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Development of a Lab-on-a-Chip Device for Rapid Nanotoxicity Assessment In Vitro

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

DEVELOPMENT OF A LAB-ON-A-CHIP DEVICE FOR RAPID NANOTOXICITY
ASSESSMENT IN VITRO

A dissertation submitted in partial fulfillment of

the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOMEDICAL ENGINEERING

by

Pratikkumar Shah

2015

To: Dean Amir Mirmiran
College of Engineering and Computing

This dissertation, written by Pratikkumar Shah, and entitled Development of a Lab-on-a-Chip Device for Rapid Nanotoxicity Assessment In Vitro, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Date of Defense: December 11, 2014

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University Graduate School

Florida International University, 2015

DEDICATION

Dedicated to my family - without their love, support and sacrifice this dissertation would not have been completed.

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ABSTRACT OF THE DISSERTATION

DEVELOPMENT OF A LAB-ON-A-CHIP DEVICE FOR RAPID NANOTOXICITY

ASSESSMENT IN VITRO

by

Pratikkumar Shah

Florida International University 2015

Miami, Florida

Professor Chenzhong Li, Major Professor

Increasing use of nanomaterials in consumer products and biomedical applications creates the possibilities of intentional/unintentional exposure to humans and the environment. Beyond the physiological limit, the nanomaterial exposure to humans can induce toxicity. It is difficult to define toxicity of nanoparticles on humans as it varies by nanomaterial composition, size, surface properties and the target organ/cell line. Traditional tests for nanomaterial toxicity assessment are mostly based on bulk-colorimetric assays. In many studies, nanomaterials have found to interfere with assay-dye to produce false results and usually require several hours or days to collect results. Therefore, there is a clear need for alternative tools that can provide accurate, rapid, and sensitive measure of initial nanomaterial screening. Recent advancement in single cell studies has suggested discovering cell properties not found earlier in traditional bulk assays. A complex phenomenon, like nanotoxicity, may become clearer when studied at the single cell level, including with small colonies of cells. Advances in lab-on-a-chip techniques have played a significant role in drug discoveries and biosensor applications, however, rarely explored for nanomaterial toxicity assessment. We presented such cell-

integrated chip-based approach that provided quantitative and rapid response of cell health, through electrochemical measurements. Moreover, the novel design of the device presented in this study was capable of capturing and analyzing the cells at a single cell and small cell-population level. We examined the change in exocytosis (i.e. neurotransmitter release) properties of a single PC12 cell, when exposed to CuO and TiO₂ nanoparticles. We found both nanomaterials to interfere with the cell exocytosis function. We also studied the whole-cell response of a single-cell and a small cell-population simultaneously in real-time for the first time. The presented study can be a reference to the future research in the direction of nanotoxicity assessment to develop miniature, simple, and cost-effective tool for fast, quantitative measurements at high throughput level. The designed lab-on-a-chip device and measurement techniques utilized in the present work can be applied for the assessment of other nanoparticles' toxicity, as well.

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ABBREVIATIONS AND ACRONYMS

NP/NPs	Nanoparticle/Nanoparticles
LOC	Lab-on-a-chip
COC	Cell on chip
MEMS	Micro-electro-mechanical systems
DA	Dopamine
NE	Norepinephrine
BBB	Blood brain barrier
CNS	Central nervous system
ROS	Reactive oxygen species
CNT	Carbon nanotubes
SWCNT	Single walled carbon nanotubes
FCM	Flow cytometry
DEP	Dielectrophoresis
pDEP	Positive dielectrophoresis
nDEP	Negative dielectrophoresis
Ag-NPs	Silver nanoparticles
CuO-NPs	Copper oxide nanoparticles
TiO ₂ -NPs	Titanium dioxide nanoparticles
ITO	Indium tin oxide
PC12	Pheochromocytoma 12
F-12K	Kaighn's modification of ham's F-12
PBS	Phosphate buffered saline

SAM	Surface assembled monolayer
PEG	Polyethylene glycol
CV	Cyclic voltammetry
CFM	Carbon fiber microelectrode
LFIA	Lateral flow immuno assay
DLS	Dynamic light scattering
PDMS	Polydimethylsiloxane
SRB	Sulforhodamine B
EIS	Electrical impedance sensing
ECIS	Electric cell-substrate impedance sensing

CHAPTER 1- INTRODUCTION

This chapter can be cited as; **Pratikkumar Shah**, Ajeet Kaushik, Xuena Zhu, Chengxiao Zhang and Chen-Zhong Li, “*Chip Based Single Cell Analysis for Nanotoxicity Assessment.*” **Analyst**, **2014**. 139(9): p. 2088-98

1.1 Motivation

Nanomaterials have been utilized extensively in everyday life related consumable products and in technology because of their tunable properties and excellent performances. Exposure of excess nanomaterials, beyond a physiological range, can cause health risks via affecting the function of an organ, genomic system, or even the central nervous system. Ever growing applications of nanomaterials with various properties create a huge matrix of nanomaterials that need to be tested and validated for the amount of nanotoxicity. Traditional ways of studying nanomaterials toxicity rely on observing the response of a cell-population to the nanomaterials and averaging the results to each cell in the study. However, recent advancements in single cell studies have presented cell-properties not known earlier. The availability of proper tools to study nanomaterial toxicity rapidly at single and multiple cell levels simultaneously will enhance the information obtained. Thus, new analytical approaches for nanotoxicity assessment to verify the feasibility of nanomaterials for future use are in demand.

Chip based single cell nanotoxicity approach has been reported rarely. Here, we presented a cell integrated lab-on-a-chip (LOC) approach to provide quantitative and rapid cell response upon exposure to various nanoparticles. The device presented in this study is capable of capturing and analyzing cells at a single cell level; which can be a critical parameter to understand the complex nanomaterial toxicity phenomena at a single cell level. The obtained results can be used as supporting document to demonstrate the influence of nanoparticles and cell's surroundings on cell response. The study can be a reference to future research in the direction of nanotoxicity assessment to develop

miniature, simple, and cost effective tool for rapid, quantitative measurements at high throughput level.

1.2 Hypothesis

Our hypothesis is that a single-cell integrated lab-on-a-chip (LOC) device will provide real-time measurements of the effects of nanomaterials on cell health using electrochemical techniques. This will provide an early indication of nanomaterial toxicity on cells, and can speed up the nanotoxicity assessment of nanomaterials for future use in targeted applications. Moreover, a controlled study from a single cell to a fixed population of cell will help us understand the difference in response of single and multiple cells, which might open new questions about the role of cell-cell communication in the spread of toxicity.

1.3 Objective of the presented research

The overall objective of the presented research is to design and develop a Lab-on-a-chip (LOC) device to as an investigating tool for the assessment of nanomaterial toxicity at a single cell and small cell-population level. The overall goal is divided into 3 specific aims as follows;

1.3.1 Development of a lab-on-chip device for single cell trapping

Approach

- a) Pattern gold electrodes on a Pyrex wafer in order to get the sensing electrodes and connection pads. Pattern SU-8 on top of the gold electrodes to construct the dielectric layer as well as cell capturing microwells.
- b) Characterize the device optically and electrochemically to verify for diffusion limited response behavior.

- c) Functionalize the surface of the device to assist in cell trapping by creating cytophilic and cytophobic regions using paper stamp technique.
- d) Optimize a well-known dielectrophoresis technique for noninvasive and controlled single PC12 cell trapping.

1.3.2 Rapid nanotoxicity assessment using single cell integrated chip

Approach

- a) Use the developed LOC device for electrochemical detection of PC12 cell exocytosis (i.e. neurotransmitters dopamine (DA) and norepinephrine (NE), primarily for PC12 cells) at a constant applied potential using amperometry technique.
- b) Analyze the amperometry data collected from different conditions in Igor software using a peer reviewed signal analysis procedure file for parameters, such as maximum current, half maximum time, quantal release and burst frequency.
- c) Perform exocytosis recordings of PC12 cells for CuO and TiO₂ exposures, and compare them with the control measurements.

1.3.3 Comparison of single cell and multiple cell behavior under nanoparticle exposure

Approach

- a) Upgrade the existing LOC device to incorporate microwells, which allow different number of cells trapping.
- b) Investigate the real-time response of single cells and a small cell-population when exposed to nanoparticles (NPs) using electrochemical impedance sensing technique.

1.4 Thesis outline

Chapter 1 provides a comprehensive literature review of investigations that has been done by other researchers in the field of *in vitro* assessment of nanotoxicity. It also provides ideas for research areas that can be further explored. Chapter 2 presents a strategy for the design and development of a lab-on-chip device. This chapter provides insights on the chosen design parameters and detailed process for lab-on-chip device fabrication. Also, the chapter provides details of successful trapping of single cells in microwells using a noninvasive dielectrophoresis approach, which is further utilized in the experiments discussed in following chapters. Chapter 3 describes the application of the cell-on-chip device to measure and understand the real-time effects of nanomaterials on the exocytosis function of a single PC12 cell. Chapter 4 describes the nanotoxicity study on PC12 cells using an impedance spectroscopy approach and further highlights the differences in response of cells when cells are isolated and in small colonies. Conclusions and a scope for future research for this study are discussed in Chapter 5.

1.5 Nanotoxicity

Nanotechnology has been one of the hot topics in not only research, but also the consumer market for the last 20 years. The ongoing trend and success suggest an ever-increasing use of the nanotechnology in every sector. At the nano-scale ($\sim 1\text{-}50\text{ nm}$), NPs demonstrate unique physico-chemical properties in comparison to their bulk form. The unique features, such as size, shape, surface properties, etc., have inspired the production of nanomaterials, not only for research, but also at the industrial scale for various applications. NPs are increasingly used in cosmetics, food, electronics, paint, material science, medicine, biotechnology, and energy technologies. The Nanotechnology

Consumer Products Inventory list currently contains 1628 consumer products (not comprehensive), a 24% increase from the 2010 update [1] and perhaps thousands of nanomaterials are handled at the research level without knowing proper details about their properties. With increased production and use of nanomaterials lies the risk of increased intentional/unintentional exposure to these particles [2-4].

Figure 1.1: Schematic of NP localization in various body organs (Figure is reused with permission from reference [5]).

Despite the positive aspects of the use of nanoparticles in many applications, they do have adverse effects on human, animals and environment living due to their own toxicity. NP toxicity has been studied and assessed for years; however a gold standard technique to assess toxicity is absent due to several limitations. First and foremost, even the toxicity of nanoparticles does not have a common agreement [5]. Since the NPs behavior may change based on targeted organ, understating of the NPs potential interaction with

biological systems [6, 7] are not unanimous, and there are no specific tools which can provide rapid nanotoxicity assessment.

NPs with many novel properties are used in various applications and come in contact with complex and dynamic biological systems. It is challenging to characterize NPs throughout their biological interaction and to quantify the uptake rate and localization inside organs and cells. Cells exposed to nanomaterials may undergo necrosis or repairable oxidative DNA damage and recover from it eventually, or may undergo apoptosis. Nanotoxicity may alter cell differentiation, proliferation, morphology, or cell-cell communication. Upon exposure, NPs can easily get access to the systemic circulation in a human body, and even can localize into different organs and tissues to further induce organ targeted toxicity/disease. Figure 1.1 highlights the possible accumulation of NPs in various organs. Some NPs can cross the blood-brain barrier (BBB) [8] (Figure 1.2a [9]) and hence have been proposed for diagnostic and therapeutic applications [10, 11]. Their smaller size and larger surface area provide NPs' unique properties in their translocation to the systemic circulation and central nervous system (CNS). Once inhaled, nanoparticles can pass through epithelia of the respiratory track and access the bloodstream directly or via lymphatic pathways [12, 13]. NPs also can be translocated in the CNS by nerve endings embedded in airway epithelia and nerve endings of the olfactory bulb [12, 14]. NPs can damage cells by reactive oxygen species (ROS) formation, mechanical damage of intracellular organelles, and an imbalance in cytosolic Ca^{2+} concentration [15-17] (Figure 1.2b). NPs' can also affect ion channels and synapses, thereby impeding neuronal communication. To understand the role of the NP interaction

with neurons is critical to sense any dis-functionality in cell behavior and to assess any toxic effects.

Figure 1.2: (a) Illustration of possible pathways in which NPs can cross blood brain barrier. The pathway depends on the material and size of NPs. (b) Illustration of cell-NP interaction and nanotoxicity generation. Event 1 represents the extracellular ROS generation outside and inside the cell. Event 2 represents the damage to the cell membrane integrity. Event 3 is particle dissolution or ion leaching affecting the cell function. Event 4 signifies mechanical damage to intracellular organelles due to NP intrusion. Event 5 is indicating the role of NPs surface properties (roughness, charge, and active groups) whereas event 6 is highlighting the role of NP size in toxic effects to a cell. Event 7 is representing the NPs shape induced toxicity as different shapes of NP may interact with the cell differently. Event 8 is dissolution or leaching of nanoparticle outside the cell membrane and making it easier to penetrate the cell membrane for nanoparticle and affect the cell function. Figures 1.2a and 1.2b are reused with permission from references [9] and [15], respectively.

The research on the potential health risks of NP exposure lags behind the rapid development of nanotechnology [18-21]. The federal agency of the United States alone has invested \$750 million (from 2006-2014) in research to establish “risk assessment” of environment, health and issues related to the use of nanomaterials [22]. The National Institute for Occupational Safety and Health (NIOSH) has identified risk assessment as one of the 10 critical areas that it wants to “guide in addressing knowledge gaps, developing strategies, and providing recommendations” [23]. Strategic efforts of US and Europe are continuing to establish risk assessment approaches of nanoparticle exposure.

1.5.1 Causes of nanotoxicity

The physico-chemical properties of materials play an important role when NPs interact with biomolecules or biological systems. These properties not only define the uptake and excretion of NPs, but also explain the interference (toxicity) with the biological system [24]. The most discussed properties of a NPs causing toxicity are its chemical composition, shape, size, surface charge, surface functional group, reactivity, and ability to be stable in the biological system (Figure 1.3). Also, the longevity of NP-cell interaction and the exposed dose are definitely deciding factors in the adverse effects of nanomaterials.

The intrinsic property (chemical composition) of a material defines the basic character of the material. For that reason, some nanomaterials (e.g., CdO, CuO) are toxic, whereas some materials are biocompatible (less or non-toxic, e.g., Au, FeO₃) [25]. In an experiment performed by our group earlier, we found that gold NPs are nontoxic while cadmium oxide nanoparticles of the same size and dose are highly toxic, whereas silver (Ag) and carbon nanotubes (CNT) showed some sign of toxicity [26]. Shape and size of

NPs become important factors to provide large surface to volume ratio of the nanoparticles. It is generally believed that the smaller the NP, more toxic it is [27]. Due to a larger surface to volume ratio, NPs have more molecules on the surface and possess higher reactivity that can enhance the intrinsic toxicity [28]. In a recent study by Wang et al., it was found that longer single wall carbon nanotubes were more toxic to PC12 cells than short single wall carbon nanotubes [29]. Another study conducted by Napierska et al. [30] showed that the mono-dispersed amorphous silica NPs exhibit size dependent toxicity on endothelial cells with smaller size of NPs of the same morphology being highly toxic compared to the larger size of NPs at the same concentration. The surface properties of NPs play a great role in defining their reactivity in biological systems. Sometimes, the toxicity of NPs can also be tuned by changing their surface properties, such as by decorating a compatible functional group or by changing the surface charge (zeta potential). Huang et al. showed that the shape of the mesoporous silica NPs affects cellular functions [31]. A study conducted by Marques et al. [32] to verify the surface charge effect on internalizations of noble NPs, Au (~26.5 nm) and Ag (~33.3 nm), showed that the positively charged NPs (Au⁺ and Ag⁺) were more susceptible to internalization by mast cells compared to their negatively charged counterparts (Au⁻ and Ag⁻).

