation/emission maximum at 492/518 nm) signals, respectively.

5.2.6 Flow Cytometry

We carried out flow cytometry to quantitatively determine the proportion of MiaPaCa-2 and Panc-1 cells that were successfully transfected with IL-8 siRNA using the designed nanoassay. For the flow cytometry experiments, the cells were washed twice with PBS and harvested by trypsinization. Though the siRNA was stained with FAM, filter set for FITC was applied (as both the fluorescent markers possess same emission wavelength) to determine the transfection efficiency quantitatively. The samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Mississauga, CA). Transfected cells were identified in terms of FITC-fluorescent intensity signal obtained by flow cytometry experiments, and a statistical comparison of different cell groups was prepared subsequently.

5.2.7 Gene Expression Analysis

We conducted gene expression analysis to determine the relative IL-8 mRNA levels in cancer cell lines following the nanoassay administration. 48 hours after transfection, total RNA was extracted from MiaPaCa-2 and Panc-1 cells using TRIzol reagent (Invitrogen) and the amount was quantitated by a spectrophotometer (Nano-Drop ND-1000). Reverse transcription was conducted in a 20 μ L reaction mixture and cDNA was synthesized from 2 μ g of total RNA using the reverse transcriptase kit

(Promega) following the procedure described in [153]. The relative IL-8 mRNA expression level was determined using semi-quantitative PCR normalized to the expression of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), serving as the control housekeeper gene for evaluation of gene expression. The primer sequences for different genes were as follows: IL-8, forwards 5'-CTTCTAGGACAAGAGCCAGGAAGAAACCAC-3' and reverse 5'-GTCCAGACAGAGCTGTCTTCCATCAGAAAG-3'; GAPDH, forwards 5'-ACCACAGTCCATGCCATCAC-3' and reverse 5'-TCCACCACCCT-GTTGCTGTA-3', as reported previously [46]. The PCR products were electrophoresed on 1.2 % ethidium bromide-stained agarose gel and observed under a UV transilluminator (Bio-Rad). The concentration of IL-8 in cell culture supernatants was assayed using standard sandwich ELISA Kits (EH2IL8, Thermo Scientific) according to the manufacturer's instructions.

5.2.8 Cell proliferation, Migration, Invasion, and Apoptosis Assays

To examine the capacity of cell migration under the influence of the AuNR-siRNA nanoassay, we conducted the wound healing assay. Briefly, MiaPaCa-2 cells (1×10^4) were seeded into 24-well plates, then transfected with AuNRs-siRNA (weight ratio 10:1) and other formulations for 48 hours to reach confluence. A wound was generated by scratching the surface of the plates with a sterile middle pipette tip. Thereafter, the cells were incubated in complete culture medium. Subsequent cellular migration towards the scratched area as part of the wound healing process was assessed at 0 and 72 hours post treatment (0h is the time at the instant of scratching) by imaging with a Nikon Eclipse-Ti microscope. The distances of migration from the cellular monolayer to the wounded area during this time period was measured by NIS Elements Br Analysis 4.0 software.

The invasive capacity of MiaPaCa-2 and Panc-1 cells treated with AuNRs-siRNA and other formulations was tested by using 8.0- μ m BD BioCoatMatrigel invasion chambers Assay System (BD Biosciences). Briefly, the transfected cells (5×10^4) with the FBS-free medium were seeded into the upper chamber of the system and the bottom chamber of the system was filled with 10 % FBS medium as a chemoattractant. After 72 hours of incubation, the cells in the upper chamber were removed with swabs, and the cells that invaded through the Matrigel matrix membrane were stained with 1% crystal violet in PBS for 30 minutes. Thereafter, the invaded cells were viewed under the microscope, and counted in 5 random fields of vision.

The apoptosis assay was conducted by Annexin V: FITC Apoptosis Detection Kit I (BD PharmingenTM) according to the manufacturer. Briefly, approximately 1×10^6 MiaPaCa-2 cells were seeded in a six-well plate. Then, the cells were treated with the AuNRs-siRNA (10:1) nanoplex and other formulations, and incubated for 72 hours. The cells were then collected and washed twice with cold PBS and suspended in binding buffer. The induction of the apoptosis process enables the FITC-labeled Annexin V to bind with phosphatidylserine (PS) as it appears on the outer surface of cell membrane due to onset of apoptosis, and is otherwise confined to the inner boundary of the cell membrane in healthy cells. The cell nuclei were stained with propidium iodide (PI). The stained cells were analyzed by flow cytometry to determine the apoptotic cells. The cells with positive fluorescent intensity signals for both FITC and PI were representative of the apoptotic cell count. The same procedure was followed for determining apoptosis in AuNRs-IL-8-siRNA transfected Panc-1 cells.

5.2.9 Statistical Analysis

We performed statistical analysis using analysis of variance (ANOVA). All data are presented as mean $\pm$ standard deviation (mean $\pm$ SD). All statistical calculations

were performed with the SigmaPlot (SPSS 11.0, Chicago, IL, USA) software package. When two comparisons were obtained, Student's unpaired two-tailed t-test was used. A *p*-value less than 0.01 was regarded as statistically significant.

5.3 Results and Discussion

Over-expression of IL-8 and/or its receptors in pancreatic cancer cells activates signaling pathways that have profound effect on the tumor microenvironment and malignant progression of cancer [106]. These signaling pathways are known to affect the expression of several transcription factors, e.g. NF- κ B, AP-1, β -Catenin, that regulate a host of activities like angiogenesis, cell survival, cell proliferation and cell invasion [106]. Hence, *in vitro* experiments were performed to assess the gene expression of IL-8 associated with MiaPaCa-2 and Panc-1 pancreatic cancer cell lines.

Figure 5.2a shows the reverse transcription polymerase chain reaction (RT-PCR) result showing the gene expression of IL-8 mRNA in both MiaPaCa-2 and Panc-1 cells relative to the common control, GAPDH. We also measured the IL-8 protein levels in the cell culture supernatant using the ELISA assay (**Figure 5.2a** right). Both results characterize the dominant gene expression of IL-8 in MiaPaCa-2 cells as compared to Panc-1 cells.

Figure 5.2b represents the comparison of the invasive potential of both cancer cells (stained in blue). It is evident from both the stained images and statistical summary that, IL-8 may possibly influence the cell invasion characteristic of MiaPaCa-2 cells more than that of the Panc-1 cells. Similar findings are observed in case of cell migration abilities of both MiaPaCa-2 and Panc-1 cells by the wound healing test (**Figure 5.2c**).

Figure 5.2: IL-8 gene expression, cell invasion and migration in the pancreatic cancer lines (MiaPaCa-2 and Panc-1). (a) The IL-8 mRNA expression in the MiaPaCa-2 and Panc-1 cells determined by RT-PCR, and the IL-8 protein concentration in the cell culture supernatant detected by ELISA assay. (b) The invasiveness of MiaPaCa-2 and Panc-1 cells detected by the BD Bio-Coat Matrigel invasion assay system (BD Biosciences). Invading cells were stained with trypan blue, and were counted in 5 random microscopic fields (200 $\times$). (c) The cell migration ability of MiaPaCa-2 and Panc-1 cells assessed by wound healing assay. The relative values shown are mean $\pm$ SD; *, $p < 0.01$ compared to Panc-1 cell group.

From these experiments, it is convincingly clear that IL-8 mRNA has distinct expression patterns in different pancreatic cancer cell lines, and it may exert a probable effect on the metastasis and development of cancer, perhaps variably in two pancreatic cancer patients with distinct gene expression patterns. We have hence attempted to target IL-8 gene silencing in pancreatic cancer cells through the molecular therapy of RNAi.

First of all, to examine the functions and effects of IL-8 on MiaPaCa-2 and Panc-1, we need a low-toxicity, biocompatible and efficient nanocarrier for delivery of siRNA. Our previous reports indicate that AuNRs possess aforementioned advantages [108, 154]. Therefore, we synthesized AuNRs and functionalized them with PSS and PAH polyelectrolyte polymers. Notably, these synthesized AuNRs have positive zeta potential which enables them to interact with negatively charged siRNA by electrostatic binding. Meanwhile, we estimated the optimum ratio of AuNRs-siRNA nanoplex for high transfection efficiency into cells. Thereafter, we investigated the suitability of these non-viral AuNRs for the efficient uptake and delivery of IL-8 siRNA in realizing gene knockdown *in vitro*.

The gel electrophoresis images, as shown in **Figure 5.3a**, confirmed the binding of siRNA on the surface of AuNRs. High contrast images with respect to unbound AuNRs were obtained as result of effective binding of siRNA to AuNRs. The inset of **Figure 5.3b** shows TEM image of the synthesized AuNRs. PAH-treated AuNRs and siRNA were mixed in different ratios by weight, in determining the best formulation for effective delivery to the tumor cells.

We confirmed the complexation of siRNA molecules with AuNRs (different OD ratios) by size estimation and zeta potential measurements. We recorded changes in the size of AuNRs as siRNA bound to the surface of AuNRs, as shown in **Figure 5.3b**. We observed that as the quantity of AuNRs increases, siRNA molecules find more binding sites on the surface of AuNRs, eventually resulting in a larger size AuNRs-siRNA nanoplex. This gradual increase in size continued till AuNRs were mixed with siRNA in the ratio of 10:1, beyond which siRNA molecules were trapped onto very few sites on AuNRs.

Also seen in **Figure 5.3b**, a zeta potential of 35.01 ± 5.81 mV was measured for naked-AuNRs, whereas it yielded negative values as the concentration of siRNA in the AuNRs-siRNA nanoplex was increased. A value of -25.35 ± 2.69 mV was obtained for AuNRs-siRNA nanoplex of ratio 0.625:1, indicating that the anionic siRNA molecules are bound to the surface of PAH-treated positively-charged AuNRs.

Figure 5.3: (a) Agarose gel electrophoresis of AuNRs-siRNA nanoplexes. (b) Monitoring of the change in particle hydrodynamic size and zeta potential with different OD ratios of AuNRs-siRNA. Inset: TEM images of the synthesized AuNRs. The OD dose of siRNA molecules was fixed at 0.008 OD.

Before conjugating AuNRs with siRNA, we first assessed the cell toxicity of AuNRs on MiaPaCa-2 and Panc-1 cells. Potential cytotoxic effects of the nanoparticles

were investigated by an MTT assay and this experiment was conducted on MiaPaCa-2 and Panc-1 cells treated with AuNRs at different concentrations for 24 and 48 hours. We found that over 90 % of both MiaPaCa-2 and Panc-1 cells are viable across a large dosage range (up to 1.28 OD mL⁻¹) even after 48 hours of treatment, indicating that the AuNRs are highly biocompatible and non-toxic (**Figure 5.4**). Thus, it can be concluded that AuNRs provide a biocompatible, flexible and simple way to transfect siRNA into cancer cells with very low cytotoxicity.

Figure 5.4: Cell viability of MiaPaCa-2 and Panc-1 cells treated with AuNRs at different concentrations for 24 and 48 hours assessed by MTT assay. Results are represented as mean $\pm$ SD, n = 6.

We measured (qualitatively) the uptake of the prepared AuNRs-siRNA nanoplexes in MiaPaCa-2 cells by inverted fluorescence microscopy. The MiaPaCa-2 cell nuclei were stained with DAPI (blue color) and the siRNA was labeled with FAM (green) to measure fluorescence. **Figure 5.5** shows the color-coded fluorescence images of MiaPaCa-2 cells treated with different formulations at 4 hours post treatment. MiaPaCa-2 cells treated with AuNRs-siRNA demonstrated strong FAM fluorescence as opposed to MiaPaCa-2 cells treated with only-siRNA in which no FAM signal was observed.

These observations illustrate the necessity for a viable nanoassay to assist siRNA to penetrate the cell membrane effectively, which is otherwise challenging for naked siRNAs. It is clearly distinguishable from the fluorescent images that, as the ratio of AuNRs-siRNA increases, a larger proportion of the AuNRs-siRNA nanoplex gets transfected into the MiaPaCa-2 cells, resulting in higher siRNA transfection efficiency. We also used a commercial transfection agent, Oligofectamine, which acted as a positive control. It is important to note that AuNRs-siRNA nanoplex ratios of 10:1 and 20:1 showed comparable fluorescence intensities with that of Oligo-siRNA formulation (Figure 5.5).

Figure 5.5: Fluorescence microscopy images of MiaPaCa-2 cells treated with different materials. Cell nuclei are stained with DAPI and rendered in blue, and the fluorescent signal from the FAM labeled-siRNA is rendered in green. A commercial transfection reagent, Oligofectamine, served as a positive control. For efficient delivery, the ratio between the siRNAs and AuNRs carriers is an important parameter to optimize. In this study, six different

OD ratios (AuNRs:siRNA), 0.625:1, 1.25:1, 2.5:1, 5:1, 10:1 and 20:1 were evaluated, where the OD dose of siRNA molecules was fixed at 0.032 OD.

In order to decide the optimum ratio of AuNRs-siRNA nanoplex and estimate the corresponding transfection efficiency of siRNAs by AuNRs into the cancer cells, we utilized flow cytometry. **Figure 5.6a-j** shows the cytometric plots of the FAM intensity in MiaPaCa-2 cells treated with free siRNA, only-AuNRs, blank cell groups, and different formulations of AuNRs-siRNA nanoplexes. For all the treatment groups, a reference gate was chosen to identify the normal cell events (**Figure 5.6a**). Based on the intensity of the fluorescent signals, all the gated events were divided into two populations of P2 and P3 (**Figure 5.6b-h**), where cells in P2 have low fluorescent signals, primarily from autofluorescence, while cells in P3 express strong fluorescence from the FAM label, representative of cells transfected with AuNRs-siRNA. The transfection efficiency was defined as the fraction of P3 which is normalized by the total gated events. **Figure 5.6k-l** is the statistical summary of results obtained from flow cytometry.

Consistent with the fluorescence microscopy results, we observed negligible transfection in cases of free siRNA (0.22 ± 0.10 %), naked-AuNRs (0.16 ± 0.05 %), and blank cell groups (0.19 ± 0.08 %) whereas high transfection efficiencies are obtained with formulations of AuNRs-siRNA nanoplexes in the ratio 10:1 (88.40 ± 2.14 %) and 20:1 (87.10 ± 2.23 %), indicating significant penetration of nanoplexes inside the cells. In addition to the transfection efficiency, the average fluorescent intensity per cell count, shown in **Figure 5.6l**, is indicative of average siRNA delivery efficiency to cells. The results show a sequential rise in fluorescent intensity with increasing AuNRs-siRNA ratio. They also present similarities in fluorescent intensities between the 10:1, 20:1 AuNRs-siRNA nanoplexes and the Oligo-siRNA group, suggesting that the loading

capacity of AuNRs gets saturated around the 10:1 ratio. Increasing the AuNRs-siRNA weight ratio beyond 10:1 leads to an insufficient siRNA loading per nanoparticle and consequently there was no significant difference in the overall average fluorescent intensity between the 10:1 and 20:1 groups. Based on these observations, the 10:1 AuNRs-siRNA nanoplex was chosen as the optimum ratio for effective IL-8 siRNA delivery into cancer cells.

Figure 5.6: Transfection efficiency of MiaPaCa-2 cells evaluated by flow cytometry. (a)-(j) are statistical counts of cells treated with PBS, siRNA^{FAM}, AuNRs, AuNRs-siRNA^{FAM} at different OD ratios of AuNRs:siRNA at 0.625:1, 1.25:1, 2.5:1, 5:1, 10:1 and 20:1, and Oligo-siRNA^{FAM}, respectively. Accordingly, the OD dose of siRNA was fixed at 0.032 OD per well. (k)-(l) are the quantitative evaluation of the results from (a)-(j) showing the transfection efficiency and average fluorescent intensity of each group respectively. Average fluorescence intensity presents the FAM intensity in the cells. The transfection efficiency is defined as the ratio

between the cell counts of P3 (transfected cell counts) to P2+P3 (total cell counts). The relative values shown are mean $\pm$ SD, n = 5; *, P<0.01 compared to Oligo-siRNA group.

The flow cytometry data also indicates that AuNRs allow siRNA transfection with efficiencies even greater than that of the commercial transfection agent, Oligofectamine. More importantly, the AuNRs formulation exhibits a much lower cytotoxicity when compared to Oligofectamine. In addition, we carried out similar fluorescence imaging and flow cytometry experiments on Panc-1 cells conjugated with the 10:1 AuNRs-siRNA and other different formulations to evaluate transfection efficiency of the nanoassay. Interestingly, we observed comparable fluorescent images of Panc-1 cells indicating similar siRNA transfection in the cases of 10:1 AuNR-siRNA and Oligo-siRNA, as shown in **Figure 5.7a**. Moreover, the quantitative fluorescent intensity signals as measured by flow cytometry are shown in **Figure 5.7b**. These results suggest that AuNRs can be used to successfully transfect siRNA into Panc-1 cells with a high transfection efficiency of 86.35 ± 4.39 % (**Figure 5.7c**) and yield an overwhelming average fluorescent intensity per cell count when compared with control groups (**Figure 5.7d**).

Post-transfection with AuNRs-siRNA nanoplex, we systematically evaluated the cellular activities of MiaPaCa-2 and Panc-1 cells to assess gene silencing of the mutant IL-8 mRNA. We determined the gene knockdown capability of the synthesized nanoplex in terms of the regulated expression of the IL-8 mRNA, normalized to the mRNA expression of the commonly used housekeeping gene, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). **Figure 5.8a** is a histogram showing the relative expression levels of mutant IL-8 mRNA in MiaPaCa-2 and Panc-1 cells treated with different formulations of AuNRs-siRNA nanoplex, obtained using quantitative real time RT-PCR. We observed that, while the cases of blank cell groups, free siRNA, only-

AuNRs, and AuNRs-NC-siRNA showed negligible change in the relative gene expression, there was a significant reduction in the gene expression level of the IL-8 mRNA in MiaPaCa-2 cells transfected with AuNRs-siRNA and Oligo-siRNA.

