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Understanding the Biological and Environmental Implications of Nanomaterials

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UNDERSTANDING THE BIOLOGICAL AND ENVIRONMENTAL IMPLICATIONS
OF NANOMATERIALS

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Materials Science and Engineering

by
Sijie Lin
May 2010

Accepted by:
Dr. Pu-Chun Ke, Committee Chair
Dr. John Ballato
Dr. Jian Luo
Dr. Igor Luzinov
ABSTRACT

The last two decades have witnessed the discovery, development, and large-scale manufacturing of novel nanomaterials. While nanomaterials bring in exciting and extraordinary properties in all areas of materials, electronics, mechanics, and medicine, they also could generate potential adverse effects in biological systems and in the environment. The currently limited application of nanomaterials in biological and ecological systems results from the insufficient and often controversial data on describing the complex behaviors of nanomaterials in living systems. The purpose of this dissertation intends to fill such a knowledge void with methodologies from the disciplines of biophysics, biology, and materials science and engineering.

Chapter 1 of this dissertation provides a comprehensive review on the structures and properties of carbon nanomaterials (CBNMs), metal oxides, and quantum dots (QDs). This chapter also details the state-of-the-art on the biological applications, ecological applications, and toxicity of nanomaterials.

With Chapter 1 serving as a background, Chapters 2-5 present my PhD research, an inquiry on the fate of nanomaterials in biological and ecological systems, on the whole organism and cellular levels. Specifically, CBNMs are introduced to rice plant seedlings and the uptake, translocation and generational transfer of fullerene C\textsubscript{70} in the plant compartments are imaged and characterized. The interactions between CBNMs and rice
plants on the whole organism level are initiated by the binding between CBNMs and natural organic matter (NOM), driven by the transpiration of water from the roots to the leaves of the plants and mediated by both the physiochemical properties of the CBNMs and plant physiology.

In Chapter 3, semiconducting nanocrystals quantum dots (QDs) are introduced to green algae *Chlamydomonas* to probe the interactions of nanomaterials with ecological systems on the cellular level. The adsorption of QDs onto the algal cell wall is quantified by UV-vis spectrophotometry and fitted with the Freundlich isotherm. Effects of the adsorption of QDs on the photosynthetic activities of the algae are evaluated using O$_2$ evolution and CO$_2$ depletion assays, and the ecological impact of such adsorption is discussed.

To understand the effects of nanomaterials on the cell membrane, nanoparticles (Au, TiO$_2$, and QDs) of different surface charges and chemical compositions are introduced to HT-29 mammalian cells in Chapter 4. The polarization of the cell membrane is investigated using a FLIPR membrane potential kit. The phase of the cell membrane, in the presence of both positively and negatively charged nanoparticles, are examined using laurden, a lipophilic dye that serves as a molecular reporter on the fluidic or gel phase of the host membrane.

To address the effects of nanomaterials on biological and ecological systems within the same context, Chapter 5 offers a first parallel comparison between mammalian and plant
cell responses to nanomaterials. This study is conducted using a plant cell viability assay, complimented by bright field, fluorescence, and electron microscopy imaging. Discussions of this study are presented based on the hydrophobicity and solubility of C_{60}(OH)_{20} and of supramolecular complex C_{70}-NOM, hydrophobicity and porous structure of the plant *Allium cepa* cell wall, and the amphiphilic structure and endocytosis of the plasma cell membrane of both *Allium cepa* and HT-29 cells.

Chapter 6 summarizes and rationalizes results obtained from the entire dissertation research. Future work inspired by this research is presented at the end of the chapter.

Specifically, this dissertation is structured to embody the following essential and complementary chapters:

- Chapter 1: Literature review
- Chapter 2: Nano-Eco interactions at the whole organism level;
- Chapter 3: Nano-Eco interactions at the cellular level;
- Chapter 4: Nano-Bio interactions at the cellular level;
- Chapter 5: Parallel comparison of Nano-Eco and Nano-Bio interactions at the cellular level.
- Chapter 6: Conclusions and future work

The overarching goal of this research is to advance our understanding on the fate of nanomaterials in biological and ecological systems. Knowledge obtained from this
dissertation is expected to benefit future research on the implications and applications of engineered nanomaterials.
DEDICATION

This dissertation is dedicated to my parents and beloved ones.
ACKNOWLEDGMENTS

I would like to thank many people who have helped and supported me throughout my Master and PhD studies at Clemson.

I own my deepest gratitude to my advisor Dr. Pu-Chun Ke, for his endless guidance and support. He brought me into this exciting field of biophysical nanoscience, he guided me through every step in the lab, and he trained me on each process of research from literature review, project design, experimental execution, data analysis, to paper writing. His passion about science, his intelligence on envisioning research projects, his broad knowledge, and studious working style have inspired me tremendously and this dissertation would not be possible without him.

I am grateful to my PhD committee members Dr. Jian Luo, Dr. Igor Luzinov, and Dr. John Ballato. I have greatly benefited from their comments on my research proposal, thoughtful suggestions on my research designs, and fruitful discussions on various occasions. I am honored to have such an outstanding group of scientists serving on my committee.

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I wish to thank all who have helped me find my place at Clemson.
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CHAPTER ONE
LITERATURE REVIEW

1.1 Introduction

“There is plenty of room at the bottom.” This famous line stated by Richard Feynman on December 29th, 1959 at the annual meeting of the American Physics Society opened the door towards nanotechnology and has recruited millions of brilliant minds to research nanostructure materials synthesis, applications of nanomaterials, and the development of nanoscale devices covering every aspect of human life [1]. What Feynman envisioned was further realized by the Foresight Institute in 1989 as the bottom-up approach for manufacturing nanotechnology products. This approach uses the chemical properties of a single atom or molecule to self-organize or self-assemble into some useful conformation. The forces commonly involved in such an assembly include covalent bonding, hydrogen bonding, metal chelating, hydrophobic forces, van der Waals forces, pi-pi stacking, and electrostatic effects. In contrast, the top-down approach uses the traditional micro-fabrication methods where externally-controlled tools are used to cut, mill, and shape materials into the desired shape and order. These two methods (bottom-up and top-down) are nowadays frequently adopted by researchers in nanotechnology [2].

The uniqueness of nanomaterials originates from their nanoscale size and hence millions or even billions times larger surface area than their bulk materials. As illustrated by
figure 1-1, a solid cube of 1 cm³ in volume has a surface area equal to 6 cm², if the cube was divided equally into one thousand cubes of 1 mm³ in volume, the surface area would increase to 60 cm². If further milled down into cubes of 1 nm³, the surface area would increase to ten millions times more. The dramatically increased surface area results in a much higher surface energy and much more reactive surfaces. On the nano-scale, the mechanical, electronic, optical, chemical, and other properties may differ significantly from the properties of bulk materials [3].

![Figure 1-1. Illustration of correlation between surface area and particle size [4].](image)

1.1.1 Carbon based nanomaterials (CBNMs)

Carbon based nanomaterials (CBNMs) are one of the most studied and extensively used nanomaterials. They possess excellent electrical, mechanical, thermal and biological properties. Since the discovery of fullerene C₆₀ in 1985 and the single-wall carbon
nanotube (SWNT) in 1991 [5, 6], CBNMs nowadays have many different forms of nanostructures including spheres (fullerenes), tubes (single-wall and multi-wall), coils, wires, etc (Figure 1-2). Depending on the methods of synthesis and properties, they can be used as drug delivery systems [7-10], bio-imaging agents [11-13], therapeutic materials [14, 15], fiber reinforcement agents [16, 17], or thermal or electrical conductivity enhancers [18-19]. Due to the inherent hydrophobicity of CBNMs, they readily aggregate and require surface modification to be able to disperse well in an aqueous environment. Studies have shown that by covalently bonding with hydrophilic functional groups [20-22] or physically adsorbing with amphiphilic amoities [23-25], a suspension of CBNMs can be obtained.

Figure 1-2. Six allotropes of carbon. a, diamond. b, graphite. c, graphene. d, amorphous carbon. e, C_{60}. f, single-wall carbon nanotube [26].
1.1.2 Nanosized metals and metal oxides

Nanosized metals and metal oxides, such as Au nanoparticles (AuNPs) and TiO₂, are additional examples of major forms of nanomaterials developed over the past decades. AuNPs were highly attractive due to their inert surfaces, low-cytotoxic effects and capability in inducing unique optical properties such as surface plasma resonance (SPR) [27-29]. The application areas of AuNPs include nanomedicine, molecular imaging, contrast agents, and drug delivery systems [30-33].

Two major crystal forms of TiO₂ are known: anatase and rutile, each with different toxicity effects. These forms have been extensively used in the cosmetic industry due to their nanoscale size and excellent UV absorbing capacity to effectively block damaging light sources [34, 35]. However, studies have also shown that their highly reactive surfaces initiate the generation of harmful reactive oxygen species (ROS) that would damage cells and tissues [36-38].

1.1.3 Quantum dots (QDs)

QDs, also known as the semiconducting nanocrystals, were discovered by Louis Brus when he was at the Bell Labs. They are usually crystals composed of materials from the II-VI, III-V, or IV-VI periodic groups. The uniqueness of QDs comes from their nanoscale size (2 ~10 nm in diameter) and tunable fluorescence emission spectra. This
flexibility of tuning QDs depending upon their size is based on the concept of quantum confinement.

