Real time monitoring of Cell-Nanoparticles interaction and tracking internalization process by mechanical probing using Atomic Force Microscopy

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REAL TIME MONITORING OF CELL-NANOPARTICLES INTERACTION AND TRACKING INTERNALIZATION PROCESS BY MECHANICAL PROBING USING ATOMIC FORCE MICROSCOPY

by

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ABSTRACT

With extensive development of nanotechnology in last few years, scientists have discovered that nanoparticles (NPs) can be used as an efficient Drug Delivery System (DOS). In order to develop better NPs based drug delivery tool, it is imperative to understand the interaction between the NPs and the cell membrane. In our earlier studies, cerium oxide nanoparticles (CNPs) have been reported to have therapeutic properties, specifically against abnormalities associated with oxidative stress. Therefore, CNPs with different sizes and morphology were selected to understand the interaction with cell. We analyzed the mechanical property of human nasal septum tumor cells membranes using Atomic Force Microscopy (AFM) with and without CNPs. In particular, Force-Distance spectroscopy mode was used to estimate the elasticity of cells membrane. Different concentrations (0, 50, 125 and 250 μM) of CNPs were added to the cells (squamous cells; CCL30) and incubated for different time periods (0, 15, 30 and 60 minutes). Cell membrane elasticity/Young’s modulus was calculated using a modified Hertz model. Changes in the cell elasticity were observed in high concentration of CNPs when treated with one hour. Significant changes in cell elasticity were observed at high concentration of CNPs for one hour of incubation. No significant change in cell elasticity was observed over one hour time period for 50 μM of CNPs. Moreover, by using selected inhibitors to block different cell mediated internalization pathways, we also investigated the correlation between the cellular uptake and the tracking of NPs with their size. Specifically, similar change in cell elasticity was observed after blocking the cell energy production for CNPs with smaller diameter (3-5 nm). On the other hand, bigger size NPs (20-30 nm) showed no change in cell elasticity after
blocking the cell energy production. These results indicate that 3-5 nm particles internalize cell by non-energy dependent pathway i.e. passive diffusion whereas 20-30 nm particles entered in cell by energy dependent pathways i.e. endocytosis of particles. Further, we have also identified the cellular uptake of 20-30 nm particles is by enclosing those CNPs in membrane vesicles in caveolae-mediated endocytosis mechanism. In summary, these results indicate that the nanoparticles-cell interaction has pronounced influence on the shape and size of the nanoparticles. These interactions can be further monitored by real time mechanical property measurement of cell membrane.
To my parents
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CHAPTER ONE: INTRODUCTION

1.1. Introduction to Nanomedicine

The year 1959 is the important year for nanotechnology and its application to medicine more specifically. Given at the California Institute of Technology in that year, R. Feynman had his famous lecture “There’s Plenty of Room at the Bottom, An Invitation to Enter a New Field of Physics” to show the promises to future of nanotechnology [1]. When the sizes of the materials are reduced, their properties change dramatically. In nano-scale, materials have many special properties which are not normally present in bulk material, such as: quantum confinement in semiconductor materials, surface plasmon resonance in metallic materials, and superparamagnetism in magnetic materials.

For half of the century, the development of nanotechnology has been and continues changing every field of science, technology and healthcare. According to the National Institutes of Health, the applications of nanotechnology for treatment, monitoring, diagnosis, and control of the biological systems has been referred as Nanomedicine [2].

For over 30 years, Nanomedicine has been stated to be one of the most important applications of nanotechnology. Today, Nanomedicine is not a futuristic vision anymore and its products are already entering the market for the benefit of the patients and society [3].

Applications of Nanotechnology in Medicine:

- Implantable Drug Delivery nanostructures
- Nanostructured scaffolds for tissue engineering
• Cell expansion
• Targeted nanoparticles for disease diagnosing
• Nanoparticle for the efficiency, sensitivity, and throughput improvement of bioassays
• Nano drug form formation for efficacy improvement, and side effects reduction
• Rapid wound healing dressings using nanofibrils [3]

Nowadays, Nanomedicine is a big term including many aspects and areas such as: Drug delivery, therapies, diagnostics, bio-sensors, biomaterials, and implants. With the numbers of publications rising from some ten per year in 80s to more than 1000 in 2004, Nanomedicine has seen an escalation in research activity over the past decade [4].

The content of this thesis, however, is only related with Nanomedicine in Drug Delivery.

1.2. Drug Delivery Systems

A drug delivery system (DDS) is defined as - “a formulation or a device that enables the introduction of a therapeutic substance in the body”. Few examples are the therapeutic products administration, the active ingredients release, and the active ingredients subsequent transport across the biological membranes to the place of an action [5]. Drug delivery study is the subject aiming to improve the effectiveness and reliability by controlling the rate, time, and location of the delivering of drugs inside the body. Thus, DDS is a bridge connecting the patient and the drug.