Figure 5.7: The Panc-1 cells were transfected with the optimized ratio of AuNRs:siRNA, and transfection was assessed by fluorescence microscopy and flow cytometry. a) Fluorescence microscopy images of Panc-1 cells treated with different materials, and AuNRs-siRNA nanoplex at 10:1 OD ratio. b) Transfection efficiency of Panc-1 cells treated with different materials determined by flow cytometry analysis. c,d) Quantitative evaluation of the results from (b) showing the transfection efficiency (left) and average fluorescent intensity (right) of each group. The relative values shown are mean $\pm$ SD, $n = 5$; *, $p < 0.01$ compared to Blank group.

Both AuNRs-siRNA and Oligo-siRNA show comparable gene silencing effect with knockdown efficiencies of $81.02 \pm 10.14\%$ and $76.04 \pm 6.03\%$ (**Figure 5.8a**), respectively. At the same time, it is worth noting that albeit the IL-8 mRNA expression in Panc-1 cells was very low in blank cell groups, free siRNA, only-AuNRs, and AuNRs-NC-siRNA, it was further reduced upon AuNRs-siRNA transfection. The reduced IL-8 protein concentrations in both MiaPaCa-2 ($595.69 \pm 81.18 \text{ pg ml}^{-1}$) and Panc-1 cells ($121 \pm 35.74 \text{ pg ml}^{-1}$), shown in **Figure 5.8b** as estimated by ELISA assay, supported the preceding results. These findings strongly support that AuNRs can be used as an effective non-viral vector for the release of siRNA to cancer cells and induce gene suppression of the target mRNA.

Figure 5.8: The IL-8 mRNA expression in MiaPaCa-2 and Panc-1 cells, and IL-8 protein concentration in the supernatant in the different treatment groups. a) IL-8 mRNA relative expression levels detected by quantitative real time RT-PCR in MiaPaCa-2 and Panc-1 cells. b) The IL-8 protein concentration in the cell culture supernatant detected by ELISA assay. The relative values shown are mean $\pm$ SD, $n = 4$; *, $p < 0.01$ compared to Blank group.

Cell invasion and cell apoptosis are crucial prognostic indicators that outline the progress of cancer and determine the line of treatment to be adopted. The presence of metastasis at the time of diagnosis is attributed to the highly invasive properties of pancreatic cancer cells [155]. The cell invasion pathway and its development at different

durations define the dimensions and severity of the metastasis cycle. Aggressive invasive phenotype of pancreatic cancer cells leads to tumor extension into the peripancreatic tissues and metastasis to local lymph nodes [156]. Thus the study of cell invasion is a vital part of cancer metastasis and prognosis evaluation.

We conducted a cell invasion assay, based on an *in vitro* matrigel invasion chambers assay [157], on MiaPaCa-2 and Panc-1 cells after treatment with different formulations. Cells were stained with trypan blue. In this assay, invasion is quantified in terms of cell numbers crossing an extracellular matrix barrier [158]. **Figure 5.9a** shows the bright-field microscope images of cells that penetrated through the Matrigel-coated membrane in the transwell chamber.

Figure 5.9: Cell invasion assay based on a transwell chamber model of MiaPaCa-2 and Panc-1 after treatment with different formulations after 72h. a) Bright-field microscope images of cells that penetrated through the Matrigel-coated membrane in the transwell chamber. b) Quantitative cell counts of the cells penetrated through the Matrigel-coated membrane. Each bar represents five random microscopic fields under 200 $\times$ magnification. Error bars indicate SD, n = 5; *, $p < 0.01$ compared with Blank group.

A comparative study of these images indicates that the IL-8 siRNA transfection into pancreatic cancer cells inhibited cell invasion considerably. It can be quantitatively shown (**Figure 5.9b**) that the AuNRs-siRNA nanoplex suppressed cell invasion in MiaPaCa-2 and Panc-1 cells by 64.30 % and 31.42 % respectively. Therefore, the invasive capability and metastatic potential of both cell lines can be significantly controlled by the IL-8 mRNA gene silencing mechanism mediated by AuNRs-siRNA nanoplex.

Metastasis can also be studied through the measurement of phenotypic changes in cell migration [159]. In the wound healing migration assay, the cells are incubated to near confluence, and an artificial wound empty of cells is created [159]. The cells gradually migrate towards the wound site following a traveling wave-like behavior. **Figure 5.10a** depicts the phase-contrast microscopic images of the movement of cells as part of the wound healing process of 72 hours post treatment.

In the non-treated blank group, the cells started to migrate between the wound margins as the wound healing progresses and eventually the window closes to around 83.54 ± 4.47 % and 37.28 ± 2.02 % of the initial wound window width for the MiaPaCa-2 and Panc-1 cells, respectively. Similar observations were found in the naked-AuNRs and free-siRNA treated groups. On the contrary, we observed a significantly suppressed healing process in the cells treated with the AuNRs-siRNA nanoplex and Oligo-siRNA. The reduction in window width was much slower and the wound closed to around 31.75 ± 5.45 % and 35.81 ± 4.73 % of the initial window width for MiaPaCa-2 cells treated with AuNRs-siRNA nanoplex and Oligo-siRNA respectively, even 72 hours post treatment. For Panc-2 cells transfected by the AuNRs-siRNA complex and Oligo-siRNA, the percent of the wound closed was only 25.49 ± 4.69 % and 27.56 ± 4.95 % respectively. **Figure 5.10b** represents the wound healing process of each group

quantified as the normalized percentage of the wound closure. These findings support the fact that the AuNRs-siRNA nanoplex can greatly reduce the migration ability of pancreatic cancer cells as is depicted by wound healing assay.

Figure 5.10: Wound healing assay in MiaPaCa-2 and Panc-1 cell culture treated with different formulations. a) Phase-contrast microscope images of the wound healing process monitored for 72 hours post treatment. b) Quantitative evaluation of the percentage of window closed after treatment, values were normalized by the initial wound window width. The relative values shown are mean $\pm$ SD, $n = 4$; *, $p < 0.01$ compared to Blank group.

The effect of AuNRs-siRNA nanoplex on cell migration was similar as that of the control Oligo-siRNA group. These results suggest the active role of AuNRs-siRNA complex in the gene knockdown capability of the mutant IL-8 mRNA in MiaPaCa-2 and Panc-1 cells. Significant correlation between IL-8 and Vascular endothelial growth factor (VEGF) mRNA levels supports the promotion of angiogenesis and cell proliferation in pancreatic cancer [103]. The study of cell migration abilities of cancer cells is an important component in cancer diagnosis as tumor cells infiltrate the peritoneal cavity and enter the blood vessels to mediate metastasis [160]. Thus, the

inhibition of cell migration by silencing the mutant IL-8 gene in MiaPaCa-2 and Panc-1 cells can provide a potential therapeutic way for the treatment of pancreatic cancer.

Finally, we assessed the apoptosis of MiaPaCa-2 and Panc-1 cells treated with different formulations by FITC-labeled Annexin V and PI staining. **Figure 5.11a** shows the flow cytometric data of the apoptotic cells stained with Annexin V/PI. Apoptotic cells showed positive signal for both Annexin V and PI fluorescence. Positive Annexin V staining indicates that PS has transgressed the boundary of the otherwise impermeable cell membrane, and bound to Annexin V, and positive PI staining signals the disintegration of cellular membrane upon induction of apoptosis, since PI is known to stain with double-stranded nucleic acids which can only be realized after cell membrane dissolution [161]. The AuNRs-siRNA nanoplex-treated cell culture showed no obvious increase in the apoptotic cell count with respect to the non-treated blank group as well as to the only-AuNRs and free-siRNA treated groups for both cell lines, as shown in **Figure 5.11a**. **Figure 5.11b** gives the quantitative measurement of the apoptotic cells in the Q2 zone of the cells treated with different formulations. It is apparent that the AuNRs-siRNA nanoplex has weak influence on the cell apoptosis of both MiaPaCa-2 and Panc-1 cells. This whole range of experiments highlight the role of IL-8 mRNA expression in different pancreatic cancer cells, and outline the need to pre-screen pancreatic cancer patients for IL-8 expression as a major step towards personalized cancer therapy. IL-8 mRNA silencing through RNAi is a suitable alternative to conventional therapies in the treatment of pancreatic cancer.

Hence, we suggest through *in vitro* experiments, the substantial role of AuNRs as a potential vector for siRNA uptake and delivery to bring about gene knockdown- thus serving the *therapeutic* part, and the role of AuNRs in determination of transfection efficiency in terms of fluorescence and flow cytometry- thus serving the *diagnostic* part

of the theranostics nanoassay. The encouraging performance of this theranostics nanoassay could possibly be extended to *in vivo* examination and clinical trials as a major push towards RNAi-based gene therapy.

Figure 5.11: FITC-Annexin V and PI contained apoptosis assay on MiaPaCa-2 and Panc-1 cells treated with different formulations at 72 h post treatment. a) Apoptotic cells stained with Annexin V/PI were measured by flow cytometry. b) Late apoptotic cell counts in the upper right quadrant (Q2) of different treatment groups. Error bars indicate SD, $n = 5$; *, $p < 0.01$ compared with Blank group.

5.4 Summary

In this chapter, we proposed and demonstrated a novel nanoassay for RNAi-based pancreatic cancer theranostics as a non-viral, non-toxic and flexible platform for targeted IL-8 siRNA delivery. Here, we investigated the possibility of using AuNRs for transfecting IL-8 siRNA into two pancreatic cancer cell lines, MiaPaCa-2 and Panc-1, by synthesizing AuNRs-siRNA nanoplexes. We experimentally demonstrated the formation of stable and non-toxic AuNRs-siRNA nanoplexes. Both MiaPaCa-2 and Panc-1 cell lines were probed for IL-8 mRNA gene expression levels, and subsequently

examined for gene silencing after transfection of cells with AuNRs-siRNA nanoplexes. We illustrated the ability of AuNRs as potential nanocarriers for siRNA through fluorescence microscopy and flow cytometry experiments. Finally, we presented the effect of IL-8 siRNA gene knockdown through *in vitro* studies of gene expression, cell invasion, cell migration and cell apoptosis assays. These studies form the basis for *in vivo* utility of AuNRs-siRNA nanoplexes for targeting IL-8 mRNA gene knockdown in pancreatic cancer cell lines. Moreover, with easy sample preparation procedure, this nanoassay can be used siRNA delivery in MiaPaCa-2 and Panc-1 cells, and these cells (which are 14-16 μm in diameter) can be readily focused in our engineered portable flow cytometry set-up, and the transfection efficiency can be determined by measuring the output fluorescent signal.

Chapter 6 A biodegradable charged polyester vector-based theranostic nanoassay for Chronic Myeloid Leukemia

In chapter 5, we described an AuNRs-based theranostics nanoassay for siRNA delivery in pancreatic cancer cells. We focused on the vital role of flow cytometry to extract both therapeutic as well as diagnostic signals from the targeted tumor cells when subjected to the designed nanoassay. In this case, the pancreatic cancer cells were essentially adherent cells, and hence were easy to transfect with AuNRs as non-viral and stable nanocarriers of IL-8 siRNA. In this chapter, we introduce a non-adherent or suspension cancer cell line – Chronic Myeloid Leukemia (CML) cell line (K562 cells), and investigate the effect of a biodegradable polymer-based theranostics nanoassay to (i) enable BCL-ABL siRNA transfection and induce gene silencing (*therapeutics*) and (ii) facilitate qualitative and quantitative gene delivery determination studies (*diagnostics*).

6.1 Introduction

RNAi technology, initiated by a double-stranded siRNA molecule targeting a sequence-specific gene at the post-transcriptional level, has attracted great interest for leukemia treatment in the past decade [123, 162-164]. However, the application of RNAi for leukemia treatment faces some critical bottlenecks because siRNA is easily degraded and cannot cross the cell membrane by itself. Various nanoparticles have been developed as nanovehicles for siRNA delivery into cells, which typically pertain to adherent cell lines. However, there are rare reports that outline desired siRNA transfection efficiency by nonviral transfection agents on the suspension cells, such as K562, a model cell line of human leukemia. Few reports describe BCR-ABL mRNA targeting in K562 suspension cells but state fairly low transfection results [122, 126,

162, 165]. A major underlying reason is that the reduced contact probability between nanoplexes and cells due to 3D cultivation condition as compared to the adherent cells [166]. Hence, designing polymers that can efficiently deliver siRNA into suspension cells would accelerate the development of novel treatment for leukemia cancer, and even for acquired immunodeficiency syndrome (AIDS). Herein, we explore the use of a recently synthesized polymer - biodegradable charged polyester-based vector (BCPV) to overcome the challenges of siRNA transfection in suspension cells. This cationic polymer with a structure shown in **Figure 2.15**, has previously been used as an highly efficient transfection agent for siRNA delivery in pancreatic cancer cells [89]. Furthermore, we also investigate the properties of this polymer to serve as a theranostics nanoassay for a non-adherent cancer cell line (K562). We study the stability of the BCPV-siRNA nanoplexes, even in the presence of FBS, and perform *in vitro* siRNA transfection in the non-adherent K562 cells.

Figure 6.1: Overview of the BCPV-siRNA nanoplex formation and the ABL-BCR fusion gene knockdown by siRNA transfection on leukemia cell.

Figure 6.1 pictorially describes the BCPVs-mediated BCL-ABL transfection in K562 cells. In addition, we also study the effect of gene transfection on downregulation

of BCR-ABL, cell proliferation and cell apoptosis in K562 cells. Moreover, we compare all results with a commercial transfection reagent, Lipofectamine2000TM, which serves as a positive control.

6.2 Material and Methods

6.2.1 Synthesis of BCPVs and Characterization of BCPV-siRNA nanoplexes

We synthesized BCPVs by ring-opening polymerization (ROP) of allyl functionalized lactide (LA) and L-LA monomers followed by a thiol-ene modification, in accordance with the method reported previously [167]. The resulting chemical structure of the synthesized BCPV was characterized using ¹H NMR and gel permeation chromatography (GPC). A number-average degree of polymerization (DP_n) of 95 was determined experimentally. The number-average molecular weight (M_n^{NMR}) and charge density of the BCPV were calculated to be M_n^{NMR} =19.3 kDa and 28% amine mole ratio respectively. GPC results indicated that the BCPV had a polydispersity index (PDI^{GPC}) of 1.38 relative to polystyrene. Thereafter, we prepared BCPV-siRNA nanoplexes by mixing BCR-ABL siRNA (10 μl, 1.25 μg) with BCPV (1 mg/ml) at different weight ratios (0.5:1, 1:1, 2:1, 4:1, 8:1) followed by gentle vortexing for 10 s. The solutions were left undisturbed for 30 min to enable the formation of stable BCPV-siRNA nanoplexes. To verify the effective encapsulation of the siRNA with BCPVs and determine the optimum dosage of BCPVs required to fully interact with siRNA, we performed agarose gel electrophoresis. After 20 minutes of incubation at room temperature, we loaded the BCPV-siRNA nanoplexes on a 1.2% agarose gel and electrophoresed at 100V for 15 minutes. We then observed the gels under UV transilluminator (Bio-Rad). We studied the structure of the BCPV-siRNA nanoplexes observed under JEM-2010 TEM (JEOL USA, Inc., Peabody, MA, US). Meanwhile, we verified the formation of stable complexation by measuring the hydrodynamic diameter and zeta potential (surface

potential) of BCPV-siRNA nanoplexes in ultrapure DI water at 25 °C using a 90-Plus particle size analyzer (Brookhaven Instrument Corp., Holtsville, NY, USA).

6.2.2 Cell Culture

We maintained human CML cell line, K562, obtained from ATCC, in Roswell Park Memorial Institute medium (RMPI, Hyclone), supplemented with 10% FBS (Hyclone), 100 U/ml penicillin (Gibco) and 100 µg/ml streptomycin (Gibco). Cells were cultured at 37°C in a humidified atmosphere with 5% CO₂, and maintained routinely. We also purchased MiaPaCa-2, PC-3, SMMC7721, T47D and T98G cell lines were from ATCC, and maintained them in DMEM culture medium. The other conditions for culturing these cells were the same as K562.

6.2.3 Transfection of cells with BCR-ABL siRNA

We obtained a custom-synthesized BCR-ABL siRNA from Shanghai GenePharma Co., Ltd (Shanghai, P.R.China) with the following sense and antisense sequences – sense: 5'-GCAGAGUUCAAAAGCCCTT-3'; antisense: 5'-TTCGUCUCAAGUUUUCGGG-3', in accordance with a previous report [168]. We purchased negative control siRNA (NC-siRNA, sense: 5'-UUCUCCGAACGUGUCACGUTT-3; antisense: 5'-ACGUGACACGUUCGGAGAATT-3') was Sigma (All stars negative control, Sigma, UK). First, we seeded K562 cells (1×10^4) in 6-well plates in the RMPI medium without antibiotics (FBS) to give 30% - 50% confluence at the time of transfections. For experiments with high levels of FBS, we supplemented the culture medium with 30% FBS, with other culture conditions remaining the same as the normal culture process of K562 cells. We then replaced the culture medium with OPTI-MEM (Invitrogen) prior to transfection. Second, we prepared BCPV-siRNA nanoplexes (ratio 8:1) by mixing BCPVs with Cy3-labeled BCR-ABL-siRNA^{Cy3} (20 µl, 10 µM) or NC-siRNA^{Cy3} (20 µl,

10 μM), and incubated them for 20 minutes. We then added the assembled nanoplexes to the 6-well plate wells and continuously cultured the cells for eight hours. The commercial transfection reagent Lipofectamine2000™ (Invitrogen) coupled-siRNA^{Cy3} (Lip2000-siRNA^{Cy3}) was used as a positive control. Additionally, we also used the blank K562 cells (Blank) and cells transfected with free-siRNA^{Cy3} (siRNA^{Cy3}) as controls. Lastly, we separated the different groups of transfected cells into two parts each, one for imaging under inverted fluorescence microscope and the other for flow cytometry for detection of transfection efficiency. Transfection efficiency examination and fluorescence imaging were performed 8 hours post-treatment. The gene expression was monitored at 48 hours post-transfection.