Quantum confinement describes a physical regime where the electron energy levels of semiconducting materials can no longer be treated as continuous. For bulk semiconducting materials with a fixed composition, their energy levels are continuous and bandgap energies are fixed. Because of the fixed bandgap energies, transitions from the conducting level to the valence level result in fixed emission frequencies. For QDs whose sizes approach Exciton Bohr Radius, the excitons are confined in the three spatial dimensions. As a result, the electron energy levels are no longer continuous, but discrete. The bandgap energies of QDs are not only dependant on the composition but also the size of the crystals. As the sizes of the crystals decrease, the bandgaps of the quantum dots increase, causing blue shifts in the emission wavelengths (Figure 1-3). Besides the fluorescence properties being tunable, QDs’ anti-bleaching property makes them more robust imaging agents than traditional organic fluorophores [39, 40].
1.2 Bio-applications of nanomaterials

One way to better understand nanomaterials is to examine the connections between their structures, properties, and applications. The aim of this section is to review the bio-applications of nanomaterials based on their surface, optical, electric, thermal, and mechanical properties.

1.2.1 Applications based on surface properties

Pristine CBNMs have been proven difficult to solubilize in aqueous solutions due to their...
intrinsic hydrophobicity, which limits their use in biological application. However, surface modification has shown great promise not only to disperse them in the aqueous phase but also equip them with new functionalities.

Pantarotto et al. demonstrated that fluorescently-labeled SWNTs covalently bound with bioactive peptides could penetrate cellular and nuclear membranes [42]. Confocal fluorescence microscopy was used to slice through the cells optically to localize the SWNTs (Figure 1-4). In this case, the hydrophobic surfaces of the SWNTs were more favorable for initiating their contact with the amphiphilic cell membranes. The resulting uptake and transport of the bioactive peptides were attributed to both passive diffusion and endocytosis of the cells. Besides bioactive peptides, Pantarotto et al. also demonstrated that by covalently modifying the surfaces of SWNTs or MWNTs with a pyrrolidine ring bearing a free amino-terminal oligoethylene glycol moiety attached to the nitrogen atom, the resulting positively charged ammonium functionalized nanotubes could bind to the phosphate groups of plasmid DNA and be delivered into HeLa cells (Figure 1-5) [43].
Figure 1-4. Epifluorescence (A) and Confocal microscopy (B) images of 3T3 cells incubated with SWNT, respectively. Epifluorescence microscopy images (C, D, E and F) of 3T6 cells incubated with SWNT. The nucleus is stained with DAPI (blue) and the SWNT is functionalized with FITC dye (green) [42].
Figure 1-5. TEM images of ultrathin section of Hela cells treated with functionalized MWNT. A, the entire cell. B and C, two subsequent magnifications. D, a MWNT crossing the cell membrane [43].

Ke et al. demonstrated the delivery of RNA polymer poly(rU) to MCF-7 breast cancer cells using SWNTs as a gene transporter [44]. In this study, the surface modification was done by physical adsorption and supramolecular assembly. The binding between an SWNT and the RNA polymers was elicited by the hydrophobic interaction and pi-stacking between the external surface of the SWNT and the nitrogenous bases of the
RNA. The translocation of RNA across the cell membranes and their distribution within the cytoplasm and nuclear membranes were monitored by confocal microscopy.

Towards this direction of gene and drug delivery, my previous work demonstrated that SWNTs could also be used to deliver phospholipids, the major component of the cell membrane, into cancer cells [45]. The amphiphilicity of the Rhodamine-labeled phospholipids and the hydrophobic SWNT formed a supramolecular complex through hydrophobic interaction. Due to the partially overlapping spectra between the Rhodamine dye emission and the SWNT light absorption, and the small spatial separations between the phospholipids and the SWNT, fluorescence resonance energy transfer (FRET) was observed in such supramolecular complexes upon light excitation. After incubation with the cancer cells, fluorescence signals were found to recover inside the cells. These results suggested that after entering the cell membranes, some of the phospholipid molecules were stripped off from the SWNT surface due to the relatively weak binding strength between them. Such easy release could be further exploited for the purposes of drug delivery.
Figure 1-6. Confocal images of MCF-7 cell incubated with SWNT. a, control cells without SWNT. b, SWNT without cells. c-f, MCF-7 cells incubated with SWNT. The red spots suggest the dissociation of Rhodamine labeled lipids from SWNT [45].

The specificity in nanoparticles drug delivery systems has been realized by recruiting antibody-antigen binding, one example of such is the biotin-streptavidin system. Ojima et al. developed a novel SWNT-based tumor-targeting drug delivery system, in which SWNTs served as a platform conjugated with tumor-recognition modules (biotin and a spacer) and prodrug modules of an anticancer agent (taxoid with a cleavable linker) [46]. After the SWNT-based drug delivery system was introduced to cancer cells, the biotin recognized and bound to the streptavidin on the cancer cell surfaces and induced receptor-mediated endocytosis. Once inside cells, the linker between the taxoid and the
SWNTs was cleaved off by endogenous thiols such as glutathione, thirredoxin, or other intracellular thiols, and the active cytotoxic agent, taxoid, was released to cause tumor cell death. Such surface modifications eliminated the non-specific binding between the SWNTs and non-tumor cells (Figure 1-7).

These bio-inspired applications reviewed above utilized the surface properties of the nanomaterials such as hydrophobicity and high surface area, and the ability of the nanomaterials to conjugate through multiple surface functionalizations.

Figure 1-7. Schematic illustration of the SWNT-based tumor-targeted drug delivery system [46].
1.2.2 Applications based on optical properties

Many bio-applications of nanomaterials employ their intrinsic optical properties. Researchers often probe the Raman scattering and infrared (IR) fluorescence signals of carbon nanotubes to track them inside the biological samples. Weisman et al. studied the uptake of pristine SWNTs into macrophage-like cells using the intrinsic near-infrared fluorescence of nanotubes (Figure 1-8) [47]. The fluorescence spectrum of the SWNTs ranged from 950 to 1450 nm and had several signature peaks at 985, 1060, 1150 and 1280 nm. The near-infrared fluorescence microscopy at wavelengths above 1100 nm provided high contrast images which indicated that nanotubes were located in numerous intracellular vesicles. Strano et al. further monitored the nonphotobleaching SWNTs as they were incorporated into and expelled from NIH-3T3 cells in real time on a perfusion microscope stage [48]. The rates of endocytosis and exocytosis of the cells closely matched (Figure 1-9).
Figure 1-8. Near-infrared images of cells incubated with SWNT. Line scan of the fluorescence intensity of SWNT [47].
Figure 1-9. a, a subset of observed trajectories extracted using Particle Tracker. b-i images show the process of endocytosis. j-m images show the process of exocytosis. n-m images show the aggregation and movement inside the cells [48].

Colloidal AuNPs have been considered as “plasmonic nanorulers” because they contain free electrons that can be collectively and resonantly excited at optical frequencies, leading to a large enhancement of the electromagnetic field near the particles’ surfaces [49]. Due to this unique optical property, AuNPs are frequently employed to enhance the surface sensitivities of fluorescence emission and Raman scattering. Nie et al. recently developed stimuli-responsive surface-enhanced Raman scattering (SERS) nanoparticles based on colloidal gold nanocrystals with a class of thiolated block copolymers consisting
of a pH-responsive polymer segment (Figure 1-10) [50]. The resulting complexes demonstrated that the SERS signals could be switched on and off by changing the solvent pH. Instead of metallic AuNPs, Rajh et al. used semiconducting nanoparticles TiO$_2$ for their SERS experiments [51]. The Raman scattering of biomolecules (dopamine etc.) adsorbed on the surfaces of the TiO$_2$ nanoparticles yielded an enhancement factor up to $~10^3$ (Figure 1-11). This field enhancement was associated with the asymmetric vibrations of attached molecules that lowered the symmetry of the charge transfer complex. The intensity and the energy of selected vibrations were also dependent on the size and shape of TiO$_2$ nanoparticles.
Figure 1-10. Schematic illustration of AuNPs based pH sensing materials [50].
QDs have been tested in numerous biotechnological applications that utilize fluorescence, including immunofluorescence assays [52], DNA array [53], and cellular trafficking studies [54, 55]. Researchers have shown that QDs can also be used to label membrane proteins [56], microtubules [57], and actin filaments [58], etc. The tunable fluorescence spectra of the QDs have enabled researchers to circumvent the autofluorescence from the biological samples; meanwhile the fluorescence signal of the QDs is still stable and strong enough for real-time imaging. In the animal study by Larson et al, two-photon excitation confocal microscopy was used to image blood vessels in live mice that had received QDs by intravenous injection [59]. The QDs showed higher contrast and...
imaging depth at a lower excitation power than organic fluorophores. To make such QDs target specific, Gao et al. functionalized the QDs surfaces with PEG polymers and antibodies to a prostate-specific membrane antigen [60]. The QDs rapidly migrated to nearby lymph nodes and were imaged directly (Figure 2-12). These applications suggest that functionalized QDs could possibly aid surgical procedures in animals and humans.

Figure 1-12. QDs served as an optical probe for tumor detection [59].