The use of Drug Delivery has been there thousands years since the day human discovered drugs or other medical treatment for sickness, diseases, virus, cancer and so on.
With the development of nanotechnology, there are many types of DDS have already been in drug industry. DDS based on particles are normally nano-sized uniformly dispersed particles, and composed of biocompatible and biodegradable synthetic polymers, self-assembled lipids, or inorganic materials. These nanoparticles (NPs) can carry therapeutics small molecules, drugs peptides, proteins, nucleic acids and deliver the payloads in a controlled manner to a desired site of action. They may carry drug molecules to the tumor site by encapsulation or as covalently linked drug molecules on the nanoparticle surface [8]. Comparing to other DDS, DDS based on NPs offer some unique advantages:

- High solubility as well as pharmacokinetics and pharmacodynamics profiles of the drugs.
- Drug targeting controllable (passive and active): passive targeting to increase drug concentration in tumor tissue and active targeting to reduce the drug concentration in normal tissue and toxic side effects.
- High drug efficiency by decreasing drug release during transit.
- Improving drug stability by reducing its degradation in the systemic circulation
- Improving cellular internalization and organelle-specific delivery of the loaded drug that results from adoption of various surface functionalization strategies [6].

Due to these advantages, over last 2 decades, a large number of nanoparticular DDS have been evaluated for medical science and industry, especially for cancer therapy.
Figure 1-1: Various types of nanoparticles used in biomedical research and drug delivery [7]

Figure 1-1 shows the different types of DDS based NPs recently studied and developed by research groups all around the world. Liong et al. claimed about the potential of inorganics NPs for simultaneous imaging and therapeutic applications [8]. Soppimath and his group in their review paper well presented the applications of biodegradable polymeric nanoparticles in DDS which is one of the most outstanding contributions in the field [9]. Gohla et al. reviewed the present state of the art regarding production techniques for solid lipid nanoparticles and its advantages over the traditional DDS [10]. There are also liposome, dendrimer, nanocrystal and nanotube have gained a lot of interest for their pharmaceutical and medicinal chemistry recently [11] [12] [13] [14].
1.3. Cell-NPs interaction

1.3.1. Importance of understanding Cell-NPs interaction

Recently, there are numerous articles and publications stated the important of understanding the interaction between NPs and their host systems. Since those NPs used as drug carriers come to contact with humans and the environment, characterizing the interaction between NPs and cells is very important from the perspective of safe use of nanomaterials. It is well established that NPs offer huge potential benefits to medicine through their applications as diagnostic and therapeutic agents. However, there is a need of in depth understanding of the possible risks to human health before NPs are used routinely, and yet this remains a comparatively underexplored aspect of nanomaterial science. Several papers have reviewed aspects of nanoparticle toxicology and cytotoxicity recently [15] [16] [17] [18].

This section therefore focuses on the review of the cell and NPs uptake pathway, and the impact of NPs on their biological host systems.

As drug delivery carriers, NPs are prepared with specific surface charges, morphologies and hydrophobicity depending on their purposes and application systems. Basically, NPs interact with the cells through electrostatic and/or hydrophobic interactions, hydrogen bonding and van der Walls forces and so on [19].

1.3.2. Cell membrane - NPs Mechanism

From a microscopic and physical point of view, the interaction of NPs with the cell is the combination of various specific physical or chemical interactions at the nano-bio interfaces at molecular level. In able to enhance solubility and biocompatibility, typically the NPs used for
biological applications have chemical/polymer coatings. The biological environment the NPs target is also vastly complex, consisting of proteins, peptides, nucleic acids, and lipids, etc. [20]. Cell contains intracellular organelles which are separated and protected by the cell membrane from the outside environment. Cell membrane is soft and fluid structure constructed by phospholipid bilayer, with hydrophobic fatty acid tails shielded in the middle of the layer and hydrophilic heads pointing out toward the aqueous environment. This membrane envelops the cell, for both prokaryotes and eukaryotes, surrounding the cell nucleus and other sub-cellular structures.

The integrity of cell membrane is extremely essential for cellular functions since it covers cells internal organelles, regulating bimolecular and ion trafficking to maintaining the electric potential and osmotic pressure of the cell [21]. Surrounding the cytoplasm, the cell membrane of living cells physically separates the intracellular components from the extracellular environment. It also plays an important role for providing shape of the cell by anchoring the cytoskeleton, and helps forming tissues by helping the cells to attach and group with the extracellular matrix or other cells [22].

For any interaction between cells and other extracellular subjects, the cell membrane presented the most important factor determining the mechanism of the interaction. Since the membrane allows which could able to enter and exit the cell, it is selectively permeable and thus catalyzing to carry of materials needed for survival. It works as a filter which has function filtering things which go in or out of the cell. The selective substances may transport through the cell membrane by “passive” way, which occurs without using cell energy or “active” way which requires energy expendability. [22].
Depending on the integration and the combination of conditions, in the presence of NPs, the interactions between those NPs and the cells membrane may follow different pathways.