6.2.4 Cytotoxicity

First, we seeded K562 cells at 5×10^3 per well in 96-well plates and incubated them for 48h with different concentrations of BCPV. After incubation, we measured the cell viability in terms of cell counts with an Eclipse Ti-U Inverted Microscope System (Nikon, Singapore) after 48 hours under the BPCV environment. It should be highlighted here that the conventional MTT assay for evaluating the cell viability could not be used in our experiments, as the K562 CML cell line is essentially non-adherent, and it is not feasible to fix the cells on the bottom of the wells. As a result, we could not use the MTT assay for observing absorbance and determining cell viability. Hence, we determined the cell viability by normalizing the cell counts of the sample well against that from the control well and expressed it as a percentage of non-treated cells (Blank).

6.2.5 Fluorescence imaging

For the visual inspection of the proportion of cells that may have succeeded in siRNA transfection, we performed *in vitro* fluorescence microscopy images using the Eclipse Ti-U Inverted Microscope System (Nikon, Singapore). After 8 hours of

incubation, the transfected cells were washed with PBS twice, and fixed with 4% formaldehyde. Prior to imaging, the cells were mixed with DAPI (Sigma) for nuclear counterstaining. Filter sets for DAPI (405/450 nm) and Cy3 (550/570 nm) were applied for cell imaging. (DAPI and Cy3 are fluorescent dyes that are abbreviated forms of (4',6'-diamidino-2-phenylindole) and Cyanine3 respectively.)

6.2.6 Flow Cytometry

We performed flow cytometry to quantitatively determine the transfection efficiency of the K562 cells that were successfully transfected with BCR-ABL siRNA. The cells were washed twice with PBS and harvested by trypsinization. The samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Mississauga, CA), using Cy3 as the luminescent marker. We estimated the transfection efficiency in terms of Cy3-fluorescent intensity signal obtained from the flow cytometry results, and subsequently performed a statistical comparison of different groups.

6.2.7 Post-transfection Gene expression analysis

After 48 hours of transfection, we detected the BCR-ABL gene expression by real-time RT-PCR in terms of mRNA levels. First, total RNA was extracted from K562 cells using TRIzol reagent (Invitrogen) and the amount was quantitated by a NanoDrop 1000 Spectrophotometer (Wilmington, DE, USA). Next, 1 µg of RNA was reverse transcribed to cDNA in a 20 µl reaction mixture following the procedure described in [169] using the M-MLV reverse transcriptase kit (Promega). The cDNA products were then used as templates for subsequent real-time PCR with gene-specific primers to determine the relative BCR-ABL mRNA expression level. Specific forward and reverse primers' sequences were - BCR-ABL: 5'-CATTCCGCTGACCATCAATAAG-3'; 5'-GATGCTACTGGCCGCTGAAG-3', β-actin (housekeeping endogenous gene): 5'-CCACCCCACTTCTCTCTAAGGA-3' and 5'-GATGCTACTGGCCGCTGAAG-3',

as mentioned previously by Valencia-Serna et al. [170] . Real-time RT-PCR was performed using SYBR Select Master Mix (Invitrogen), with the following cycling conditions: 94°C for 2 min, followed by 40 cycles of denaturation (95°C for 15 s) and an annealing/elongation step (60°C for 15 s). The difference in gene expression was estimated as $2^{-\Delta\Delta CT}$, and gene expression of BCR-ABL was normalized against the internal control (β -actin).

6.2.8 Cell proliferation and apoptosis assay

For estimation of cell proliferation, we incubated all the transfected cell groups, control samples, blank and free-siRNA formulations for 72 hours and counted under the microscope. Secondly, we followed the Annexin V: FITC Apoptosis Detection Kit I (BD Pharmingen™, BD Biosciences, Singapore) to realize the effect of BCPV-siRNA transfection on the induction of K562 cell apoptosis. Briefly, approximately 1×10^6 K562 cells were seeded in a six-well plate. Then, the cells were incubated for 72 hours after treatment with the BCPV-siRNA (8:1) nanoplex and other formulations. The cells were then washed twice with cold PBS and resuspended in binding buffer. The onset of the apoptosis process enables the FITC-labeled Annexin V to bind with PS as it externalizes from the inner boundary of the cell membrane. The cell nuclei were stained with PI. We performed flow cytometry on the stained cells to determine the apoptotic cell count. Positive fluorescent intensity signals for both FITC and PI were representative of the total apoptotic cell count.

6.2.9 Statistical analysis

All data are presented as mean $\pm$ SD. Statistical analysis was performed using ANOVA. All statistical calculations were performed with the SigmaPlot software (SPSS 11.0, Chicago, IL, USA). When two comparisons were obtained, Student's unpaired

two-tailed t-test was used. A *p*-value less than 0.01 was regarded as statistically significant.

6.3 Results and discussion

BCPVs, a class of cationic polylactides, are synthesized by ring-opening polymerization technique and thiol-ene click functionalization, where the mol fraction can be precisely controlled for facilitating the loading of interested siRNA cargos. BCPVs interact with negatively-charged siRNA to form stable nanoplexes and the net positively-charged surface facilitates endocytosis of resulting nanoplexes by interacting with the negatively-charged cell membrane. Considering the importance of zeta potential and size of the BCPV-siRNA nanoplex for efficient siRNA delivery, we first optimized the weight ratio of siRNA and BCPV before transfecting the BCR-ABL mRNA with the nanoplex. Firstly, BCPVs diluted in ultrapure DI water were mixed with siRNA at different weight ratios (0.5:1, 1:1, 2:1, 4:1 and 8:1, where the weight of the siRNA molecule was fixed at 1.25 μ g), and left undisturbed for 30 min to form BCPV-siRNA nanoplexes. We confirmed the encapsulation of siRNA by BCPVs and formation of stable nanoplexes by agarose gel electrophoresis. Equal amounts of the siRNA were mixed with the BCPVs at different weight ratios and loaded into the electrophoresis gel. In this process, as seen in **Figure 6.2a**, the bright color bands signify the bonding of ethidium bromide with the siRNA that were not conjugated with BCPV. Compared with the pure siRNA channel, we observed decreased fluorescence intensities as the ratio of the BCPV was increased. When ethidium bromide is bound to a nucleic acid, its intensity gets amplified. In the cases of high BCPV: siRNA ratios, the excess amount of the BCPVs “buried” the siRNAs and impeded the contact with ethidium bromide, which resulted in decreased intensities. Nevertheless, this served as a clear indication of siRNA–BCPV conjugation. Also, we observed that the BCPV formulation

is able to retard most of the siRNA when the BCPV–siRNA weight ratio is above 8:1. At an 8:1 feeding ratio, the BCPV weight was estimated to be 10.25 μg , siRNA weight being 1.25 μg . Based on these values, the drug loading content was calculated to be around 11.1 wt% at an 8:1 feeding ratio. As the concentration of BCPV was increased (up to 8:1 ratio of BCPV-siRNA), the staining turned less bright, and eventually disappeared, indicating that most of the siRNA macromolecules were encapsulated by BCPVs (**Figure 6.2a**). Additionally, the Dynamic Light Scattering (DLS) results showed that BCPV-siRNA nanoplexes had a different hydrodynamic diameters at different weight ratios (81.2 nm for 8:1 weight ratio) while blank BCPVs particles possessed a diameter of 9.8 nm. The change in particle size after complexing with siRNA confirmed the successful loading of siRNA in BCPVs. Notably, when the particle size was over 20 nm, the nanoplexes are able to prevent rapid renal clearance *in vivo* [171]. Interestingly, zeta potential measurements revealed that BCPV particles bear strong positive charge (63.87 ± 1.53 mV). After loading the siRNA with BCPV particles at different weight ratios, the surface potential of siRNA-BCPV nanoplex decreased to 39.34 ± 5.10 mV at 8:1 weight ratio. The positive surface charge of resulting nanoplexes facilitate easy permeation through the negatively-charged cell membrane of targeted cells. The aforementioned information demonstrates 8:1 as an optimum loading ratio for BCPV-siRNA nanoplexes. TEM images further revealed the spherical morphology of the assembled BCPV-siRNA nanoplexes (**Figure 6.2b inset**) with around 100 nm diameter. The negatively-charged siRNA encapsulated with positively-charged BCPV molecule to form stable polyplexes through electrostatic interaction and led to the formation of capsule-like structures. This encapsulation of BCPV-siRNA could provide continuous release of siRNA over longer time periods and prevent siRNA degradation from hydrolysis [172].

Figure 6.2: Formation of BCPV-siRNA nanoplexes. (a) Gel retardation assay revealed the conjugation of siRNA with BCPV at different weight ratios. The staining becomes less pronounced as the concentration of BCPV is increased, indicating more uptake of siRNA and stable BCPV-siRNA nanoplexes. (b) The apparent change in the polydispersive hydrodynamic diameter of BCPV alone (9.8 nm) and BCPV-siRNA (8:1) nanoplexes (81.2 nm), as measured by a 90-Plus particle size analyzer, confirmed the formation of stable nanoplexes. Inset: TEM images of the assembled nanoplexes illustrate spherical morphology with average diameter measuring around 100 nm.

Before exploring the transfection in suspension cells, we first investigated the capacity of BCPVs to deliver siRNA into adhesive cell lines. FAM- and Cy3-labeled siRNA were mixed with BCPV at 8:1 weight ratio (where the weight of the siRNAs was fixed at 2.5 μ g) for 30 min, and were then cultured with various adherent cancerous cell lines (MiaPaCa-2, PC-3, SMMC7721, T47D and T98G) for 4 hours, following which

they were observed under inverted fluorescence microscopy to assess the ratio of transfected cells. Substantial and strong green (FAM), red (Cy3) and yellow (overlay) fluorescence signals were observed, revealing the high transfection ratios of selected cells (**Figure 6.3**) [89, 173].

Although the BCPVs have presented very high efficiency for siRNA delivery in adherent cells, BCPV-assisted siRNA transection in non-adherent cells has not been reported to date. K562 CML is a suspension cell line and represents a unique source of malignant leukemic cells overexpressing the BCR-ABL oncogene due to t(9;22) chromosome aberrations [174]. Hence, this is a perfect model to demonstrate a successful gene therapy of CML by knockdown this fusion oncogene. Moreover, K562 CML cells are well known to be difficult-to-transfect with current available non-viral carriers [126, 162, 175, 176]. Aggressive methods such as lentiviral vectors, lipid-based transfection reagents and electroporation have been implemented for the siRNA delivery in the K562 cells [162, 165, 177].

In our work, we propose BCPVs as nanocarriers for BCR-ABL siRNA delivery into K562 cells. Prior to the complexation of BCR-ABL with siRNA, innate cytotoxic effects of BCPVs on K562 cells were evaluated by counting the cell numbers under an inverted microscope (**Figure 6.4**). We observed that more than 90% of cells numbers were viable over a wide dosage range (up to 80 $\mu\text{g/ml}$) 48 hours post treatment, and no significant difference was seen among these groups. In addition, BCPVs also showed negligible cytotoxicity even at dosages higher than 160 $\mu\text{g/ml}$. The results indicated that BCPVs are biocompatible and have insignificant cytotoxicity to K562 cells at a wide dosage range of BCPV.

Figure 6.3: BCPV as an effective transfection agent for adherent cells. Various types of adherent cancer cells, namely MiaPaCa-2 (pancreatic cancer), PC-3 (prostate cancer), SMMC7721 (hepatocellular carcinoma), T47D (ductal carcinoma), and T98G (glioblastoma multiforme) were transfected by BCPV-siRNA^{FAM}-siRNA^{Cy3}. The observance of green (FAM), red (Cy3) and yellow (Overlay of FAM and Cy3) colors in the images obtained by inverted fluorescence microscope confirm the BCPV-assisted transfection of siRNA into these cell lines.

Figure 6.4: Cytotoxic effect of BCPV on K562 leukemic cells. The cells were treated with different concentrations of BCPV, and the intrinsic cytotoxicity was evaluated by counting cell numbers under a microscope 48 hours post treatment with BCPV. Blank cells were untreated. Results are represented as means $\pm$ SD, n=3.

Drawing motivation from the results on adhesive cells, we transfected the K562 suspension cells with BCPV-siRNA^{Cy3} and other groups (Blank, free-siRNA^{Cy3}, BCPV-siRNA^{Cy3}, and Lip2000-siRNA^{Cy3}), and left for 8 hours incubation. In the BCPV-siRNA group, the weight ratio was fixed at 8:1, with the siRNA weight being 2.5 µg. Because the total volume one well was 1ml, 20 µg/ml of BCPV was chosen as a safe loading concentration. On the other hand, as compared with the solid tumor treatment in which siRNA has to survive in the blood stream and go through the complicated extracellular matrix before it can reach the tumor tissue, treatment of leukemia presents an obvious advantage as siRNA can be directly exposed to leukemia cells of the blood vessels by intravenous injection. For mimicking conditions of blood stream, the K562 cells were treated with BCPV in the culture medium with 10% FBS (the main component of serum), and the attempts were denoted as BCPV-siRNA^{Cy3}-FBS. To obtain a positive control, a commercialized siRNA transfection reagent Lipofectamine2000TM was used under the same condition of FBS (Lip2000-siRNA^{Cy3}-FBS). The fluorescence microscopic images showed a relatively bright Cy3 fluorescence (red color) of BCPV-

siRNA^{Cy3} and BCPV-siRNA^{Cy3}-FBS in K562 cells, as compared with the positive control groups (**Figure 6.5**), indicating that BCPVs can successfully deliver siRNA into K562 cells, and prevent siRNA from rapid degradation by RNase present in FBS medium. The untransfected cell group (“Blank”) displayed only blue fluorescence arising from the DAPI stained-cell nuclei.

Figure 6.5: BCPV-assisted transfection of Cy3-labeled siRNA into K562 cells. The Blank and siRNA^{Cy3} group show only blue fluorescence, indicating the DAPI-stained cell nuclei without siRNA transfection. The BCPV-siRNA^{Cy3} and BCPV-siRNA^{Cy3}+FBS, exhibit both blue and red fluorescence indicating siRNA transfection (Cy3-stained) into the cells. The images are compared with the standard transfection agent Lipofectamine2000TM (Lip2000-siRNA^{Cy3} and Lip2000-siRNA^{Cy3}+FBS).

To further quantify the transfection efficiency of the siRNAs by the designed BCPV-based theranostics nanoassay, we employed flow cytometry to determine transfection efficiency of BCPV- and control-assisted transfection in non-adherent K562 cells. Quantitative values regarding transfection in terms of the Cy3-fluorescent signal strength were obtained by flow cytometry assessments (Figure 6a). We obtained extremely high transfection rates (Figure 6.6b) via BCPV-siRNA^{Cy3} ($96.7 \pm 3.13\%$), which was comparable to Lip2000-siRNA^{Cy3} ($88.46 \pm 2.42\%$). Notably, there was a significant difference in the average fluorescent intensity per cell count (Figure 6.6c) between the BCPV-siRNA^{Cy3} (>46000) and Lip2000-siRNA^{Cy3} (3400) groups. Such result indicated enhanced siRNA transfection efficiency of BCPV in non-adherent K562 cells. Additionally, the transfection efficiency of BCPV-siRNA^{Cy3}+FBS ($97.8 \pm 2.55\%$) was very close to that of BCPV-siRNA^{Cy3} ($96.7 \pm 3.13\%$), implying that the transfection efficiency of BCPVs was not influenced by the antibiotics in the cell culture medium (Figure 6.6a,b). In contrast to BCPVs, Lip2000-siRNA^{Cy3}+FBS exhibited a considerably decreased transfection efficiency ($52.67 \pm 0.83\%$) as compared with Lip2000-siRNA^{Cy3} ($88.46 \pm 2.42\%$), suggesting that Lipofectamine2000TM was quite sensitive to the serum components and thereby weakened its gene delivery functions.

The efficacy of cancer gene therapy through RNAi mechanism is generally assessed by the expression of the targeted mRNA. The BCR-ABL mRNA was targeted to be suppressed by the exogenously-mediated siRNA. In this study, we subjected the treatments of K562 cell line with BCPV-siRNA and other control groups (Blank, free siRNA^{Cy3}, BCPV, BCPV-siRNA^{Cy3}+FBS, Lip2000-siRNA^{Cy3} and Lip2000-siRNA^{Cy3}+FBS) to RT-PCR 48 hours post transfection for the quantitative determination of BCR-ABL mRNA levels. Figure 6.6 shows that all the negative control groups including untransfected cells, free siRNA and BCPVs exhibit minimal

effect on knocking down BCR-ABL mRNA expression (100%, 93.25%, 100.35%), suggesting that a potential vector is required for the uptake of therapeutic siRNA in K562 cells, and subsequent gene silencing.

Figure 6.6: Estimation of transfection efficiency in the K562 cells were detected by flow cytometry. a) Cell groups treated with different formulations (siRNA^{Cy3}, BCPV, BCPV-siRNA^{Cy3}, BCPV-siRNA^{Cy3}+FBS, Lip2000-siRNA^{Cy3} and Lip2000-siRNA^{Cy3}+FBS) were subjected to flow cytometry and fluorescent signal intensities showing positive staining for Cy3-labeled siRNA were representative of siRNA transfection into the cells. The higher fluorescent signals for groups BCPV-siRNA^{Cy3} and BCPV-siRNA^{Cy3}+FBS indicate higher transfection efficiencies. Blank cells were untreated. b) Statistical calculation of transfection efficiency. The ratio of cells in P3 to the total P2+P3, yielded the transfection efficiency. c) The average fluorescent signal intensities resulting from (a), depicting the performance of BCPV as an

outstanding transfection agent for non-adherent leukemic cells. Results are represented as mean $\pm$ SD, n=3; *, $p<0.05$; **, $p<0.01$.

As compared with the positive control group Lip2000-siRNA^{Cy3} (51.38% mRNA expression), the BCPV-siRNA^{Cy3} group demonstrated a substantial enhancement in gene knockdown efficiency (21.83% mRNA expression) post transfection (**Figure 6.7**). Additionally, the Lip2000-siRNA^{Cy3}-FBS group demonstrated a significant decrease in the gene knockdown efficiency (75.7% mRNA expression) due to the strong interference by the FBS. In contrast to Lip2000-siRNA^{Cy3}-FBS, the BCPV-siRNA^{Cy3}+FBS group exhibited 30.7% mRNA expression, which essentially describes the ability of BCPVs to withstand the reduced knockdown efficiency caused by the presence of FBS in the culture medium.