1.2.3 Applications based on electrical properties

Lieber’s group has pioneered the area of semiconducting nanowires based field-effect transistors (FETs) [61-63]. Such devices exhibit a conductivity change in response to variations in the electric field or potentials at the surfaces of the device. Adsorption of
biomolecules onto the nanowires can lead to an increase or decrease in the device conductance depending on the net charge of the biomolecules. By functionalizing with certain binding receptors, the devices can be used to detect specific biomolecules in real time.

1.3 Eco-applications of nanomaterials

The advanced properties of the nanomaterials suggest that they can also be utilized in the ecological systems - many of the current problems involving water quality could be resolved or greatly ameliorated using nanomaterials. Nanomaterials have two key properties that make them particularly attractive as nanosorbents. One is that they have much larger surface areas than bulk materials, and the other is that their surfaces can be functionalized with various chemical groups to increase their affinity for target compounds. For example, Li et al. investigated the sorption capability of MWNT to heavy metal ions (Pb(II), Cu(II) and Cd(II)) in aqueous solution [64]. They found that the sorption capacities of MWNT were 3~4 times larger than that of the sorbents (powder activated carbon and granular activated carbon) routinely used in water purification. Fugetsu et al. encapsulated MWNTs inside cross-linked alginate vesicles, and the caged MWNT could be used to absorb four water-soluble dyes (acridine orange, ethidium bromide, eosin bluish and orange G) [65]. Cheng et al. demonstrated that fullerenes had a high affinity for polycyclic aromatic hydrocarbons (PAHs) and could be used to absorb major contaminants in water sources, such as naphthalene and phenanthrene [66].
Nanomaterials can also be used to promote the growth of plants or reduce the effects of bacteria in the environment. Studies have shown that TiO\textsubscript{2} nanoparticles (anatase) could promote photosynthesis and greatly improve spinach growth by facilitating the electron transfer during photosynthesis [67]. Liu et al. also demonstrated that other crystal forms of TiO\textsubscript{2} nanoparticles (rutile) could increase the seed germination rate of spinach and increase the dry weight of the plants during growth [68]. The promotion on growth was related to N\textsubscript{2} fixation by TiO\textsubscript{2} nanoparticles. Biris et al. found MWNTs could penetrate tomato seeds and affect their germination and growth rates [69]. The germination was found to be dramatically higher for seeds that germinated on the medium containing MWNTs (10~40 µg/mL) compared to the control. They concluded that the MWNT were able to penetrate the thick seed coats and support water uptake inside seeds, thereby affecting seed germination and growth (Figure 1-13).
Recent studies have suggested that the antiviral activity of fullerols is originated from its ability to generate singlet oxygen in the presence of UV light or superoxide in the presence of both UV and an electron donating molecule [70]. Such nanomaterials also exhibited a potent antibacterial activity toward physiologically diverse bacteria over a range of environmental conditions. Elemelech et al. also demonstrated that SWNTs also possessed antibacterial properties, and direct contact with SWNTs could result in membrane disruption of Escherichia coli (E. coli) K12 [71]. Such antimicrobial and antiviral properties of nanomaterials may provide exciting new engineering solutions to the challenging problems of bacterial colonization and biofilm development in drinking
water systems.

Transgenic researches have employed nanomaterials in the use of molecular cargo transportation across both cell wall and cell membrane. Wang et al. have successfully synthesized mesoporous silica nanoparticles that delivered DNA and chemicals into plants simultaneously (Figure 1-14) [72]. In this study, protoplasts (plant cells with cell wall removed) were incubated with the mesoporous silican nanoparticles. DNA and chemicals that were imbedded inside the nanoparticles were released once they entered the plant cells. The expression of the green fluorescence protein (GFP) gene under laser excitation indicated a successful delivery without damage to the plant cells. However, protoplasts are not a perfect model for plants as they are not walled like in their native states. Alternatively Fang et al. have used SWNTs as a molecular transporter for walled plant cells [73]. In their study Nicotiana tobacum L.cv. Bright Yellow (BY-2) suspension cells were treated with SWNTs loaded with fluorescein isothiocyanate. After translocation across the plant cell wall and cell membrane, the binding between SWNTs and fluorescein isothiocyanate was averted by the enzymes and green fluorescence was subsequently emitted (Figure 1-15). The translocation of the SWNTs was attributed to endocytosis of the plant cells and the nanoscale size of the SWNTs enabled them to diffuse through the porous structure of the plant cell wall.
Figure 1-14. Schematic illustration of mesoporous silica nanoparticles delivering multiple substances into plant cells (protoplasts) [72].
Figure 1-15. Confocal images of plant (tobacco) cells treated with SWNT. The green fluorescence signals inside the cells demonstrate the success of delivery by SWNT [73].

1.4 Toxicity of nanomaterials

As introduced above, the applications of nanomaterials have touched every aspect of human life. Intentionally or unintentionally biological and ecological systems would be
exposed to these nanomaterials. Consequently understanding the potential adverse effects of nanomaterials has become a great concern for researchers in the fields of cytotoxicity and ecotoxicity.

1.4.1 Cytotoxicity of nanomaterials

Cytotoxicity refers to the quality of being toxic to cells. For nanomaterials, the cytotoxic effects might be attributed to their small size (large surface area) and size distribution, chemical composition (purity, crystallinity, electronic properties, etc.), surface structure (surface reactivity, surface groups, surface coatings), solubility, shape, and aggregation, etc [74].

Colvin et al. demonstrated that the cytotoxicity of water-soluble fullerene species was closely related to their surface derivatization [75]. In their study, pristine $C_{60}$, $C_3$ (three carboxyl groups attached to one $C_{60}$ molecule), $Na^{+}2.3[C_{60}O_{7.9}(OH)_{12.15}]^{(2-3)-}$ and $C_{60}(OH)_{24}$ were employed to two different human cell lines to test their cytotoxic effects. Among these four types of nanomaterials, pristine $C_{60}$ was the most toxic and caused severe cell membrane disruption. $C_{60}(OH)_{24}$, on the other hand, did not induce any toxic effect to both cell lines up to its solubility limit (Figure 1-16). The cytotoxicity of the pristine $C_{60}$ was attributed to its high electron affinity and ability to generate radicals to oxidize lipid molecules and hence disrupt the cell membrane. We have demonstrated that physical adsorption and aggregation of fullerene $C_{70}$ onto the cell membrane caused
cell death at 20 mg/L and above [76]. The aggregation of C$_{70}$ on the cell membrane resulted in cell membrane contraction overtime and loss of membrane integrity at high dosages of the C$_{70}$ (Figure 1-17). Similar results were found by Sayes et al. on the study of SWNTs [77]. While the pristine SWNTs exhibited some toxic effects, well-coated and solubilized SWNTs showed no signs of cell death up to 30 mg/L and 48 hours incubation. The toxic effects of SWNTs or MWNTs were also shown to be caused by the impurities of these nanomaterials, such as amphotous carbon and metallic nanoparticles used as catalysts during nanomaterials synthesis [78, 79].
Figure 1-16. Different cytotoxicity based on different functionalized of C$_{60}$ [75].
Like carbon nanoparticles, TiO$_2$ nanoparticles have also been shown as toxic to cells in a study reported by Lu et al [80]. The toxicity of the TiO$_2$ nanoparticles was attributed to the generation of ROS and lactate dehydrogenase. Both of these chemicals caused oxidative stress to cells and resulted in cell death. Schwaller et al. studied the shape effects of TiO$_2$ [81]. They synthesized TiO$_2$-based nanofilaments with dimensions of 20 nm in diameter and a half micron in length. The internalization of such needle shape nanomaterials altered the cell morphology as observed under the microscope (Figure 1-18).
Figure 1-18. Bright field images of cells incubated with TiO$_2$ filaments. The images show the morphological change of cells due to the internalization of TiO$_2$ filaments [81].
QDs, the semiconducting nanocrystals usually contain elements such as Cd, Se, Te, and Pb. Such elements are a cause of concern as they are known to be toxic. Derfus et al. tested the cytotoxicity of QDs made of CdSe and found that when incubated with rat primary hepatocytes, bare CdSe QDs underwent surface oxidation, resulting in the release of free cadmium ions [82]. To minimize such toxic effects, QDs are often synthesized with an additional layer of ZnS which can effectively suppress the toxicity of the nanocrystals [83]. Researchers have also been examining additional surface coatings on the core-shell structure of the QDs, and a variety of organic coating have been developed, including two major types of MUA and PEG polymers [84, 85].

1.4.2 Ecotoxicity

With the rapidly expanding and advancement of nanotechnology, more and more nanomaterials have come to existence each year. For example, for the most commonly used TiO$_2$, the amount produced in 2005 reached 2 million tons [86]. Nowack et al. predicted in 2008 that the world production of nano TiO$_2$ was 5000 T/yr, for nano Ag 500 T/yr, and for carbon nanotubes 350 T/yr [87]. Accordingly, assessments on the fate of nanomaterials in living organisms and plant species have become very active in the recent years.