Figure 1.3: Illustration of basic properties of NPs responsible for toxicity. (a) Illustration of the size effect of NPs, smaller NPs (<5 nm) not only can cross the cell membrane, but also can interfere and damage the intracellular organelles. (b) Illustration of different shapes of nanoparticles; it is proven that a sphere shape NP is less toxic than a rod or star shape NP. (c) Illustration of NP surface functionalization; besides the intrinsic surface property i.e. surface roughness and surface charge, the functionalized group on the surface plays a major role in dictating the behavior of NPs in the biological system.

1.6 Single cell nanotoxicity measurement

Traditional methods of analyzing cells are based on the averaging of results studied from multiple cells in an assay, also referred to bulk assay. The results are correlated and assumed to be equally contributed to by all cells of the population under study. However, recent advancements in single cell studies have shown that individual cells behave differently from the population even under identical environmental conditions.

Figure 1.4: Gene expression of GAPDH for Jurkat cells treated with GAPDH siRNA relative to normal untreated cells. The levels of GAPDH expression roughly fall into two categories: 0% and 50% of normally untreated cells. However, the average GAPDH expression from bulk assay (50 cells) is 21 ± 4 %, which is not representative of any individual cell. Figure adapted from [33].

Cell heterogeneity has been studied by researchers recently, suggesting that the cells in a sub-population may have different behavior from the general population results [34-36]. In a published study by Toriello et al. [33] (Figure 1.4), mRNA expression of GAPDH from individual Jurkat cells after siRNA knockdown, the levels fall into roughly two categories: 50% and 100% knockdowns (i.e. 50% and 0% expression remained). Importantly, the average GAPDH expression obtained from the measurement of 50 cells ($21 \pm 4\%$) was not representative of any individual cell. This means that the cell population studies in aggregation may hinder some critical cell mechanisms which are only visible at the single cell level. To study the true functionality of a cell, it is really important to study a cell independently without interference from neighboring cells.

Studying multiple cells in a single assay might obscure some important information that can only be understood by a single cell study. In bulk assays, the difficulties to distinguish the result from a smaller response of a homogeneous population or a larger response from a small subpopulation of cells have been discussed earlier [37]. Therefore, single cell analysis can be an equivalent and complementary strategy to existing approaches. Especially for neuronal cells, the physiological function can be monitored by recording the pattern of electrical activity and any disturbance in these patterns of electrical activity could serve as a highly sensitive way to measure the functional toxicity as interference of the functional activity can be observed before any other changes are monitored, and much before the cell death. Figure 1.5 illustrates the reasons for the selection of a single cell based assay for nanotoxicity assessment.

Figure 1.5: Highlights of the single cell study. Single cell behavior differs due to heterogeneity, which is a resultant of one of the genetic, biochemical/metabolic, physiological or behavioral heterogeneity. Additional benefits such as observation of discrete and dynamic events of cells during their life-time, cellular pathways study without interference of neighbor cells, comparison or relating microscopic to macroscopic (single cell to a large population of cell) and study or rare and transient cell states can only be achieved by single cell analysis.

1.7 Assessment of nanotoxicity: state-of-the-art

Since, various characteristics or properties of a NP can induce toxicity in numerous ways, many toxicity assays from chemical toxicity assays are available for toxicity assessment. Principally, the toxicity of NPs is measured in terms of change in viability of cells or functional changes in the cells (i.e. DNA damage, gene alteration, ROS generation). The detailed explanation of conventional nanotoxicity assessment techniques have been explained in many reports [38, 39]. Figure 1.6 illustrates the adopted strategies for nanotoxicity assessment. The brief introduction and state-of-the-art features of these techniques are described in the next section.

MTT, LDH, and Spectrophotometric Assays
➤ Measure the activity of enzyme in living cell
➤ Not possible at single cell level
➤ Final time point measurement
➤ Many times require cell lysing
➤ Mostly, labeling required
➤ Time consuming and costly
➤ Specificity and sensitivity can be argued
Electrochemical Assays
Impedance Spectroscopic Assessment
➤ Real time kinetics of the nanoparticle interaction with the cells
➤ Can not provide information about cell signaling at single exocytosis/vesicle resolution
Amperometric Assessment
➤ Sensitive and provide cell signaling kinetics information at single exocytosis/vesicle level
➤ Complex setup and require a trained personnel.

Figure 1.6: Table highlighting the common limitations associated with dye/optical based assay and electrochemical approach as an alternative mechanism for nanotoxicity assessment.

1.7.1 Viability assays

Metabolic rate, cellular membrane integrity, apoptotic/necrotic cell death or the rate of proliferation properties of cells is utilized to evaluate cell viability. The parameters may overlap and is presented only as a classical way of cell viability demonstration. Dye based viability assays mostly work on the principle of inclusion, exclusion or conversion of an added dye in live versus dead cells that can further be identified by colorimetric or fluorescent assays [40]. Trypan blue exclusion assay [40-43] is used to characterize the viability of cells. This assay is based on a diazo dye, which can only be taken up by dead cells and is excluded by live cells. Alamar Blue assay [40, 42, 44] is another common cell viability assay. The Alamar Blue reagent is a non-toxic, water-soluble resazurin dye that yields a fluorescent signal and a colorimetric change when incubated with metabolically active cells. There are many other dye based (fluorescent and non-fluorescent) live-dead assays, such as calcein AM and propidium iodide based assays [45], neutral red assay [46], and Live/Dead (Invitrogen, Carlsbad, CA) [47].

LDH (Lactate dehydrogenase) assay [30, 40, 48, 49] has been used to understand the integrity of cellular membrane. LDH is an indicator of lytic cell death, as soluble LDH is released into the extracellular medium through damaged cell membrane. Tetrazolium salt-based assays (MTT, MTS, WST) [48, 50, 51] are widely used for the proliferation rate measurement of cells under the influence of NPs. In this metabolic assay, MTT is reduced by cells into blue/purple color non-soluble formazan dye. Formazan dye is then solubilized in DMSO to get the average idea of cell viability. Higher metabolic rate (more blue color) is an indication of more viable cells in the population. In a ^3H Thymidine-based assay [52], uptake of ^3H by newly synthesized DNA can be used as the

detection of cell proliferation. Alamar Blue is comparatively simpler and more sensitive method for cell metabolism detection than MTT; however, possibilities of false positives or negatives cannot be nullified [53]. A study conducted by Riviere and Zhang [54] showed that the obtained results of viability with different dyes for different materials were inconsistent and this inconsistency could be contributed by nanomaterial/dye interaction and nanomaterial adsorption of the dye. Thus, it is always recommended to use more than one type of dye-based assays for the confirmation of results. Casey et al. [55] found that the CNT interfering with MTT, Alamar Blue and neutral red dyes giving false measurements and so pressed the urgent need of developing alternative techniques to quantify nanotoxicity. These studies raised concern about the widely used viability/cytotoxicity assays for nanomaterial toxicity screening and thoughtfully suggested the need of alternative techniques for nanomaterial and biological system interaction evaluation. This also highlights the basic limitation of the conventional and new dye-based assays in general.

The above-mentioned assays are forms of bulk-assays, require a huge population of cells to experiment and a consensus is built for every cell. Since, the role of a single cell study is found to be important, there are a few single-cell, dye-based cell viability assays which can further be incorporated with flow-cytometer for high throughput detection. Annexin V is a marker of externalization of phosphatidylserine on the outer surface of plasma membrane, is an early sign of apoptosis. Labeling of annexin V [56] with fluorescent or radioactive molecules makes it possible to identify the binding of annexin V on the surface of dead (apoptotic) cells. TUNEL assay [57] can be used to identify the cell death (apoptotic) by detecting fragmented DNA by labeling the terminal end of

nucleic acids. Colony forming assay [58] does not require any additional tagging. Here, the ability of a single (or very few cells) to form a colony is measured as an indication of being healthy over the period of few days.

1.7.2 Functional assays

Functional assays are more specific to detect nanotoxicity at the genomic level, change in gene formation, DNA damage, reactive oxygen species (ROS) generation, etc.

Oxidative stress is commonly observed due to the influence of NPs [40, 56]. This phenomenon is linked with toxicity as un-repairable oxidative stress generates free intracellular radicals and damages the lipid, protein, and nucleic acids. Direct/indirect intracellular ROS measurement assays include the glutathione (GSH) assay [59], which is a luminescent-based assay and has been used for the detection and quantification of glutathione in cells. Lipid peroxidation measurement assay measures increasing concentrations of end products of lipid peroxidation indicating increased oxidative damage in the cells [38, 40, 41, 44, 60]. For example, 2, 7-dichlorofluorescein (DCFH) assay which detects intracellular DCFH oxidation due to the presence of hydrogen peroxides [40, 42, 44, 46, 56, 59, 61] to measure free radicals in cells and tissue. Besides stress, cellular inflammation response also can be used as a measure of cytotoxicity by detecting specific biomarkers. Cytokines are particularly related with the cell proliferation and inflammation. Immunoassays (i.e. ELISA [62]) are used to detect secreted cytokines such as detection of interleukin 6 (IL-6) [63], interleukin 8 (IL-8) [63, 64] or monocyte chemotactic protein-1 (MCP-1) [65]. However, ELISA tests are usually time consuming and require multiple operations. Detection of DNA damage can be sensed at the single cell level by comet assay (single cell gel electrophoresis) [66].

However, mitochondrial DNA damage and smaller DNA fragments (<50 Kb) are hard to be detected by comet assay, and some apoptotic cells that can be washed during the lysis will not be detected by the assay. Mutation or the change in a particular gene can be identified by several assays for oxidized guanine bases. These modifications are often the resultant of oxidative stresses and traditionally are identified by immunohistochemistry or HPLC techniques. PCR/RT-PCR array [62] can be used to identify the panel of genes. Karyotype analysis [67] can provide the information about number and integrity of chromosome by detecting the micronucleus of the cell undergoing cell-division, under the NP influence.

Traditionally, we have seen dye based assays for the nanotoxicity assessment and it has been shown that NPs interfere with dye and many times give false positive results. Moreover, many times these techniques possess limitations of being end point measurements instead of dynamic and real-time measurements, requiring cell lysing and cannot be performed on single cell level without an additional labeling system. Recent advances in nanotoxicity assessment have tried to address some of the problems faced by these traditional techniques and are discussed in the following section.

1.8 Recent advances in nanotoxicity assessment

The growth of nanotechnology has offered not only the nanomaterials with unique properties, but also presented advanced analytical tools which exhibit highly sensitive sensing mechanisms. Efforts have been put together to develop either device based or new technique based approaches to evaluate the hidden parameters of NPs-biological interaction (Figure 1.7).

1.8.1 Lateral flow immunoassay

ROS induced oxidative DNA damage is a well-known trait of nanotoxicity. 8-hydroxyguanine and its nucleoside 8-hydroxy-2'-deoxyguanosine (8-OHdG) are the most studied oxidized guanine bases [68]. Commonly used techniques for 8-OHdG analysis are high-performance liquid chromatography (HPLC) with electrochemical detection (ECD), gas chromatography-mass spectrometry (GCMS), HPLC tandem mass spectrometry, and Enzyme linked Immunosorbent Assay (ELISA). However, most of these techniques are time-consuming, expensive, and require special techniques and equipment. Our group has recently developed a novel lateral flow immunoassay (LFIA) [69] to overcome these challenges and to measure the concentration of 8-OHdG and thus reveal the nanotoxicity on the genomic level. The LFIA approach can be simple, scalable, and inexpensive analytical tool for nanotoxicity detection. However, cell lysing was a compulsion for this end point measurement technique.

1.8.2 Atomic force microscopy

Atomic force microscopy (AFM) is a powerful force sensitive technique and has been successfully applied in single cell studies to gather the information on cell structure, topography, membrane nanostructures, and mechanics (e.g., adhesion force, elasticity) of mammalian cells at a nano-scale resolution under physiological conditions or near physiological conditions [70]. In a recent study by Blechinger et al. [71], the uptake and localization of SiO₂ NPs were scanned by using AFM combined with fluorescence microscopy. An atomic force microscope can be used to study the mechanics of cell under the influence of nanoparticles. Recently, Wu et al. [70] used AFM to study the biophysical properties of vascular endothelial cells at a single cell level upon diesel

exhaust particles exposure. By using AFM, Wu et al. measured the mechanical properties (young's modulus and adhesion force) and the topography of the cell membrane.

Figure 1.7: Recent advancement for nanotoxicity assessment. (a) Lateral flow immune-strip (FLIS) is very common for pregnancy test and some other biomarker application. LFIS was first time used for evaluation of genotoxicity/ DNA damage detection upon NP exposure in reference [69]. (b) AFM is used to measure the adhesion force and stiffness of the cell membrane upon diesel NP exposure on human aortic endothelial cells [70]. (c) Carbon fiber microelectrode is a very sensitive technique with very high temporal resolution and has been used recently to identify the change in exocytosis behavior of endocrine or immune cells upon NP exposure [72-74].

1.8.3 Carbon fiber microelectrode

Carbon fiber microelectrodes (CFM) of tip size $\sim 5\text{-}10\ \mu\text{m}$ exhibit high sensitivity and low noise levels for single cell analysis. Their ability to detect diffusion limited current at very high scan rates allows better temporal resolution [75]. CFM amperometry technique is used to explore biophysics of exocytosis, and has been proved as an important tool to understand cellular communication under the influence of NPs. Marquis et al. [76] used

CFM amperometry to characterize serotonin exocytosis from murine peritoneal mast cells co-cultured with fibroblasts for 48 hours on interaction with Au nanoparticles (12-46 nm). A decrease in granule transport and fusion events along with increments in intracellular matrix expansion and a higher number of serotonin exocytosis per granule was observed. The effect on cell viability when NP exposure was extended for 48 and 72 hours [74] was also studied. The effect of citrate reduced noble NPs, Au (28 nm) and Ag (61 nm), on neuroendocrine cells has been evaluated by Love and Haynes [72]. In this work, the uptake quantifications were measured using inductively coupled plasma-atomic emission spectroscopy (ICP-AES). The uptake rate for Ag and Au NPs (1 nM) was found different for each type after 24 hours of exposure, 3.4×10^4 versus 7.5×10^5 NPs per cell, respectively. This suggests higher internalization of the Au NPs. The observed different rate of NP internalization was dependent on factors like size, surface charge, and functionalization. CFM amperometry exposed the changed exocytosis behavior of chromaffin cells in this study.

Metal oxide NPs (MONP) such as nonporous SiO_2 , porous SiO_2 , and nonporous TiO_2 are being used in consumer products of everyday life. CFM amperometry technique has explored the nanotoxicity effect of MONP on immune cells by Maurer-Jones et al. [73]. The outcomes of their studies revealed functional changes in chemical messenger secretion from mast cell granules. The surface properties of NPs are known to play a major role in deciding the nature of the interaction with biological systems. Love et al. [77] further utilized this technique to evaluate the changes in cellular communication in neuroendocrine cells on exposure of size dependent Ag NPs and surface functionalized Au NP for 24 hours. Authors conclude that Ag NPs of 15 - 60 nm did not alter cell

viability but exhibited size dependent cellular uptake and increase in the speed of exocytosis release kinetics. Beside this, PEG-functionalized Au NPs did not change the cell viability; however, they decreased the number of molecules released from each vesicle.

1.8.4 Fluidic based cell-on-chip (COC) approach

Micro-chip-based biosensors show a promising future for monitoring cellular nanotoxicity as they allow rapid, real-time and multi-sample analysis creating a versatile, noninvasive tool that is able to provide quantitative information with respect to alteration in cellular function under various nanomaterials exposures. Most of the COC-based approaches for nanotoxicity assessment at present are based on multiple cell screening. As the importance of single cell analysis besides the multiple cells screening already highlighted, some efforts have already been in the direction. In a recently published study by our group [78], we presented a chip-based biosensor capable of selective trapping of single cell dielectrophoretically in a micro-well and the same electrode can be used for further electrochemically study the captured cell. These electrodes are independently addressable for capturing a single cell in an individual microwell as well as study an independent cell on order. This COC can be used for nanomaterial based toxicity assessment. Cell behavior can be monitored in terms of catacalomine release from cell vesicles.