Figure 6.7: Downregulation of BCR-ABL mRNA post siRNA transfection. The cell groups were treated with different formulations and the gene knockdown of the BCR-ABL oncogene was evaluated by real time RT-PCR assay 48h post treatment. BCPV-siRNA-treated cell group showed considerable gene suppression, down to 20%, compared to 51% in the case of commercial transfection agent, Lipofectamine2000TM. These results correlate with the high transfection efficiency of BCPV-siRNA nanoplexes. Results are represented as mean $\pm$ SD, n=3; **, $p<0.01$.

Abnormal gene expression of BCR-ABL prevents leukemia cells from apoptosis and promotes cell proliferation by activating the cell survival signaling such as STAT5 and Erk signal pathway. In this study, we systematically evaluated the cellular activities of the K562 cells after the downregulation of BCR-ABL mRNA expression post BCPV-siRNA transfection. We examined the cell proliferation based on the total cell count 72 hours after transfection with treatments of BCPV-siRNA^{Cy3} and other positive and negative control groups. As expected, the cell count was considerably reduced to around 2.78×10^4 by BCPV-siRNA^{Cy3} and to 3.23×10^4 by BCPV-siRNA^{Cy3}+FBS (**Figure 6.8**), much lower than that inhibited by Lip2000-siRNA^{Cy3} (3.54×10^4) and Lip2000-siRNA+FBS (2.58×10^4).

Figure 6.8: Control of cell proliferation as a consequence of BCR-ABL oncogene suppression. The cell groups were treated with different formulations and the cell numbers were counted under a microscope post 72 hours of incubation. Consistent with the gene knockdown results in **Figure 6.6**, BCPV-siRNA-treated cell group yielded the lowest cell count, revealing the efficient performance of BCPV-assisted siRNA transfection in non-adherent leukemic cells. Blank cells were untreated. Results are represented as mean $\pm$ SD, $n=3$; *, $p<0.05$; **, $p<0.01$.

Results showed that post treatments with BCPV-siRNA and BCPV-siRNA-FBS, the viable cell percentages (relative to untreated cells) were decreased to 44.76% and 52.02% while Lip2000-siRNA and Lip2000-siRNA^{Cy3}-FBS had 57.06% and 79.04% viable cell percentages. Correspondingly, BCPV had a larger effect on suppressing the proliferation of K562 cells than Lipofectamine2000TM due to its higher transfection efficiency. These results demonstrated the active role of BCPVs (even in FBS) in transfecting more siRNA in the K562 cells, which positively correlates to greater inhibition of cell growth, and considerable suppression in cell proliferation.

Finally, we assessed the indicative results regarding the induction of apoptosis resulting from knockdown of BCR-ABL siRNA expression in K562 cells by FITC labeled Annexin V and PI staining. As shown in **Figure 6.9a**, the FITC- and PI-positive apoptotic cell counts in the Q2 and Q4 zone represent the number of early apoptosis cells of BCPV-siRNA treated group and other control groups. The quantitative data (**Figure 6.9b**) indicated that all negative control groups including untransfected cells, siRNA and BCPV have no influence on induction of apoptotic cells. However, BCPV-siRNA treatments (78.17% gene knockdown efficiency) resulted in 12.4% of apoptotic cells while Lip2000-siRNA^{Cy3} (48.63% gene knockdown efficiency) exhibit 5% of apoptotic cells. Results indicated that the cell apoptosis in K562 cells can be induced by knockdown of mRNA expression, and the proportion of apoptotic cells was positively correlated with the corresponding transfection efficiency. Moreover, in the presence of FBS, BCPV-siRNA^{Cy3}-FBS and Lip2000-siRNA^{Cy3}-FBS decreased their portions of apoptotic cells to 6.3% and 2.6%, implying that the reduced gene transfection efficiency arising from FBS had a direct effect on the decrease in apoptosis. The decrease in cell proliferation and apoptosis can be attributed to a decrease in cyclin D expression as a direct consequence of BCR-ABL mRNA silencing, which leads to cell cycle arrest in

G1 phase [178]. The downregulation of the anti-apoptotic BCR-ABL gene activates the required signaling components for re-opening of the apoptotic pathway [179].

Figure 6.9: Enhanced cell apoptosis as a consequence of high BCPV-siRNA transfection. a) The cell groups were treated with different formulations and subjected to Annexin V: FITC Apoptosis assay, 72 hours post treatment. Cells presenting higher fluorescent signals for both FITC and PI staining in the flow cytometry micrographs were representative of the apoptotic cell count. b) The BCPV-siRNA-treated cell group records the highest proportion of apoptotic cells. Blank cells were untreated. Results are represented as mean $\pm$ SD, $n=3$; **, $p<0.01$.

6.4 Summary

In this report, we demonstrated the knockdown of BCR-ABL fusion oncogene by RNAi using a BCPVs-based theranostics nanoassay with outstanding siRNA

transfection efficiency (up to 96%), followed by subsequent inhibition of cell proliferation and induction of cell apoptosis in K562 cells *in vitro*. The transfection and release of BCR-ABL siRNA from BCPV-siRNA nanoplexes, and subsequent cell apoptosis have been confirmed through fluorescent imaging and quantitative flow cytometry. This is the first report describing the use of BCPVs as vectors to transfect BCR-ABL siRNA into non-adherent K562 cells, and induce gene silencing. Therefore, we demonstrate another theranostic nanoassay capable of gene delivery and gene silencing in suspension cells, and yielding qualitative and quantitative diagnostic information using the powerful flow cytometry technique. The results in this study can be regarded as a breakthrough for treating leukemia diseases via gene therapy.

Moreover, with the results obtained in Chapters 5 and 6, we can conclude that the role of flow cytometers is pivotal in measuring quantitative theranostics effects in cancer therapies.

Chapter 7 Conclusions and Future Directions

Flow cytometry is a powerful analytical tool that has cast a major footprint on disease diagnosis and cell biology, among other applications. Sample characterization using flow cytometry technique yields well-differentiated clustered classifications based on size, shape, texture and fluorescence, making it a preferred choice over microscopy in the sense that it gives quantitative information as well. Yet, the mechanical and optical complexity, bulkiness and high-cost of conventional cytometers have restricted their use to state-of-the-art research laboratories and high-end clinical settings. Flow cytometers are designed to interrogate single cells or particles at a time, which are guided in the fluidic nozzles by the surrounding sheath fluid to form a hydrodynamically focused profile of single-stream particles/cells, the conventional macro-scale cytometers require large volumes of sheath fluid for the analysis of few milliliters of samples. Thus, the need for affordable, low-cost and accessible point-of-care is driving researchers towards a common aim of designing and developing miniature flow cytometers.

7.1 Summary of the presented work

To this end, the *first research objective* of this thesis revolves around building microfluidic particle focusing systems that can be subsequently engineered to lab-on-a-chip flow cytometers. Researchers have primarily exploited two basic particle focusing mechanisms: i) the sheath flow-assisted and ii) the inertial forces-assisted to realize single-position particle focusing in microchannels. The first mechanism, though relatively popular, yields low-throughput and is a bit cumbersome to operate due to the presence of multiple inlet ports. On the other hand, the latter mechanism is a high-throughput, sheathless particle-focusing approach but requires meticulous design methodologies to achieve single-stream particle focusing.

Thus, towards the first objective, we have proposed and investigated a unity AR, single-layer, symmetric microchannel design that precisely focuses particles and cells to a single equilibrium position for a wide Re range ($20 < Re < 153$) using inertial lift forces and Dean Drag forces in Chapter 3. We have described particle focusing mechanism in the given flow conditions with detailed theoretical analysis and experimental validations. Furthermore, we have demonstrated a novel technique to integrate optical fibers within the microfluidic chip and shown proof-of-concept performance of the miniature flow cytometry set-up using a beads-based size assay.

In Chapter 4, we have presented an in-depth study of the effect of inertial forces in sheath-assisted hydrodynamic particle focusing at higher Re ($\gg 1$) and identified the significance of inertial forces in single-position particle focusing. These findings are useful for increasing the throughput of the sheath-assisted particle focusing technique, which is otherwise low at lower Re (< 1) where only viscous forces dominate. We have also outlined the differences between inertial forces-assisted and sheath-assisted particle focusing in microfluidic channels.

In particular, flow cytometers play an instrumental role in the qualitative and quantitative estimation of drug/gene delivery to cancer cells, which are useful for cancer diagnosis and therapeutic modalities. Nevertheless, designing nanoassays that meet both diagnostic (capable of yielding fluorescent or output signals) and therapeutic (capable of drug/gene delivery) aims are imperative for robust in-the-field applications of miniature flow cytometers in the future.

Hence, for the *second research objective* of our work, we have proposed non-toxic and biocompatible nanoassays for cancer theranostics utilizing the RNAi phenomenon for gene delivery into cancer cells to bring about oncogene suppression in Chapters 5 and 6. We have presented our studies on two different cancer cell lines:

pancreatic cancer (MiaPaCa-2 and Panc-1) and CML (K562) and established the immense utility of flow cytometers in cancer theranostics-based nanoassays. Moreover, the performance of our miniature flow cytometer as compared to conventional flow cytometer is promising for such theranostics nanoassays to be useful for measuring quantitative theranostics effects in cancer therapies.

7.2 Challenges to realize miniaturized portable flow-cytometry for cancer theranostics: Present and Future

Although flow cytometry is not a new field to engineers and biologists, yet its cross-disciplinary architecture comprising optical, mechanical and electrical, and its footprint on biology and medicine, continue to fuel motivation and have propelled us to think and re-think of new ways of restructuring it so as to put it to the best and larger use in new application areas. We have attempted to address the research objectives with detailed investigations and studies, nevertheless, the outcomes of this multi-disciplinary research theme have culminated in some critical challenges which prospective researchers might want to pursue:

1. Improving the clusterization of the output of the miniature flow cytometer. By the term clusterization, we mean lowering of the coefficient of variation (CV) of the output for a specific sample population under investigation. In simpler words, the difference between the maximum output signal and the minimum output signal of the output from the particular sample of particle/cell under investigation should be as low as possible. This will in turn lead to higher signal-to-noise ratio (SNR) and hence a higher throughput.
2. Increasing the size range of the proposed microfluidic device for single-position 3D focusing of particles/cells. The proposed microfluidic device is able to align and focus 10-20 μm spherical particles/cells to a single-position. Modifications in the

geometrical parameters and a study of the interacting inertial and Dean Drag forces to focus even smaller particles ($< 10\ \mu\text{m}$) would broaden the application domain of the miniature flow cytometer.

3. Study of different shapes and densities of cells on focusing performance of the microfluidic device. Many cells, such as the red blood cells, change their shape dynamically according to space constraints. Hence, it would be interesting to study the lateral migration behavior of these shape-changing cells in the influence of inertial and Dean Drag forces in microfluidic channels.
4. Study on beam shaping of tapered optical fibers on the efficiency of the flow cytometer. Studies on the spot diameter of the input light source inside the microchannel, and optimization of the distance of the optical fibers from the edge of the microchannel so as to avoid multiple signals from the same particle/cell. Moreover, modifications of the fiber end face, for e.g., Fresnel lens shaping to get the optimum spot diameter might also be interesting to investigate.
5. Sorting and quantifying different types of cells in a sample population. Exploring the possibility of sorting and quantifying different types of cells or particles in a sample population based on different biomarkers or different morphologies with minimum SNR and high efficiency is also a requirement in various practical applications.
6. Exploring more nanomaterials that can be used as nanoassays with flow cytometry applications. Various types of drugs or genes need to be applied as therapeutic targets in different types of cells. Therefore, studies on other nanomaterials for cancer theranostics targeting other types of cancer cells or targeting other overexpressed oncogene mutations can yield novel theranostic nanoassays. Furthermore, illustrating the compatibility of different types of cells and associated

nanoassays with the microfluidic chip, and the studying the effect of flow parameters on the focusing performance would further establish the suitability of the miniature flow cytometer system for cancer theranostics. Finally, innovative approaches to determine the transfection efficiency of the theranostic nanoassays in real-time using the portable optofluidic flow cytometer would strengthen point-of-care cancer theranostics.

7.3 Current ongoing projects

We are currently working on further structural optimization of the microfluidic channel to accommodate a broader size range for 3D single-position focusing. This would enable us to investigate the miniature flow cytometer performance with smaller cell types, for e.g., RBCs. In addition, we are conducting experiments to evaluate the transfection efficiency of our theranostics nanoassays and compare the performance of the miniature flow cytometer with that of the conventional flow cytometer results.

Further, we are working on extending the microfluidic design to incorporate the sorting functionality as well for enabling cell sorting applications.

References

- [1] H. M. Shapiro, *Practical flow cytometry*: John Wiley & Sons, 2005.
- [2] S. Howard, "A History of Flow Cytometry and Sorting," in *The Microflow Cytometer*, ed: Pan Stanford Publishing, 2010.
- [3] W. R. Gowers, "On the numeration of blood-corpuscles," *Lancet*, vol. 110, pp. 797-798, 1877.
- [4] A. Moldavan, "Photo-electric technique for the counting of microscopical cells," *Science*, vol. 80, pp. 188-189, 1934.
- [5] F. T. Gucker, Jr., C. T. O'Konski, and et al., "A photoelectronic counter for colloidal particles," *J Am Chem Soc*, vol. 69, pp. 2422-31, 1947.
- [6] P. J. Crosland-Taylor, "A Device for Counting Small Particles suspended in a Fluid through a Tube," *Nature*, vol. 171, pp. 37-38, 1953.
- [7] W. H. Coulter, "High speed automatic blood cell counter and cell size analyzer," *Proc Natl Electron Conf*, vol. 12, pp. 1034-1040, 1956.
- [8] L. Hallermann, R. Thom, and H. Gerhartz, "Elektronische Differentialzählung von Granulocyten und Lymphocyten nach intravitaler Fluochromierung mit Acridinorange," in *Verhandlungen der Deutschen Gesellschaft für Innere Medizin*. vol. 70, B. Schlegel, Ed., ed: J.F. Bergmann-Verlag, 1964.
- [9] M. A. Van Dilla, T. T. Trujillo, P. F. Mullaney, and J. R. Coulter, "Cell microfluorometry: a method for rapid fluorescence measurement," *Science*, vol. 163, pp. 1213-1214, 1969.
- [10] W. Dittrich and W. Göhde, "Notizen: Impulsfluorometrie bei Einzelzellen in Suspensionen," in *Zeitschrift für Naturforschung B* vol. 24, ed, 1969.
- [11] L. A. Herzenberg, R. G. Sweet, and L. A. Herzenberg, "Fluorescence-activated cell sorting," *Sci Am*, vol. 234, pp. 108-117, 1976.
- [12] R. Curbelo, E. R. Schildkraut, T. Hirschfeld, R. H. Webb, M. J. Block, and H. M. Shapiro, "A generalized machine for automated flow cytology system design," *J Histochem Cytochem*, vol. 24, pp. 388-395, 1976.
- [13] H. M. Shapiro, E. R. Schildkraut, R. Curbelo, R. B. Turner, R. H. Webb, D. C. Brown, *et al.*, "Cytomat-R: a computer-controlled multiple laser source multiparameter flow cytophotometer system," *J Histochem Cytochem*, vol. 25, pp. 836-844, 1977.
- [14] M. Brown and C. Wittwer, "Flow Cytometry: Principles and Clinical Applications in Hematology," *Clinical Chemistry*, vol. 46, pp. 1221-1229, 2000.

References

- [15] D. L. Garner, "Flow cytometric sexing of mammalian sperm," *Theriogenology*, vol. 65, pp. 943-957, 2006.
- [16] X. Huang, H. D. Halicka, F. Traganos, T. Tanaka, A. Kurose, and Z. Darzynkiewicz, "Cytometric assessment of DNA damage in relation to cell cycle phase and apoptosis," *Cell proliferation*, vol. 38, pp. 223-243, 2005.
- [17] C. Banning, J. Votteler, D. Hoffmann, H. Koppensteiner, M. Warmer, R. Reimer, *et al.*, "A flow cytometry-based FRET assay to identify and analyse protein-protein interactions in living cells," *PLoS One*, vol. 5, p. e9344, 2010.
- [18] G. Vereb, P. Nagy, and J. Szollosi, "Flow cytometric FRET analysis of protein interaction," *Methods Mol Biol*, vol. 699, pp. 371-92, 2011.
- [19] B. N. G. Giepmans, S. R. Adams, M. H. Ellisman, and R. Y. Tsien, "The Fluorescent Toolbox for Assessing Protein Location and Function," *Science*, vol. 312, pp. 217-224, 2006.
- [20] A. Sigal, R. Milo, A. Cohen, N. Geva-Zatorsky, Y. Klein, Y. Liron, *et al.*, "Variability and memory of protein levels in human cells," *Nature*, vol. 444, pp. 643-646, 2006.
- [21] A. L. Givan, *Flow cytometry: first principles*: John Wiley & Sons, 2013.
- [22] C.-H. Lin and G.-B. Lee, "Micromachined flow cytometers with embedded etched optic fibers for optical detection," *Journal of Micromechanics and Microengineering*, vol. 13, p. 447, 2003.
- [23] X. Mao, A. A. Nawaz, S. C. Lin, M. I. Lapsley, Y. Zhao, J. P. McCoy, *et al.*, "An integrated, multiparametric flow cytometry chip using "microfluidic drifting" based three-dimensional hydrodynamic focusing," *Biomicrofluidics*, vol. 6, pp. 24113-241139, 2012.
- [24] S. Y. Yang, K. Y. Lien, K. J. Huang, H. Y. Lei, and G. B. Lee, "Micro flow cytometry utilizing a magnetic bead-based immunoassay for rapid virus detection," *Biosens Bioelectron*, vol. 24, pp. 861-8, 2008.
- [25] H. Choi, K. B. Kim, C. S. Jeon, I. Hwang, S. Lee, H. K. Kim, *et al.*, "A label-free DC impedance-based microcytometer for circulating rare cancer cell counting," *Lab Chip*, vol. 13, pp. 970-7, 2013.
- [26] D. Spencer, F. Caselli, P. Bisegna, and H. Morgan, "High accuracy particle analysis using sheathless microfluidic impedance cytometry," *Lab on a Chip*, vol. 16, pp. 2467-2473, 2016.
- [27] S. Joo, K. H. Kim, H. C. Kim, and T. D. Chung, "A portable microfluidic flow cytometer based on simultaneous detection of impedance and fluorescence," *Biosens Bioelectron*, vol. 25, pp. 1509-1515, 2010.