Oberdorster et al. have conducted the first study on the toxicity of fullerene C$_{60}$ to the aquatic living organisms, the juvenile largemouth bass [88]. They found that the exposure
of C$_{60}$ to fish resulted in significant lipid peroxidation inside the brains of largemouth bass after 48 h of exposure at 0.5 mg/L. The C$_{60}$ used in this study was suspended by tetrahydrofuran that is known to be toxic to living organisms. In collaboration with Klaine et al. we have studied the biomodification of SWNTs by aquatic organism *Daphnia magna* [89]. In this study, the SWNTs were coated by phospholipids and well dispersed in moderate hard water to simulate the freshwater environment of the organism. During exposure, daphnia showed the ability to ingest the SWNTs, digest the lipid molecules as their food source and excrete the SWNTs outside their bodies (Figure 1-19). Acute toxicity was only found at or above 20 mg/L of the SWNTs. It should be noted that although SWNTs alone showed minimum toxic effects, the combination of SWNTs and heavy metals could induce higher toxicity than either the SWNTs or heavy metals alone. In collaboration with Kim et al. we have found that by mixing SWNTs and Cu(II), SWNTs could serve as a carrier for Cu to enter daphnia and trigger acute toxicity [90]. In addition to these studies on fullerenes and SWNTs, Gauthier et al. have tested the toxicity of double-wall carbon nanotubes (DWNTs) to the amphibian *Xenopus laevis* [91]. The toxicity observed in this study was mainly due to the blockage of gills and the digestive track; no genotoxicity was found for the DWNTs.
Research regarding the toxicity of nanomaterials in plants remains limited and inconsistent. As introduced above, nanomaterials have been reported to have positive effects on plants growth including promoting root growth and water uptake, and increasing the plant’s dry weight and enhance photosynthesis. However, some other studies have shown reported the negative effects of nanomaterials. Xing et al. conducted extensive studies on the toxicity of nanomaterials (MWNT, aluminum, alumnia, zinc and zinc oxide) to plants (radish, rape, ryegrass, lettuce, corn and cucumber) [92]. They found the seed germination was affected by the nanosized zinc on ryegrass and zinc oxide on corn at 2 mg/mL concentration. Watts et al. evaluated the effects on plants of model nanoparticles, including nano-Au, nano-Ag, and Fe$_3$O$_4$ [93]. They found that the introduction of nanomaterials to the plants induced the growth of large roots, which is an indication of plant stress caused by the environment. Therefore, long term exposure would certainly result in harmful effects to the plants. They also suggested that since engineered nanomaterials are always affiliated with solvents or stabilizers, the toxicity
effects observed could be attributed to the combined effects of both the nanomaterials and the solvents. It seems unclear as how these two aspects might be combined to elicit such dynamic results as discovered on the growth and regeneration of plant species. One major motivation of this dissertation is to offer an insight on the vast complexity associated with the behaviors of the nanomaterials in plant systems.
Chapter two aims to understand the interactions between carbon-based nanomaterials (CBNMs) and plants at the whole organism level. CBNMs are one of the most commonly used nanomaterials and were chosen for this study based on their bioavailability in the environment. Such bioavailability was provided by natural organic matter (NOM), the most abundant heterogeneous mixture of plant and animal degradation products in the environment. The binding between CBNMs and NOM was facilitated through van der Waals interaction, hydrophobic-hydrophobic interaction and pi-pi stacking. The resulting supramolecular complexes (fullerene C$_{70}$ and MWNTs) were suspended in aqueous solution to introduce plants exposure to the nanoparticles. *Oryza sativa* (rice) was chosen as the model system for this study due to its extensive consumption by humans. The translocation, transmission, and generational transmission of CBNMs in the rice plant was investigated using the techniques of light/fluorescence microscopy, electron microscopy, Fourier transform infrared spectroscopy (FTIR) and Fourier transform Raman spectroscopy.

2.1 Introduction

The recent development of nanotechnology has reshaped the landscape of modern
science and engineering and shown vast promises for bettering people’s life. As we strive to explore benefits of the exciting technologies, efforts should also be paid to the investigation of the potential adverse effects of nanomaterials. Understanding the interactions between nanomaterials and their end points is essential, since such knowledge facilitates the design of new technologies and mitigates the potential harm of these nanomaterials.

Carbon based nanomaterials (CBNMs) comprise a large majority of produced nanomaterials and have been extensively applied in both research labs and industries. The highly inert properties make them difficult to be broken down over a significant long period. Due to their hydrophobic external surface, they readily aggregate and are usually not considered potential contaminants in the liquid phase. However, when discharged into the environment, the hydrophobicity of nanomaterials can be altered through their interactions with the NOM. In 2007, a group of environmental scientists at Georgia Institute of Technology discovered that the NOM extracted from the Suwannee River was able to disperse MWNTs in aqueous solution, with the suspension remained stable over months [94]. The vast possibilities of mobile carbon nanoparticles migrating in the water column and interacting with ecological plant systems were the motivation for the studies described in this chapter.

Plants, the most widely spread living organisms in the environment, range from the single cellular algae to massive trees. Since they lie at the very bottom of the food chain, they
play an essential role in human and animal diet, the living environment, and the conversion from CO\textsubscript{2} to O\textsubscript{2} in photosynthesis. Vascular plants, such as rice, the major food crop in Asia and many parts of the world, have lignified tissues for water, mineral, and photosynthetic products conduction. Nutrients and water from the soil and photosynthetic products from the leaves are distributed to specific areas in the plant through xylem and phloem (Figure 2-1) [95]. While the xylem takes charge of drawing water and nutrients up from the roots to the upper sections of the plant’s body, the phloem transports other materials that give the plant energy to keep it growing and seeding.

Figure 2-1. Stem cross-section of vesicular plant. a. xylem; b. phloem [95].
As previous studies have shown, plants undergo numerous biotic and abiotic stresses during their whole life cycle. However, in literature, the impact of nanomaterials on high plants has been scantily examined. Among the studies available, none have used major food crops or CBNMs for their evaluations. Although both enhanced and inhibited growth have been reported for vegetations exposed to nanomaterials at various developmental stages, including seed germination, root growth, and photosynthesis [67-69], fundamental questions remain regarding the uptake, accumulation, translocation, and transmission of nanomaterials in plant cells and tissues and the impact of these processes on plant reproduction.

2.2 Structures and surface property of carbon based nanomaterials

Carbon-based nanomaterials (CBNMs) are mostly comprised of the only element of carbon. Depending on the structures and shapes they represent, CBNMs can be classified as fullerenes (C_{60} and C_{70} as the most abundant), nanotubes (SWNTs, MWNTs and DWNTs), nanowires, and nanocoils, etc. The electrons in CBNMs mostly take the form of sp2 orbitals, which provide them with the unique strength. Due to their hydrophobic surfaces, CBNMs usually clump to minimize their surface area when exposed to a hydrophilic environment such as water.

Fullerene C_{70} is comprised of seventy carbon atoms and forms an elongated soccer ball shape. The diameter of the structure is around 0.7 to 0.8 nm. Both SWNTs and MWNTs
can be considered as further elongated structures when compared to the fullerences. The diameter of an SWNT is approximately 0.8 to 1.4 nm. The structure of a MWNT consists of concentric rings of SWNTs and its diameter ranges from ~10 to 100 nm depending on the number of the SWNTs enclosed. The length of a carbon nanotube ranges from tens of nanometers to a few micrometers.

2.3 Structures of natural organic matter and humic acid

NOM, a heterogeneous mixture of decomposed animals and plants, occurs in all natural water sources. The major components of NOM are humic acids (Figure 2-2a), tannic acids (Figure 2-2b), proteins, lipids, amino acids, hydrocarbons, etc. The aromatic structures and hydrophobic moieties of the NOM provide numerous binding sites for CBNMs through which hydrophobic interaction and pi-pi stacking may occur.
Figure 2-2. Building blocks of humic acids (a) and tannic acids (b) [96, 97].

Humic acids, the most abundant component of NOM, are a mixture itself of phenols and
other alcohols, ketones/quinines, aldehydes, carboxylic acids, amino- and nitro-groups, and sulfur containing entities such as mercaptans, sulfates, and sulfonates in the environmental water column. The detergent qualities and potential uses of humic acids in pollution remediation due to their amphiphilic structure have been reported before [98]. The relatively simple structures of humic acids were a substitute for the more complex NOM in some of the studies included in this chapter.

2.4 Synthesis and characterizations of supramolecular complexes of CBNMs-NOM

Fullerene $C_{60}$, $C_{70}$, SWNTs, and MWNTs with diameters of 10–15 and 40–70 nm were applied to test the suspendability of NOM for different types of CBNMs. Nordic NOM was directly dissolved in Milli-Q water at 100 mg/L concentration to mimic the water sources in the environment. An equal amount of CBNMs was added to the NOM solution at 1 mg/mL concentration. The mixtures were then probe-sonicated for 30 min to break apart the CBNMs bundles and promote their binding with the NOM. After sonication, the suspensions appeared dark yellow for $C_{60}$, dark brown for $C_{70}$ and black for MWNTs and all samples remained stable for days. The SWNT sample, however, quickly aggregated after sonication and settled to the bottom of the container, leaving the supernatant clear in color (Figure 2-3).
Figure 2-3. Photograph showing CBNMs-NOM suspensions. From left to right: Milli-Q; Nordic NOM (100 mg/L); C60-NOM; C70-NOM; SWNT-NOM; MWNT-NOM (10–15 nm); MWNT-NOM (40–70 nm); Carbon nanowire-NOM.