Electrochemical and optical measurement based fluidic chip platform have been explored for cell analysis over the last decade [79] which hold the potential to be used for nanotoxicity assessment. Zheng et al. [80] evaluated cytotoxicity of cadmium containing quantum dots on multicellular (HEK293 cells) events using a microfluidic chip with a

fluorescent microscope. In another study by Hosokawa et al. [81], a microfluidic chip based high throughput single cell array device was developed to study the gradient generated cytotoxic effect of a toxin. Similar platform could be applied to study nanoparticle concentration dependent toxicity in a single cell. However, optical assays are not label-free and variation in batch to batch measurement due to die selection limits the application of these assays. These challenges are being addressed using electrochemical based nanotoxicity assessment.

In a recent experiment by Kim et al. [82], a chip-based electrochemical approach was used for the assessment of nanotoxicity. On a lithographically patterned chip platform, a gold electrode modified with RGD-MAP-C to enhance cell (SH-SY5Y) adhesion on the chip was used as the sensing electrode. Silica NPs of various sizes and surface chemistries were examined to understand the effects of induced nanotoxicity on SH-SY5Y cells by studying cell viability at different concentrations of NPs ranging from 50 $\mu\text{g/ml}$ to 400 $\mu\text{g/ml}$ at various time points. Electrochemical measurements of nanotoxicity were recorded using differential pulse voltammetry and were compared with absorption and fluorescence-based techniques to evaluate the benefits of electrochemical measurements to assess nanotoxicity. In another experiment to overcome the limitation of statics models, Kim et al. [83] studied the cytotoxicity of mesoporous silica NPs (< 50 nm) to human endothelial cells under microfluidic flow conditions to mimic more of a blood vessel environment. This study tried to cover the missing factor of the shear-stress component, missing in most of the *in vitro* nanotoxicity studies, to mimic the actual NP toxicity in blood vessels. In their study, it was found that unmodified mesoporous silica NPs induced larger loss of cell viability under shear stress conditions than static

conditions whereas organo-modified NPs did not have a significant difference in toxicity of both conditions. The biggest advantage of the chip-based approach is that it provides the opportunity to measure the real-time kinetics (dynamic) of a cell rather than only providing the end point result (static) after a certain time, a disadvantage of major conventional nanotoxicity techniques. In a published study by our group, Hondroulis et al. [26], a whole cell based electrical impedance sensing (EIS) approach was developed for rapid and real-time assessment of nanomaterials (gold and silver NPs, single walled carbon nanotubes, and cadmium oxide) toxicity. This technique has huge advantages over the traditional nanotoxicity assessment techniques. EIS is cheaper, faster, and quantitative, and allows real time sensing of cell behavior. The study showed that gold nanoparticles to be nontoxic and cadmium oxide to be highly toxic, whereas, smaller size of silver nanoparticle (10 nm) were more toxic than larger (100 nm) and the length of SWCNT did not change the toxic effects. Recently, Alexander Jr. et al. [84] experimented silica nanowire toxicity on epithelial breast cancer cells and found dose dependent toxicity using an array based real-time impedance measurement chip. Impedance-based sensing approach is a highly sensitive method to monitor cell behavior (growth, health, motion, etc.) on top of the electrode in a very simple setup. The constraints of monolayer cell populations have also been highlighted recently in their limitation of not being able to mimic the 3D tissue culture and emphasis on in vitro 3D cell culture for nanotoxicity assessment. Luongo et al. [85] recently published a study on a microfluidic device fabrication for trapping and monitoring 3D multi-cell spheroid using real-time electrical impedance spectroscopy (Figure 1.8). Also, the proposed designs of 3D cellular/spheroid monitoring designs used for drug studies can be utilized

for nanotoxicity evaluation [86-88]. The role of NPs for drug delivery is highlighted many times, however, a recently published study by Albanese et al. [89] discussed NP transport kinetics and NP tissue accumulation in a tumor spheroid mounted on a microfluidic chip for different size, and surface modified NPs for drug delivery and diagnostic application. Similar designs in nanotoxicity evaluation will definitely curtail the gap of nanomaterial-biological interaction.

Figure 1.8: (A) is a microfluidic chip for spheroid capture and real-time impedimetric detection. (B) is zoomed area of the microfluidic channel and the trap mechanism for spheroid. (C) is the image of a multicellular spheroid before perfusion and (D) is the entrapment of spheroid between two impedimetric electrodes. (E) Another tumor on chip platform placed on an inverted microscope, the highlighted part is live spheroid and (F) is schematic representation showing accumulation of smaller NPs (40 nm) in interstitial tissue whereas escape of bigger NPs from penetration in spheroid matrix. Images A-D are reused with permission from [85] and E-F from [89].

Looking at the recent advancements in nanotoxicity assessment, an ideal platform would look like the illustration in Figure 1.9 wherein, real-time cell analysis can be performed on a microfluidic chip platform. The new generation of microfluidic chips should include not only bulk assay, but also single cell analysis in order to evaluate and

clearly identify the role of cell communication in spreading or deterring of nanotoxicity. The next section briefly discusses the associated challenges in nanotoxicity assessment with conclusion.

Figure 1.9: Schematic of an ideal microfluidic COC device for nanotoxicity assessment. The presented COC can assess toxicity at single cell level and at group of cells. It can compare the microscopic and macroscopic effects and can present a clear role of NP interaction with single cell by blocking other stimulating factors, such as cues from neighbor cells, and with the overall integrative effects produced by NP and the cell communication in a population of cells.

1.9 Challenges and conclusion

Looking at the growth of nanotechnology, number of nanomaterials (metallic, metal oxide based, ceramic, carbon based, polymeric, polymer based, biomolecule based, magnetic and composites) developed along with various sizes of NPs makes the matrix of nanomaterials to be tested very huge. Screening of the huge number of nanomaterials at an *in vivo* level cannot be done expeditiously, would be costly and faces ethical concerns.

A large number of *in vitro* tests are available, and we need to perform more than one type experiment to be sure about the results. In such a condition, we need very sensitive, rapid, cost effective and easy to operate analytical tools in labs. Single cell has been shown to be more sensitive towards a smaller change than population of cells in a shorter time [90].

To analyze single cells, sorting of a single cell from population of cell is necessary. One of the most frequently used methods to quickly and efficiently sort, count and/or measure the characteristics of single cells in large volume (large throughput) is flow-cytometry (FCM). However, it requires cells to be tagged with fluorescent probes that may interfere with the natural behavior of the cell. A technique that measures cell behavior in its natural state would be ideal. Patch clamp and carbon fiber microelectrode based techniques are good for single cell analysis; however, they require cumbersome and expensive equipment, as well as, a trained professional to conduct the experiments. Performing these techniques can be very time consuming and yield a low throughput. On the other hand, using a chip based approach to sort and analyze single cells out of a population could be more cost effective, simpler and yield a high throughput performance. Microelectrodes on the chip integrated with microfluidic control and automation would not only make the initial nanotoxicity screening easy, but also increase the participation of various research and regulatory authorities, and encourage nanotoxicity assessment efforts throughout the world. A COC-based assay will provide more dynamic information of cell and particle interaction. Currently, not all the labs that work with nanomaterials have the facilities and equipment to perform traditional nanotoxicity assays. The COC-based assay can be an initial screening point for nanotoxicity.

CHAPTER 2- DESIGN AND FABRICATION OF A LAB-ON-CHIP DEVICE FOR SINGLE CELL TRAPPING

Major portion of this chapter can be cited as; **Pratikkumar Shah**, Xuena Zhu, Chunying Chen, Ye Hu, and Chen-Zhong Li, “*Lab-on-Chip Device for Single Cell Manipulation and Analysis.*” **Biomedical Microdevices**, 2014. 16(1): p. 35-41

2.1 Introduction

To analyze a single cell, sorting of a single cell from a population of cells is necessary. One of the most frequently used methods to quickly and efficiently sort, count and/or measure the characteristics of single cells in large volume (large throughput) is flow cytometry (FCM). Cells can be tagged with different fluorescent markers and simultaneous measurement of multiple fluorescent signals, as well as light scatter-induced illumination of single cells or microscopic particles in suspension can also be detected [91]. However, this technique cannot support real time measurements of cells in their natural environment. The capacitance based patch clamp is a very sensitive technique to detect dynamic cell signaling, but the electrode interferes with the cell membrane and requires complex set-up [92]. A needle electrode based technique is also a very sensitive approach for spatio-temporal single cell analysis, however, like patch clamp, it also requires complex and time consuming set-up, need for a trained professional to operate and manipulate precise positioning of electrodes and pipettes, and has low throughput [93, 94].

Today, several lab-on-a-chip (LOC) cell immobilization and manipulation methods have been developed, such as, microwell, micro chambers, dams, traps or single cell adhesion through functionalized surfaces. These LOC devices have employed acoustic [95], magnetic [96, 97], optical [98, 99], hydrodynamic [100, 101], mechanical [102, 103], and electrical [104, 105] approaches to aid trapping of cells. Acoustic, magnetic and optical cell sorting techniques require additional labeling with antibody conjugated micro/nanoparticles for cell sorting. However, additional labeling of cells may induce changes in physiological property of cells. A hydrodynamic approach is a good passive

approach for cell sorting where cells flow through the microchannel at a controlled flow rate. The main challenge in hydrodynamic capturing is that it requires a precise microfluidic control of multiple streams to employ cell sorting. Mechanical cell sorting approaches are based on the microfabricated structural filters where cells are separated or captured based on its morphology. The filter structures can be blocked and intensive surface interactions during the filtration process can cause significant shear forces on sorted cells [106] in such designs. Surface functionalization to capture high-throughput array-based single cell employs modification surfaces with cytophilic and cytophobic materials to attract and repel cells, respectively. Dielectrophoresis is an effective and noninvasive technique to manipulate single cells efficiently. It allows label-free, shear stress-free, and strong deflection as well as fast response times. Microwell structure can support time elapsed study of a single cell. Dielectrophoresis integrated with microwell structure has been used before [104, 105] where it was applied in an array format and control of the individual electrodes was not functional. Here, we present an active microwell LOC device for controlled capturing of cells and the same microwell electrode can be used to measure the cell behavior without any external detection mechanism. Individual control of each microwell on the chip allows capturing of a single cell inside a chosen microwell or all microwells simultaneously in less than 30 seconds. Sensitive microelectrodes allow the device to be used for high throughput applications with precise and easy control for single cell analysis in drug screening, and cytotoxicity or nanotoxicity studies.

2.2 Experimental Section

2.2.1 Chemicals and reagents

Hydrogen peroxide (H_2O_2), Sulfuric Acid (H_2SO_4), Acetone, Methanol, and Isopropanol were purchased from Fisher Scientific Inc. and used as received. 0.01 M (1X) Phosphate Buffered Saline (PBS) (Ph =7.4), cysteamine hydrochloride (Santa Cruz Biotechnology Inc.), F-12K with L-glutamine Medium (ATCC, VA, USA), sucrose (BDH, VWR, PA, USA), dopamine hydrochloride (Sigma-Aldrich, MO, USA), mPEG-NH₂ (Nanocs Inc., NY, USA).

2.2.2 Cell culture and solutions

The rat pheochromocytoma cell-line, PC-12 (CRL-1721.1) was obtained in a frozen vial from the American Type Culture Collection (Manassas, VA, USA). PC-12 cells were cultured in a 75 cm² culture flask at 37 °C in a humidified atmosphere containing 5% CO₂ in an incubator.

We used F-12K Medium (ATCC, VA, USA) supplemented with 2.5% Fetal Bovine Serum (Gibco, Life Technologies, NY, USA), 15% horse serum (Gibco, Life Technologies, NY, USA) and 1% Penicillin-Streptomycin (Sigma-Aldrich, MO, USA) as the culture medium for PC-12. The growth medium was changed at every 48 hours. Once the cell culture was confluent (after 2-3 days) the cell-culture was trypsinized and centrifuged to collect the pellet of cells. Cells in the culture medium were centrifuged at 1700 rpm for five minutes. The supernatant culture medium was carefully removed and the cell pellet was gently washed two times with 0.2 M sucrose buffer before finally re-suspending the cells in 0.2 M sucrose buffer at a concentration of 2×10^5 cells/ml for dielectrophoresis (DEP). The average diameter of PC12 cells in sucrose media was

noticed to be about $12 \pm 3 \mu\text{m}$. The cells are also re-suspended in fresh F-12K for other experiments carried out in chapter 4.

Isotonic saline bath solution was used as an electrolyte during the single-cell exocytosis experiment. Isotonic saline bath solution consisted of (in mM): 150 NaCl, 4 KCl, 0.7MgCl₂, 10 HEPES, and 11 glucose, pH 7.4).

The depolarizing buffer (high K⁺ solution) used to stimulate cell exocytosis consisted of 100 mM KCl, 55 mM NaCl, 5 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES and 10mM glucose.

2.2.3 Design and fabrication of the LOC device (version 1a and 1b)

I followed the traditional lithography technique for fabrication of LOC device. It is an optical means of transferring a pattern on a mask to the surface of a silicon/glass wafer. In the process, the patterns are first transferred to the photoresist (photoresist is a liquid film that can be spread out on a substrate, exposed with a desired pattern, and developed in the developer solution). Detailed steps involved in the fabrication process are explained as follows with information about the design of the device.

For the LOC device version 1a, 2 rows of 4 electrodes were designed such that they maintain a distance of 200 μm between them to avoid any cross talk. A bigger size reference electrode of 400 μm was created on the chip to avoid any need of an external electrode in a microfluidic chip. The reference/counter electrodes are designed on both sides of the sensing electrodes and only one reference electrode is used at a time during the electrochemical measurements. The size of the reference electrode was very big (20 times larger) as compared to working electrodes, which avoids any capacitive effects.

Figure 2.1: Schematic of lithography process layout. (a) Step by step representation of the lithography process to fabricate microchip; Cr/Au thin film deposition on a glass wafer, patterning of Cr/Au films for desired sensing electrodes and connection pads, spin coating dielectric layer of SU-8 photoresist, and patterning of SU-8 photoresist for the desired microwell size. (b) Schematic representation of the chip assembly (version 1a), and (c) microchip (version 1a) wire-bonded to PLCC adapter.

Glass (borosilicate) wafers (University wafers, USA) of 4 inch diameter were cleaned in piranha solution ($\text{H}_2\text{SO}_4:\text{H}_2\text{O}_2$ in a ratio of 3:1) (Sigma-Aldrich) for 20 minutes and thoroughly rinsed in running deionized (DI) water for at least 5 minutes. The wafer was blow-dried with a nitrogen stream and left on a hotplate at 105°C for five min to remove the moisture. Chromium (Cr) adhesion layer of $250\text{ \AA}$ thickness and subsequently $2500\text{ \AA}$ thickness of gold (Au) layer was deposited using Ion beam evaporator (CHA Industries, CA, USA). The conductive thin films were patterned using photolithography. A positive photoresist, AZ1518 (AZ Electronic Materials, NJ, USA) was spin coated (Headway Research, Inc., TX, USA) onto the glass wafer for $1.5\text{ }\mu\text{m}$ thickness in two steps; first at 500 rpm with an acceleration of 100 rpm for five seconds and the second at 4000 rpm

with an acceleration of 1000 rpm for 30 seconds. The wafer was then heated at 115 °C on a hotplate for five minutes and cooled down to room temperature. The chrome based photomask (Nanofilm, CA, USA), already prepared for the desired pattern designed in Layout Editor (<http://www.layouteditor.net/>), was used with a mask aligner (OAI Model 800 MBA, CA, USA) and illuminated with $\sim 10.5 \text{ mW/cm}^2$ UV light for 8 seconds. The photoresist was developed with AZ400K: DI water in a proportion of 1: 4, washed with DI water, and blown dry with a nitrogen stream. The photoresist was then post baked on the hotplate for about three min. The unwanted Au layer was etched for ~ 30 seconds using the gold etchant and the wafer was thoroughly rinsed in DI water. The unwanted Cr layer, exposed from the etched gold, was etched with Cr etchant for 90 seconds. The wafer was thoroughly washed with DI water and then dipped in acetone to lift-off the photoresist and to expose the patterned gold electrodes and connection pads.