References

- [28] O. Jakobsson, C. Grenvall, M. Nordin, M. Evander, and T. Laurell, "Acoustic actuated fluorescence activated sorting of microparticles," *Lab on a Chip*, vol. 14, pp. 1943-1950, 2014.
- [29] C. Grenvall, C. Antfolk, C. Z. Bisgaard, and T. Laurell, "Two-dimensional acoustic particle focusing enables sheathless chip Coulter counter with planar electrode configuration," *Lab on a Chip*, vol. 14, pp. 4629-4637, 2014.
- [30] Y. Chen, A. A. Nawaz, Y. Zhao, P.-H. Huang, J. P. McCoy, S. J. Levine, *et al.*, "Standing surface acoustic wave (SSAW)-based microfluidic cytometer," *Lab on a Chip*, vol. 14, pp. 916-923, 2014.
- [31] R. Afshar, Y. Moser, T. Lehnert, and M. A. M. Gijs, "Three-Dimensional Magnetic Focusing of Superparamagnetic Beads for On-Chip Agglutination Assays," *Analytical Chemistry*, vol. 83, pp. 1022-1029, 2011.
- [32] L. Liang and X. Xuan, "Diamagnetic particle focusing using ferromicrofluidics with a single magnet," *Microfluidics and Nanofluidics*, vol. 13, pp. 637-643, 2012.
- [33] Z. Jian, C. Chen, V. Pallavi, B. Vincent, J. T. Tzuen-Rong, and X. Xiangchun, "Three-dimensional magnetic focusing of particles and cells in ferrofluid flow through a straight microchannel," *Journal of Micromechanics and Microengineering*, vol. 22, p. 105018, 2012.
- [34] Y. Song, C. Wang, M. Li, X. Pan, and D. Li, "Focusing particles by induced charge electrokinetic flow in a microchannel," *ELECTROPHORESIS*, vol. 37, pp. 666-675, 2016.
- [35] D. Li, X. Lu, Y. Song, J. Wang, D. Li, and X. Xuan, "Sheathless electrokinetic particle separation in a bifurcating microchannel," *Biomicrofluidics*, vol. 10, p. 054104, 2016.
- [36] S. Song and S. Choi, "Field-free, sheathless cell focusing in exponentially expanding hydrophoretic channels for microflow cytometry," *Cytometry Part A*, vol. 83, pp. 1034-1040, 2013.
- [37] S. Yan, J. Zhang, H. Chen, G. Alici, H. Du, Y. Zhu, *et al.*, "Making a hydrophoretic focuser tunable using a diaphragm," *Biomicrofluidics*, vol. 8, p. 064115, 2014.
- [38] S. Song and S. Choi, "Inertial modulation of hydrophoretic cell sorting and focusing," *Applied Physics Letters*, vol. 104, p. 074106, 2014.
- [39] Y. Choongho, J. Vykoukal, D. M. Vykoukal, J. A. Schwartz, S. Li, and P. R. C. Gascoyne, "A three-dimensional dielectrophoretic particle focusing channel for microcytometry applications," *Journal of Microelectromechanical Systems*, vol. 14, pp. 480-487, 2005.

References

- [40] I.-F. Cheng, C.-C. Chung, and H.-C. Chang, "High-throughput electrokinetic bioparticle focusing based on a travelling-wave dielectrophoretic field," *Microfluidics and Nanofluidics*, vol. 10, pp. 649-660, 2011.
- [41] Y.-C. Kung, K.-W. Huang, W. Chong, and P.-Y. Chiou, "Tunnel Dielectrophoresis for Tunable, Single-Stream Cell Focusing in Physiological Buffers in High-Speed Microfluidic Flows," *Small*, vol. 12, pp. 4343-4348, 2016.
- [42] J. P. Golden, J. S. Kim, J. S. Erickson, L. R. Hilliard, P. B. Howell, G. P. Anderson, *et al.*, "Multi-wavelength microflow cytometer using groove-generated sheath flow," *Lab Chip*, vol. 9, pp. 1942-50, 2009.
- [43] X. Mao, S.-C. S. Lin, C. Dong, and T. J. Huang, "Single-layer planar on-chip flow cytometer using microfluidic drifting based three-dimensional (3D) hydrodynamic focusing," *Lab on a Chip*, vol. 9, pp. 1583-1589, 2009.
- [44] J. Guo, X. Ma, N. V. Menon, C. M. Li, Y. Zhao, and Y. Kang, "Dual Fluorescence-Activated Study of Tumor Cell Apoptosis by an Optofluidic System," *IEEE Journal of Selected Topics in Quantum Electronics*, vol. 21, pp. 392-398, 2015.
- [45] F. Liang-Liang, H. Xu-Kun, H. Yu, Z. Jiang, and Z. Liang, "Continuous 3D particle focusing in a microchannel with curved and symmetric sharp corner structures," *Journal of Micromechanics and Microengineering*, vol. 25, p. 035020, 2015.
- [46] C. Chih-Chang, H. Zhi-Xiong, and Y. Ruey-Jen, "Three-dimensional hydrodynamic focusing in two-layer polydimethylsiloxane (PDMS) microchannels," *Journal of Micromechanics and Microengineering*, vol. 17, p. 1479, 2007.
- [47] M. Rosenauer, W. Buchegger, I. Finoulst, P. Verhaert, and M. Vellekoop, "Miniaturized flow cytometer with 3D hydrodynamic particle focusing and integrated optical elements applying silicon photodiodes," *Microfluidics and Nanofluidics*, vol. 10, pp. 761-771, 2010.
- [48] X. Mao, A. A. Nawaz, S.-C. S. Lin, M. I. Lapsley, Y. Zhao, J. P. McCoy, *et al.*, "An integrated, multiparametric flow cytometry chip using "microfluidic drifting" based three-dimensional hydrodynamic focusing," *Biomicrofluidics*, vol. 6, pp. 024113-1-024113-7, 2012.
- [49] G. K. Batchelor, *An introduction to fluid dynamics*: Cambridge: U.P., 1967.
- [50] G. Segré and A. Silberberg, "Behaviour of macroscopic rigid spheres in Poiseuille flow Part 2. Experimental results and interpretation," *Journal of Fluid Mechanics*, vol. 14, pp. 136-157, 1962.
- [51] B. P. Ho and L. G. Leal, "Inertial migration of rigid spheres in two-dimensional unidirectional flows," *Journal of Fluid Mechanics*, vol. 65, pp. 365-400, 1974.
- [52] P. Vasseur and R. G. Cox, "The lateral migration of a spherical particle in two-dimensional shear flows," *Journal of Fluid Mechanics*, vol. 78, pp. 385-413, 1976.

References

- [53] L. G. Leal, "Particle Motions in a Viscous Fluid," *Annual Review of Fluid Mechanics*, vol. 12, pp. 435-476, 1980.
- [54] J. A. Schonberg and E. J. Hinch, "Inertial migration of a sphere in Poiseuille flow," *Journal of Fluid Mechanics*, vol. 203, pp. 517-524, 1989.
- [55] E. S. ASMOLOV, "The inertial lift on a spherical particle in a plane Poiseuille flow at large channel Reynolds number," *Journal of Fluid Mechanics*, vol. 381, pp. 63-87, 1999.
- [56] B. Chun and A. J. C. Ladd, "Inertial migration of neutrally buoyant particles in a square duct: An investigation of multiple equilibrium positions," *Physics of Fluids*, vol. 18, p. 031704, 2006.
- [57] D. Di Carlo, D. Irimia, R. G. Tompkins, and M. Toner, "Continuous inertial focusing, ordering, and separation of particles in microchannels," *Proceedings of the National Academy of Sciences*, vol. 104, pp. 18892-18897, 2007.
- [58] A. A. S. Bhagat, S. S. Kuntaegowdanahalli, and I. Papautsky, "Enhanced particle filtration in straight microchannels using shear-modulated inertial migration," *Physics of Fluids*, vol. 20, p. 101702, 2008.
- [59] J. Zhou and I. Papautsky, "Fundamentals of inertial focusing in microchannels," *Lab on a Chip*, vol. 13, pp. 1121-1132, 2013.
- [60] D. R. Gossett and D. D. Carlo, "Particle Focusing Mechanisms in Curving Confined Flows," *Analytical Chemistry*, vol. 81, pp. 8459-8465, 2009.
- [61] J. M. Martel and M. Toner, "Particle Focusing in Curved Microfluidic Channels," *Scientific Reports*, vol. 3, p. 3340, 2013.
- [62] H. Ramachandraiah, S. Ardabili, A. M. Faridi, J. Gantelius, J. M. Kowalewski, G. Mårtensson, *et al.*, "Dean flow-coupled inertial focusing in curved channels," *Biomicrofluidics*, vol. 8, p. 034117, 2014.
- [63] S. C. Hur, H. T. K. Tse, and D. Di Carlo, "Sheathless inertial cell ordering for extreme throughput flow cytometry," *Lab on a Chip*, vol. 10, pp. 274-280, 2010.
- [64] A. A. S. Bhagat, S. S. Kuntaegowdanahalli, N. Kaval, C. J. Seliskar, and I. Papautsky, "Inertial microfluidics for sheath-less high-throughput flow cytometry," *Biomedical Microdevices*, vol. 12, pp. 187-195, 2010.
- [65] J. M. Martel and M. Toner, "Inertial focusing dynamics in spiral microchannels," *Physics of Fluids*, vol. 24, p. 032001, 2012.
- [66] J. Oakey, R. W. Applegate, E. Arellano, D. D. Carlo, S. W. Graves, and M. Toner, "Particle Focusing in Staged Inertial Microfluidic Devices for Flow Cytometry," *Analytical Chemistry*, vol. 82, pp. 3862-3867, 2010.

References

- [67] A. J. Chung, D. Pulido, J. C. Oka, H. Amini, M. Masaeli, and D. Di Carlo, "Microstructure-induced helical vortices allow single-stream and long-term inertial focusing," *Lab on a Chip*, vol. 13, pp. 2942-2949, 2013.
- [68] A. J. Chung, D. R. Gossett, and D. Di Carlo, "Three Dimensional, Sheathless, and High-Throughput Microparticle Inertial Focusing Through Geometry-Induced Secondary Flows," *Small*, vol. 9, pp. 685-690, 2013.
- [69] J. Zhang, M. Li, W. H. Li, and G. Alici, "Inertial focusing in a straight channel with asymmetrical expansion-contraction cavity arrays using two secondary flows," *Journal of Micromechanics and Microengineering*, vol. 23, p. 085023, 2013.
- [70] X. Wang, M. Zandi, C.-C. Ho, N. Kaval, and I. Papautsky, "Single stream inertial focusing in a straight microchannel," *Lab on a Chip*, vol. 15, pp. 1812-1821, 2015.
- [71] A. E. Reece, K. Kaastrup, H. D. Sikes, and J. Oakey, "Staged inertial microfluidic focusing for complex fluid enrichment," *RSC Advances*, vol. 5, pp. 53857-53864, 2015.
- [72] D. Jiang, W. Tang, N. Xiang, and Z. Ni, "Numerical simulation of particle focusing in a symmetrical serpentine microchannel," *RSC Advances*, vol. 6, pp. 57647-57657, 2016.
- [73] Q. Zhao, J. Zhang, S. Yan, D. Yuan, H. Du, G. Alici, *et al.*, "High-throughput sheathless and three-dimensional microparticle focusing using a microchannel with arc-shaped groove arrays," *Scientific Reports*, vol. 7, p. 41153, 2017.
- [74] H. Amini, W. Lee, and D. Di Carlo, "Inertial microfluidic physics," *Lab on a Chip*, vol. 14, pp. 2739-2761, 2014.
- [75] J. M. Martel and M. Toner, "Inertial Focusing in Microfluidics," *Annual Review of Biomedical Engineering*, vol. 16, pp. 371-396, 2014.
- [76] J. Zhang, S. Yan, D. Yuan, G. Alici, N.-T. Nguyen, M. Ebrahimi Warkiani, *et al.*, "Fundamentals and applications of inertial microfluidics: a review," *Lab on a Chip*, vol. 16, pp. 10-34, 2016.
- [77] D. Di Carlo, J. F. Edd, K. J. Humphry, H. A. Stone, and M. Toner, "Particle segregation and dynamics in confined flows," *Phys Rev Lett*, vol. 102, p. 094503, 2009.
- [78] E. Moczko, E. M. Mirkes, C. Cáceres, A. N. Gorban, and S. Piletsky, "Fluorescence-based assay as a new screening tool for toxic chemicals," vol. 6, p. 33922, 2016.
- [79] X. He, F. Chen, A. Dagan, S. Gatt, and E. H. Schuchman, "A fluorescence-based, high-performance liquid chromatographic assay to determine acid sphingomyelinase activity and diagnose types A and B Niemann-Pick disease," *Analytical Biochemistry*, vol. 314, pp. 116-120, 2003.

References

- [80] X. Yan, W. Zhong, A. Tang, E. G. Schielke, W. Hang, and J. P. Nolan, "Multiplexed Flow Cytometric Immunoassay for Influenza Virus Detection and Differentiation," *Analytical Chemistry*, vol. 77, pp. 7673-7678, 2005.
- [81] M. K. Wagner, F. Li, J. Li, X.-F. Li, and X. C. Le, "Use of quantum dots in the development of assays for cancer biomarkers," *Analytical and Bioanalytical Chemistry*, vol. 397, pp. 3213-3224, 2010.
- [82] J. Yan, M. C. Estévez, J. E. Smith, K. Wang, X. He, L. Wang, *et al.*, "Dye-doped nanoparticles for bioanalysis," *Nano Today*, vol. 2, pp. 44-50, 2007.
- [83] B. L. Davidson and P. B. McCray, "Current prospects for RNA interference-based therapies," *Nat Rev Genet*, vol. 12, pp. 329-340, 2011.
- [84] S. Y. Wu, G. Lopez-Berestein, G. A. Calin, and A. K. Sood, "RNAi Therapies: Drugging the Undruggable," *Science Translational Medicine*, vol. 6, p. 240ps7, 2014.
- [85] M. P. Czech, M. Aouadi, and G. J. Tesz, "RNAi-based therapeutic strategies for metabolic disease," *Nat Rev Endocrinol*, vol. 7, pp. 473-484, 2011.
- [86] G. R. Devi, "siRNA-based approaches in cancer therapy," *Cancer Gene Ther*, vol. 13, pp. 819-829, 2006.
- [87] S. M. Elbashir, W. Lendeckel, and T. Tuschl, "RNA interference is mediated by 21- and 22-nucleotide RNAs," *Genes & Development*, vol. 15, pp. 188-200, 2001.
- [88] R. L. Boudreau and B. L. Davidson, "RNAi therapeutics for CNS disorders," *Brain Res*, vol. 1338, pp. 112-21, 2010.
- [89] C. Yang, R. Hu, T. Anderson, Y. Wang, G. Lin, W.-C. Law, *et al.*, "Biodegradable nanoparticle-mediated K-ras down regulation for pancreatic cancer gene therapy," *Journal of Materials Chemistry B*, vol. 3, pp. 2163-2172, 2015.
- [90] K. A. Whitehead, R. Langer, and D. G. Anderson, "Knocking down barriers: advances in siRNA delivery," *Nat Rev Drug Discov*, vol. 8, pp. 129-138, 2009.
- [91] A. C. Bonoiu, S. D. Mahajan, H. Ding, I. Roy, K. T. Yong, R. Kumar, *et al.*, "Nanotechnology approach for drug addiction therapy: gene silencing using delivery of gold nanorod-siRNA nanoplex in dopaminergic neurons," *Proc Natl Acad Sci U S A*, vol. 106, pp. 5546-5550, 2009.
- [92] M. R. Lares, J. J. Rossi, and D. L. Ouellet, "RNAi and small interfering RNAs in human disease therapeutic applications," *Trends Biotechnol*, vol. 28, pp. 570-579, 2010.
- [93] M. S. Shim and Y. J. Kwon, "Efficient and targeted delivery of siRNA in vivo," *The FEBS journal*, vol. 277, pp. 4814-27, 2010.
- [94] E. Devroe and P. A. Silver, "Therapeutic potential of retroviral RNAi vectors," *Expert Opin Biol Ther*, vol. 4, pp. 319-327, 2004.