2.4.1 Absorbance measurement of CBNMs-NOM

UV-vis absorbance spectra of materials dissolved or suspended in aqueous solutions can be treated as their optical fingerprints. Different molecules or molecular complexes absorb light at different wavelengths due to their characteristic vibrational energy levels. For example, DNA molecules usually absorb UV light peaked at 260 nm, while proteins usually absorb at 280 nm [99, 100]. The vertical axis of a UV-vis absorbance spectrum displays the intensity of light being absorbed by the molecules or complexes under study.
These intensities are directly proportional to concentrations of the materials in aqueous solutions (Beer-Lambert Law).

A UV-vis spectrophotometer (Biomate 3) was used to measure the absorbance spectra of the NOM solution and CBNMs-NOM suspensions. The absorption spectrum of NOM in Milli-Q at neutral pH has a signature peak centered at 256 nm (Figure 2-4), with a full width at half maximum (FWHM) of 60 nm. A range of NOM solutions were prepared at 2.5, 10, 50 and 100 mg/L, whose absorbance values at 256 nm followed the Beer-Lambert Law precisely. (Figure 2-5)

![N-NOM absorbance 100 mg/L](image)

Figure 2-4. Absorbance spectrum of Nordic NOM at 100 mg/L.
Figure 2-5. Absorbance concentration dependence of Nordic NOM (at 256 nm).

To obtain the equilibration of each CBNMs-NOM suspension, the samples were left at room temperature (20°C) overnight and the supernatants were used for the absorbance measurements. As shown in Figure 2-6, the spectra of C$_{60}$-NOM, C$_{70}$-NOM and MWNT-NOM all display broadened and red-shifted peaks centered around 256 nm with increased baselines, indicating binding between the CBNMs and the NOM. The peak wavelength and FWHM for each suspension are listed in Table 2-1.
Table 2-1. UV-vis absorbance of NOM and CBNMs-NOM.

<table>
<thead>
<tr>
<th>Suspensions</th>
<th>Peak Value (nm)</th>
<th>FWHM (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOM</td>
<td>256</td>
<td>60</td>
</tr>
<tr>
<td>C60-NOM</td>
<td>262</td>
<td>115</td>
</tr>
<tr>
<td>C70-NOM</td>
<td>259</td>
<td>106</td>
</tr>
<tr>
<td>SWNT-NOM</td>
<td>n/a*</td>
<td>n/a*</td>
</tr>
<tr>
<td>MWNT-NOM (10~15 nm)</td>
<td>265</td>
<td>190</td>
</tr>
<tr>
<td>MWNT-NOM (40~70 nm)</td>
<td>263</td>
<td>182</td>
</tr>
</tbody>
</table>

* n/a: unable to obtain data due to negligible absorbance.
Rapid aggregation and settlement of the SWNT-NOM sample was again observed after probe sonication. The absorbance measurement of SWNT-NOM yielded negligible signal, indicating neither SWNTs nor NOM were suspended in the water column. The ability of SWNTs to absorb most of the NOM in the solution and pull the NOM out of the liquid resulted in a nearly zero absorbance from the supernatant.

The distinctive behaviors from SWNTs and the other types of CBNMs were attributed to their physical structures, especially their morphology. NOM, consists of mostly aromatic rings, has a mostly planar configuration. The binding between NOM and individual SWNTs was energetically unfavorable due to the mismatch between their surface curvatures (illustrated in Figure 2-7). However, bundling of SWNTs in the aqueous phase resulted in much reduced curvatures to allow their binding with the NOM. In this case, the weight of large SWNT bundles could overwhelm the buoyancy force of water. As a result, the NOM was pulled out of the water column together with the SWNT bundles.

Fullerenes \( C_{60} \) and \( C_{70} \) also possess highly curved surfaces like SWNTs. However, the small sizes of the fullerenes enabled them to be completely held within the hydrophobic moieties of the NOM to render suspendability (illustrated in Figure 2-8). MWNTs, on the other hand, have a much larger diameter and a flatter surface to match the NOM curvature. As a result, MWNTs were well suspended in the NOM solution as individuals or small bundles (illustrated in Figure 2-9). This explanation, also provided by computational simulation work done by Dr. Emppu Salonen at the Aalto University School of Science and Technology (TKK), Finland, is consistent with the work by
Kaneko regarding the curvature dependence of polycyclic aromatic hydrocarbons adsorbed onto an SWNT.

Figure 2-7. Illustration of SWNT-NOM complex. Circle represents the cross-section of SWNT.
Figure 2-8. Illustration of C$_{60}$-NOM complex.
Figure 2-9. Illustration of MWNT-NOM complex.
2.4.2 Size measurement of CBNMs-NOM and CBNMs-humic acid

After having successfully obtained CBNM suspensions in NOM, I have examined the size distributions of the nanoparticles. This information is critically needed since the physical dimensions of nanoparticles would directly impact on their interactions with the ecological systems. To determine the hydrodynamic size of particles in aqueous solution a dynamic light scattering device is commonly used. Since smaller particles diffuse faster than larger ones, the hydrodynamic size of a particle can be calculated based on the rate of its Brownian motion.

A NanoSizer S90 (Malvern) was used to measure the hydrodynamic size of NOM and CBNMs-NOM in aqueous solutions. NOM alone had a wide size distribution curve ranging from 1 nm to 1 µm (Figure 2-10) much due to its heterogeneous nature and rich biological origin. Compared to the NOM, the size distribution of C\textsubscript{70}-NOM displayed a slight shift towards the larger sizes, as shown in Figure 2-11. The difference between each peak from C\textsubscript{70}-NOM to NOM alone was attributed to the presence of the C\textsubscript{70}. Unlike C\textsubscript{70}-NOM, the MWNT-NOM suspension only had one peak centered at 200 nm. Although the average diameter of the MWNTs was around 40-70 nm, their lengths ranged from hundreds of nanometers to microns. When dispersed in aqueous solution, the MWNTs would coil to form polymer-like blobs with a diameter around 200 nm. The signal from the smaller sized NOM, in this case, was negligible.
Due to the complexity with the size distribution of the NOM, humic acids were also used to suspend the CBNMs instead to avoid data misinterpretation. Very similar trends were observed for the CBNMs-humic acid complexes, with SWNTs settling down quickly.
while other types of the CBNMs remaining suspended in the liquids. From the size measurements, humic acids alone had one hydrodynamic size of 18 nm in diameter (Figure 2-12). The CBNMs-humic acid complexes were measured both immediately after probe sonication and after equilibration overnight.

![Statistics Graph (1 measurements)](image)

**Figure 2-12. Size distribution of humic acid.**

For MWNT-humic acid, there were two hydrodynamic sizes centered at 55 nm and 170 nm immediately after sonication (Figure 2-13). After equilibration, the hydrodynamic size of MWNT-humic acid was centered at 180 nm (Figure 2-14). The absence of the smaller peak over time can be explained as a result of slight aggregation of the MWNTs. However, the MWNTs remained stable in the aqueous solution.
Figure 2-13. Size distribution of MWNT-humic acid right after sonication.

Figure 2-14. Size distribution of MWNT-humic acid overnight.

For SWNT-humic acid, the size measurement right after probe sonication displayed a hydrodynamic size at 400 nm (Figure 2-15). However, due to the rapid sedimentation of the SWNTs, a second attempt made immediately after the first test could not resolve any
size distribution. The software also reported that there were large or sedimenting particles present and that the sample was too polydispersed for cumulant analysis. The supernatant of the SWNT-humic acid suspension was also measured and showed absence of peaks, indicating that humic acid was absorbed and pulled out of the water column by the SWNTs.

![Statistics Graph](image)

**Figure 2-15.** Size distribution of SWNT-humic acid right after sonication.

### 2.4.3 Zeta-potential measurement of CBNMs-NOM

When dispersed in aqueous solution, most of the particles would acquire a surface charge either by their surface groups or ionization of their surfaces. In this case, the ions in the aqueous solution would redistribute to accommodate the new surfaces. Ions with opposite charges bind tightly on the surfaces of the suspended particles to form a layer called the Stern layer. Outside the Stern layer, there is another loosely bound layer called the
diffusive layer, which contains ions with the same charge as the particle's surface. This interface is known as the electrical double-layer (Figure 2-16). Zeta potential refers to the electrical potential at the diffusive layer and is commonly used to describe the stability of the dispersion system. Particles that have a surface change higher than +30 mV or lower than -30 mV are considered as stable in the aqueous suspension.
Figure 2-16. Schematic representation of zeta-potential [101].
Following the principle of electrical double layer, the zeta potential is designed to measure the electrical potential at the diffusive layer. When applying an electric field across the suspension, particles with a surface charge will migrate toward the electrode of opposite charge with a velocity proportional to the magnitude of the zeta potential. The velocity can be obtained by measuring the mobility of particles in the suspension using the same principle described in section 2.4.2.