*Figure 1-2: NPs interacting with a patch of non-uniform and dynamic cell membrane [19].*

Depending on the mechanism of the internalization, substances may create different impact to the biological functions of the cell. The impact could be much stronger when the internalization is made through membrane comparing to the internalization by mere adsorption onto the surface of cell membrane. The mechanism of internalization however, is complex with the involvement of many physicochemical interactions and biological activities.

Generally, there are two uptake pathways that cells internalize extracellular materials inside the cytoplasm: “energy-independent” pathway and “energy-dependent” pathway or also called endocytosis [23]. For energy-independent pathway cellular membrane uptakes NPs without any energy consumption. For example: a class of cationic peptides that contain protein transduction domains such as the TAT, penetratin, and VP22 peptides could be taken up by cells without energy generation. It was initially suggested that these peptides are able to penetrate through cell membranes directly by a non-endocytosis route. In the opposite side,
energy-dependent endocytosis pathway has been demonstrated as the main mechanism for the cells internalization.

Endocytosis refers to the internalization of macromolecules and solutes into cellular membrane-bound vesicles engulfed by the plasma membrane [24]. Endocytosis can also be subdivided into two main categories: the uptake of large NPs - phagocytosis or cell eating and the uptake of fluids and solutes - pinocytosis or cell drinking [24]. Pinocytic pathway is divided into three morphologically distinct sub-pathways, namely: clathrin- mediated endocytosis, caveolae- mediated endocytosis, and macropinocytosis.

- **Clathrin-mediated endocytosis (CME)** is the best well-known among all the endocytosis pathways [25]. CME occurs in the turnover of plasma membrane proteins and lipids in all mammalian cells and conducts the continuous uptake of nutrients, antigens, plethora of growth factor, and pathogens.

- **Macropinocytosis** usually takes place in highly ruffled religion of plasma membrane and indicates to the formation of large endocytic vesicles. It refers as a novel route for some pathogenic bacteria internalizing into the cells.

- **Caveolae-Mediated Endocytosis**: Caveolae are hydrophobic membrane consist of the cholesterol-binding protein caveolin and glycosphingolipids. It was described as flask-shaped invaginations of the plasma membrane and is essential for establishment of caveolar endocytic vesicles. Caveolae exists in many types of cells and rich in endothelial cells especially.
Figure 1 -3: Endocytosis mechanisms of nanoparticle uptake by cells. The four primary modes of NP uptake are illustrated [26]: “Clathrin-mediated endocytosis”, “Macropinocytosis”, “Caveolae-mediated endocytosis” and “Phagocytosis”.

Till now, in able to study the Cell – NPs internalization, the methods that scientists mostly use is labeling NPs with fluorescent molecule and use confocal microscopy to characterize. There is still no record of a real-time method which can monitor the fate of NPs when they interact with cells. In this study, we demonstrate the new approach to understand cell-NPs interaction by measurement of real-time mechanical property of cell membrane.
1.4. Cells mechanics measurement

As mentioned in many publications, understanding the mechanics of the cells became more and more significant, since many cellular activities have been discovered to be related by, or linked to changes of the cells mechanical properties [27].

Recently, there are several techniques have been using for studying cells mechanical properties (Fig. 1-4).

![Figure 1-4: Different types of experimental technique used to probe living cells. (a) Atomic force microscopy (AFM); (b) Magnetic twisting cytometry; (c) Micropipette aspiration; (d) Optical trapping; (e) Shear flow; (f) Substrate stretching; (f) Methods are capable of evaluating the mechanical response of a population of cells. [28]](image-url)
1.5. Atomic Force Microscopy

1.5.1. Introduction

AFM is a very powerful tool for materials science and has many applications acquired in biological science. Its precursor, scanning Tunneling Microscope (STM), was discovered and developed by Binning and Rohrer in 1980 at an IBM-Research in Zurich. Six years later, they invented the first AFM that was commercially available in 1989. While STM could only work with conductive samples, AFM could work with any kinds of surfaces which is much more applicable and effectual. Unlike other classical microscopes, AFM is operated by monitoring the force between its sharp probe and the specimen surface. AFM also has many advantages comparing to other microscopy, especially for biological science. Through its tip, AFM could topographically characterize samples which have resolution that is not able to achieve by optical microscopy. Also, AFM is one of the few instruments that have the ability to perform the characterization in liquid environment with minimal sample preparation [29].

For many years, AFM has been mainly used for samples characterization by scanning their surfaces in high resolution to achieve the three-dimensional images of the surface. However, AFM is not only a surface-imaging tool but also a powerful force measurement spectroscopy that can be used to probe the physical properties of the samples such as surface charges, molecular interactions, mechanical properties and so on [30].