The wafer was cleaned again on spin coater with acetone and methanol, dried at 115 °C for five minutes on a hotplate and allowed to cool down to warm temperature (above room temperature) before spin coating SU-8 photoresist. The slightly increased temperature of a glass wafer was required for improvement of the SU-8 adhesion on a glass wafer. SU-8 2025 negative photoresist (Microchem Co., MA, USA) was spun coated (Headway Research, Inc., TX, USA) onto the wafer for 20 μm thickness in two steps; first at 500 rpm with an acceleration of 100 rpm for five seconds and the second at 3500 rpm with an acceleration of 350 rpm for 35 seconds. The wafer was then soft baked at 65 °C on a hotplate for 1 minutes and then the temperature was ramped to 95 °C and the wafer was allowed to be baked for five minutes at 95 °C. The wafer was then allowed to cool down and rest for 60 minutes before the exposure. Another, chrome based

photomask (Nanofilm, CA, USA), prepared for SU-8 patterning, was used with a mask aligner (OAI Model 800 IR, CA, USA) and illuminated with $\sim 10.5 \text{ mW/cm}^2$ UV light for 27 seconds. After exposure, the wafer was then post baked at 65°C on a hotplate for 1 minutes and then the temperature was ramped to 95°C and the wafer was allowed to be baked for five minutes at 95°C . The wafer was allowed to cool down and rested for 20 minutes before the development. The photoresist was developed with SU-8 developer (Microchem Co., MA, USA) for three minutes, rinsed with Isopropanol, and blown dry with a nitrogen stream. The SU-8 pattern was created such that only the sensing part ($20 \mu\text{m}$ cell capturing microwells and reference electrode), and connection pads were exposed, while the rest of the SU-8 layer served as a dielectric passivation layer to avoid any cross talk. The wafer was cleaned by sonication in DI water for five minutes and in oxygen plasma RIE for removal of any organic contaminant and uncross-linked SU-8. Oxygen plasma RIE also improves the hydrophilicity of the SU-8 photoresist surface. The wafer was hard baked on a hotplate at 150°C for 30 minutes to make the SU-8 chemical resistant. Thickness of SU-8 was measured by a profilometer (Alpha-step 200, Tencor, CA, USA). The processed glass wafer was then diced to collect individual chip by first making marks with CO_2 laser and eventually by glass cutter diamond scribe. The individual chip die was cleaned in ethanol and then again in DI water (5 minutes sonication) before the experiment.

The second design, version 1b is fabricated using the identical design and fabrication parameters used for the development of the chip version 1a. The size of the reference electrodes and the sensing electrodes also remain the same. The only difference is that the version 1b has 8 electrodes in 2 rows and the chip connection pads are of larger size to fit

with the clip connection as depicted in Figure 2.2c. During the absence of wedge bonding machine, version 1b served the same sensitivity of version 1a.

Figure 2.2: Schematic of the LOC device, version 1b (a). Microscopic photograph, scale bar 50 μm (b). Picture of the LOC device connected to the holder (c).

2.2.4 Electrochemical characterization of the chip

The cell analyzing devices were electrochemically characterized by cyclic voltammetry (CV). The LOC version 1b is used for all cellular experiments explained in following chapter, hence, electrochemical characterization of LOC version 1b is only summarized here. CV experiments were performed in PBS (pH = 7.4) solutions containing 5 mM potassium ferricyanide ($[\text{Fe}(\text{CN})_6]^{3-/4-}$), using a two electrode electrochemical cell (on chip gold electrode was used as the reference/auxiliary electrode). The potential was scanned from either -0.4 to 0.4 V or -0.6 to 0.6 V, versus gold reference electrode at 100 mV/s using a CHI660C potentiostat (CH Instruments, TX, USA). All electrochemical measurements were performed at room temperature (~ 25 $^{\circ}\text{C}$).

2.2.5 Surface modification

Surface functionalization provides the benefits of the selective modification of the surface. A cytophilic surface will promote the cell attachment and a cytophobic surface will halt any non-specific cell adhesion on the surface that may create noise during measurements. A surface modification method used in an earlier study was applied [107] with a minor modification. SU-8 resist surface was modified using paper-stamp technique sequentially by poly dopamine (for 8 hours) and mNH₂ linked PEG (1 hour) whereas gold surface was modified with 100 μ M cysteamine or cysteine (30 minutes). In paper-stamp technique, a Whatman grade 1 paper of a size that covers the essential electrodes and surrounding regions on the chip was wicked in chemical solution (either poly dopamine or mNH₂ linked PEG) and placed on the chip. The chip containing the paper strip was secured in a petri dish covered with paraffin film for appropriate time. –SH group of cysteine will covalently bind to the gold surface and develops self-assembled monolayer (SAM) and the free amine group will favor the cell attachment by attracting carboxylic groups available on the cell membrane [108].

2.2.6 Microfluidic channel

For DEP application, an approach similar to earlier studies was used [105, 109]. A microfluidic channel of cross-section of 0.4 mm² (4 mm wide and 0.1 mm height) and 1 cm length was prepared using double sided adhesive spacer. An ITO electrode (Delta-technologies Inc., CO, USA) was placed on top of the microchannel for generating non-uniform AC field in the microchannel and supporting the flow of cells. Cell suspension of 2×10^5 cell/ml prepared in 0.2M sucrose buffer was allowed to flow at a rate of 5-10 μ l/min while DEP force is applied for single cell trapping. Once the cells are captured

(within 1 minute), the remaining cells in the microchannel were cleaned using plain sucrose buffer by maintaining the flow-rate of 30-50 $\mu\text{l}/\text{min}$. The final assembled chip is depicted in Figure 2.1c. The prepared microchip was then wire-bonded to the PLCC adapter to provide a robust platform and easy connections to analyzer and signal generator. The schematic of the final assembled chip is depicted in Figure 2.1b and Figure 2.1c shows the actual assembly.

2.3 Dielectrophoresis (DEP) Theory

DEP is generated due to interaction between any dielectric particle's dipole movement and spatial gradient of the electric field [110]. DEP is illustrated in Figure 2.3. In a uniform electric field, neutral particle feels the dipole moment but do not move in either electrode direction due to the zero net force, whereas charged particles are attracted towards the opposite electrode as illustrated in Figure 2.3a. When a neutral particle is placed in a nonuniform electrical field, two halves of the induced dipole experiences different force magnitude and thus the net force is produced as shown in Figure 2.3b. DEP phenomenon can be used to move and manipulate polarizable micro-particles such as cells, markers, etc. suspended in liquid medium [111]. The ability of DEP forces to manipulate suspended particles remotely without any contact has a significant potential for applications in μTAS (micro total analysis systems) [111]. The nonuniform AC field can be generated by microelectrodes. Several designs, ranging from simple planar electrodes to complex 3-D structures have been investigated for biomolecules manipulations. Cells, cellular particles, DNA particles, and synthetic marker particles treated with biochemical tags can be separated, collected, concentrated using

microelectrodes [111]. DEP force does not depend on the polarity of the electric field and thus can be produced by either DC or AC electric field.

Figure 2.3: Illustrations of Dielectrophoresis (DEP) forces.

The time averaged dielectric field, F_{DEP} exerted on cells suspended in liquid may be approximated by following equation;

$$F_{DEP} = 2\pi R^3 \epsilon_m \text{Re}[f_{CM}(\omega)] \nabla E^2 \quad (2.1)$$

Where R = radius of cell; ϵ_m = absolute permittivity of suspending medium ($\epsilon_r \times \epsilon_0$), ϵ_c = permittivity of cell; ∇E = rms value of applied A.C. field, ω = angular velocity of the applied field, $\text{Re}[f_{CM}(\omega)]$ is the real part of the Clausius–Mossotti (CM) factor (polarization factor) given by the equation;

$$f_{CM}(\omega) = (\epsilon_c^* - \epsilon_m^*) / (\epsilon_c^* + 2\epsilon_m^*) \quad (2.2)$$

here, ϵ_m^* and ϵ_c^* are the complex electrical permittivity of suspended medium and cell, respectively. $\epsilon^* = [\epsilon - (j\sigma)/\omega]$, where σ is electrical conductivity and $j = \sqrt{-1}$.

Observation of equation 2.1 clarifies the dependence of F_{DEP} on frequency is only resides in CM, and spatial dependence in applied electric field. R^3 suggest that the force

value increases significantly with the increase in particle size. The exerted dielectric field on the cell can be either positive or negative and the real part of $\text{Re}[f_{\text{CM}}(\omega)]$ suggests that, it can be controlled by adjusting the conductivity of suspending medium and frequency of the applied field. The cell will be attracted or repelled from the electric field region depending on real the part of the polarization factor $\text{Re}[f_{\text{CM}}(\omega)]$ value. When the relative polarizability of a particle (i.e. cell here) is greater than that of suspending medium, the particle will experience positive DEP (pDEP) and will be attracted towards the maxima of the electrical field. On the other hand, when the polarizability of suspending medium is higher than that of suspending particle, then the particle will experience negative DEP (nDEP) and it will be repelled from the maxima of the electrical field gradient. Simply, When $\text{Re}[f_{\text{CM}}(\omega)] > 0$ ($\epsilon_c > \epsilon_m$), it refers to attraction; if $\text{Re}[f_{\text{CM}}(\omega)] < 0$ ($\epsilon_c < \epsilon_m$), it corresponds to repulsion.

Equations 2.2 is valid for a homogeneous sphere, however, biological cells generally possess cell membrane (and more intracellular layers) or a shell outside the core sphere. In such conditions, equation 2 needs to be updated with an effective permittivity of a cell, ϵ_c^* instead of ϵ_c for a single shell sphere model [110, 112]. For a cell with a cell membrane, the effective permittivity can be given by;

$$\epsilon_c^* = \epsilon_{\text{mem}}^* \left[\frac{\left(\frac{R}{R-d}\right)^3 + 2\left(\frac{\epsilon_{\text{cyto}}^* - \epsilon_{\text{mem}}^*}{\epsilon_{\text{cyto}}^* + 2\epsilon_{\text{mem}}^*}\right)}{\left(\frac{R}{R-d}\right)^3 - \left(\frac{\epsilon_{\text{cyto}}^* - \epsilon_{\text{mem}}^*}{\epsilon_{\text{cyto}}^* + 2\epsilon_{\text{mem}}^*}\right)} \right] \quad (2.3)$$

Where d = cell membrane thickness, $\epsilon_{\text{mem}}^* = \epsilon_{\text{mem}} - \frac{j\sigma_{\text{mem}}}{\omega}$ and $\epsilon_{\text{cyto}}^* = \epsilon_{\text{cyto}} - \frac{j\sigma_{\text{cyto}}}{\omega}$

CM factor will be dominated by relative conductivities at low frequencies and by relative permittivities at high frequencies. At low frequencies CM can be represented as;

$CM = (\sigma_c - \sigma_m) / (\sigma_c + 2\sigma_m)$, whereas, at high frequencies, $CM = (\epsilon_c - \epsilon_m) / (\epsilon_c + 2\epsilon_m)$.

The relaxation time between separating these two limits is given by Maxwell-Wagner interfacial polarization factor, $\tau_{MW} = (\epsilon_c + 2\epsilon_m) / (\sigma_c + 2\sigma_m)$.

This Maxwell-Wagner interfacial polarization causes the frequency variations in the CM factor. It is due to the competition between the charging process in the particle and medium, resulting in charge buildup at the particle/medium interface.

We suspend our cells ($\sim 2 \times 10^5$ per ml) in 0.2 M sucrose buffer ($\sigma_m = 0.002$ S/m), which is very resistive in nature. Parameters of PC12 cells are assumed from various references [113] with changes in $R = 6 \times 10^{-6}$ m, and $\sigma_m = 0.002$ S/m. The parameters are given in the Table 2.1 and the CM for these values is shown in Figure 2.4b for single-shell cell shown in Figure 2.4a. The CM calculation suggests a pDEP force at 1 MHz frequency and will attract the cell towards the field maxima.

Table 2.1: PC12 cell parameters assumed for CM calculation

Medium permittivity, ϵ_m	$80 \times \epsilon_0$	Medium conductivity, σ_m	0.002 S/m
Cell membrane permittivity, ϵ_{mem}	1.8E-12	Cell membrane conductivity, σ_{mem}	10^{-8} S/m
Cell cytoplasm permittivity, ϵ_{cyto}	7.1E-10	Cell cytoplasm conductivity, σ_{cyto}	0.75 S/m

$$\epsilon_0 = 8.854 \times 10^{-12} \text{ F/m}, R = 6 \times 10^{-6} \text{ m}, d = 5 \times 10^{-9} \text{ m}$$

Figure 2.4: Diagram of a single-shell spherical cell in suspension (a), and the spectrum of real part of CM factor for a cell in suspension, calculated from parameters provided in Table 2.1, showing positive dielectrophoresis effect from frequencies higher than 500 KHz (b).

2.4 Results

2.4.1 Optical characterization of microelectrodes

Figure 2.5a and 2.5b show the optical bright and dark field images, respectively of electrodes and the SU-8 pattern. The exposed circular area (cell capturing/sensing well) is seen in the image as a contrast circle is responsible for contributing to the signal. Figure 2.5c and 2.5d show the bright and dark field images, respectively of a single cell capturing electrode.

Figure 2.5: Optical characterization of microchip; a) and b) bright and dark field image at 5X magnification, respectively; scale bar 100 μm . c) and d) bright and dark field images at 100X magnification, respectively; scale bar 5 μm .

2.4.2 Electrochemical characterization of microelectrodes

The microelectrodes responses were recorded for three times and the average of three potential sweeps is depicted in Figure 2.6a for each microelectrode, when recorded at 100 mV/s scan rate in 5 mM potassium ferricyanide solution ($[\text{Fe}(\text{CN})_6]^{3-/4-}$). These responses from individual electrodes are plotted simultaneously for comparison in Figure 2.6b and the average of these 8 superimposed cyclic voltammetry responses is plotted in Figure 2.6c with standard error of mean. The average peak current was recorded to be of 3.44 nA. Figure 2.7a shows the cyclic voltammetry response of a single electrode at various scan rates between 100-500 mV/s where the increment in peak current is evident with the increased voltage scan rate with a standard reversible behavior of a microelectrode in the ferricyanide solution. Figure 2.7b shows the Randles-Sevcik plot of the anodic and cathodic peak current values with respect to the square root of the scan rate. A linear response of peak current values with respect to the square root of voltage scan rate is noticed here. It is evident that the electrode possesses a very good linearity and electron transfer is surface controlled.

Figure 2.6: Electrochemical characterization of microchip; (a) Simultaneous cyclic voltammetry of 8 electrodes (b) Cyclic voltammetry of an electrode at different scan rate from 0.1 V - 0.5 V; (c) Anodic and cathodic peak current vs square root of voltage scan rate.

Figure 2.7: (a) Cyclic voltammetry of an electrode at different scan rate from 0.1 V/s - 0.5 V/s (b) Anodic and cathodic peak current vs square root of voltage scan rate.

Dopamine and norepinephrine are two major components of PC12 cell exocytosis. Measurements of PC12 cell-exocytosis are explained in chapter 3. Figure 2.8a and 2.8b shows cyclic voltammograms for dopamine and norepinephrine, respectively.

Figure 2.8: Representative steady-state cyclic voltammograms recorded on the microwell electrode in isotonic buffer containing (a) 100 μ M dopamine at scan rate of 10 mV/s and (b) 100 μ M of norepinephrine with scan rate of 100 mV/s.