To evaluate the stability of each CBNMs-NOM suspension, zeta potential measurements were conducted using a ZetaSizer nano ZS (Malvern). NOM alone had a zeta potential equal to -38 mV, which was contributed to the carboxyl and phenol surface groups. Both $C_{70}$-NOM and MWNT-NOM established zeta potentials lower than -30 mV, which served as more evidence that NOM bound to the surfaces of these nanomaterials to form supramolecular complexes (Figure 2-17 and 2-18). I was not able to measure the zeta potential of the SWNT-NOM due to its fast sedimentation.
Figure 2-17. Zeta potential of C$_{70}$-NOM (-34.3 mV)

Figure 2-18. Zeta potential of MWNT-NOM (-43 mV)
2.5 Uptake, transmission and generational transfer of CBNMs

Based on the studies above for CBNMs-NOM complexes, fullerene $C_{70}$ and MWNTs were chosen as the model CBNMs to study their interactions with rice plants.

2.5.1 Experimental scheme

Newly harvested rice seeds (*Oryza sativa* L. ssp. *japonica*, cv Taipei 309) were randomly chosen and soaked in 70% ethanol for 30 seconds, surface sterilized twice in 10% (v/v) Clorox® bleach plus two drops of Tween-20™ (Polysorbate 20), and stirred for 30 minutes. After sterilization, these seeds were incubated in Petri dishes that contained 15 mL of incubation buffer. A rice germination buffer (half-strength MS basal salts, vitamins, and 7.5g/L sucrose with pH=5.7) was used as the negative control. Different concentrations of $C_{70}$-NOM and MWNT-NOM from 2.5 mg/L to 800 mg/L were mixed with the germination buffer to treat the seeds. Identical amounts of NOM for each concentration that were used for $C_{70}$-NOM and MWNT-NOM were used as the non-CBNMs controls. For example, the NOM concentration in “NOM400” was identical to that in $C_{70}$-NOM or MWNT-NOM of 400 mg/L (Figure 2-19).
Figure 2-19. Experimental scheme [102].
Seeds incubated with different nanoparticles suspensions were incubated at 25±1 °C for 2 weeks. After germination, they were transplanted to soil in big pots and grown in a greenhouse to maturity without any addition of nanomaterials. For each sample concentration, 5 pots of plants were maintained for statistical analysis. These plants are referred to as the first generation.

To investigate the generational transfer of nanomaterials, mature seeds from the control plants and CBNMs treated plants were harvested after 6 months. The seeds were germinated in Petri dishes filled with only rice germination buffer and kept at 25±1°C for 2 weeks. These plants without any addition of nanomaterials are referred to as the second generation.

2.5.2 Uptake of CBNMs by the first-generation plants - light microscopy

To examine the uptake of CBNMs by the plants, germinated plants were carefully taken out of the Petri dishes and thoroughly washed using distilled water to remove any surface attachment. Plant tissues of rice plants at various sites including the seed, root, stem and leaf were cut, sectioned to make thin layers, and imaged on glass slides using a bright field microscope (Imager A1, Zeiss). Figure 2-20 shows bright field images of the plant tissues acquired one week after incubating in C70 of 20 mg/L. Black aggregates were frequently found in the seeds and roots, and less frequently in stems and leaves, indicating that the sequence of nanomaterial uptake was from the plant seeds and roots to
the stems and leaves. The appearance of black aggregates mainly in and near the stem’s vascular system suggests that the transport of $C_{70}$ occurred simultaneously with the uptake of water and nutrients in the xylem. The water flow inside xylem also pushed the black aggregates to further penetrate across the xylem and into the plant tissues.
Figure 2-20. Uptake of CBNMs observed by light microscope [102].
In contrast to C\textsubscript{70}, the uptake of MWNTs at concentrations of 20 to 800 mg/L was found to be insignificant, with few black aggregates appearing in the vascular system and almost none in the plant tissues. The significantly reduced uptake of MWNTs by the plants is mostly due to their relatively larger size that inhibits penetration through plants seeds and roots.

To confirm that the plant roots have the ability to take up C\textsubscript{70} through water uptake, another experimental setup was conducted as shown in figure 2-21. The seeds were naturally germinated without introducing CBNMs-NOM suspension to the germination buffer. One week after germination, the plants were transported into boxes that only allowed the roots to be exposed to the liquid column. This setup eliminated the exposure of nanomaterials to other parts of plants, and limited them only to the roots. Same observations were found for this setup.
2.5.3 Uptake of CBNMs by the first generation plants - electron microscopies

Due to the resolution limit of light microscopy, which can only resolve structures in the range of hundreds of nanometers, electron microscopy techniques were applied to visualize the interaction between CBNMs and plants. However, the vacuum environment for electron microscope operation required the biological samples to be fixed and
dehydrated before imaging. For scanning electron microscopy, samples were also coated by a thin metal layer to ensure electron conductivity.

Prior to electron microscopy imaging, glutaraldehyde with 3.5% concentration was used as the fixative. Tissue samples were cut in small pieces and put in glutaraldehyde overnight at 4°C. After fixation, the tissue samples were washed a couple times and kept in the rinsing buffer overnight. The dehydration process was conducted using a gradient concentration of ethanol from 15% to 100%. The tissue samples were kept in each ethanol concentration for 15 min and then kept in 100% ethanol overnight. Finally, the chemical critical point dry process was done by using hexamethyldisilozane (HMDS) on the tissue samples.

For MWNT treated samples, most of the MWNTs aggregated and adhered to the surface of plant roots. Therefore, scanning electron microscope (SEM) was used to focus on the surfaces of the roots and root hairs. Ten samples of the plant roots were dehydrated as described above and evenly coated with a thin layer of platinum (~5 nm) using a Hummer® 6.2 sputtering system. SEM imaging was performed using an FESEM, Hitachi 4800, microscope operating at 10 kV. As shown in figure 2-22, the plants roots were heavily coated by the MWNTs. The diameter of MWNTs ranges from 40 to 70 nm and the length ranges from hundreds nanometers to microns.
For $C_{70}$ treated samples, black aggregates were observed inside the vascular structures of the rice plants. Therefore, transmission electron microscope (TEM) was used to examine the plant compartments and tissues. Ten samples of the roots and leaves of rice plants underwent the same dehydration process as described above and then embedded in the LR White resin. The LR white resin was allowed to polymerize overnight at 60°C. The samples were sectioned into 60 to 100 nm thin films using an Ultracut E microtome. TEM images were acquired using a Hitachi H7600 microscope operated at 80 and 100
kV. As shown in Figure 2-23, most of the C$_{70}$ translocated through the cell wall and cell membrane and remained in the vacuole. The lattice spacing of C$_{70}$ particles acquired by high resolution TEM was analyzed by performing Fast Fourier Transform (FFT) using the attached software “Diffractogram”.

Figure 2-23. TEM images showing the uptake of C70 inside plant cells [102].
2.5.4 Existence of fullerene C\textsubscript{70} inside both first and second-generation plants - FT-Raman and FTIR

As shown by both optical and electron microscopes, it is suggested that C\textsubscript{70} nanoparticles were taken up by the plants along with water uptake. The nano-scale sizes of the C\textsubscript{70} particles enabled them to translocate across plant cell walls and cell membranes and remained in the vacuoles of the plant cells. Our imaging also suggested that C\textsubscript{70} could be further transferred to the second-generation plants (Figure 2-24). Since C\textsubscript{70} has signature Raman scattering signals as well as a distinct infrared absorbance spectrum, both Raman and infrared absorbance measurements were conducted to further confirm the existence of such nanomaterials inside the plants.
Figure 2-24. Generation transfer of C\textsubscript{70}. a. Fluorescence image showing the existence of C\textsubscript{70} inside plant tissue; b. TEM images showing the existence of C\textsubscript{70} inside plant cells; c. High resolution TEM image showing the lattice structure of C\textsubscript{70} [102].

Fourier Transform (FT) Raman and infrared (IR) spectra were acquired at room temperature for both the first and second-generation rice plants. Typical FT-Raman (red traces) and IR-spectral (blue traces) finger prints are presented in Figure 2-25 for C\textsubscript{70}, control, first-generation seeds and leaves, and second-generation leaves. Clearly, the dominant FT-Raman (indicated by “+”) and FTIR (indicated by “diamonds”) features of C\textsubscript{70} were observed in the first-generation seeds and leaves and in the second-generation leaves, thus confirming the uptake and transmission of C\textsubscript{70}. 
To quantify the dynamics of C\(_{70}\) uptake, a detailed FTIR study was carried out for the roots, stems, and seeds of the first-generation rice plants when the concentration of C\(_{70}\) was increased from 20 to 800 mg/L. After collecting the absorption spectrum, each of the C\(_{70}\) peaks was fitted to a Lorentzian line shape, and the area under the peak (integrated intensity) was calculated via equation:

\[
I = \frac{A}{\pi},
\]
where $A$ is the amplitude, and $\Gamma$ is the FWHM. This area was then converted into a percent uptake of $C_{70}$ by dividing it by the total area of all the combined samples. As shown in Figure 2-25c, $C_{70}$ particles were prevalent in the roots as well as in the stems and leaves of the 2 week-old plants, while the distribution of $C_{70}$ in these plants showed no significant concentration dependence. The prevalence of $C_{70}$ in plant leaves and roots is also evident in Figures 2-20. For the mature (six-month-old) plants, however, $C_{70}$ was predominantly present in or near the stems’ vascular systems, less in the leaves, and understandably even less in the seeds due to the multiplied uptake rates (green bars). Furthermore, no $C_{70}$ was left in the roots of the mature plants, suggesting robust transport of nanomaterials from the plant roots to the leaves.