1.5.2. Working Mechanism

As the same with other SPMs, by using a sharp tip, AFM raster scans across the sample surface to generate an image by applying a constant force. Typically the probe tip is made of
silicon or silicon nitride and is mounted on a cantilever spring. By focusing a laser beam on the back of the cantilever, all the movements of the AFM tip will be monitored and recorded by the laser reflection on a photodiode detector (Figure 1-5). The image is topographically measured by the up and down scanning motion of the AFM tip.

Figure 1-5: Schematic of an AFM operation. The cantilever scans the surface and bends due to the interaction force between the tip and the surface. The deflection of the cantilever is recorded as the laser beam displaced on the photodiode [31].

1.5.3. AFM Operation

1. Imaging modes: there are several AFM imaging modes available such as contact mode, non-contact mode and tapping mode. Typically, contact mode is widely used as a sample surface topography method. In the contact mode, the tip constantly contacts with the sample surface. In non-contact mode, the tip is scanned over the sample surface at 5-15 nm range. In tapping mode the
cantilever is oscillated at high distance (>20nm) and rastered across the sample, help reducing the lateral forces and the damage on the surface. Thus, for soft materials, the tapping mode is preferable, especially in fluid environment. While an optical microscope is limited by the diffraction limit of light, the AFM resolution is limited by the shape and size of the tip which is in the range one to few nanometers [29].

2. Force measurement: measuring the force acting between the AFM probe and the sample by achieving force-distance curves. This feature opens a new approach to study the heterogeneities of mechanical properties of the surface and subsurface layers of biological samples like cells at nano-level [32]. Its ability is not limited in obtaining a local elastic modulus of a surface but can measure the modulus difference across a sample surface and the ligand-receptor interactions. Force-distance curve is analyzed by monitoring the vertical deflections of AFM cantilever while the probe approaches and detaches the surface. A force curve is plotted based on the recorded signal of the photodiode voltage versus the piezo scanner position. A real-time force-distance curve can be obtained after by applying appropriate corrections [30].

1.6. Statement of Purpose

In order to develop advance drug delivery systems, one has to understand the interaction between the cells as a system and the NPs as a drug carrier. Without this understanding, it is really difficult to improve the efficiency of the delivery systems and to minimize the side
effects. Recently, there is an amount of research in understanding the interaction between NPs and cells. However, due to the small size of micro-organism, it is difficult to investigate their physical properties. In most of the researches, scientists obtained information by using some techniques such as: X-ray photoelectron spectroscopy, contact angle, electrophoretic mobility [33] or recently using confocal microscopy by adding fluorescence molecules to NPs. All of those methods, however, require manipulating the cells prior to examination, which may seriously compromise the validity of the analysis. In addition, by using those methods we could only generally obtained the information from large numbers of cells instead of individual ones. Hence, there is clearly a need for a new, nondestructive method which is capable of probing single cell at high resolution.

In this thesis, the NPs and the cell membrane interaction was studied using Atomic Force Microscopy in cellular real-time condition. Cerium dioxide nanoparticle (CNPs) or ceria was used in this study as model system.

1.6.1. Research Objective

The objective of this research is to understand cell-NPs interaction by mechanical point of view: monitor the changing of cellular Young’s Modulus with and without the presence of CNPs. Moreover, the NPs - cellular uptake pathways were also monitored by using AFM.

1.6.2. Hypothesis

It is hypothesized that:

- By using AFM, it is possible to measure any changes in cell membrane mechanical property due to interaction with NPs.
• Due to the different sizes and shapes, NPs may create different effects to the cell membrane structure lead to any change in cell elasticity.

• By adding particular cellular internalization inhibitor and using AFM to measure the elasticity, we may determine the exact the pathways that NPs enter inside the cells.
CHAPTER TWO: METHODOLOGY

Cerium dioxide nanoparticles or ceria (CeO$_2$) is a rare earth oxide material known widely for its unique properties and various biological applications. There have been many articles about ceria antioxidant behaviors which included superoxide dismutase activity [34], catalase mimetic activity [35], nitric oxide radical scavenging [36], and hydroxyl radical scavenging [36]. In our group, we have been developing an advanced drug delivery carrier based on this material. Thus, CeO$_2$ was selected as a model material in this thesis.

Cerium Nanoparticles (CNPs) with the following sizes and morphologies: nanoparticle with diamater 3-5 nm, nanoparticle with diamater15-20 nm, nanocube 20-30 nm and CeO$_2$ nanorod 50-200 nm were prepared wet-chemical or by hydrothermal method [37]. Several characterization techniques such as Transmission Electron Microscopy (TEM) and Dynamic Light Scattering (DLS) were used to confirm their sizes and morphologies of these nanostructures.