References

- [95] R. Tomanin and M. Scarpa, "Why do we need new gene therapy viral vectors? Characteristics, limitations and future perspectives of viral vector transduction," *Curr Gene Ther*, vol. 4, pp. 357-372, 2004.
- [96] S. Spagnou, A. D. Miller, and M. Keller, "Lipidic Carriers of siRNA: Differences in the Formulation, Cellular Uptake, and Delivery with Plasmid DNA," *Biochemistry*, vol. 43, pp. 13348-13356, 2004.
- [97] S. Zhang, B. Zhao, H. Jiang, B. Wang, and B. Ma, "Cationic lipids and polymers mediated vectors for delivery of siRNA," *J Control Release*, vol. 123, pp. 1-10, 2007.
- [98] O. Boussif, F. Lezoualc'h, M. A. Zanta, M. D. Mergny, D. Scherman, B. Demeneix, *et al.*, "A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine," *Proc Natl Acad Sci U S A*, vol. 92, pp. 7297-301, 1995.
- [99] M. A. Zanta, O. Boussif, A. Adib, and J. P. Behr, "In vitro gene delivery to hepatocytes with galactosylated polyethylenimine," *Bioconjug Chem*, vol. 8, pp. 839-44, 1997.
- [100] C. Nie, C. Liu, G. Chen, J. Dai, H. Li, and X. Shuai, "Hepatocyte-targeted psiRNA delivery mediated by galactosylated poly(ethylene glycol)-graft-polyethylenimine in vitro," *J Biomater Appl*, vol. 26, pp. 255-275, 2011.
- [101] M. S. Rubin, S. Clouston, and B. G. Link, "A fundamental cause approach to the study of disparities in lung cancer and pancreatic cancer mortality in the United States," *Social Science & Medicine*, vol. 100, pp. 54-61, 2014.
- [102] D. T. Graves and Y. Jiang, "Chemokines, a family of chemotactic cytokines," *Crit Rev Oral Biol Med*, vol. 6, pp. 109-18, 1995.
- [103] M. Li, Y. Zhang, L. W. Feurino, H. Wang, W. E. Fisher, F. C. Brunicardi, *et al.*, "Interleukin-8 increases vascular endothelial growth factor and neuropilin expression and stimulates ERK activation in human pancreatic cancer," *Cancer Sci*, vol. 99, pp. 733-737, 2008.
- [104] Q. Shi, J. L. Abbruzzese, S. Huang, I. J. Fidler, Q. Xiong, and K. Xie, "Constitutive and inducible interleukin 8 expression by hypoxia and acidosis renders human pancreatic cancer cells more tumorigenic and metastatic," *Clin Cancer Res*, vol. 5, pp. 3711-3721, 1999.
- [105] X. Le, Q. Shi, B. Wang, Q. Xiong, C. Qian, Z. Peng, *et al.*, "Molecular regulation of constitutive expression of interleukin-8 in human pancreatic adenocarcinoma," *J Interferon Cytokine Res*, vol. 20, pp. 935-946, 2000.
- [106] D. J. Waugh and C. Wilson, "The interleukin-8 pathway in cancer," *Clin Cancer Res*, vol. 14, pp. 6735-41, 2008.
- [107] A. C. Bonoiu, S. D. Mahajan, H. Ding, I. Roy, K.-T. Yong, R. Kumar, *et al.*, "Nanotechnology approach for drug addiction therapy: Gene silencing using delivery of

References

gold nanorod-siRNA nanoplex in dopaminergic neurons," *Proceedings of the National Academy of Sciences*, 2009.

[108] A. C. Bonoiu, E. J. Bergey, H. Ding, R. Hu, R. Kumar, K. T. Yong, *et al.*, "Gold nanorod--siRNA induces efficient in vivo gene silencing in the rat hippocampus," *Nanomedicine (Lond)*, vol. 6, pp. 617-630, 2011.

[109] M. M. Ow Sullivan, J. J. Green, and T. M. Przybycien, "Development of a novel gene delivery scaffold utilizing colloidal gold-polyethylenimine conjugates for DNA condensation," *Gene Ther*, vol. 10, pp. 1882-1890, 2003.

[110] K. K. Sandhu, C. M. McIntosh, J. M. Simard, S. W. Smith, and V. M. Rotello, "Gold nanoparticle-mediated transfection of mammalian cells," *Bioconjug Chem*, vol. 13, pp. 3-6, 2002.

[111] R. Ren, "Mechanisms of BCR-ABL in the pathogenesis of chronic myelogenous leukaemia," *Nat Rev Cancer*, vol. 5, pp. 172-83, 2005.

[112] J. R. McWhirter and J. Y. Wang, "Activation of tyrosinase kinase and microfilament-binding functions of c-abl by bcr sequences in bcr/abl fusion proteins," *Molecular and Cellular Biology*, vol. 11, pp. 1553-1565, 1991.

[113] S. Salesse and C. M. Verfaillie, "BCR/ABL-mediated increased expression of multiple known and novel genes that may contribute to the pathogenesis of chronic myelogenous leukemia," *Mol Cancer Ther*, vol. 2, pp. 173-82, 2003.

[114] A. McGahon, R. Bissonnette, M. Schmitt, K. M. Cotter, D. R. Green, and T. G. Cotter, "BCR-ABL maintains resistance of chronic myelogenous leukemia cells to apoptotic cell death," *Blood*, vol. 83, pp. 1179-87, 1994.

[115] P. Ramaraj, H. Singh, N. Niu, S. Chu, M. Holtz, J. K. Yee, *et al.*, "Effect of mutational inactivation of tyrosine kinase activity on BCR/ABL-induced abnormalities in cell growth and adhesion in human hematopoietic progenitors," *Cancer Res*, vol. 64, pp. 5322-31, 2004.

[116] R. C. Zhao, Y. Jiang, and C. M. Verfaillie, "A model of human p210(bcr/ABL)-mediated chronic myelogenous leukemia by transduction of primary normal human CD34(+) cells with a BCR/ABL-containing retroviral vector," *Blood*, vol. 97, pp. 2406-12, 2001.

[117] C. Barthe, P. Cony-Makhoul, J. V. Melo, and J. R. F.-X. Mahon, "Roots of Clinical Resistance to STI-571 Cancer Therapy," *Science*, vol. 293, pp. 2163, 2001.

[118] S. Branford, Z. Rudzki, S. Walsh, A. Grigg, C. Arthur, K. Taylor, *et al.*, "High frequency of point mutations clustered within the adenosine triphosphate-binding region of BCR/ABL in patients with chronic myeloid leukemia or Ph-positive acute lymphoblastic leukemia who develop imatinib (STI571) resistance," *Blood*, vol. 99, pp. 3472-3475, 2002.

References

- [119] N. von Bubnoff, F. Schneller, C. Peschel, and J. Duyster, "BCR-ABL gene mutations in relation to clinical resistance of Philadelphia-chromosome-positive leukaemia to STI571: a prospective study," *The Lancet*, vol. 359, pp. 487-491.
- [120] M. Azam, R. R. Latek, and G. Q. Daley, "Mechanisms of Autoinhibition and STI-571/Imatinib Resistance Revealed by Mutagenesis of BCR-ABL," *Cell*, vol. 112, pp. 831-843.
- [121] A. Hochhaus, S. Kreil, A. S. Corbin, P. La Rosee, M. C. Muller, T. Lahaye, *et al.*, "Molecular and chromosomal mechanisms of resistance to imatinib (STI571) therapy," *Leukemia*, vol. 16, pp. 2190-6, 2002.
- [122] A. H. Elmaagacli, M. Koldehoff, R. Peceny, L. Klein-Hitpass, H. Ottinger, D. W. Beelen, *et al.*, "WT1 and BCR-ABL specific small interfering RNA have additive effects in the induction of apoptosis in leukemic cells," *Haematologica*, vol. 90, pp. 326-34, 2005.
- [123] W. Glienke, L. Maute, U. Koehl, R. Esser, E. Milz, and L. Bergmann, "Effective treatment of leukemic cell lines with wt1 siRNA," *Leukemia*, vol. 21, pp. 2164-70, 2007.
- [124] M. A. Mintzer and E. E. Simanek, "Nonviral Vectors for Gene Delivery," *Chemical Reviews*, vol. 109, pp. 259-302, 2009.
- [125] A. C. Rinkenauer, A. Schallon, U. Günther, M. Wagner, E. Betthausen, U. S. Schubert, *et al.*, "A Paradigm Change: Efficient Transfection of Human Leukemia Cells by Stimuli-Responsive Multicompartment Micelles," *ACS Nano*, vol. 7, pp. 9621-9631, 2013.
- [126] W. He, M. J. Bennett, L. Luistro, D. Carvajal, T. Nevins, M. Smith, *et al.*, "Discovery of siRNA lipid nanoparticles to transfect suspension leukemia cells and provide in vivo delivery capability," *Mol Ther*, vol. 22, pp. 359-70, 2014.
- [127] J. D. Love, W. M. Henry, W. J. Stewart, R. J. Black, S. Lacroix, and F. Gonthier, "Tapered single-mode fibres and devices. I. Adiabaticity criteria," *IEE Proceedings J - Optoelectronics*, vol. 138, pp. 343-354, 1991.
- [128] A. A. S. Bhagat, S. S. Kuntaegowdanahalli, and I. Papautsky, "Continuous particle separation in spiral microchannels using dean flows and differential migration," *Lab on a Chip*, vol. 8, pp. 1906-1914, 2008.
- [129] J. Sun, M. Li, C. Liu, Y. Zhang, D. Liu, W. Liu, *et al.*, "Double spiral microchannel for label-free tumor cell separation and enrichment," *Lab on a Chip*, vol. 12, pp. 3952-3960, 2012.
- [130] D. Di Carlo, J. F. Edd, D. Irimia, R. G. Tompkins, and M. Toner, "Equilibrium Separation and Filtration of Particles Using Differential Inertial Focusing," *Analytical Chemistry*, vol. 80, pp. 2204-2211, 2008.

References

- [131] J. Hansson, J. M. Karlsson, T. Haraldsson, H. Brismar, W. van der Wijngaart, and A. Russom, "Inertial microfluidics in parallel channels for high-throughput applications," *Lab on a Chip*, vol. 12, pp. 4644-4650, 2012.
- [132] A. A. S. Bhagat, S. S. Kuntaegowdanahalli, and I. Papautsky, "Inertial microfluidics for continuous particle filtration and extraction," *Microfluidics and Nanofluidics*, vol. 7, pp. 217-226, 2009.
- [133] J.-P. Matas, J. F. Morris, and E. Guazzelli, "Inertial migration of rigid spherical particles in Poiseuille flow," *Journal of Fluid Mechanics*, vol. 515, pp. 171-195, 2004.
- [134] B. H. Yang, J. Wang, D. D. Joseph, H. H. Hu, T.-W. Pan, and R. Glowinski, "Migration of a sphere in tube flow," *Journal of Fluid Mechanics*, vol. 540, pp. 109-131, 2005.
- [135] L. Gwo-Bin, C. Chih-Chang, H. Sung-Bin, and Y. Ruey-Jen, "The hydrodynamic focusing effect inside rectangular microchannels," *Journal of Micromechanics and Microengineering*, vol. 16, p. 1024, 2006.
- [136] J. B. Knight, A. Vishwanath, J. P. Brody, and R. H. Austin, "Hydrodynamic Focusing on a Silicon Chip: Mixing Nanoliters in Microseconds," *Physical Review Letters*, vol. 80, pp. 3863-3866, 1998.
- [137] T. Stiles, R. Fallon, T. Vestad, J. Oakey, D. W. M. Marr, J. Squier, *et al.*, "Hydrodynamic focusing for vacuum-pumped microfluidics," *Microfluidics and Nanofluidics*, vol. 1, pp. 280-283, 2005.
- [138] M. Islam, H. Brink, S. Blanche, C. DiPrete, T. Bongiorno, N. Stone, *et al.*, "Microfluidic Sorting of Cells by Viability Based on Differences in Cell Stiffness," *Scientific Reports*, vol. 7, p. 1997, 2017.
- [139] H. Shafiee, M. B. Sano, E. A. Henslee, J. L. Caldwell, and R. V. Davalos, "Selective isolation of live/dead cells using contactless dielectrophoresis (cDEP)," *Lab on a Chip*, vol. 10, pp. 438-445, 2010.
- [140] S. Patel, D. Showers, P. Vedantam, T.-R. Tzeng, S. Qian, and X. Xuan, "Microfluidic separation of live and dead yeast cells using reservoir-based dielectrophoresis," *Biomicrofluidics*, vol. 6, p. 034102, 2012.
- [141] A. M. Lennon, C. L. Wolfgang, M. I. Canto, A. P. Klein, J. M. Herman, M. Goggins, *et al.*, "The Early Detection of Pancreatic Cancer: What Will It Take to Diagnose and Treat Curable Pancreatic Neoplasia?," *Cancer Research*, vol. 74, pp. 3381-3389, 2014.
- [142] Y. Li, D. Kong, A. Ahmad, B. Bao, and F. H. Sarkar, "Pancreatic cancer stem cells: Emerging target for designing novel therapy," *Cancer Letters*, vol. 338, pp. 94-100, 2013.
- [143] G. Antoniou, P. Kountourakis, K. Papadimitriou, V. Vassiliou, and D. Papamichael, "Adjuvant therapy for resectable pancreatic adenocarcinoma: Review of

References

the current treatment approaches and future directions," *Cancer Treatment Reviews*, vol. 40, pp. 78-85, 2014.

[144] M. J. Moore, D. Goldstein, J. Hamm, A. Figer, J. R. Hecht, S. Gallinger, *et al.*, "Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group," *J Clin Oncol*, vol. 25, pp. 1960-6, 2007.

[145] S. Gysin, M. Salt, A. Young, and F. McCormick, "Therapeutic Strategies for Targeting Ras Proteins," *Genes & Cancer*, vol. 2, pp. 359-372, 2011.

[146] A. S. Paulson, H. S. Tran Cao, M. A. Tempero, and A. M. Lowy, "Therapeutic advances in pancreatic cancer," *Gastroenterology*, vol. 144, pp. 1316-26, 2013.

[147] A. Neesse, P. Michl, K. K. Frese, C. Feig, N. Cook, M. A. Jacobetz, *et al.*, "Stromal biology and therapy in pancreatic cancer," *Gut*, vol. 60, pp. 861-868, 2011.

[148] E. C. Hayden, "Personalized cancer therapy gets closer," *Nature*, vol. 458, pp. 131-2, 2009.

[149] K. S. Garman, J. R. Nevins, and A. Potti, "Genomic strategies for personalized cancer therapy," *Hum Mol Genet*, vol. 16 Spec No. 2, pp. R226-32, 2007.

[150] J. W. Tyner, "Functional Genomics for Personalized Cancer Therapy," *Science translational medicine*, vol. 6, pp. 243fs26-243fs26, 2014.

[151] K.-T. Yong, Y. Sahoo, M. Swihart, P. Schneeberger, and P. Prasad, "Templated Synthesis of Gold Nanorods (NRs): The Effects of Cosurfactants and Electrolytes on the Shape and Optical Properties," *Topics in Catalysis*, vol. 47, pp. 49-60, 2008.

[152] G. Lin, C. Yang, R. Hu, C.-K. Chen, W.-C. Law, T. Anderson, *et al.*, "Interleukin-8 gene silencing on pancreatic cancer cells using biodegradable polymer nanoplexes," *Biomaterials Science*, vol. 2, pp. 1007-1015, 2014.

[153] C. Yang, X. Mo, J. Lv, X. Liu, M. Yuan, M. Dong, *et al.*, "Lipopolysaccharide enhances FcεRI-mediated mast cell degranulation by increasing Ca²⁺ entry through store-operated Ca²⁺ channels: implications for lipopolysaccharide exacerbating allergic asthma," *Experimental Physiology*, vol. 97, pp. 1315-1327, 2012.

[154] R. Masood, I. Roy, S. Zu, C. Hochstim, K.-T. Yong, W.-C. Law, *et al.*, "Gold nanorod-sphingosine kinase siRNA nanocomplexes: a novel therapeutic tool for potent radiosensitization of head and neck cancer," *Integrative Biology*, vol. 4, pp. 132-141, 2012.

[155] T. J. Farrell, D. J. Barbot, and F. E. Rosato, "Pancreatic resection combined with intraoperative radiation therapy for pancreatic cancer," *Annals of Surgery*, vol. 226, pp. 66-69, 1997.

References

- [156] F. Marchesi, P. Monti, B. E. Leone, A. Zerbi, A. Vecchi, L. Piemonti, *et al.*, "Increased survival, proliferation, and migration in metastatic human pancreatic tumor cells expressing functional CXCR4," *Cancer Res*, vol. 64, pp. 8420-7, 2004.
- [157] N. Kramer, A. Walzl, C. Unger, M. Rosner, G. Krupitza, M. Hengstschläger, *et al.*, "In vitro cell migration and invasion assays," *Mutation Research/Reviews in Mutation Research*, vol. 752, pp. 10-24, 2013.
- [158] A. Albin, Y. Iwamoto, H. K. Kleinman, G. R. Martin, S. A. Aaronson, J. M. Kozlowski, *et al.*, "A rapid in vitro assay for quantitating the invasive potential of tumor cells," *Cancer Res*, vol. 47, pp. 3239-45, 1987.
- [159] A. Q. Cai, K. A. Landman, and B. D. Hughes, "Multi-scale modeling of a wound-healing cell migration assay," *Journal of Theoretical Biology*, vol. 245, pp. 576-594, 2007.
- [160] A. Mantovani, P. Allavena, A. Sica, and F. Balkwill, "Cancer-related inflammation," *Nature*, vol. 454, pp. 436-44, 2008.
- [161] F. S. Ligler and J. S. Kim, *The Microflow Cytometer*: Pan Stanford, 2010.
- [162] Y. Arthanari, A. Pluen, R. Rajendran, H. Aojula, and C. Demonacos, "Delivery of therapeutic shRNA and siRNA by Tat fusion peptide targeting BCR-ABL fusion gene in Chronic Myeloid Leukemia cells," *J Control Release*, vol. 145, pp. 272-80, 2010.
- [163] M. Merkerova, H. Klamova, R. Brdicka, and H. Bruchova, "Targeting of gene expression by siRNA in CML primary cells," *Mol Biol Rep*, vol. 34, pp. 27-33, 2007.
- [164] H. Ohba, Z. Zhelev, R. Bakalova, A. Ewis, T. Omori, M. Ishikawa, *et al.*, "Inhibition of bcr-abl and/or c-abl gene expression by small interfering, double-stranded RNAs," *Cancer*, vol. 101, pp. 1390-1403, 2004.
- [165] M. Wilda, U. Fuchs, W. Wossmann, and A. Borkhardt, "Killing of leukemic cells with a BCR/ABL fusion gene by RNA interference (RNAi)," *Oncogene*, vol. 21, pp. 5716-24, 2002.
- [166] J.-P. Behr, "Synthetic gene transfer vectors II: back to the future," *Accounts of chemical research*, vol. 45, pp. 980-984, 2012.
- [167] C. H. Jones, C.-K. Chen, M. Jiang, L. Fang, C. Cheng, and B. A. Pfeifer, "Synthesis of Cationic Polylactides with Tunable Charge Densities as Nanocarriers for Effective Gene Delivery," *Molecular Pharmaceutics*, vol. 10, pp. 1138-1145, 2013.
- [168] M. Wilda, U. Fuchs, W. Wössmann, and A. Borkhardt, "Killing of leukemic cells with a BCR/ABL fusion gene by RNA interference (RNAi)," *Oncogene*, vol. 21, pp. 5716-5724, 2002.
- [169] C. Yang, X. Mo, J. Lv, X. Liu, M. Yuan, M. Dong, *et al.*, "Lipopolysaccharide enhances FcεRI-mediated mast cell degranulation by increasing Ca²⁺ entry through

References

store-operated Ca^{2+} channels: implications for lipopolysaccharide exacerbating allergic asthma," *Experimental physiology*, vol. 97, pp. 1315-1327, 2012.