To compare the uptake capacity of plant seeds vs. roots, we germinated two sets of rice seeds: one set in rice germination buffer and one set in $C_{70}$-NOM mixed rice germination buffer (20 mg/L). Within 3 days, these seeds started germination to produce shoots first and then roots. One week after shooting at three-leaf stage, the seeds were no longer able to provide sufficient nutrients for the newly germinated plants and detached from the seedlings. At this point, we transferred the seedlings into rice germination buffer to be in contact with $C_{70}$ suspensions (20 mg/L) for one week, prior to FTIR study of the roots, stems and leaves of these plants. This set of samples is termed “roots exposed”. The other set of samples, which had been exposed to nanoparticles from the beginning of germination, is termed “seeds+roots exposed”. FTIR study was conducted for the roots, stems, and leaves of these plants at the end of the second week. Since shoots usually
come out 1-2 days earlier than roots during seed germination, C_{70} taken up by the seeds could first be transported to the shoots (stems and leaves) and then to the roots. This may have led to more accumulation of nanoparticles in the leaves than in the roots (Figure 2-25d, “seeds+roots exposed”, dark red bars). The “roots exposed” samples showed a different trend of nanoparticle translocation possibly because C_{70} first entered the roots and then was transported to the stems and leaves (Figure 2-25d, “roots exposed”, brown bars).

2.6 Discussion and summary

The accumulation and transformation of nanomaterials in plant tissues and cells suggests a plausible mechanism for nanomaterials uptake: a dynamic competition between nanotransport driven by water and nanomaterials convections and the physical hindrances of plant tissues and nanomaterials aggregation. Individual C_{70} nanoparticles may enter plant roots through osmotic pressure, capillary forces, pores on cell walls (3.5~5 nm), and intercellular plasmodesmata (50~60 nm at midpoint), or via the highly-regulated symplastic route. Once in the plant roots and stems, individual C_{70} nanoparticles may share the vascular system with water and nutrients and may be transported via transpiration, the evaporation of water from the plant leaves. Individual C_{70} nanoparticles may also form aggregates or even clog the vascular system (Figure 2-26) due to hydrophobic interaction, or may leak into nearby tissues and cells (Figure 2-26) via the mechanisms that are discussed above for plant roots. At high concentrations, C_{70}
aggregation within the vascular system and in plant tissues and cells is expected to interfere with nutrients and water uptake, and hinder plant development. It is also very likely that plant cell-nanoparticle interaction could lead to the modification of plant gene expression and related biological pathways, and consequently impacting plant development. Indeed, flowering of the rice plants incubated with C\textsubscript{70}-NOM (400 mg/L) was delayed by at least one month and their seed setting rate reduced by 4.6% (Figure 2-27), compared to the controls or the NOM-fed plants. MWNTs, meanwhile, are larger one-dimensional nanostructures and, unless oriented approximately perpendicular to plant tissues, are less likely to enter plants (Figure 2-27). Our bright field (not shown) and scanning electron microscopy imaging (Figure 2-26 inset) showed that MWNTs adsorbed to the plant root surfaces, possibly because of the high affinity of the tubes for the epidermis and the waxy casparian strips of the roots. At high MWNT concentrations, uptake of water, nutrients, and NOM as well as plant development could be impeded due to increased blockage of the plant roots and root hairs by surface-adsorbed nanotubes. In our experiment, flowering of the rice plants incubated with MWNT-NOM (400 mg/L) was delayed by at least one month and their seed setting rate reduced by 10.5%, compared to the controls or the NOM-fed plants (Figure 2-27). Accordingly, the weight per 100 seeds was reduced by 8.59% (p<0.05) and 11.2% (p<0.05) for the plants incubated with C\textsubscript{70}-NOM (400 mg/L) and MWNT-NOM (400 mg/L) respectively, while no statistically significant change was found for the plants treated with NOM400, as compared with the controls.
Figure 2-26. Images showing the presence of C\textsubscript{70} inside plant tissue [102].
In summary, the dynamic uptake, compartment distribution, transformation, and generational transfer of fullerene C$_{70}$ in rice plants have been observed and characterized. The mobility of the hydrophobic C$_{70}$ was elicited by NOM, a collection of organic substances abundant in nature. The integration of nanoparticles by plant species may result from the nanoparticles’ small dimension and self assembly and from the nanoparticles’ interactions with plant organelles and the NOM. Another type of CBNMs, MWNT was found mostly glued on the external surface of rice plants due to their larger size. The potential impacts of these processes on both food safety and the environment are important subjects to understand. Future research needs to address questions as to
what extent molecular and genetic mechanisms may mediate plant responses to nanoparticle exposure and, furthermore, how to control such responses for mitigating the adverse effects of nanomaterials on plant development.
CHAPTER THREE
NANO-ECOSYSTEM INTERACTION
- CELLULAR LEVEL

Chapter two was focused on the interaction between carbon-based nanomaterials (CBNMs) and the ecological system (rice plants) at the whole organism level. Natural organic matter (NOM), the naturally occurring heterogeneous mixture of degraded animal and plant material, served as the connection between CBNMs and the rice plants. The transport, transmission, and generational transfer of fullerene $C_{70}$ in the rice plants were observed, while the uptake of multi-wall carbon nanotubes (MWNT) was mostly inhibited due to their larger hydrodynamic size.

Chapter three aims at understanding Nano-Ecosystem interaction at the cellular level. For this study, single-celled green algae, *Chlamydomonas*, were introduced as the model plant system. Quantum dots (QDs), the semiconducting nanocrystals that have been extensively produced and used for imaging and biosensing, were employed as a model for engineered nanoparticles. The interaction between QDs and algae was investigated using UV-vis spectrophotometry, bright-field and confocal fluorescence microscopy and scanning electron microscopy. The effect on algal photosynthesis induced by QDs was evaluated based on the rates of $O_2$ evolution and $CO_2$ depletion.
3.1 Introduction

Numerous types of engineered nanoparticles (ENPs) have been developed and applied to the applications of nanotechnology. Exposure of living systems to ENPs is inevitable due to the dramatic increase in the release of the nanomaterials into the environment from anthropogenic sources. The extremely small size of the ENPs may facilitate their tissue and cellular uptake by both plants, as presented in chapter two, and animals, resulting in either positive (drug delivery, antioxidation) [6-8] or negative (toxicity, cellular dysfunction) effects [37, 38].

QDs, the major class of semiconducting nanocrystals, possess unique optical, electrical, and chemical properties. Since their early development in the 1980s, QDs have been used extensively in such biological applications as cell labeling, in situ hybridization, pathogen detection, ligand binding, genomic and proteomic detection, and high-throughput screening of biomolecules. The toxicity of QDs has also been examined, and strategies—though far from optimal—have been developed to improve the biocompatibility of QDs through ligand exchange, hydrophobic interaction, and encapsulation. Like other classes of engineered nanomaterials, QDs may eventually be discharged through industrial and research outlets and impact living organisms in the environment. Despite their broad use in many applications, fundamental research on the biological and their environmental fate is severely lacking.
Single-celled plant species, such as *Chlamydomonas sp.*, commonly called green algae, are found in both soil and fresh water and serve as the primary producer of aquatic food webs. They have the simplest plant cell structure, while retaining the ability to undergo photosynthesis (Figure 3-1). Hence, studies on the interaction between QDs and algae cells may provide essential information on the tolerance of living organisms and the environment in general towards the potential effects of nanotechnology. Obtaining such understanding will benefit the biological, medicinal, and environmental applications of nanotechnology. Additionally, research in this area will guide the design and production of nanomaterials to minimize their potential adverse effects on human health and the environment.
3.2 Structure and optical properties of QDs

QDs are usually synthesized from precursor compounds dissolved in solution, undergo crystal growth, and are stopped at the size of nanometers. They are one of the most exciting colloidal semiconductor nanocrystals because of their excellent optical properties. The elements commonly used to make QDs include cadmium, selenide, sulfide, indium, arsenide and phosphide. Due to the high toxic nature of these elements, most of the commonly used QDs have a core-shell structure containing a fluorescent core.
material such as CdSe and a shell layer such as ZnS that prevents the leakage of core materials. Since QDs are frequently used in biological environments, an extra layer that contains either hydrophilic polymers or hydrophilic functional groups is needed to render water solubility.

In this study, commercialized yellow fluorescent CdSe/ZnS core/shell QDs (Ex: <550 nm, Em: 570~585 nm) were purchased from NN-Labs, LLC and used directly without modification. The QDs were rendered water-soluble by coating mercaptoundecanoic acid (MUA) ligands on the QDs surfaces (Figure 3-2). The average hydrodynamic diameter of the QDs was determined by dynamic light scattering to be 11.69 nm (Figure 3-3), and the dimensions of dried QDs were determined to be 5~9 nm electron microscopies (Figure 3-4). The relatively smaller size of QDs was due to the collapse of MUA ligands on the surface in the dry state. The fluorescence spectrum of QDs was obtained by a fluorophotometer (Figure 3-5). When excited in the range of 400~500 nm, the QDs emitted a stable bright yellowish fluorescence center at 570 nm. The fluorescence wavelength of the QDs did not overlap with algal autofluorescence, which ensured the distinction between QDs and algal cells under a confocal fluorescence microscope (Zeiss 510).