Cells were seeded on cover slips and then cultured for overnight. CNPs prepared were then added in each chamber glass well to obtain final concentrations: 0 µM, 50 µM, 125 µM and 250 µM. The cells were then incubated for different time periods (15, 30 and 60 minutes).

Following the incubation of cell with the nanoparticles, AFM was used to conduct a Force-Distance measurement on the cells in Phosphate Buffered Saline environment (PBS). PBS is a physiological buffer according osmolality and pH which is not harmful to the cells (at least not at short term) and may use for remove other excess staining substances. For each NP treated cells, the cells elasticity was calculated based on Hertz contact mechanics model [38].
2.1. Materials Preparation

In able to obtain CeO$_2$ nanostructures, we started from Ce(NO$_3$)$_3$.6H$_2$O used as a cerium source. Technically, 0.45-0.6 M of Ce(NO$_3$)$_3$.6H$_2$O and an appropriate amount of NaOH (10-22.5 M) were dissolved in 20 ml of deionized water separately. Then these two solutions were placed and mixed in a Teflon bottle. After stirring for 30 min, the Teflon bottle with the as-obtained milky slurry was transferred into a temperature-controlled electric oven for hydrothermal treatment. After 6-72 h process with temperature in the range of 80 - 180 °C, an obtained fresh white precipitates were separated by centrifugation and then washed with deionized water for several times. The sample was brought to dry at 80 °C in air overnight. The final products obtained were yellow powders [37].

2.2. Cell Culture

The human squamous cell carcinoma (RPMI, 2650; ATCC CCL 30) were used as model cells in this research. Cells were removed from the culture flasks with 0.25% trypsin/EDTA. We also counted and approximately calculated the concentration of cells before seeding. These cells were then seeded on German glass coverslips coated with Poly-D-lysine (PDL) in media environment for at least 24 hours incubation. The cell line experiment was designed so that the number of cells was around 300,000 cells per cover slip.
The incubator maintains the temperature of 37 °C which is the normal body temperature requirements simulating the human body condition for the survival of cell. Before doing experiments, the cover slips were washed several times with PBS to maintain a constant pH and to remove other excess staining substances.

2.3. Cell uptake investigation

In order to investigate the uptake mechanisms, through which these nanoparticles are endocytosed by cells, several pharmacological inhibitors for defined endocytotic pathways were used.

- To examine whether CNPs interact with cells by energy dependent or independent pathway, 2-Deoxy-D-glucose (DOG) and Sodium Azide (NaN3) inhibitor were used to stop cell energy production (with concentration 50 mM & 0.1% respectively)
• To monitor which pathway of endocytosis of cellular uptake of nanoparticle, 3 known inhibitors were used to block each of these pathways one by one, respectively:

I. 5-(N,N-Dimethyl) Amiloride hydrochloride: stock 100 µg/ml (final concentration 10 µg/ml) was used to block Macropinocytosis pathway.

II. Genistein synthetic: Stock 1 mg/ml (final concentration 100 µg/ml) to block the Clathrin-mediated endocytosis pathway.

III. Lovastatin: stock 100 µg/ml (10 µg/ml final concentration) was used to block the Caveolae-Mediated Endocytosis pathway.

Cells were incubated with inhibitors for 1 hr in serum free media to limit their uptake mechanism by specified pathway. Nanoparticles were introduced to the cells and incubated for appropriate time and then washed with PBS before carrying out the force curve measurements.

2.4. Force-Distance Measurement

The AFM instrument used to carry out this study was Solver Scanning Probe Microscope 2006 purchased from NT-MDT (Russia). The measurements were conducted in AFM Force-Distance mode.
Figure 2-2: The AFM cantilever is moved up and down with the z-piezo. When the AFM tip is brought to touch and indent the cell, the cantilever will bend. A laser beam points to head of the AFM tip and reflects onto a split photodiode measuring the bending of the cantilever [27].

First, in order to calibrate instrument, the probing experiment was carried out on a hard and smooth surface. On hard surface, the bending of the AFM tip equals with the displacement of z-piezo while the tip is touching the surface, so we could calculate and estimate the ratio between the bending and displacement which are not in the same unit. After, a coverslip containing the cells was mounted on a substrate inside the AFM chamber. A cell was selected using an optical microscope which is connected with the screen. The motion of the AFM tip was recorded as well as the applied force when tapping to the cell (Fig. 2-2).
In our experiment, MLCT AFM tip purchased from Bruker was chose to do the Force-curve measurement. With spring constant from 0.07 N/m to 0.14 N/m, this Bruker's microlever AFM probe have soft Silicon Nitride cantilever and tip is ideal for contact imaging modes, force modulation microscopy, and liquid operation. The range in force constants enables users to probe extremely soft samples in contact/tapping mode as well as high load versus distance spectroscopy.