2.4.3 Single cell trapping

PC12 cells have shown poor adhesion to the surface. It is important to have a better adhesion of PC12 cell on the gold electrode to minimize the distance between cell and electrode and avoid the cell to be washed away in the microchannel. Better adhesion of the cell to the electrode will result in lesser noise.

Figure 2.9: Single cell capturing using pDEP technique; (a) at 10 s, (b) 15 s, (c) 19 s and (d) 20 s. Images were taken from a cell capturing video.

When DEP force was not applied and only the surface modification approach was in practice, 50 μl of the cell suspension in isotonic medium (2×10^5 cells/ml) were incubated on top of the LOC device surface for 10-60 minute range. We found poor adhesion of PC12 cells to the gold electrode surface for a short incubation time (~ 10 -30 minutes) and the cells were readily washed away when we applied a gentle cleaning buffer (isotonic medium) to get rid of the extra cells surrounding the electrode area. Whereas for a higher incubation period (~ 30 -60 minutes) we observed clumps of PC12 cells aggregated near the electrode surface. We noticed the ability of PC12 cells to form clumps readily and make it harder for a single cell to be captured in a microwell. Clumps of PC12 cells have also been noticed in earlier studies [114, 115]. Cell trapping due to gravitation method usually takes several minutes and the cell retention rate in the microwell is also very low [116]. Cell trapping using surface modification feature also takes few minutes to capture a cell on the electrode surface and it will not be selective for individual electrode. To overcome these challenges we applied DEP principle, in addition to the surface modification to capture single PC12 cells. With the DEP, we can control cell capture on an individual electrode separately and simultaneously for selective trapping of a cell in a particular microwell.

In the microfluidic channel, a cell suspension of 2×10^5 cell/ml prepared in 0.2 M sucrose buffer was applied at one end and allowed to flow through the other end via capillary action. To generate DEP, sinusoidal waveforms of 3 V_{pp} at 1 MHz frequency were applied to the sensing microwell electrode and top Indium Tin Oxide (ITO) plate electrode with a common ground and 180° out of phase alignment. This difference in size of microwell sensing electrode (1256 μm^2) and top ITO plate electrode (30 mm^2) creates

a non-uniform AC field between two electrodes. Permittivity of the medium and cell make the DEP force in the direction of microwell electrode. The cell will be trapped on top of the microwell electrode when it travels from the pDEP region. Within 30 seconds (Figure 2.9), we achieved the single cell capturing on individual electrodes with this technique. Additional cells in the micro-channel were cleaned by added sucrose buffer flow from the channel. After cleaning, the sucrose medium was replaced by isotonic buffer and the cells were allowed to rest for 15 minutes in the incubator for better cell attachment to the electrode. The top ITO electrode was removed carefully, and the positions of the cells were confirmed by an upright microscope before taking any amperometric measurements.

We used trypan blue in order to verify the cell viability after capturing cells using pDEP. 10 μ l of trypan blue solution was inserted after 15 minutes of cell capturing. Cells captured in the microwells were confirmed alive under the microscope as they did not stained blue. Rare dead cells were observed outside the microwell in the microfluidic channel or outside the microfluidic channel on the chip.

2.5 Discussion

When a metal electrode is covered by polymer coating, the resultant capacitance can be defined as

$$C = (A * \epsilon_0 * \epsilon_r) / d \quad (2.4)$$

Here, $\epsilon_0 = 8.86 \times 10^{-14}$ F/cm (permittivity of free space), ϵ_r is the relative permittivity of the polymer coating, A is the area of coating exposed to the electrolyte, and d is the coating thickness. When the coating thickness is less, the effect of capacitive component increases in impedance spectroscopy measurement.

The design criteria were chosen carefully so that the SU-8 coating thickness does not create a coating capacitance during the measurement of cell response. Earlier, it has been established [117] that the ratio of the coating area (cm²)/ coating thickness (cm) should be less than 5.5 to avoid the interference of coating capacitance during impedance spectroscopy measurements. The electrical connections between bond-pad/connection-pad and reference/working electrodes were fabricated by 20 µm wide gold patterned traces on the chip. The maximum area of electrical connections covered by SU-8 photoresist exposed to the media at any time does not exceed 0.00204400 cm². For 20 µm thick photoresist, the ratio is calculated to be 1.02, which is less than 5.5 and hence, satisfactory to neglect the coating capacitance effect during the impedance spectroscopy measurements. This ratio for version 1b chip was calculated to be less than 3.4.

Electrochemical testing immediately before the measurement provides the opportunity to remove damaged chips or electrodes and improves reliability of experiments. Characterization of reproducibility and electrochemical activity was carried out by cyclic voltammetry (CV) in potassium ferricyanide solution ([Fe(CN)₆]^{3-/4-}), prepared in PBS (pH=7.4) buffer. CV is the most widely used technique for acquiring qualitative information about electrochemical reactions.

It is evident from Figure 2.6a that the cyclic voltammetry response of microelectrodes is steady state current limiting sigmoidal shapes, a typical sign of a microelectrode. During the potential sweep, the potentiostat measures the redox current resulting from the applied potential using the Randles–Sevcik equation [75];

$$i_p = (2.69 \times 10^5) n^{3/2} A C D^{1/2} \nu^{1/2} \quad (2.5)$$

where i_p is the peak current, n is the number of electrons, A is the surface area of the working electrode, C is the bulk concentration of the electroactive species (5 mM), D is the diffusion coefficient of the electroactive species ($\sim 7.2 \times 10^{-6} \text{ cm}^2/\text{s}$ for potassium ferricyanide [118]), and v is the scan rate of voltammograms. The theoretical value of the peak current calculated from the equation 2.5 is 3.59 nA and the recorded value of the fabricated chip was found to be 3.44 nA, only 4% lower than the theoretical value, suggesting a good fabrication of the microelectrodes. The variability between different electrodes of the same chip was calculated as a standard error of a mean to the average and found to be only of ± 0.09 nA. The peak current of microelectrode was found to be proportional to the squared root of voltage sweep rate as suggested in equation 2.5 and shown in Figure 2.7. The linearity indicates a good microelectrode surface controlled electron transfer.

Non-functionality of the wire bonding instrument at FIU forced us to redesign the connection pads as shown in schematic of Figure 1.2a. Instead of 4 electrodes in a row as in the design version 1a, the second design includes 8 microwell-electrodes in a row and the reference electrodes in the left and right corners. The connection pads of the second design are modified to accommodate the clip connection instead of wire bonding as shown in Figure 1.2c. The design includes the reference electrode at the corner and the distance of the reference electrode varies from the working electrode, which resulted in intra electrode limiting current variation of ± 90 pA when recorded in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ solution at 100 mVs^{-1} scan rate. The electrodes showed very good stability for repetitive runs with less than 10% variation in the measured values of currents. An

updated design would require for comparison of single and small cell population, which is explained further in chapter 4.

Frequency and applied voltage play a critical role in the entrapment of cells. 1 MHz frequency is proven to be noninvasive for many cell lines, including PC12 cells [104, 105, 119, 120]. The calculations for the chosen parameters of PC12 cells and sucrose medium suggest that 1 MHz frequency is capable of generating pDEP effect on the cells as explained in section 2.3 of this chapter. The applied voltage was optimized to 3 V_{pp} as when we applied the voltage lower than 3 V_{pp}, the pDEP force generated was not high enough (or was lower than the capillary drag force) to capture the cell. When a single cell is captured in a 20 μm diameter microwell, the chances of another cell being able to go in the same microwell are scarce because there will be no physical space for another cell and the occupied cell will also reduce the pDEP force by blocking the electric field.

2.6 Conclusion

In this study, a novel DEP based microfluidic array device was successfully used to capture single cells. Cyclic voltammetry (CV) was used to characterize microelectrodes and linear diffusion controlled reaction was measured. The challenging task of single cell trapping showed a high success rate of precise control capturing of single cells in the chosen microwell in less than 30 seconds. Such new concept of active microwell array combined with highly sensitive electrodes promises high throughput assay of single cells in a controlled manner. The application of this lab-on-chip platform holds potential to be used in drug screening, biomarker detection and cytotoxicity analysis and has been used with an updated design for nanotoxicity measurement described in the following chapters.

CHAPTER 3- RAPID ASSESSMENT OF NANOTOXICITY ON QUANTAL EXOCYTOSIS FUNCTION IN A SINGLE PC12 CELL

3.1 Introduction

Metal containing nanoparticles (NPs) are commonly utilized in consumer products. The utilization of NPs is also being extensively proposed for diagnostic and therapeutic applications associated with brain. Therefore, nanotoxicity assessments of these NPs are crucial for appropriate use.

In traditional methods, the results are correlated and assumed to be equally contributed to all cells of the population under study. However, this theory is weakened by recent advancements in single cell studies, and it has been shown that individual cells behave differently from the population even under identical conditions [33, 36, 121]. The study of single cell dynamics could help us better understand the complex processes, such as, neurotransmitter kinetics, ion channel functions, and cell communications [36, 121]. The study of single cells are often recommended for a clear understanding of the heterogeneous cell populations, such as, neurons, stem cells and cancer cells. Therefore, single cell analysis can be an equivalent and complementary strategy to existing approaches.

Carbon fiber microelectrodes (CFM) have been used to study the impact of nanoparticles on critical cell functions, *i.e.* exocytosis. Exocytosis is a process whereby transmitter-loaded intracellular vesicles fuse with the cell membrane and release their contents to the outside of the cell [107]. Studies of exocytosis behavior of neuronal cells are often associated with its functional and structural health [122]. Changes in exocytosis of PC12 cells have been linked to various disease-conditions and neuronal toxicity of heavy metal ions, organic solvents, and environmental pollutants [122], therefore, the

study of exocytosis upon NPs exposure could provide a sensitive means of measuring nanotoxicity.

A recent advancement in nanotoxicity assessment using CFM amperometric method has provided real-time dynamic vesicle kinetics of cells upon nanomaterial exposure [73, 74, 77]. CFM amperometric method has been used to test the effect of different nanoparticles' properties, such as different size of nanoparticles [77], surface charge [32] and surface functional groups [77], porosity and the composition of nanomaterials [73]. CFM is a highly sensitive technique but requires complex set-up and provides low throughput [123]. Moreover, a smaller difference in placement of the electrode distance from the cell can generate large differences in the signal. To overcome the basic limitations of traditional dye-based assays, and improve the efficiency and throughput of CFM amperometric method, we have employed single cell integrated Lab-on-chip (LoC) approach to explore the results of metal-containing nanomaterial composition on cell signaling (*i.e.* exocytosis) kinetics. The utilization of NPs is being extensively proposed for diagnostic and therapeutic applications associated with brain. In this study, we selected neuronal model cell line [122], PC12 cells, to study real-time spatiotemporal measurements of cell kinetics and measure the change in cell behavior upon nanomaterial exposure, in a single cell mode.

Metal containing NPs synthesized in different compositions of tunable properties using various routes are of particular importance because of their widespread applications. Specifically, CuO-NPs and TiO₂-NPs are the focus of this work for their extensive use in consumer products. CuO NPs have immense usage in electronics and technology as CuO-NPs hold excellent thermophysical properties [124]. CuO-NPs have

been used in gas sensor [125], solar cells [126], batteries [127, 128], and for catalytic processes [129]. CuO-NPs are used as pigments in ceramic and glass industries to produce different glazes. CuO is also used as a source of copper in dietary supplements against copper deficiency. Due to its ability to inhibit microorganism growth, CuO-NPs have been widely used as an antibacterial agent in different consumer products such as fabrics [130], sox [131], facemasks, and wound dressings [132]. TiO₂-NPs are widely used in cosmetics due to their ability to absorb ultraviolet (UV) rays. TiO₂-NPs are also used as antibacterials in textiles [133].

CuO offers a wide range of reactions and makes it very difficult to predict its role in neuron excitability. Copper can modulate several voltage gated (*i.e.* K⁺, Na⁺, and Ca²⁺ channels) and ligand-gated (*i.e.* glutamate receptors, GABA_A receptors and P2X receptors) ion channels [134]. Previous experimental studies have proven CuO-NPs to be extremely toxic compared to other metal oxide nanoparticles [43]. *In-vitro* studies by Wang *et al.* have shown a reduction in cell viability and increased reactive oxidative species upon CuO-NPs interaction [135]. These studies have shown that nanoparticles may not only affect the viability of the PC12 cells but can also affect their functionalities. CuO-NPs have displayed ROS generated toxicity in PC12 cells in a recently published study [136]. CuO NPs have shown to interfere and block sodium [137] and potassium [138] channels' current in CA1 neurons. However, these studies were performed as bulk assays, and the effect on single cell functionality was not considered. For the first time, we are trying to measure the effects of NPs on a single PC12, and verify that if we can collect any significant effect in a limited time period. To examine the immediate impact of nanoparticles, PC12 cells were exposed to 100 µg/ml CuO-NPs.

TiO₂-NPs have shown mixed results for cytotoxicity assays. It has been found that TiO₂-NPs can alter the exocytosis function of mast cells [139]. Increased intracellular ROS decreases the number of molecules released and the exocytosis frequency. TiO₂-NPs of 24 to 697 nm diameter size showed dose dependent decrement in cell viability of PC12 cells [140]. On the other hand, nanoporous TiO₂-NPs were nontoxic on MPMC/3t3 co-culture [73]. A single cell based study of cell-exocytosis mechanism might help us understand initial reaction of PC12 cell and consequent reactions.

Endocytosis and exocytosis of NPs is a common process when NPs comes in contact with cells, and the process highly depends on the property of NPs and cells [71, 141]. Regardless; it is a slow process (i.e. hours) and difficult to be monitored in real-time, consequently, requires a long duration study to identify the effects of NPs on cell's health/function. When studying the effects of NPs toxicity towards a neuronal cell's function, it is highly dependent on cells internalization of NPs. Co-application of NPs with 100mM K⁺ buffer will stimulate the exocytosis, consequently, endocytosis as well. Here, we try to amplify the effects of NPs to be visible immediately when comes in contact with a cell using stimulated cell exocytosis recording by amperometric approach. Toxicity assays are usually performed over a longer period, ranging from a couple of hours to few days. We intend to shorten this time by analyzing the immediate impact of nanomaterial exposure on cell function using a microelectrode array chip to make the initial toxicity screening simple and fast.

3.2 Experimental Section

The common approaches for cell culture, chip fabrication and characterization, and single PC12 cell trapping are explained in the experimental section of Chapter 2, section 2.2. The plain and single cell trapped LOC device is depicted in Figure 3.1.

Figure 3.1: Image of a cell on chip device when (a) empty (b) after capturing single cell; inset is a larger picture of a microwell containing a cell and circled microwell lost its hold of single cells during washing cycle. Scale bar is 50 μm .

3.2.1 Characterization of nanoparticles

TiO₂ and CuO NPs were purchased from Sigma- Aldrich in dry powder form (Cat No. 637254 and 544868 respectively). The NPs powder was suspended in isotonic buffer (1 mM) and then sonicated using a sonicator bath at room temperature for 20 minutes to form a homogeneous suspension. For size measurement, sonicated NPs stock solutions (1 mM) were then diluted to 0.1 mM solutions. The Dynamic light scattering (DLS) system was used to measure the hydrodynamic size and zeta potential of the NPs in the suspension.

3.2.2 Amperometric detection and data analysis

Amperometry measurements were performed using a CHI 660C (CH Instruments, TX, USA) as a two-electrode potentiostat. The working electrode was held at potential of 700 mV relative to the on chip gold reference electrode. All readings were taken at room temperature ($\sim 25^{\circ}\text{C}$) in a faradic cage to avoid external electromagnetic noise and used isotonic bath solution (PH=7.4) as an electrolyte. Cell's positions were confirmed on the microwell electrode by viewing it under an upright microscope before taking measurements. Amperometric signals were digitized at 1000 Hz and were digitally filtered using automatic filter selection in CHI user interface before saving them on computer hard-drive for further analysis. Baseline current for reference was measured for each cell without any stimulus. The exocytosis events were only accounted when the spike currents exceeds at least 3 times the baseline current.