[170] J. Valencia-Serna, H. Gul-Uludağ, P. Mahdipoor, X. Jiang, and H. Uludağ, "Investigating siRNA delivery to chronic myeloid leukemia K562 cells with lipophilic polymers for therapeutic BCR-ABL down-regulation," *Journal of Controlled Release*, vol. 172, pp. 495-503, 2013.

[171] H. Lee, A. K. Lytton-Jean, Y. Chen, K. T. Love, A. I. Park, E. D. Karagiannis, *et al.*, "Molecularly self-assembled nucleic acid nanoparticles for targeted in vivo siRNA delivery," *Nat Nanotechnol*, vol. 7, pp. 389-93, 2012.

[172] R. Misra, S. Acharya, and S. K. Sahoo, "Cancer nanotechnology: application of nanotechnology in cancer therapy," *Drug Discovery Today*, vol. 15, pp. 842-850, 2010.

[173] C. K. Chen, W. C. Law, R. Aalinkeel, B. Nair, A. Kopwithaya, S. D. Mahajan, *et al.*, "Well-defined degradable cationic polylactide as nanocarrier for the delivery of siRNA to silence angiogenesis in prostate cancer," *Adv Healthc Mater*, vol. 1, pp. 751-61, 2012.

[174] J. D. Rowley, "A New Consistent Chromosomal Abnormality in Chronic Myelogenous Leukaemia identified by Quinacrine Fluorescence and Giemsa Staining," *Nature*, vol. 243, pp. 290-293, 1973.

[175] A. Eguchi, B. R. Meade, Y. C. Chang, C. T. Fredrickson, K. Willert, N. Puri, *et al.*, "Efficient siRNA delivery into primary cells by a peptide transduction domain-dsRNA binding domain fusion protein," *Nat Biotechnol*, vol. 27, pp. 567-71, 2009.

[176] A. K. Shalek, J. T. Gaubblomme, L. Wang, N. Yosef, N. Chevrier, M. S. Andersen, *et al.*, "Nanowire-Mediated Delivery Enables Functional Interrogation of Primary Immune Cells: Application to the Analysis of Chronic Lymphocytic Leukemia," *Nano Letters*, vol. 12, pp. 6498-6504, 2012.

[177] L. Wohlbold, H. van der Kuip, A. Moehring, G. Granot, M. Oren, H. Vornlocher, *et al.*, "All common p210 and p190 Bcr-abl variants can be targeted by RNA interference," *Leukemia*, vol. 19, pp. 290-292, 2005.

[178] J. Rangatia and D. Bonnet, "Transient or long-term silencing of BCR-ABL alone induces cell cycle and proliferation arrest, apoptosis and differentiation," *Leukemia*, vol. 20, pp. 68-76, 2005.

[179] D. Cortez, L. Kadlec, and A. M. Pendergast, "Structural and signaling requirements for BCR-ABL-mediated transformation and inhibition of apoptosis," *Molecular and Cellular Biology*, vol. 15, pp. 5531-41, October 1, 1995.

List of Figures

Figure 1.1: Research scope of this thesis	2
Figure 2.1: Application domains of a flow cytometer.	8
Figure 2.2: Schematic representation of flow cytometer instrumentation depicting the four major architectural sections.....	9
Figure 2.3: a) Schematic of the hydrodynamic focusing process used to align the cells or particles fed to the flow cytometer into a focused stream of single cells. The fluidic unit consists of a nozzle that has inlets for sample and sheath fluid fluids. The sheath fluid surrounds and flows past the sample at a higher pressure such that the sample cells or particles get focused into a single-cell line as they traverse the length of the nozzle. b) The optical signals are acquired by a signal acquisition unit and processed to give distributions of output intensities in terms of cell or particle count.	11
Figure 2.4: a) Statistical trend depicting the increasing relevance of flow cytometers. b) Research output for microfluidic flow cytometers during the last two decades.....	13
Figure 2.5: Parameters of a typical sheath flow-assisted hydrodynamic focusing in a microfluidic channel layout.	15
Figure 2.6: a) Embedded etched optical fiber based detection of different-size particles on a lithographically fabricated substrate [22]. b) Groove-generated sheath flow for particle focusing [42]	16
Figure 2.7: a) 3D hydrodynamic focusing and detection of particles using microfluidic drifting technique [48]. b) Dual-sheath hydrodynamic focusing and integrated optical signal detection [44].....	17
Figure 2.8: a) Schematic representation of particle ordering in curved channels. b) Images of 9 μm diameter particles flowing in a 50 μm square channel, showing distinct (four) number of equilibrium position at different Re_p . c) Two equilibrium positions in a symmetric curving channel the symmetry. d) A single equilibrium position in an asymmetric curving system [57].....	22
Figure 2.9: Schematic of a spiral microfluidic design illustrating sheathless 3D particle focusing under the action of inertial lift forces and Dean Drag forces for flow cytometry applications [64].	24
Figure 2.10: Schematic of a single-stream particle focusing microfluidic channel design consisting of a low AR straight channel with stepped, orthogonally-arranged constrictions [68].	25
Figure 2.11: Sheathless particle focusing in a microfluidic channel with arc-shaped grooves on the top surface [73].....	26

Figure 2.12: a) Publication trend for sheathless inertial focusing in microfluidic devices. b) Publication trend for sheathless inertial forces-based lab-on-a-chip flow cytometers.	28
Figure 2.13: The constituents of the term theranostics.	29
Figure 2.14: Illustration of the RNAi phenomenon for IL-8 mRNA suppression in MiaPaCa-2 pancreatic cancer cells. AuNRs act as nanocarriers for transfecting IL-8 siRNA inside the cell. Upon successful delivery into the cytoplasm, siRNA forms an assembly with RNA-induced silencing complex (RISC) and gets dissociated with its antisense strand. Thus, degradation of IL-8 mRNA results when it encounters the sense strand of siRNA.	33
Figure 2.15: The chemical structure of BCPV.....	35
Figure 3.1: Schematic of the stepwise soft-photolithography process for fabrication of the microchannel chip. a) Printing of microchannel design on glass wafer. b) Wafer view of the fabrication processes: cleaning and spin coating SU-8 photoresist on a Si wafer (1, 2), UV exposure on the SU-8 coated-Si-wafer (3), post-baking, developing and hard-baking the wafer-‘master’ wafer (4), curing of the PDMS-based molding suspension poured over the master wafer at 70°C (5), Removal of patterned PDMS chip from the master wafer (6), irreversible bonding of the chip to a clean glass slide by plasma treatment for performing flow experiments (7).	42
Figure 3.2: Snapshots of single mode optical fiber during the tapering process. a) Diameter of the cladding region at the taper waist, b) diameter of the core region at the taper waist, c) diameter of the cladding region between the taper waist and untapered part, d) diameter of the cladding region at the untapered part, and e) diameter of the core region at the untapered part.....	46
Figure 3.3: a) Schematic drawing of the 3D-printed wall. b) The microfluidic chip integrated with optical fibers.	47
Figure 3.4: Lab-on-a-chip flow cytometry instrumentation. a) Schematic representation of the microchannel chip with auxiliary instrumentation to detect output photo-signals. b) Amplified views of the fiber interrogation part, showing the tapered single mode fiber (Fiber IN) and tapered multimode fiber (Fiber OUT). c) The complete experimental set-up. d) Amplified photo of the microfluidic channel. e) Microscope image of the optical fibers integrated inside the PDMS microfluidic chip.	48
Figure 3.5: a) The complete dimensional drawing of the microchannel geometry for 3D focusing of cells or particles. b) Pictorial schematic of the functionality of the microchannel design. Randomly-ordered cells or particles are fed at the inlet at pre-determined flow rates. As they traverse through the linear and curved parts of the channel, interplay of transverse inertial lift forces ‘ F_I ’ and Dean Drag forces ‘ F_D ’ leads to 3D single-position focusing of cells or particles at the outlet.....	50
Figure 3.6: The dimensional representation of the three designs D1, D2 and D3 used to demonstrate 3D single-position inertial focusing. The dimensions in each design represent the diameter of each series of the semi-arcs.....	51

Figure 3.7: (a-c) Behavior of lift coefficient (f_L) in a square channel. Poiseuille flow of randomly distributed particles of diameter, a in the square channel of width, w_{ch} with maximum velocity at the center, U_{max} . Pictorial representation of a straight square channel with four particle equilibrium positions, one along each of the face center. (d-e) Variation of f_L as a function of flow rate and particle size near the channel center and near the walls [77].	52
Figure 3.8: Graphical representation of the extent of wall induced-inertial lift force (d_{wl}) measured from the wall as a function of Re , highlighting the significant of wall-induced shift in particle focusing in confined flows.	54
Figure 3.9: (a-b) Schematic illustration of particle focusing as they traverse the linear and curved regions of the channel; (c-d) Pictorial representation of the interplay of inertial lift forces and Dean Drag force for cell or particle focusing at the channel outlet.	56
Figure 3.10: Theoretical condition for inertial focusing of cells or particles in a symmetrical, periodically increasing, curvilinear square microchannel. The shaded area in the curve represents the range of geometrical and flow parameters that will lead to 3D focusing of cells or particles in the microchannel design.	58
Figure 3.11: a) Simulation result for particle velocity profile and particle trajectory under the influence F_I and F_D in the curved and b) linear regions. (The color bar indicates increasing magnitude of velocity, red color being the highest).	59
Figure 3.12: a) Flow rate-dependent lateral shift in equilibrium position. The data shown are fluorescent streaks of 15 μm spherical polystyrene beads at the outlet part of the microfluidic channel (Design 3) for Re from 21 through 153 (Scale bar, 80 μm). b) The effect of F_D is responsible for the biasing of equilibrium position to the channel side and thus, establish single-position inertial focusing. Inset shows the fluorescent signal from flowing beads at 300 $\mu\text{l/min}$ in the arcs (Scale bar, 80 μm). c) Normalized intensity distribution of fluorescent 20 μm polystyrene beads across the micro-channel outlet showing single-position focusing.	61
Figure 3.13: High speed images of the outlet section taken at 25000 fps showing a single 20 μm bead flowing in D3 at 300 $\mu\text{l/min}$. The series of images (i)-(ix) capture particle trajectory for a time interval of 0.36 msec. The equilibrium position remains uniform through the entire outlet (Scale bar, 50 μm).	62
Figure 3.14: Snapshots of the fluorescent images of the microfluidic channel outlet demonstrating the focusing of polystyrene particle from concentrations of $10^3 \sim 10^6$ particles/mL. For each sample concentration, the flow rate was set to 300 $\mu\text{l/min}$. (Scale bar, 80 μm).	62
Figure 3.15: a) Confocal microscopy images for verifying the single-position 3D focusing of spherical polystyrene beads at the microchannel outlet. 3D fluorescent imaging of flowing polystyrene beads (300 $\mu\text{l/min}$) in the microchannel (Scale bar, 50 μm). b) Cross-section view (y-z plane) of the microchannel with flowing beads, the dotted circle indicates the measured focusing position (Scale bar, 20 μm). c) Side-view (x-z plane) of the microchannel with flowing beads (Scale bar, 50 μm). d) Schematic	

representation of the microfluidic chip configuration. The red box indicates the section that was imaged.....63

Figure 3.16: a) Normalized intensity profiles of 15 μm fluorescent polystyrene beads flowing at different flow rates in the Design 3 microfluidic channel. The intensity profiles depict flow rate-dependent particle focusing at different positions relative to channel center. b) Schematic representation of the lateral displacement of the particles at equilibrium from the channel centerline.64

Figure 3.17: Experimental results on the effect of Re on the lateral migration of equilibrium position of 15 μm spherical polystyrene particles relative to channel center in the three designs. Design 1: $r_s=300\text{ }\mu\text{m}$; Design 2: $r_s=400\text{ }\mu\text{m}$; Design 3: $r_s=500\text{ }\mu\text{m}$65

Figure 3.18: a) Dye-stained MiaPaca-2 cells (14 μm , scale bar 50 μm) and b) QDs-stained macrophages (20 μm , scale bar 80 μm) are precisely focused at the channel outlet. c) High-speed CMOS camera images of the inlet and d) outlet region at 50000 fps. Inlet: Randomly flowing macrophages fed at a flow rate of 300 $\mu\text{l}/\text{min}$. (Scale bar, 80 μm .) Outlet: Cells getting focused at the outlet. The blue circles represent the trajectory of one cell. Images were taken continuously (Scale bar, 80 μm).66

Figure 3.19: a) Experimental comparison of the lateral migration of equilibrium position of 15 μm spherical polystyrene beads and 14 μm pancreatic cancer cells (MiaPaCa-2) relative to channel center in the D3. b) Residual errors for 15 μm beads (top) and 14 μm cells from the fitted curve.67

Figure 3.20: Output signals recorded for 1 second interval for 18.6 μm beads flowing at a $Re = 65.6$. Inset: Amplified 100 ms interval snapshot of the output voltage signal. The system gives a throughput of the system was 445 particles/sec for 18.6 μm particle size at 100KS/s.....68

Figure 3.21: The count distributions for 18.6 μm and 26 μm beads, indicating a CV of 24% and 15% respectively for 1 sec duration of the entire output signal.....69

Figure 3.22: Comparison of the performance of the FL signal of the 26 μm beads from the (a) miniature and (b) commercial flow cytometer in terms of CV70

Figure 4.1: Schematic representation of the a) single-sheath and b) dual-sheath hydrodynamic particle focusing microfluidic geometries.75

Figure 4.2: a) COMSOL simulation model of single-sheath hydrodynamic focusing. Inset: meshing domains used for calculation of velocity and concentration of flowing sample. b) Velocity profile obtained for an 80 μm (w_{ch}) wide channel and c) Concentration profile showing the focusing of the samples surrounded by sheath fluid. d) Velocity profiles for single-sheath and dual-sheath configurations across the microchannel width for $FRR = 2$. Inset: The hydrodynamic focusing effect due to single-sheath and dual-sheath flows as a function of sample concentration across the w_{ch} (50 μm). A sample could be fluorescent-labeled particles/cells or a dye solution.....78

Figure 4.3: a) Simulation results of the relative focused particle stream width in single-sheath configuration as a function of FRR at $Re \gg 1$ for $Re_p > 1$; Q_{sh} is fixed at 150 $\mu\text{l}/\text{min}$. The focused stream width obtained in this case is 8 μm for $Q_{sh}/Q_s = 5$. b) Simulation results of the relative focused particle stream width in single-sheath configuration as a function of FRR at $Re < 1$ for $Re_p \ll 1$; Q_{sh} is fixed at 12 $\mu\text{l}/\text{min}$. The focused stream width obtained in this case is 35 μm for $Q_{sh}/Q_s = 5$80

Figure 4.4: a) Inverted fluorescent microscope images of the main channel of single-sheath microfluidic chip showing the hydrodynamic focusing of 5.7 μm and 9.8 μm beads for $77 < Re < 105$ (Scale bar, 80 μm). b) Relative focused stream width plots for 5.7 μm and 9.8 μm beads as function of FRR. The fluorescent intensity distribution across the microchannel width is fitted to a Gaussian profile and the FWHM is calculated to determine the relative focused stream width.82

Figure 4.5: Effect of single-sheath and dual-sheath hydrodynamic focusing at higher Re_p . a) Schematic illustration of single-sheath and dual-sheath configurations for the studying the hydrodynamic focusing effect in confined-particle flows ($a/w_{ch} = 0.12$) at $Re \gg 1$. b) The experimentally determined relative focused stream width profile for 9.8 μm fluorescent polystyrene beads in single-sheath and dual-sheath configurations. Inset: Fluorescent intensity distribution showing the FWHM of the focused stream width in single-sheath (red) and dual-sheath (blue) configurations at $Re = 88$83

Figure 4.6: Role of sheath fluid in achieving hydrodynamic focusing in single-sheath configuration. The simulation images show particle trajectories recorded 0.2 sec after the onset of flow. In the absence of (a) sheath flow, negligible lateral migration of particles is observed. In the presence of sheath flow, (b) 5 μm and (c) 20 μm polystyrene particles laterally migrate to a narrow width at the channel center. The enlarged images of the main channel indicate that the focused stream of the smaller 5.7 μm particles consist of the bunch of individual particles and not a single-particle train.84

Figure 4.7: a) Experimentally determined FWHM of the focused stream width of 5.7 μm and 18.7 μm polystyrene beads to demonstrate the effect of Re_p at higher $Re = 88$. The FWHM of both sizes of beads is approximately the same, indicating the role of inertial forces at higher Re_p . b) The microscope images of the fluorescent streaks of (i) 18.7 μm and (ii) 5.7 μm polystyrene beads agree with the particle trajectory simulation results (Scale bar, 80 μm).85

Figure 4.8: a) Comparison of variation of Re with FRR in confined-particle flows ($a/w_{ch} > 0.1$; $AR = 1$) and point-particle flows ($a/w_{ch} \ll 0.1$; $AR = 0.016$). Increase in FRR leads to much significant changes in the flow profile for confined-particle flows (red) than in point-particle flows (blue).86

Figure 4.9: Theoretically obtained focused stream width profile at low Re according to Equation 4.5 (blue). Plot of relative focused stream width obtained by simulation of single-sheath hydrodynamic focusing at higher Re (red).87