Figure 2-3: MLCT Bruker AFM tip
CHAPTER THREE: RESULTS AND DISCUSSION

3.1 Ceria morphologies

Cerium oxide nanoparticles with different morphologies were prepared using hydrothermal method by changing the synthesis parameters such as concentration of base, temperature and the time of the reaction. Correlation of different synthesis parameters with size and shape of nanoparticles is presented in the phage diagram (Figure 3-1). With high temperatures but low concentration of NaOH, there was a shift from nanoparticles to nanorods. When temperature and caustic concentration were increased, there was high concentration of nanorods based on moderate hydrothermal conditions. When the temperature of the treatment was above 180°C, nanorods and nanocubes morphologies were dominant [37].

![Figure 3-1: Morphological phase diagram of CeO₂. The phase boundaries were estimated to](image-url)
indicate regions of nanostructure formation: nanoparticle, nanorod, and nanocube. The estimation was made based on experimental parameters of the hydrothermal treatment and other physicochemical characterizations [37].

Figure 3-2: TEM image and DLS data of (a) CeO$_2$ nanoparticles with diameter 3-5 nm; (b) CeO$_2$ nanoparticles with diameter 15-20 nm (c) CeO$_2$ nanocubes; (d) CeO$_2$ nanocubes [37].

Figure 3-2.a shows the TEM image of the uniform CNPs with diameter 3 – 5 nm and the DLS result indicates the distribution of their size in aqueous environment. Figure 3-2.b shows the TEM image of uniform CNPs with diameter 15 – 20 nm and the histogram represents the hydrodynamic radii of the nanoparticles. Figure 3-2.c exhibits the TEM image of the CeO$_2$ nanocubes, with diameters of 20 nm and and the DLS results of the distribution
of their hydrodynamic size. Figure 3-2.d shows a typical TEM image of nanorods, 50-200 nm in size and and their hydrodynamic size distribution.

Table 1: Comparison between the size of CNPs as measuring in water and in Media at 60 minutes and 250 µM

<table>
<thead>
<tr>
<th>CNPs</th>
<th>Size in Media (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanoparticle (3-5 nm)</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>Nanoparticle (15-20 nm)</td>
<td>194 ± 10</td>
</tr>
<tr>
<td>Nanocube (30 nm)</td>
<td>370 ± 3</td>
</tr>
<tr>
<td>Nanorod (50-200 nm)</td>
<td>192 ± 4</td>
</tr>
</tbody>
</table>

Since the CNPs were dropped into the cell in media environment, the sizes of CNPs then were characterized by DLS to compare with the sizes of CNPs in water. Table 1 above shows that the sizes of CNPs in media incubated for 60 minutes had the same results comparing to their size in water.
3.2 Cells Young’s Modulus Measurement

3.2.1 AFM Force Curves

The curve obtained during the approach of the AFM probe on the cell membrane is the Force-Distance (FD) curves or also called DFL (Deflection)-Height. We could observe an increasing of the DFL signal when the tip touches the surface of the samples and the bends. The signal of the cantilever’s deflection is given in nA because this is the signal from the detector. In order to recalculate it in nm, the optical registration system needs to be calibrated. There are several points should be taken into account while calibrating the instrument:

Figure 3-3: AFM Force Curve schematic
1. Once the cantilever is installed and the laser is aligned, they should not be touched/move anymore because the movement may cause the changing of a laser alignment signal. Depending on the position of the laser spot on the cantilever, the same bending of the cantilever will give different DFL signals.

2. The calibration should be made on a hard surface which the tip does not indent into the sample. In our case, the cells are attached on cover slips which are glasses and hard surfaces.

3. The force of AFM cantilever should not be too much since it may damage on hard surface and led to the incorrect results when doing experiment.

The calibration procedure is the following: AFM cantilever was installed and laser was aligned. Then FD curve was made on a hard surface. Because the tip could not indent the sample, the cantilever bended and we had linear correspondence between the bending and the movement of the piezo. In other words, the bending part on the FD curve should be linear. On the X-axis movement of the piezo (also call Z-axis) in nm and on the Y-axis- the bending of the cantilever in nA was plotted. From the plots, we could find how many nm corresponded to the deflection of 1nA were identified and this transformation coefficient was used for all the following measurements as long as the laser is not moved or touched.
As we could see, if the force curves are obtained on hard surface, since there is no penetration of the AFM tip inside the sample, the deflection of the AFM cantilever should be equal to the movement of the AFM probe or the bending of the AFM cantilever in nm should be the same with the distance of the piezo. Also, since the experiment was made on hard surface, the force curves obtained followed Hooke’s Law.
When the Force-Distance curve measurements were carried on cells, we should not obtain the same with what we had on hard surfaces (Fig. 3-4). The reason is that cells are soft and when the AFM tip approach cell, there is an indentation of this tip inside the cell. That is why instead of a straight line as on hard surfaces case; we saw a curve in Figure 3.4. Based on our experience when doing the Force – Distance measurement, the “curvier” that the Force curves showed, the “softer” the samples were. Also, the difference between the AFM cantilever deflections at the same piezo on hard surface and soft surface exactly equals with the indentation of AFM tip penetrating inside cell (Fig. 3-2). The measurement was carried out by moving the AFM tip along x-axis and y axis to the positions of cells and then did the force curves. There were 20 cells for an average measurement.
3.2.2 Cells Young’s Modulus