To study the effect of nanoparticles on exocytosis in PC12 cells, 100 $\mu\text{g/ml}$ NPs (i.e. CuO-NPs and TiO_2 -NPs) solution in high K^+ buffer was prepared freshly. 50 μl of the nanoparticle containing solution was exposed on captured PC12 cells by dropping it gently on the chip. The recording was started within 10 seconds of the exposure and recorded for 60 seconds.

Amperometry signals were analyzed using an Igor procedure file programmed for the analysis of quantal exocytosis, provided by Dr. David Sulzer lab (<http://www.sulzerlab.org/>) [142], in IgorPro 6 (Wavemetrics, Lake Oswego, OR, USA). Threshold for peak detection was three times the current trace of the baseline current (i.e. noise). The noise can be selected by two points in the software and the threshold can be defined accordingly. Out of the myriad parameters measured in the software, particular

interest is given to peak half time ($t_{1/2}$), maximum peak current (I_{max}) and area under each spike or transferred charge (Q). Average number of neurotransmitter molecules released per spike (N) was calculated using Faraday's law of $Q = nFN$. Where Q is the charge, n is the number of electrons involved in the redox reaction ($n = 2$ for catacalomines), F is the Faraday's constant. The spike parameters were only considered for analysis when the peak current was at least 3 times greater than the background current and the spikes with negative charge were not analyzed. Overlapping current spikes and signals with weird shapes were also ignored. $T_{1/2}$ of PC12 cells found to be in the range of 2 to 10 ms, so the current spikes with $T_{1/2}$ value in this ranges were only selected for analysis. The spike analysis selection criterion may hide few exocytosis events, however, they are commonly applied to avoid false consideration of noise as exocytosis events [115, 142, 143]. The data is represented as average $\pm$ standard error of mean (SEM). Pair of data sets, control and a respective NPs exposed conditions, were compared using a double tailed student's t test. The threshold for significance was either 0.05 or 0.001.

3.3 Results

3.3.1 Nanoparticles characterization

Dynamic light scattering (DLS) was used to characterize the NPs. The hydrodynamic sizes and surface charges were measured using a zetasizer in isotonic buffer. The NPs were suspended in isotonic buffer initially to make the stock of 1mg/ml and then further diluted to 100 μ g/ml in isotonic buffer for analysis of their agglomerated sizes and zeta potential. The size and surface charge features of the NPs are summarized in Table 3.1. Zeta potential measurements revealed that TiO_2 -NPs had the surface charge of -26.9 mV,

and the average particle hydrodynamic diameter was measured to be 190 nm. CuO-NPs demonstrated mean agglomerate size of 208 nm, and the surface charge was -30.4 mV. The difference in size of NPs and zeta potential of TiO_2 and CuO was minor.

Table 3.1: Nanoparticles DLS properties in isotonic buffer

Nanoparticles	DLS size (nm)	Zeta potential (mV)
CuO	208	-30.4
TiO_2	190	-26.9

3.3.2 Amperometric analysis of PC 12 cell exocytosis

Figure 3.2: (a) Background signal of a single PC12 cell without high K^+ stimulation. (b) Current spikes from a single PC12 cell upon $20 \mu\text{l}$ of 100mM high K^+ stimulation. (c) Expanded region of the amperometric spikes selected in (b).

The background signal was recorded at 0.7 V against an on-chip gold reference electrode where we did not notice any significant peak (vesicular release) in the absence of a stimulating agent (Figure 3.2a). The background amperometric signal was obtained for each cell to identify the baseline noise and peak detection of the amperometric signal under the influence of external stimulus was only considered when the maximum current was at least 3 times higher than the background. There is a possibility of small spikes being masked when selecting this region, but it is necessary to avoid any false spike counts (i.e. noise) as well. Exocytosis was induced by adding 50 μ l of depolarizing buffer, and the recording started after 10 seconds of the external stimulus. Cell membrane depolarization started to be noticed within 10 seconds of excitation from high K^+ buffer and neurotransmitters release can be noted as upward current spikes in Figure 3.2b. In an earlier study, K^+ buffer was found to induce exocytosis within 6 seconds of the exposure [144], which is close to the experimental results attained in this study. Inset (Figure 3.2c) is showing a larger image of a selected spike. Figure 3.3 represents the statistical analysis of 150 spikes collected from five PC12 cells. We found mean value of $t_{1/2}$ to be 4.02 ± 0.14 ms, Q to be 84.7 ± 5.45 fC (0.44 ± 0.03 zmole), and $I_{\max}$ to be 19.2 ± 0.63 pA. The histogram plot matches with the characteristic spike parameters studied earlier for PC12 and chromaffin cells, with half time, maximum current and quantal release. Also, the quantal release shows normalized characteristics for $Q^{(1/3)}$ as previously described [145].

Figure 3.3: Quantitative analysis of 150 spikes recorded from 5 cells. Histogram of vesicle parameters; (a) full width at half maximum ($t_{1/2}$), (b) maximum peak current (I_{max}), (c) charge per spike (Q), and (d) normalized distribution of charge cube root ($Q^{1/3}$).

3.3.3 Effects of NPs interaction on PC12 cells' exocytosis mechanism

CuO offers a wide range of reactions and makes it very difficult to predict its role in neuron excitability. Copper can modulate several voltage gated (*i.e.* K^+ , Na^+ , and Ca^{2+} channels) and ligand-gated (*i.e.* glutamate receptors, GABA_A receptors and P2X receptors) ion channels [134]. Previous experimental studies have proven CuO-NPs to be extremely toxic compared to other metal oxide nanoparticles [43]. *In-vitro* studies by Wang *et al.* have shown a reduction in cell viability and increased reactive oxidative species upon CuO-NPs interaction [135]. These studies have shown that nanoparticles may not only affect the viability of the PC12 cells but can also affect their functionalities.

CuO-NPs have displayed ROS generated toxicity in PC12 cells in a recently published study [136]. CuO NPs have shown to interfere and block sodium [137] and potassium [138] channels' current in CA1 neurons. However, these studies were performed as bulk assays, and the effect on single cell functionality was not considered. For the first time, we are trying to measure the effects of NPs on a single PC12, and verify that if we can collect any significant effect in a limited time period. To examine the immediate impact of nanoparticles, PC12 cells were exposed to 100 $\mu\text{g/ml}$ CuO-NPs.

TiO₂-NPs have shown mixed results for cytotoxicity assays. It has been found that TiO₂-NPs can alter the exocytosis function of mast cells [139]. Increased intracellular ROS decreases the number of molecules released and the exocytosis frequency. TiO₂-NPs of 24 to 697 nm diameter size showed dose dependent decrement in cell viability of PC12 cells [140]. On the other hand, nanoporous TiO₂-NPs were nontoxic on MPMC/3t3 co-culture [73]. A single cell based study of cell-exocytosis mechanism might help us understand initial reaction of PC12 cell and consequent reactions.

100 $\mu\text{g/ml}$ concentrations of NPs used in this study are found to be toxic to PC12 cells. We also found that the effect of NPs on morphology of PC12 cells can only be observed after at least 2 hours of incubation with PC12 cells grown in petri dish. The toxicity induced at the molecular level cannot be detected rapidly in NPs infected cells using traditional assays, and cellular level damage takes even longer time to be monitored. However, the sub-cellular level changes can be recorded in real-time, immediately after the cells are exposed to NPs an amperometric set-up. In efforts to minimize the incubation time with NPs, we co-applied NPs with 100 mM K⁺ solution, while stimulating the exocytosis cycles in PC12 cells, to study the effects of cell

interaction with NPs on its exocytosis behavior. Depolarized cell membrane would enhance the endocytosis process [146, 147] and hence amplify the possibility of NPs to be internalized and direct interference with cellular organelles. Using amperometric technique, adverse effects of NPs on cells' exocytosis behavior can be monitored immediately. Figure 3.4 shows representative amperometric response of a single PC12 cell when exposed with different NPs and were further quantified for 5 cells in each condition.

Figure 3.4: Representative amperometric spikes from PC12 cells following exposures to different NPs dissolved in high K⁺ buffer. (a) 100 µg/ml CuO-NPs, and (b) 100 µg/ml TiO₂-NPs.

Cell function tends to change when it comes in an interaction with different nanomaterials. To reveal potential functional changes in surviving nanoparticle-exposed PC12 cells, dynamic/real-time measurement of exocytosis function was performed following cell exposure to CuO-NPs or TiO₂-NPs. 100 µg/ml of NPs dissolved in high K⁺

buffer was exposed to the PC12 cells and the measurement was taken within a minute of the exposure. Upon analyzing the spike parameters, we see different effects on the biophysics of exocytosis in a PC12 cell after exposures at the same mass concentration (Figure 3.5). The results are summarized in table 3.2.

Figure 3.5: Average amperometric spike parameters from PC12 cells in the control condition and after exposure to 100 $\mu\text{g/ml}$ CuO and TiO2. (a) Comparison of average peak half width time ($t_{1/2}$) of spikes. (b) Comparison of average spike area or charge release per spike (Q). (c) Comparison of average spike frequency of exocytosis events. (d) Table summarizing overall effect NPs on $t_{1/2}$, Q and number of events. Error bars represent SEM, and * indicates $p < 0.05$ whereas ** indicates $p < 0.001$.

Table 3.2: Average amperometric spike parameters from PC12 cells in control and NPs exposed conditions

	Control	CuO-NPs	TiO ₂ -NPs
Average half maximum time - $t_{1/2}$ (ms)	4.02 $\pm$ 0.14	3.40 $\pm$ 0.11	4.43 $\pm$ 0.17
Average catacalomine release - Q (fC)	84. 66 $\pm$ 5.45	30.62 $\pm$ 2.0	86.57 $\pm$ 4.33
Average exocytosis spike frequency (Hz)	0.5 $\pm$ 0.025	0.35 $\pm$ 0.028	0.38 $\pm$ 0.036
Data represented in the form of average $\pm$ SEM.150 spikes for the control, 105 spikes for CuO-NPs, and 114 spikes for TiO ₂ -NPs exposed cells were analyzed from 5 cells in each condition.			

100 $\mu\text{g/ml}$ of CuO-NPs mixed in 100mM K⁺ buffer exposed to stimulate the cells. We noticed faster exocytosis release kinetics (lower $t_{1/2}$ value) and also decreased quantal release (Q) per spike. The average charge per spike was found to be 30.62 fC, a 64 % decrease from control. The average half maximum time was found to be 3.4 ms, a 16 % faster response than the control. The granule release frequency under CuO-NP influence found to be decreased by 29 % as compared to control, indicating some disruption in the process of granule trafficking, docking or cell membrane fusion.

Analyzing the amperometric spikes under the influence of TiO₂-NPs, we found a small decrement in speed of exocytosis (increased $t_{1/2}$) and a slight increase in the catacalomine release. Delays in kinetics due to TiO₂-NPs has been noticed in an earlier study [73] and was related to the high protein absorbing ability of TiO₂-NPs [73, 148]. However, the average of half maximum time under TiO₂-NPs exposure (4.43 ms), a 9% increase, was not significantly different from the average half maximum time of the control experiment (4.02 ms). Analyzing the average charge per exocytosis burst (86.57 fC) was slightly higher than the control (84.66 fC), only 2% change, and not found to be significantly different statistically. However, granule release frequency (0.38 Hz) was decreased by 23% compared to the control (0.5 Hz). The changes produced in amperometric spikes by NPs exposure were found to be non-reversible when the NPs were washed after approximately 3 minutes of exposure.

From the outcome of the present experiments and in correlation with previous literatures, it is confirmed that the nanoparticles do interfere with normal activity of neuronal cells. In *in-vitro* model/experiments, DNA damage, ROS generation and cell death are commonly noticed after few hours to days of exposure to the excess amount of

nanoparticles. Here, we captured transient effects of nanoparticle exposure on cell exocytosis function, before other cell-irregularities could be captured.

3.4 Discussion

We presented here a single cell integrated chip-based approach to identify the acute effects of nanoparticle exposure on PC12 cell exocytosis function. The effects of these nanoparticles depend on various factors, such as size and shape of the NPs, its surface charge and functional groups, solubility in liquid, leaching, *etc.* [73, 77, 149]. Here, we are paying attention to the role of nanomaterial's composition in determining its impact on PC12 cells. We recorded PC12 cell response for 60 seconds which started within 10 seconds of the NPs exposure.

Figure 3.6: Schematic representation of an exocytosis cycle, including discrete stages of vesicle formation, vesicles transport, vesicle docking, priming and fusion formation.

Exocytosis process can be divided into small steps of vesicle docking, priming, and fusion pore formation which can end up into full fusion or partial fusion (“kiss-and-run”). Both, CuO-NPs and TiO₂-NPs are found to reduce the overall exocytosis events in a PC12 cell. In exocytosis process, secretory vesicles are docked at the cell membrane

through SNARE-complex formation. Any interruption in SNARE-complex can interfere vesicle docking, priming and fusion release. Earlier, it is shown that Botulinum neurotoxin C1 light chain (BoNT/C1) cleaves the syntaxin and SNAP-25, ultimately results in the prevention of vesicles docking, priming and release [150]. When we compare the exocytosis parameters collected under the influence of CuO-NPs and TiO₂-NPs with control, we found that CuO-NPs are highly interfering. The decrease in half maximum time, $T_{1/2}$, is an indication of a faster exocytosis cycle and representing the overall reduction in exocytosis process time, from vesicle docking to fusion pore expansion. Also, the total numbers of exocytosis events were reduced more under the influence CuO-NPs as compared to TiO₂-NPs. Synaptotagmin (Syt) is a Ca²⁺ sensitive protein and is known its role in exocytosis regulation. The role of CuO-NPs interfering with the function of Syt is inferred here as earlier, it is shown that silencing Syt-I can reduce the total exocytosis events, whereas over expression of Syt-IV decreases the fusion pore open time [122, 151]. Also, a possible mutation of secretory carrier membrane protein 2 (SCAMP2) could result in inhibition of exocytosis events [152]. Delays in kinetics due to TiO₂-NPs has been noticed in an earlier study [73] and was related to the high protein absorbing ability of TiO₂-NPs [73, 148]. However, the average of half maximum time under TiO₂-NPs exposure (4.43 ms) was not significantly different from the average half maximum time of the control experiment (4.02 ms). A huge decrement in the total charge content of vesicles, Q , is noticed with CuO-NPs effect. We can say that CuO-NPs interfere in the production of catacalomines or disrupt the exocytosis cycle so that the final catacalomines release in the exocytosis spike is reduced. An earlier study showed a reduction in the flux of catacalomines and its conductance

through the fusion pores after point mutations in syntaxins [153]. We can relate our finding to earlier found results that Cu-NPs interfere with genes related to intracellular production of neurotransmitters vesicles. In a study it is shown that Cu-NPs down regulated the gene expression of Gpx1 and up-regulated the expression of Txnrd1, and MAOA, which induced neurotransmitter (i.e. dopamine-DA) depletion by increased degradation of DA, and produced dopaminergic neurotoxicity [13]. A decrease in the overall quantal release is expected after less intracellular neurotransmitter formation. The alteration in quantal size can also be possible by autoreceptors (D2) activation which might alter the amount of neurotransmitters packaged per vesicles by modulating the activity of VMAT1 [154]. Increased activity of VMAT is responsible for vesicles breakdown and the overall capacity of intracellular vesicles to store the neurotransmitters is hampered. CuO-NPs were also found to block voltage-dependent potassium channels K^+ inhibit the potassium channel (K^+) [155]. Multiple irregularities, as explained here, caused by CuO-NPs infer to interfere normal exocytosis cycle and reduced exocytosis events with smaller cycles ultimately resulted in a reduced catacholamines flux.