Figure 4.10: a) Pictorial representation of a theranostics nanoassay, consisting of a drug/gene carrier - the gold nanoparticle and the output signal provider- the fluorescent tag on the drug/gene. b) Illustration of a lab-on-a-chip flow cytometry device for theranostics applications, with the associated fluidic (inlet and outlet) and optical

instrumentation (laser diode, optical fibers, PMTs and DSO). The designed nanoassay can be fed into the inlet, and the desired drug/gene delivery information can be deduced from the two PMTs. b) Microscopic image of the optical fibers integrated with the PDMS microfluidic chip near the outlet region (Scale bar, 80 μ m). (Size of cells/particles not to scale). 90

Figure 5.1: An overview of the AuNRs-siRNA nanoplex formation and the IL-8 gene knockdown process followed by siRNA transfection. 95

Figure 5.2: IL-8 gene expression, cell invasion and migration in the pancreatic cancer lines (MiaPaCa-2 and Panc-1). (a) The IL-8 mRNA expression in the MiaPaCa-2 and Panc-1 cells determined by RT-PCR, and the IL-8 protein concentration in the cell culture supernatant detected by ELISA assay. (b) The invasiveness of MiaPaCa-2 and Panc-1 cells detected by the BD Bio-Coat Matrigel invasion assay system (BD Biosciences). Invading cells were stained with trypan blue, and were counted in 5 random microscopic fields (200 $\times$). (c) The cell migration ability of MiaPaCa-2 and Panc-1 cells assessed by wound healing assay. The relative values shown are mean $\pm$ SD; *, $p < 0.01$ compared to Panc-1 cell group. 102

Figure 5.3: (a) Agarose gel electrophoresis of AuNRs-siRNA nanoplexes. (b) Monitoring of the change in particle hydrodynamic size and zeta potential with different OD ratios of AuNRs-siRNA. Inset: TEM images of the synthesized AuNRs. The OD dose of siRNA molecules was fixed at 0.008 OD. 104

Figure 5.4: Cell viability of MiaPaCa-2 and Panc-1 cells treated with AuNRs at different concentrations for 24 and 48 hours assessed by MTT assay. Results are represented as mean $\pm$ SD, $n = 6$ 105

Figure 5.5: Fluorescence microscopy images of MiaPaCa-2 cells treated with different materials. Cell nuclei are stained with DAPI and rendered in blue, and the fluorescent signal from the FAM labeled-siRNA is rendered in green. A commercial transfection reagent, Oligofectamine, served as a positive control. For efficient delivery, the ratio between the siRNAs and AuNRs carriers is an important parameter to optimize. In this study, six different OD ratios (AuNRs:siRNA), 0.625:1, 1.25:1, 2.5:1, 5:1, 10:1 and 20:1 were evaluated, where the OD dose of siRNA molecules was fixed at 0.032 OD. 106

Figure 5.6: Transfection efficiency of MiaPaCa-2 cells evaluated by flow cytometry. (a)-(j) are statistical counts of cells treated with PBS, siRNA^{FAM}, AuNRs, AuNRs-siRNA^{FAM} at different OD ratios of AuNRs:siRNA at 0.625:1, 1.25:1, 2.5:1, 5:1, 10:1 and 20:1, and Oligo-siRNA^{FAM}, respectively. Accordingly, the OD dose of siRNA was fixed at 0.032 OD per well. (k)-(l) are the quantitative evaluation of the results from (a)-(j) showing the transfection efficiency and average fluorescent intensity of each group respectively. Average fluorescence intensity presents the FAM intensity in the cells. The transfection efficiency is defined as the ratio between the cell counts of P3 (transfected cell counts) to P2+P3 (total cell counts). The relative values shown are mean $\pm$ SD, $n = 5$; *, $P < 0.01$ compared to Oligo-siRNA group. 108

Figure 5.7: The Panc-1 cells were transfected with the optimized ratio of AuNRs:siRNA, and transfection was assessed by fluorescence microscopy and flow cytometry. a) Fluorescence microscopy images of Panc-1 cells treated with different

materials, and AuNRs-siRNA nanoplex at 10:1 OD ratio. b) Transfection efficiency of Panc-1 cells treated with different materials determined by flow cytometry analysis. c,d) Quantitative evaluation of the results from (b) showing the transfection efficiency (left) and average fluorescent intensity (right) of each group. The relative values shown are mean $\pm$ SD, $n = 5$; *, $p < 0.01$ compared to Blank group. 110

Figure 5.8: The IL-8 mRNA expression in MiaPaCa-2 and Panc-1 cells, and IL-8 protein concentration in the supernatant in the different treatment groups. a) IL-8 mRNA relative expression levels detected by quantitative real time RT-PCR in MiaPaCa-2 and Panc-1 cells. b) The IL-8 protein concentration in the cell culture supernatant detected by ELISA assay. The relative values shown are mean $\pm$ SD, $n = 4$; *, $p < 0.01$ compared to Blank group. 111

Figure 5.9: Cell invasion assay based on a transwell chamber model of MiaPaCa-2 and Panc-1 after treatment with different formulations after 72h. a) Bright-field microscope images of cells that penetrated through the Matrigel-coated membrane in the transwell chamber. b) Quantitative cell counts of the cells penetrated through the Matrigel-coated membrane. Each bar represents five random microscopic fields under 200 $\times$ magnification. Error bars indicate SD, $n = 5$; *, $p < 0.01$ compared with Blank group. 112

Figure 5.10: Wound healing assay in MiaPaCa-2 and Panc-1 cell culture treated with different formulations. a) Phase-contrast microscope images of the wound healing process monitored for 72 hours post treatment. b) Quantitative evaluation of the percentage of window closed after treatment, values were normalized by the initial wound window width. The relative values shown are mean $\pm$ SD, $n = 4$; *, $p < 0.01$ compared to Blank group..... 114

Figure 5.11: FITC-Annexin V and PI contained apoptosis assay on MiaPaCa-2 and Panc-1 cells treated with different formulations at 72 h post treatment. a) Apoptotic cells stained with Annexin V/PI were measured by flow cytometry. b) Late apoptotic cell counts in the upper right quadrant (Q2) of different treatment groups. Error bars indicate SD, $n = 5$; *, $p < 0.01$ compared with Blank group. 116

Figure 6.1: Overview of the BCPV-siRNA nanoplex formation and the ABL-BCR fusion gene knockdown by siRNA transfection on leukemia cell. 120

Figure 6.2: Formation of BCPV-siRNA nanoplexes. (a) Gel retardation assay revealed the conjugation of siRNA with BCPV at different weight ratios. The staining becomes less pronounced as the concentration of BCPV is increased, indicating more uptake of siRNA and stable BCPV-siRNA nanoplexes. (b) The apparent change in the polydisperse hydrodynamic diameter of BCPV alone (9.8 nm) and BCPV-siRNA (8:1) nanoplexes (81.2 nm), as measured by a 90-Plus particle size analyzer, confirmed the formation of stable nanoplexes. Inset: TEM images of the assembled nanoplexes illustrate spherical morphology with average diameter measuring around 100 nm. .. 128

Figure 6.3: BCPV as an effective transfection agent for adherent cells. Various types of adherent cancer cells, namely MiaPaCa-2 (pancreatic cancer), PC-3 (prostate cancer), SMMC7721 (hepatocellular carcinoma), T47D (ductal carcinoma), and T98G (glioblastoma multiforme) were transfected by BCPV-siRNA^{FAM}-siRNA^{Cy3}. The

observance of green (FAM), red (Cy3) and yellow (Overlay of FAM and Cy3) colors in the images obtained by inverted fluorescence microscope confirm the BCPV-assisted transfection of siRNA into these cell lines. 130

Figure 6.4: Cytotoxic effect of BCPV on K562 leukemic cells. The cells were treated with different concentrations of BCPV, and the intrinsic cytotoxicity was evaluated by counting cell numbers under a microscope 48 hours post treatment with BCPV. Blank cells were untreated. Results are represented as means $\pm$ SD, n=3. 131

Figure 6.5: BCPV-assisted transfection of Cy3-labeled siRNA into K562 cells. The Blank and siRNA^{Cy3} group show only blue fluorescence, indicating the DAPI-stained cell nuclei without siRNA transfection. The BCPV-siRNA^{Cy3} and BCPV-siRNA^{Cy3}+FBS, exhibit both blue and red fluorescence indicating siRNA transfection (Cy3-stained) into the cells. The images are compared with the standard transfection agent Lipofectamine2000TM (Lip2000-siRNA^{Cy3} and Lip2000-siRNA^{Cy3}+FBS). 132

Figure 6.6: Estimation of transfection efficiency in the K562 cells were detected by flow cytometry. a) Cell groups treated with different formulations (siRNA^{Cy3}, BCPV, BCPV-siRNA^{Cy3}, BCPV-siRNA^{Cy3}+FBS, Lip2000-siRNA^{Cy3} and Lip2000-siRNA^{Cy3}+FBS) were subjected to flow cytometry and fluorescent signal intensities showing positive staining for Cy3-labeled siRNA were representative of siRNA transfection into the cells. The higher fluorescent signals for groups BCPV-siRNA^{Cy3} and BCPV-siRNA^{Cy3}+FBS indicate higher transfection efficiencies. Blank cells were untreated. b) Statistical calculation of transfection efficiency. The ratio of cells in P3 to the total P2+P3, yielded the transfection efficiency. c) The average fluorescent signal intensities resulting from (a), depicting the performance of BCPV as an outstanding transfection agent for non-adherent leukemic cells. Results are represented as mean $\pm$ SD, n=3; *, $p<0.05$; **, $p<0.01$ 134

Figure 6.7: Downregulation of BCR-ABL mRNA post siRNA transfection. The cell groups were treated with different formulations and the gene knockdown of the BCR-ABL oncogene was evaluated by real time RT-PCR assay 48h post treatment. BCPV-siRNA-treated cell group showed considerable gene suppression, down to 20%, compared to 51% in the case of commercial transfection agent, Lipofectamine2000TM. These results correlate with the high transfection efficiency of BCPV-siRNA nanoplexes. Results are represented as mean $\pm$ SD, n=3; **, $p<0.01$ 135

Figure 6.8: Control of cell proliferation as a consequence of BCR-ABL oncogene suppression. The cell groups were treated with different formulations and the cell numbers were counted under a microscope post 72 hours of incubation. Consistent with the gene knockdown results in **Figure 6.6**, BCPV-siRNA-treated cell group yielded the lowest cell count, revealing the efficient performance of BCPV-assisted siRNA transfection in non-adherent leukemic cells. Blank cells were untreated. Results are represented as mean $\pm$ SD, n=3; *, $p<0.05$; **, $p<0.01$ 136

Figure 6.9: Enhanced cell apoptosis as a consequence of high BCPV-siRNA transfection. a) The cell groups were treated with different formulations and subjected to Annexin V: FITC Apoptosis assay, 72 hours post treatment. Cells presenting higher fluorescent signals for both FITC and PI staining in the flow cytometry micrographs were representative of the apoptotic cell count. b) The BCPV-siRNA-treated cell group

List of Figures

records the highest proportion of apoptotic cells. Blank cells were untreated. Results are represented as mean $\pm$ SD, n=3; **, $p<0.01$ 138

List of Tables

Table 2.1: Overview of microfluidic channel geometries on inertial focusing of microparticles.....	27
Table 3.1: Taper profile mode parameters	45
Table 3.2: Comparison of the miniature flow cytometer with the commercial flow cytometer	71
Table 4.1: Hydrodynamic Particle Focusing Techniques	88

Abbreviations and Symbols

3D	Three-dimensional
a/w	Relative particle diameter
AIDS	Acquired immunodeficiency syndrome
$a_{\min}$	Minimum particle size
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
AuNRs	Gold nanorods
BCL-ABL	Breakpoint cluster region-Abelson murine leukemia
BCPV	Biodegradable charged polyester-based vector
BMAAD	Polybutadiene-block-poly(methacrylicacid)-blockpoly(2 (dimethylamino)ethyl methacrylate)
CdSe	Cadmium selenide
c_i	Initial concentration
CML	Chronic Myeloid Leukemia
CTAB	Cetyl trimethylammonium bromide
CV	Coefficient of variation
CXC	A Chemokine sub-family
Cy3	Cyanine3
DAPI	4',6-diamidino-2-phenylindole
DAQ	Data acquisition
De	Dean number
D_h	Hydraulic diameter
DI	Deionized water
D_i	Diffusion coefficient

Abbreviations and Symbols

DLS	Dynamic Light Scattering
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DP _n	Degree of polymerization
ELISA	Enzyme-linked immunosorbent assay
F	Sum of the body forces
FAM	Carboxyfluorescein
FBS	Fetal bovine serum
F_D	Dean Drag forces
F_D	Dean Drag forces
FEM	Finite Element Method
FFR	Flow rate ratio
F_I	Inertial lift forces
F_{IS}	Shear gradient-induced lift force
FITC	Fluorescein isothiocyanate
F_{IW}	Wall-induced lift force
f_L	Lift coefficient
fps	Frames per second
FSC	Forward scatter signal
FWHM	Full width at half maximum
G	Shear rate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPC	Gel permeation chromatography
h	Microchannel height

Abbreviations and Symbols

HIV	Human immunodeficiency virus
I/O	Input/output
IL-8	Interleukin-8
KS/s	Kilo samples/second
LA	Lactide
M_n^{NMR}	Number-average molecular weight
mRNA	Messenger RNA
msec	Millisecond
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N_i	Molar flux
N-S	Navier-Stokes
OD	Optical density
p	Pressure
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate-buffered saline
PDI	Polydispersity index
PDMAEMA	Poly(2-(dimethylamino)ethyl methacrylate)
PDMS	Polydimethylsiloxane
PEG	Polyethylene glycol
PEI	Polyethylenimine
PI	Propidium iodide
PLL	Poly-L-lysine
PMT	Photomultiplier tube
PS	Phosphatidylserine
PSS	Polystyrene sulfonate

Abbreviations and Symbols

QD	Quantum Dots
Q_s	Sample flow rate
Q_{sh}	Sheath flow rate
Re_p	Particle Reynolds number
R_i	The sources or sinks in the reaction flux
RI	Refractive index
RMPI	Roswell Park Memorial Institute medium
RNAi	RNA interference
RT-PCR	Reverse transcription polymerase chain reaction
r_s	Smallest radius
SD	Standard deviation
Si	Silicon
siRNA	Small interfering RNA
SSC	Side-scatter signal
SU-8	Epoxy-based negative photoresist
T	Operating temperature
TEM	Transmission electron microscopy
t_{pw}	Pulse width
u	Velocity
v_{ch}	Average velocity of the main channel
VEGF	Vascular endothelial growth factor
v_f	Average velocity of the focused sample
w	Channel width
w_{ch}	Microchannel width,
w_f	Width of the focused particle stream in the main channel

Abbreviations and Symbols

w_f/w_{ch}	Relative focused stream widths
wt/vol	Weight/volume
δ	Curvature ratio
ϵ_{xy}	Strain rate
μ	Viscosity
$\mu\text{l/min}$	Microlitre/minute
ρ	Density
τ_{xy}	Applied shear stress

List of Publications

Journals

1. Nishtha Panwar*, Chengbin Yang*, Feng Yin, Ho Sup Yoon, Tjin Swee Chuan, and Ken-Tye Yong, *RNAi-based therapeutic nanostrategy: IL-8 gene silencing in pancreatic cancer cells using Gold nanorods delivery vehicles*, Nanotechnology 26, 2015, 365101. (Impact Factor: 3.44)
2. Chengbin Yang*, Nishtha Panwar*, Yucheng Wang, Butian Zhang, Maixian Liu, Huiting Toh, Ho Sup Yoon, Swee Chuan Tjin, Peter Han Joo Chong, Wing-Cheung Law, Chih-Kuang Chen, and Ken-Tye Yong, *Biodegradable charged polyester-based vectors (BCPVs) as an efficient non-viral transfection nanoagent for gene knockdown of the BCR–ABL hybrid oncogene in a human chronic myeloid leukemia cell line*, Nanoscale 8, 2016, 9405-9416. (Impact Factor: 7.367)
3. Yin Feng*, Nishtha Panwar*, Danny Jian Hang Tng, Swee Chuan Tjin, Kuan Wang, and Ken-Tye Yong, *The application of mesoporous silica nanoparticle family in cancer theranostics*, Coordination Chemistry Reviews 319, 2016, 86-109. (Impact Factor: 13.324)
4. Nishtha Panwar, Peiyi Song, Swee Chuan Tjin, and Ken-Tye Yong, *A sheathless, three dimensional focusing technique based on inertial microfluidics for lab-on-a-chip flow cytometry* (Submitted).
5. Nishtha Panwar, Peiyi Song, Chengbin Yang, Swee Chuan Tjin, and Ken-Tye Yong, *Sheath-assisted hydrodynamic focusing in the viscous and inertial regimes: A comparative study* (Submitted).
6. Jie Zhou, Zhonglin Cao, Nishtha Panwar, Rui Hu, Xiaomei Wang, Junle Qu, Swee Chuan Tjin, Gaixia Xu, and Ken-Tye Yong, *Functionalized Gold Nanorods for Nanomedicine: Past, Present and Future*, Coordination Chemistry Reviews, 352, 2017, 15-66 (Impact Factor: 13.324).

Conferences

1. Nishtha Panwar, Peiyi Song, Ken-Tye Yong, Swee Chuan Tjin, *Study of inertial hydrodynamic focusing in sheath-driven flows for lab-on-a-chip flow cytometry*, Asia Student Photonics Conference (ASPC-2016), 2016.

List of Publications

2. Nishtha Panwar, Peiyi Song, Chengbin Yang, Ken-Tye Yong, Swee Chuan Tjin, *An optofluidic approach for Gold nanoprobe based-cancer theranostics*, SPIE Photonics West, 2017, 10061, 100610L-1
3. Nishtha Panwar, Peiyi Song, Ken-Tye Yong, Swee Chuan Tjin, *Study of inertial hydrodynamic focusing in sheath-driven flows for lab-on-a-chip flow cytometry*, SPIE Optics+Optoelectronics, 2017, 1023114-1023114-9
4. Nishtha Panwar, Peiyi Song, Ken-Tye Yong, Swee Chuan Tjin, *Pressure-driven particle focusing in lab-on-a-chip flow cytometers: The choice between sheath-assisted and inertial focusing*, Photonics Global Conference, Singapore, 2017.

* indicates equal contribution