To measure the Young’s Modulus (elasticity) of a cell, the AFM probe was brought to indent into the cell, and the cantilever deflection is determined as a function of the piezo z position. The force F that the sample (cell) acted on the AFM cantilever was calculated through Hooke’s law: \( F = k \cdot d \), in which \( d = \alpha \cdot A \). Where k is the cantilevers spring constant, A is the measured cantilever’s deflection in nA, and \( \alpha \) is called the deflection sensitivity that converts cantilever’s deflection from nA to nm. The resulting deflection vs. indentation curve is then typically fit to the Hertz model (Fig. 3.4)

Hertz model: In order to evaluate the Young’s modulus of cells, the shape of AFM tip have to be taken into account since with different types of AFM tip lead to the different Hertzian contact model. In our case, the formula for four-side-pyramid probe:

\[
F = \frac{E \cdot \tan \alpha}{1 - \nu^2 \sqrt{2}} \delta^2
\]

With:

- F: Force acting on the probe
- E: Young’s Modulus of the Cell
- \( \nu \): Poisson’s ratio
- \( \delta \): indentation depth
- \( \alpha \): face angle of the probe
Figure 3-6: Cells and AFM probe through microscopy while scanning by AFM

Figure 3-7: Cell indentation as scanned by AFM
The cells Young’s Modulus measurement was carried out by moving the AFM tip along x-axis and y axis to the positions of cells and then did the force curves. There were 20 cells for an average measurement.

![Graph showing Young's modulus vs indentation](image)

*Figure 3-8: Cells Young’s modulus vs. the indentation of AFM tip inside*

As stated in the experimental section, the Force Curve measurement was carried in a PBS environment. The reason we want to do experiment in PBS instead of cell culture medium is that it lower the chance of contamination as AFM was carried in non-sterile open environment and in absence of external stimuli (FBS component) present in the medium. Moreover PBS can support cell survival for one to two hours, which is sufficient to correct the force data. In able to determine if there is any effect of PBS environment to the cell elasticity, we carried Force-distance Spectroscopy DFL (Height) in both environments for comparison.
Figure 3-9: Cells Young’s modulus in PBS vs. Medium Environment after adding 50µM CNPs following by 60mins incubation.

In able to determine if there is any effect of PBS environment to the cells elasticity, we did the Force-distance Spectroscopy DFL (Height) in both environments for comparison. Figure 3-9 shows the cells elasticity results in control and after 50 µM CNPS treatment for 60 minutes of incubation both in PBS and cell culture medium. As we could see in both environments, Young’s Modulus of the cells remained the same around ~ 20 kPa.
Figure 3-10: Cells Young’s modulus vs. incubation time of different CNPs nanomorphologies: with different concentrations and incubation times

The results show that when the concentration and the incubation time is increased, the Young’s Modulus of cells decreased. However, the trend is not the same with all CNPs morphologies. Irrespective of size and shape of the nanoparticles at low concentration there is minimum influence on cell membrane. However at high concentration $\geq 250 \mu M$ there is significant effect on cells membrane elasticity, which may indicate that higher concentration
of nanoparticles can damage the cell membrane. As seen in Figure 3-8, CNPs based nanoparticles with diameters from 3-5nm showed the slower decreasing rate comparing to other morphologies. These results may state that the smaller size CNP has minimum influence in the cell membrane. The higher the concentration of CNPs and the longer of the incubation time took, the lower the cells Young’s Modulus. However, the rate of changing of cells elasticity also depends on the size and shape of the materials.

![Figure 3-11: Cells Young’s modulus vs. incubation time of different CNPs nanomorphologies and incubation times when adding 50 µM CNPs](image)

This graph shows the cells Young’s Modulus when adding 50 µM of CNPs to the cells for incubation. As we could see, the cells elasticity when adding different morphologies of CNPs does not change. They stayed around 20 kPa, which is the same with the controlled
cells without NPs. This result indicated that in low concentration (50 µM) of CNPs added, there is no significant influence on the cell membrane elasticity.