3.5 Conclusion

We studied two commonly used metal-containing nanoparticles to evaluate their toxicity on the exocytosis behavior of PC-12 cells. A new approach of co-applied NPs with high K^+ buffer provided a rapid response of NPs interferences on PC-12 cell functions within minutes. Both the NPs under tests are found to be interfering with the natural exocytosis mechanism of PC-12 cells and reduced the total number of exocytosis events. CuO-NPs highly decreased the overall quantal release from individual events and

made the release cycle faster, whereas TiO₂-NPs interfered slightly by increasing the time of an individual exocytosis cycle. In initial experiments, we found that the chip-based approach for nanotoxicity assessment can provide an initial screening of the nanomaterial for interference with its exocytosis function that is difficult and time-consuming with traditional assays. The protocols developed in this study would facilitate broad matrix of materials for its toxicity assessment for the future use.

CHAPTER 4-NANOTOXICITY ASSESSMENT AT SINGLE-CELL AND SMALL CELL-POPULATION USING ELECTROCHEMICAL IMPEDANCE SPECTROSCOPY

This chapter is submitted for publication with minor changes; **Pratikkumar Shah**, Xuena Zhu, Xueji Zhang, and Chen-Zhong Li, “*MEMS Based Sensing Array for In Vitro Nanotoxicity Assessment at Single Cell and Small Cell Population Using Electrochemical Impedance Spectroscopy.*” **Nanomaterials**, 2015. (In review process)

4.1 Introduction

Lab-on-a-chip based technologies are in use for multiplex assays for several toxic material findings and drug discoveries. However, their use for nanomaterial toxicity assessment is new. Microfluidic chip-based approach is becoming the latest trend in nanotoxicity evaluation after their extensive applications in biosensing, drug discovery and stem-cell research.

Our lab has invested in developing a chip based approach for providing dynamic information of nanomaterials effect on cells [26]. The earlier approach of chip-based nanotoxicity assays were focused on cell populations, either monolayer or 3-D spheroids. The role of single cell study has been emphasized for neurological cells, stem cells and cancer cells, which are complex to characterize [156]. Nanomaterial toxicity has already shown complexity while studied on large population of cells or *in vivo*, and hence, single cell study might give complementary information about the cell behavior which could be missed in cell population study [37, 157].

To address the issue, earlier we developed chip based nanotoxicity evaluation at a single cell level [78], which is profoundly explained in Chapter 2 and 3 of this dissertation. In these studies, only single cell behavior was recorded, and we had to compromise the cell population studies. Our focus is on developing a chip based approach to provide a solution to this issue by developing a platform that can provide single cell behavior, including the behavior of a small cell population under the influence of nanomaterials. Here, we aim to present a LOC device which highlights the cell kinetics behavior of a single PC12 cell and a small population of PC12 cells. We designed different size of microwells to hold a different number of cells, i.e. from 1 cell to 20 cells.

Especially, impedance based measurement offers rapid, simple, label free and non-invasive means of cell analysis. Impedance based sensors have been in studies for many applications, such as detection of cell migration [158, 159], cell growth and proliferations [160], cell health [161, 162], cytotoxicity [163], nanotoxicity [164], drug effects [165], and circulating tumor cell detection [166]. Many chip based electrochemical measurement devices for single cell analysis have also been fabricated earlier, and the challenge of capturing single cell has been mentioned. We used a combined approach of surface functionalization and dielectrophoresis (DEP) for controlled cell trapping as profoundly explained in Chapter 2, section 2.2 and 2.3. Here, we present a comparative study of single and a small cell population, which will be useful for the future studies in selecting the electrode size and the number of cells for nanotoxicity study using a LOC device. Here, we present a comparative study of single and a small cell population, which will be useful for the future studies in selecting the electrode size and the number of cells for nanotoxicity study using a LOC device.

4.2 Experimental

4.2.1 Bulk cell viability assay

Sulforhodamine B (SRB) is a colorimetric assay commonly used to quantify the cell density by measuring the cellular protein content of live cells. Approximately 10000 cells were plated in each well of a 96 well plate by adding 100 μ L of cell suspension solution (10^5 cells/ml concentration) and allowed to adhere and grow for 24 hours. After 24 hours of cell attachment, 100 μ L of media containing different concentration of NPs ranging from 1 ng/ml to 100 μ g/ml were added to the wells, and cells were allowed to interact with NPs for 1, and 24 hours in two different well plates. After NPs-cell interactions,

cells were washed one time with PBS buffer. After washing, the cells were fixed by using ice-cold 50% (wt/vol) Trichloroacetic acid and stored for 1 hour at 4 °C. After fixation, cells were washed with DIW 5 times and dried before staining with 0.4 % SRB solution for 30 minutes at room temperature, after which the excess dye was removed by 5 times wash with 1% Acetic Acid. The microplate was allowed to dry completely before adding 10 mM Tris base solution to the micro-plate wells, and the micro-plate was placed on a shaking bed for 10 minutes to dissolve the protein-bound dye. The optical density was determined at 565 nm using a Micro-plate reader (Synergy HT, BioTek Instruments). The data were exported to Excel and later analyzed.

4.2.2 Cell morphology study in bulk assay

5×10^4 cells suspended in 1 ml of growth media were cultured for 24 hours in each well of a 12X well-plate. After 24 hours, the cells were cleaned with PBS and NPs dissolved in fresh growth media was exposed to cells. Time-lapsed observations were conducted under an upright microscope and images were collected using a microscope-mounted camera.

4.2.3 LOC device

The second generation LOC device, version 2, was fabricated as explained earlier in section 2.2.3 of chapter 2 with a modified design of cell trapping microwells as shown in Figure 2.2. 8 microwells are designed such that it can be fitted to chip holder of ECIS impedance monitoring device (Applied Biophysics, NY, USA) for real-time impedance measurement. Microwells were designed in various diameter sizes (i.e. 20, 30, 50 and 80) to capture different number of cells (i.e. ~ 1 , 4 ± 1 , 10 ± 2 , 18 ± 4 , respectively) for a comparative study.

4.2.4 Instrumentation for impedance spectroscopy

Nanotoxicity at the whole cell level was tested using impedance spectroscopy technique. In this method, cell morphological change was quantified at a single cell and multi-cell (i.e. small population) level using the LOC device. PC12 cells were allowed to grow on chip-electrodes for about 6 hours, and then the media was changed with 100 μ L of fresh media before impedance measurement. The chip was placed in an incubator and allowed to rest for at least 10 minutes before the measurement to minimize the baseline-impedance variations. In the impedance measurement unit, ECIS model 1600RE, the counter/reference electrode and measuring electrodes were connected to a phase-sensitive lock-in amplifier, and an AC signal was applied through 1 M Ω resistor. The recording of impedance was conducted at 0.025 V and 10 KHz frequency. After about 30 minutes of recording, 100 μ L of NPs suspended media was added to the resultant concentration of 100 μ g/ml. Three runs for each condition were performed, and the data were exported to Excel for further analysis.

PDMS well of the size (10 x 5 x 4 mm³) to contain about 200 μ l media was placed on a chip. The chip was covered by a damp cover to delayed the evaporation of media and the drying of well was noticed in about 5 hours. Addition of the extra media created a deflection and was noticed to come back to the original position in about 30 minutes. To avoid the repetitive deflection of baseline impedance, the fresh media was added before the experiment and then only the NPs containing media added during the experiment. The measurement of impedance was conducted for a continuous 5 hours.

4.2.5 Measurement of cell impedance response

To quantify the toxicity effects at the whole cell level, cell morphological changes of a single and small population of cells were recorded. PC12 cells were captured in predefined microwells and were allowed to adhere for 6 hours on the surface of microwell electrodes. After 6 hours, the growth medium was replaced with fresh medium and cells were allowed to rest for 10 minutes before starting the measurements to minimize the baseline noise. Various mediums, containing 500 μM H_2O_2 , 1 M H_2O_2 , 100 $\mu\text{g/ml}$ of CuO , and 100 $\mu\text{g/ml}$ TiO_2 added. Cell-impedance was recorded for an additional 5 hours.

4.3 Results

4.3.1 Nanoparticles characterization

The NPs were characterized using DLS method as explained in section 3.2.1 of chapter 3. However, here, the stock solution of 1 mg/ml was prepared in DIW (DIW) and further diluted in measurement solutions (DIW and cell growth medium) to 100 $\mu\text{g/ml}$ to measure their agglomerated-hydrodynamic size and zeta potential. The results of the size and surface charge measurements of NPs are summarized in Table 4.1. The size of CuO -NPs was recorded 492 nm while suspended in DIW with surface charge of -1.3 mV, whereas, when suspended in cell growth medium, they were 587 nm and -0.8 mV, respectively. TiO_2 were recorded 552 nm in size with -11.5 mV surface charge when suspended in DIW, whereas they were of 597 nm and -9.97 mV, respectively, when suspended in the medium.

Table 4.1: DLS properties of NPs

Nanoparticles	<u>DLS size (nm)</u>		<u>Zeta potential (mV)</u>	
	DIW	Media	DIW	Media
CuO	492	587	-1.3	-0.8
TiO₂	558	597	-11.5	-9.97

4.3.2 Cell viability studies using SRB assay

SRB assay measures the average live cells by measuring the total protein content in cell population. After NPs exposure in different concentrations, 1 ng/ml, 10 ng/ml, 100 ng/ml, 1 µg/ml, 10 µg/ml, and 100 µg/ml, for one hour, change in cell viability due to NPs toxicity was minimal and only 100 µg/ml of CuO-NPs was slightly toxic, responsible for about 10% cell death.

Figure 4.1: Effect of CuO-NPs and TiO₂-NPs on cell viability recorded using SRB assay after 1 hour and 24 hour of exposure.

During one hour of NPs exposure to PC12 cells, cells did not reveal any significant cell death. However, SRB assay results after 24 hours of TiO₂-NPs exposure revealed

that only 100 µg/ml NPs were significantly damaging the viability of PC12 cells and almost killed 75 % of cells, whereas the 10 µg/ml TiO₂-NPs were slightly toxic and killed about 5 to 7 % of cells. All other lower concentrations of TiO₂-NPs were non-toxic after 24 hours exposure to PC12 cells. CuO NPs were more toxic than TiO₂-NPs and significant cell death was noticed at 10 and 100 µg/ml concentrations after 24 hour. However, lower concentrations of CuO (from 1 ng/ml to 100 ng/ml) were nontoxic and even demonstrated cell growth after 24 hours. As per earlier findings, this response can be related to Cu being a required micronutrient promoting cell growth at lower concentrations [135]. The concentration of 1 µg/ml CuO-NPs was slightly toxic as it caused 10% cell death. All other smaller concentrations of CuO-NPs were non-toxic after 24 hours of exposure.

From SRB assay studies of 1 hour and 24 hours exposure to different NPs concentrations, we selected the concentration of higher bound (i.e. 100 µg/ml) for impedance study on single cell and small cell-population, which was more prominent to produce the change in cells' health within couple of hours of NPs exposure.

4.3.3 Cell morphology

Cell morphology changes induced by NPs exposure were studied in a 12X well-plate. After 24 hours of cell growth, the cells were cleaned with PBS buffer. The fresh growth media was added to control well, whereas, media with NPs suspension in concentration of 1 and 100 µg/ml was added to wells under study. The change in cell morphology was observed by an upright microscope and time-lapse images were captured using a microscope mounted camera as shown in Figure 4.2. The Figure 4.2 shows that the lower concentration of NPs (1 µg/ml) did not affect the cell morphology even after 6 hours of

exposure. The results of 1 $\mu\text{g/ml}$ NPs concentration aligns with the results collected from SRB assay. At this concentration, the cells survived without a noticeable difference in their morphology even after 48 hours. However, for higher concentration of NPs (100 $\mu\text{g/ml}$) the change in cell morphology was observed. At higher concentration, the change was not observed for first 1.5 hours. However, the change in cell morphology is clearly seen under CuO-NPs exposed cells after 3 hours. Cell size decreased and shape became more rounded under the influence of CuO-NPs than control cells. Similar effects were observed for TiO₂-NPs exposed cells after 3 hours, although with lesser damage as compared to CuO-NPs. The change in morphology monitored to be continued in the images collected after 6 hours of NPs exposure.

Figure 4.2: Cell morphology examined with an inverted microscope. PC12 cell morphology was studied at different time points after exposure to CuO-NPs and TiO₂-NPs.

4.3.4 Cell trapping on LOC device

The fundamental principle of cell trapping is explained thoroughly in section 2.2 of chapter 2. Here, the brief changes are mentioned in cell trapping exercise. When 20 μl of cell suspension (2×10^5 cell/ml) is introduced in the microfluidic channel at a flow rate of 10 $\mu\text{l}/\text{minute}$, the cells would be trapped in the microwells as soon as they come across the DEP field between microwell electrode and the top ITO electrode. The number of cells collected in each microwell depends on the size of microwell. From three individual cell trapping experiments, we noticed that a 20 μm microwell is capable of hosting 1 cell, a 30 μm microwell hosts 4 ± 1 cells, a 50 μm microwell hosts about 10 ± 2 cells, and a 80 μm microwell hosts about 18 ± 4 cells. We observed that the natural tendency of the cells being trapped in the 80 μm microwells under DEP force was towards the edge, which leaves the central area open for the initial 60 seconds, however, continuing the cell flow in the microchannel, in less than 3 minutes, the microwell is filled with 18 ± 4 cells. Once the cells were trapped in the microwells, they were allowed to rest for 15 minutes in the incubator and after that the top ITO electrode was carefully removed and the sucrose media was replaced with cell growth media. The cells were allowed to adhere on top of the microwell electrode for at least 6 hours before exposure to NPs and impedance measurement. The 6 hours of buffer time was enough for PC12 cells, trapped in microwells, to spread and adhere firmly on top of the microwell electrode, which is important to minimize the background noise during impedance spectroscopy measurement. The LOC device, holding cells, was always kept inside a humidified chamber within incubator to reduce the evaporation of the cell growth medium.

Figure 4.3: Microwells image captured with an optical upright microscope after controlled cell trapping. The numbers of cells captured in a microwell are controlled by the size of the microwells. A 20 μm microwell (a) hosts 1 cell, a 30 μm microwell (b) hosts 4 ± 1 cells, a 50 μm microwell (c) hosts about 10 ± 2 cells, and a 80 μm microwell (d) hosts about 18 ± 4 cells.

4.3.5 Impedance spectroscopy

The optimal frequency for our customized device and PC12 cells was determined by the impedance spectrum over the frequency range of 30 – 64000 Hz in absence of cell as well as in the presence of cells on top of electrode. When no cell is present on top of the electrode, the flow of current is free from electrode to bulk electrolyte, however, when cell is attached on the surface of a planar electrode and spreading, the flow of current will be hindered. The change in impedance is frequency dependent as the resultant current can flow from different pathways, such as, narrow gap underneath the cell, across the cell and from the uncovered electrode or between cell joints when more than one cell is present. At low frequencies, the change in impedance is very insensitive to cell spreading as the impedance is mostly dominated by electrode-electrolyte interface (i.e. Warburg impedance). At mid frequencies, the impedance is biphasic and increases slowly to a point and then starts decreasing. At higher frequencies, the capacitance becomes significant. Thus, in order to measure cell impedance on a planar electrode, it is important to record the impedance signal at the optimum frequency. Figure 4.4 shows the frequency

spectrum of a plain microwell electrode and when the electrode is covered with a cell.

The cell to cell-free ratio or cell index is represented as electrode following equation;

$$\text{Cell to cell-free ratio or Cell Index} = (Z_C - Z_0) / Z_0$$

Where Z_0 = Impedance of electrode without cell and Z_C = Impedance of electrode with cell.