Figure 3-12: Cells Young’s modulus vs. incubation time of different CNPs nanomorphologies and incubation times when adding 125µM CNPs

Figure 3-12 shows cells Young’s Modulus in presence of 125 µM of CNPs. The cell elasticity started to change at 30 minutes of incubation. The elasticity dropped from 20 kPa to around 15 kPa. We could see that at 15 minutes, the cells elasticity does not change. The change in cell elasticity may be indicative of nanoparticle- cell interaction as well as the internalization process. This result clearly indicates that the bigger size particles for example rod and cubes need more time to internalize, whereas small particles easily go inside the cell.
Figure 3-13: Cells Young modulus vs. incubation time of different CNPs nanomorphologies and incubation times when adding 250 µM CNPs

With 250 µM CNPs, the Young’s Modulus of cells dropped significantly with various time of incubation. However, the rates of these changes were different among CNPs morphologies. Those cells incubated with CNPs nanocubes and nanorods demonstrated the decreasing of elasticity from 20 kPa to around 5 kPa right after 15 minutes of incubations. CNPs based on nanocubes, nanorods and nanoparticle (20 nm diameter) all decrease significantly after 30 minutes of incubation. In contrast, cells added with CNPs based smaller nanoparticles (3 nm diameter) decreased their elasticity clearly slower than others. However, after 60 minutes, Young’s Modulus of all the cells decreased. From these results it may be concluded that smaller size nanoparticles are better for delivery system since it show less decrease in cell elasticity comparing to cell when adding other CNPs nanostructures.
Figure 3-14: ICP-MS results indicating the concentration of Ce inside the cell based on CNPs concentration vs. incubation time.

In able to confirm the concentration of Ce inside the cells depending on the CNPs concentration and incubation time, we used Inductively Coupled Plasma Mass Spectrometry (ICP-MS) which is a type of mass spectrometry to detect low concentration in cells. The result showed that the CNPs went inside the cells and the concentration of Ce inside the cell was proportional to the CNPs concentration and incubation times as predicted.
3.2.3 Cell internalization pathways

As mentioned in the introduction, eukaryotic cells internalize extracellular materials inside the cytoplasm, which are energy-independent pathway and energy-dependent pathway. As we used 2-Deoxy-D-glucose (DOG) and Sodium Azide (NaN3) inhibitor to stop cell energy production, only particles could only able to internalize inside the plasma membrane by independent pathway. We could see in the result in Fig. 3-15, the cell added smaller size CNPs with diameter 3 nm did change cell membrane elasticity while the larger nanoparticle (20 nm diameter) did not. This means small NPs could be able to interact with cell membrane by energy independent pathway while larger NPs could not be.

Figure 3-15: Cells Young’s modulus in different size nanoparticles in presence of inhibitors which inhibit cells energy production. Experiment carried out at concentration 125 µM in 60 minutes.
Chang et al. [23] also mentioned that low molecular weight solutes directly transport through the plasma membrane via the energy-independent pathway without energy consumption in carbon nanotubes. The energy-dependent pathway, also known as endocytosis for uptake of relatively large particles [23].

Figure 3-16: Cells Young’s modulus in presence of inhibitors which specifically inhibits selective pathway of endocytosis such as Amiloride hydrochloride for blocking Macropinocytosis pathway, Genistein for blocking the Clathrin-mediated endocytosis pathway and Lovastatin for blocking the Caveolae-Mediated Endocytosis pathway

In order to go further and determining which pathway the larger NPs internalize in the cells membrane, we used inhibitors to block all possible pathways that NPs could use. When we block each pathway of internalization, we want to see which pathway changing the elasticity of cells. The result showed that when we block the Caveolae-Mediated Endocytosis pathway, Young’s Modulus of cell did not change (~ 20 kPa as the same with Young’s Modulus of cells
without CNPs). In contrast when we block the other two pathways, Clathrin-mediated endocytosis pathway and Macroendocytosis pathway, the cells showed changing in the elasticity. This interesting result indicated that the larger NPs with diameter 20 nm interact with cells and internalize by Caveolae-Mediated Endocytosis pathway.
CHAPTER FOUR: CONCLUSION

This dissertation has been focused on an investigation of NPs-cell interaction and followed by monitoring the cell mechanical property using AFM. CNPs (cerium dioxide nanoparticles) were used as sample NPs for testing of the hypothesis. The most important observations and conclusions of this dissertation are summarized below.

1. Interaction between CNPs and cell membranes depends on the concentration of NPs and the time of incubation. Based on our results, with the same NP morphologies, the effects of NPs made on cells elasticity were different from 50 µm to 250 µM and from 0 minutes to 60 minutes.
   - At low concentration (50 µM) the interaction does not affect the mechanical properties of the cell membrane.
   - At high concentration CNPs (250 µM) there were significant changes in the cell elasticity.

2. Size and shape of the nanoparticles play an important role in cell interaction.

3. The correlation between the cellular uptake and the tracking of NPs with their size was investigated. By using appropriate inhibitors, we could see the difference of the cells elasticity. While small diameter CNPs (3-5 nm) could internalize cell by non-energy-dependent pathway, bigger diameter CNPs (20-30 nm) were entered in the cells by caveolae-mediated endocytosis.
LIST OF REFERENCES


715–725.