Observation of results collected in Figure 4.4a shows that the change in impedance value in the absence and presence varies with the frequency. At low frequencies, below 2000 Hz, the change is very negligible (in fact, near 0 or below). From 2000 Hz to 10 KHz, the cell index increases with frequency and reaches the maximum point (about 44 % for small microwell and 200% for larger microwell). From 10 KHz to 64 KHz, the cell index again decreases with the frequency. At 10 KHz frequency, the planar electrode shows the highest sensitivity for the cell impedance measurement on our device. Hence, 10 KHz was selected as the optimum frequency for cell impedance studies reported here.

Figure 4.4: Frequency spectroscopy plot of impedance for frequency range from 30 Hz to 64 KHz for cell free microwell electrode and when the microwells electrodes are seeded with PC12 cells, (a) for single cell microwell electrode of 20 μm and (b) for small cell-population microwell electrode of 80 μm .

4.3.6 Single cell impedance response to hydrogen peroxide

To identify the sensitivity of the LOC device for single cell impedance sensing, cell culture mediums, containing 500 μM and 1 M of H_2O_2 were exposed on a cell, and the change in impedance was recorded. H_2O_2 is a well-known reactive oxygen species, and higher exposure to it can lead to cell dysfunction and/or cell death. Figure x shows the averages response of 3 individual cells when exposed to 500 μM and 1 M of H_2O_2 . The cells exposed to the higher concentration of H_2O_2 shows an immediate decrement in impedance as the cells get damaged immediately with this dose whereas for the lower concentration we see time dependent (slower) cell death. The immediate change in impedance indicates cell death and detachment from the electrode.

Figure 4.5: Average normalized impedance response of a single cell exposed to H_2O_2 .

4.3.7 PC12 cell response after NPs exposure

Figure 4.6: Average normalized impedance signal of single cells (a) without any NPs exposure (b) when exposed to 100 $\mu\text{g/ml}$ CuO (c) when exposed to 100 $\mu\text{g/ml}$ TiO_2 and (d) Average plot of data represented in (a), (b) and (c).

Figure 4.6 shows the real-time impedance profile of nanotoxicity on single PC12 cells when exposed to 100 $\mu\text{g/ml}$ NPs. Before taking the measurements, cell inoculated device was rested for 10 minutes in the incubator with fresh media. The cell-impedance measurement was recorded for 30 minutes to collect baseline impedance before addition of NPs. After, 30 minutes of baseline measurement, the system was paused for about 2 minutes and in the meantime, NPs containing growth medium was added to the final concentration of 100 $\mu\text{g/ml}$ NPs. The cell-impedance was recorded for at least 5 hours and the results were saved on a computer. The system files were then exported to Microsoft-Excel for data analysis.

The change in impedance was normalized to the initial impedance value (i.e. before the addition of NPs). Figure 4.6 shows the results collected from a single cell experiment in different conditions. The experiment was conducted for the total of 4 times in each state. Looking at the data, we found minor cell-to-cell signal variations using impedance spectroscopy. Figure 4.6d shows the averages of the data collected in each condition with their standard error of mean values.

When comparing the results, Figure 4.6d, we see that the CuO NPs started to affect the health of a single PC12 earlier than TiO₂ NPs. The change in impedance is not immediate when exposed to CuO NPs and the cell begin to detach and shrink after about 2 hours and eventually dies within 5 hours of NPs exposure. This change of impedance is related to the change in cell morphology and cell attachment on top of the electrode surface. These impedance results were confirmed by morphology detection under the microscope. In a separate experiment of cell morphology changes in 12X well plate, it was confirmed that the cells get damaged mostly after 1.5 hours of CuO NPs exposure. We can quantify the cell kinetics using real time impedance spectroscopic technique here, which complimentary methods lack.

4.3.8 Comparison of a single cell and multiple cells

In a different size microwell electrodes (Figure 4.7), a controlled number of cells collected in individual microwells were exposed to 100 µg/ml CuO-NPs. The trend of the cell behavior is similar to that recorded earlier using only single cells, minor changes for the first two hours of exposures, decreasing current from 2 to 4 hours and plateauing during 4 to 5 hours. When carefully observed, small population of cells in 80 µm microwell-electrode responds to the external stimulus earlier than other microwells.

Average results collected from four experiments are represented in Figure 4.7 and the values of the half minimum impedance time ($Z_{1/2}$), and sensitivities are reported in Table 4.2. When calculated through half minimum impedance time, larger electrode response time was calculated to be faster than smaller electrode. We also noticed that the 80 μm electrode impedance decreased greatly from its initial impedance upon NPs exposure.

Table 4.2 Average sensitivity and response time values for different size of microwell electrode containing different number of cells.

No. of cells	Sensitivity (% change in impedance)	Response time – hours (Half minimum impedance - $Z_{1/2}$)
1	35 %	3.99 ± 0.29
4 ± 1	49 %	3.59 ± 0.13
10 ± 2	60 %	3.62 ± 0.05
18 ± 4	72 %	3.27 ± 0.26

Figure 4.7: Impedance response of different size microwell-electrode holding various number of PC12 cells, when exposed to 100 $\mu\text{g/ml}$ CuO-NPs.

4.4 Discussion

CuO and TiO_2 NPs are two of the most commonly used NPs in commercialized products due to their desirable properties, and their applications are discussed in electronics, sensors, antibacterial, cosmetics and energy sectors. Numerous applications

pose the possibility of a greater exposure to humans. It is important to verify the side-effects of these NPs besides their desirable properties.

SRB assay revealed that the change in cell proliferation is hugely reduced after 24 hour of exposure to 10 $\mu\text{g/ml}$ or higher concentration of CuO-NPs. 1 $\mu\text{g/ml}$ CuO-NPs were found to be slightly toxic after 24 hours. However, the lower concentrations of these nanoparticles are found to be helping in cell proliferation. According to earlier findings, this response can be related to Cu being a required micronutrient promoting cell growth at lower concentrations [135]. 100 $\mu\text{g/ml}$ TiO₂-NPs were found to be highly toxic after 24 hours, whereas the lower concentration did not produce high adversities to the cells. After 1 hour of exposure to these nanoparticles, only 100 $\mu\text{g/ml}$ CuO was found to induce noticeable toxicity towards PC12 cells and hence this concentration was selected for further impedance spectrometry measurements.

For morphological studies conducted in traditional 12X well-plate, the lower concentration of nanoparticles did not induce noticeable toxicity; however, toxicity was noticed under the exposure of higher concentrations of nanoparticles. The measurements are aligned with the results collected from SRB to show that the toxic effect is concentration dependent in a range from 1 to 100 $\mu\text{g/ml}$. Morphological damages to the adherent cell line, like PC12 cells, are commonly observed by monitoring the size of the cells. NPs affected cells tend to detach from the surface they were attached to and appear round in shape. The change in morphology and proliferation tend to be damaged by NPs exposure, which is a common trend in nanotoxicity where the toxicity increases with the time of exposure.

The SRB assay and other traditional assays are end point measurement based and do not present a dynamic cell response which can enhance the understanding of cellular toxicity when exposed to nanomaterials. Figure 4.6 represents the measurement of 100 $\mu\text{g/ml}$ of NPs exposure on single PC12 cells. The real time impedance data for a single cell suggest that the effect at the whole cell level is minor and not noticeable in the beginning for about 1.5 hour and maximum during 2 to 4 hours. It is confirmed by the morphological images collected that the changes in morphology are noticed between 1.5 and 3 hours. The cells are shrunk in size, appeared round-shaped and detached from the surface during this time. These changes are responsible for declining impedance values of sensing electrodes as with small and loosely bound cells on the electrode, the flow of current between two electrodes is not blocked. A constant drag of current after 4 hours suggests an occupation of a dead cell in the microwell.

Figure 4.8 Impedance response of different size microwell-electrode holding various number of PC12 cells, when exposed to 100 $\mu\text{g/ml}$ CuO-NPs. (a) Schematic demonstration of sensitivity and response time, (b) comparison of sensitivity and (c) comparison of response time.

To identify the difference in response of small cell population when exposed to NPs, the impedance response was characterized by two properties, 1) the half-minimum impedance time ($Z_{1/2}$) and 2) the sensitivity, as depicted in Figure 4.8a. $Z_{1/2}$ is the time where the value of impedance is half of the minimum value collected during the experiment and the sensitivity is the change in impedance value from its initial value at

the end of experiment (~ after 5 hours of NPs exposure). It is important to notice here that the $Z_{1/2}$ time may not be the best point to measure the highest change in sensitivity as the values of 30 μm and 50 μm diameter microwells are overlapping in this region (between 3 to 4 hour), however, the difference of 20 μm and 80 μm is clearly noticeable. As shown in Figure 4.8b, after 5 hours of NPs exposure, 35 % change from its initial impedance value was recorded for 20 μm microwell. The change was recorded to be 49 %, 59 % and 72 % for 30 μm , 50 μm , and 80 μm microwells, respectively. Hence, 80 μm microwell with 18 ± 4 cells is displaying the highest sensitivity to the NPs. A possible reason for this action is inferred to the fact that the larger size of microwell having large current carrying capacity and more number of cell-deaths would expose more area of the electrode, whereas in 20 μm microwell still possesses the unhealthy or dead cell, albeit reduced in size, ends up plateauing at a certain level.

When $Z_{1/2}$ values are considered (Figure 4.8c), 80 μm electrode response time was calculated to be 22 % faster than 20 μm electrode. The response time of 50 μm and 30 μm microwell-electrodes were found to be 10 % and 3 % faster, respectively, when compared to the response time of the single cell holding 20 μm microwell-electrodes. A possible reason for faster response of the 80 μm electrode-microwell is reasoned that there are more cells on top of the larger electrode dying simultaneously, creating a greater open space for free electron transport between medium and the electrode. Another reason for faster response of a small-cell population could be the cycle of cell-signaling between unhealthy cells making its surrounding cells unhealthy; however more studies must be conducted to elaborate this factor further.

4.5 Conclusion

We presented a combined approach of surface functionalization and dielectrophoresis for rapid and reliable capturing of a controlled number of cells in microwell arrays of a MEMS device. Using impedance spectroscopy technique, we monitored real time kinetics of cell for nanotoxicity assessment. In bulk assay experiments, NPs affected PC12 cells appeared smaller in size compared to healthy cells, along with reduced viability. We showed that the results collected from MEMS device co-aligns with results collected from qualitative analysis of morphological images and viability assays with additional quantitative information and higher sensitivity. Cell population studied here showed that the small-population (14 to 22 cells) of cells with appropriate electrode size (i.e. 80 μm diameter) has higher sensitivity for nanotoxicity as compared to a single cell with smaller microwell electrode. The response time of a single cell and a small cell-population was founded to be slightly different when exposed to CuO-NP. The microwell-electrode with a small cell-population responded faster than a single cell microwell-electrode. Results of the response time may lead us to the appropriate cell population and electrode size selection when single cell trapping is difficult.

CHAPTER 5-CONCLUSIONS AND FUTURE DIRECTION

5.1 Conclusions

The research study presented a cell integrated lab-on-chip (LoC) device approach for rapid analysis of nanotoxicity *in vitro*. Electrochemical measurement approach presented here provides highly sensitive, and real time measurement of cell functions at a single-cell level. This thesis mainly proposes an alternative approach to traditional nanotoxicity assessment as a tool for initial screening that is rapid, simple, cost-effective, and provides higher throughput.

A synopsis of this dissertation's sections is as follows:

Chapter 1 explained in detail about the available approaches for nanotoxicity detection, mostly derived from earlier assay based technique for toxic detection and drug discoveries. However, as discussed, dyes used in these techniques found to interfere with nanomaterials under the study at several occasions and the need for alternative approaches has been pressed. With the advancement in single cell studies, we know that individual cells in a population might behave differently but get unnoticed in analyzing the average response from multiple cells. Benefit of studying single cell, especially for the complexity of nanotoxicity studies, might highlight some unknown discoveries. The simplicity, ease of operation and atomization has led to several analytical applications, from drug discoveries to diagnostics, of the microfluidic chip based approach. We hypothesized that using such a device for nanotoxicity assessment will provide a rapid alternative for an initial toxicity screening. While integrating the device for single and multiple cells together would reveal any difference for response time and sensitivity of studying different number of cells.

Chapter 2 presented a design and development of single cells integrated high throughput microwell device for cells trapping and analyzing. The design criteria considered the evasion of cross talks between two single cell microwell electrodes by keeping the distance at least 200 μm between any two electrodes. Optical and electrochemical characterization confirmed the desired performance with minimum variation in the fabricated device. From the earlier research, we optimized the noninvasive dielectrophoresis cell trapping technique for precise capturing of single endothelial cells in individual microwell electrodes. A combined approach of surface functionalization and dielectrophoresis was used for single cell trapping that is harder to achieve using only surface functionalization because of the tendency of PC12 cells to form clumps. The device design was altered from version 1a to version 1b to accommodate the clip connector. A new design, version 2 of the LOC device was fabricated to serve the controlled number of cell trapping for a comparative study.

In chapter 3, we applied the LOC version 1b for a single-cell exocytosis studies. We studied two of the most commonly used metal containing NPs, CuO-NPs and TiO₂-NPs, for their toxicity towards the neurotransmitter release cycle in a single PC12 cell. We co-applied the NPs with control stimulation of high K⁺ buffer for the first time to reduce the incubation time of NPs with cells to derive NPs interference properties during exocytosis cycle of a single PC12 cell, which can be related to its ability to communicate or signal transmission. Both NPs under the study showed interference with exocytosis cycle of PC12 cells with CuO-NPs being intense, whereas TiO₂-NPs induced changes were moderate. Experimental results displayed that CuO-NPs highly decrease the average quantal release (i.e. neurotransmitter release) by 64% and made the cycle faster by

reducing the $T_{1/2}$ by 16% as compared to 2% delayed response and decrement in quantal release by TiO_2 -NPs. The overall number of events were decreased by 29% under CuO-NPs exposure whereas they reduced by 23% under TiO_2 -NPs exposure. The protocols developed in this study would facilitate broad matrix of materials for its toxicity assessment for the future use.

Chapter 4 demonstrated an updated microwell design to host controlled number of cells, from 1 cell to 22 cells. Controlled number of cell capturing microwells would allow hosting different number of cells in different microwells and studying the effect of NPs simultaneously. In many conditions, single cell trapping is challenging, compromising the single cell study to a small population study is how much different in its sensitivity and response-time were studied in this chapter. It is also important to notice that the design also provides an initial screening ability to identify any basic difference in cells response due to different number of cells (i.e. due to cell-cell interaction). Using impedance spectroscopy approach, we monitored dynamic response of single cells under NPs exposed condition, and found to match with results collected from bulk assays using SRB viability tests and morphological changes monitored under an inverted microscope. The results also aligned with previous findings and CuO-NPs showed toxic response as compared to TiO_2 -NPs. When single cell and small population of cell on the LoC device were studied together under CuO-NPs exposure, we noticed higher sensitivity in larger microwells when trapped with small population of cells while responding to exposed CuO-NPs, whereas the response time were slightly different. The larger electrode containing small colonies of cells responded faster than the single cell. This study highlights that when a single cell trapping and analyzing is challenging, a small colonies

of cell with appropriate size of microwell electrode may be provide faster response. These techniques can be applied to other applications, such as drug discoveries, toxicity analysis and biosensing, for improved sensitivity and response time.

5.2 Future direction

The role of cell to cell communication in spreading of nanotoxicity will further be evaluated on other cell lines. The LOC device presented in this work only allows us to collect 6 hours of data without addition of surplus media during the impedance spectroscopy measurements. On adding media during the experiment caused deflection in baseline current.

In future, the developed LOC device will be integrated with a microfluidic channel for automated fluid control and longer time study (> 24 hours) with high accuracy and precision. Efforts will be made to modify the electrode size and microwell design for the assessment of nanotoxicity in a neuron in a localized manner. The outcomes will allow us to identify the most sensitive area of a neuron (i.e. axon, dendrite, synapses) being affected by external nanomaterials.

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