Figure 3-2. Structure of mercaptoundecanoic acid (MUA) [104].
Figure 3-3. Size distribution of QDs.

Figure 3-4. EM images of QDs. a. TEM image; b. SEM image.
3.3 Algae *Chlamydomonas*

Plant cells and mammalian cells share a number of similar organelles such as the mitochondria, endoplasmic reticulum, Golgi apparatus, and nucleus. However, unlike mammalian cells possessing only a plasma membrane as the barrier from the extracellular space, plant cells have an additional layer outside the cell membrane known as the cell wall (Figure 3-6). The cell wall is a porous structure consists mostly of cellulose with a pore size around 5 nm in diameter. The thickness of cell wall is usually 1 to 10 µm (Figure 3-7). Due to the small pore size and rigidity of the cell wall, the process of

![Figure 3.5. Fluorescence spectrum of QDs. Excitation scan (green) and emission scan (orange).](image-url)
endocytosis for plant cells is largely reduced and only forensic molecules or particles smaller than 5 nm can penetrate through the cell wall.

Like most high plants and bacteria, *Chlamydomonas* also possess a cell wall approximately 100 nm beyond their cell membrane. The two protruding flagella afford mobility to such algae species (Figure 3-1). Such mobility is essential for the algae to gain access to light sources and CO₂ in order to conduct photosynthesis.

Figure 3-6. Structure of a plant cell [105].
**3.4 Adsorption of QDs onto algal cells**

Fresh wild type *Chlamydomonas* algae were obtained from the Clemson Research Facility (green house) and kept in growth medium at room temperature. Varied concentrations of QD suspensions, from 0.05 ppm to 5 ppm, were added directly into the growth medium to mimic the environmental exposure. The mixture of algae and QDs were incubated at room temperature for 2 h. Quadruplicate were prepared to ensure experimental repeatability and to establish error bars.
3.4.1 Bright field and confocal fluorescence imaging

After incubation, approximately 10 µL of the algae/QDs mixture was taken out and flowed into a sample channel sandwiched between a glass substrate and a cover glass (Figure 3-8). Both ends of the channel were sealed using a fingernail polish to prevent air flow and ensure image quality. Images were taken using a 100X oil immersion objective and the excitation/emission filters were set to fit the fluorescence spectrum of QDs. As shown in Figure 3-9, the size of algae was about 10 µm in diameter and the surface of the algae were heavily coated by QD aggregates.

![Figure 3-8. Illustration of the sandwiched setup for imaging.](image)
To examine the interiors of the algal cells, a laser scanning confocal microscope was used to optically section the samples. An amount of 100 µL algae/QDs mixture was put in an 8-well chamber glass and images were taken using a 40X oil immersion objective. Samples were then excited with an Argon ion laser at 488 nm, and fluorescence images were captured using a BP 570-590 filter set. The emission filter effectively blocked out the autofluorescence of algae and improved the image quality. As shown in Figure 3-10, few signals from QDs were found inside the algal cells indicating that most of the QDs

Figure 3-9. Bright-field image of algal cells [107].
were only adsorbed onto the surfaces of the algae. Since the size of QDs used for this study was around 10 nm in diameter, the algae cell wall, with a pore size of approximately 3~5 nm, efficiently prevented the uptake of the nanocrystals.

Figure 3.10. Confocal fluorescence image showing the adsorption of QDs onto algal cells. Yellow color represents the aggregates of QDs, black circles indicate the location of the algal cells [107].

3.4.2 Scanning electron microscopy imaging

Scanning electron microscopy was conducted to observe the adsorption of the QDs on
algae at the nanoscale. QDs alone were directly dried on an aluminum stub and platinum coated prior to imaging. Imaging was performed using a Hitachi S-4800 scanning electron microscope under a 15 kV accelerating voltage.

To preserve the structure of algal cells, both wild type algae and an algae/QDs mixture were fixed using 3.5% glutaraldehyde overnight, rinsed, and kept in rinsing buffer overnight and dehydrated using a gradient of ethanol from 15% to 100%. Chemical critical point dry was conducted using HMDS. Images were captured using the same Hitachi S-4800 scanning electron microscope under a relatively low accelerating voltage (10~12 kV) to prevent structural damage by the electron beams. As shown in Figure 3-11, QDs were heavily coated on the surface of the algal cells. The size of the algal cells was around 5~7 nm in diameter. This relatively smaller size compared to the light microscopy results was due to the dehydration process.
3.4.3 Quantification of QDs Adsorption

To quantify the adsorbed amount of QDs onto algae, a UV-vis spectrophotometer (Biomate 3) was used. Considering the size difference between the algal cells and the QDs, a membrane filter (Nalgene) with a pore size of 0.45 μm was used to separate the un-adsorbed QDs.

A wavelength of 545 nm was chosen to quantify the concentration of QDs because of
their absorb light primarily at this wavelength. The absorbance was measured before and after adding various concentrations of QDs into the algal growth medium with the differences denoting total concentrations of the QDs. After 2 h of incubation, 10 µL of NaOH was added to the algae/QDs solution before filtering through the membrane. The introduction of NaOH was to prevent aggregation of the negatively charged QDs in the weakly acidic algal growth medium (pH=6.45), and to ensure that all the un-adsorbed QDs passed through the membrane. The amount of adsorbed QDs was then calculated by Equation (1):

\[
\text{Abs}_{\text{adsorbed}} = (\text{Abs}_{\text{QDs+algae}} - \text{Abs}_{\text{algae}}) - \text{Abs}_{\text{filtered}},
\]

(1)

where \(\text{Abs}_{\text{QDs+algae}}\) and \(\text{Abs}_{\text{filtered}}\) denote the absorbance of the algae/QDs solution before and after filtration, while \(\text{Abs}_{\text{algae}}\) is the absorbance of the algae alone. An adsorption curve is shown in Figure 3-12 for the amount of adsorbed vs. the amount of free QDs. A logarithmic increase in the QD amount adsorbed was observed with increased equilibrium concentrations of the QDs. The surface area of a typical algal cell (~10 µm in diameter) is approximately 250,000 times larger than that of a QD (~20 nm in diameter), allowing a significant amount of the QDs to be adsorbed.

As indicated by the optical and electron microscopy images, adsorption of the QDs on the algal cells did not form single but multiple layers, Freundlich model was therefore chosen to fit the adsorption isotherms. The Freundlich model is a modification of the Langmuir adsorption scheme, and is appropriate for describing rough inhomogeneous adsorbent (i.e., algae) surfaces with multiple adsorption sites. Considering the adsorbate-adsorbate
(i.e., QDs-QDs) interactions, the empirical Freundlich equation is expressed in Equation (2)

\[ q_{eq} = k C_{eq}^n, \]  

where \( k \) is a coefficient indicating the affinity of QDs for algae, and \( n \) is a constant characteristic of the adsorption system and is related to the binding efficiency. An \( n \) value less than 1 indicates a favorable adsorption, while an \( n \) value greater than 1 reflects a weak adsorption. The parameters \( C_{eq} \) and \( q_{eq} \) represent the concentrations of non-adsorbed QDs and the QDs adsorbed on the algae at equilibrium respectively.

![Figure 3-12. Adsorbed vs. equilibrium QDs concentration. Also shown is a fitted logarithmic trendline [107].](image-url)

\[ y = 0.0213 \ln(x) + 0.133 \]
\[ R^2 = 0.9802 \]
According to the Lambert-Beer Law, absorbance is proportional to the concentration of the QDs. As such, the parameters of the Freundlich equilibrium model were fitted from a log-log plot of $C_{eq}$ vs. $q_{eq}$ (Figure 3-13). The plot slope represents the exponent $1/n$, and the value of $k$ can be read from the intercept. From our absorbance data, the value of $k$ was determined as $0.583 \text{ ppm}^{1-n}$ with its exponent $n$ fitted at 0.628. This $n$ value suggests a favorable binding of the QDs to the algae.

![Figure 3-13. Log plot of $C_{eq}$ vs. $q_{eq}$. The slope represents the exponent $1/n$, and the intercept (intercept = $\ln(1000/1000k)^{1/n}$) represents the $k$ value.](image)

3.4.4 Effects of QDs adsorption on algae photosynthesis

Light (photons) and CO$_2$ are two of the most important factors for algae to conduct
photosynthesis. As described in Equation (3), algae consume CO₂ and produce O₂ during photosynthesis.

\[
6CO_2 + 6H_2O \xrightarrow{\text{sunlight & nutrients}} C_6H_{12}O_6 + 6O_2
\]  

(3)

To evaluate the effects of QD adsorption on algal bioactivities, the rate of oxygen evolution and carbon dioxide depletion were compared between the wild type algae and QD-adsorbed algae.

3.4.4.1 CO₂ depletion rate comparison

To measure the CO₂ depletion rate of algae, a bicarbonate indicator solution was prepared. This indicator solution contains two dyes: 0.2 g of thymol blue and 0.1 g cresol red in 0.01 M NaHCO₃. As shown in Equation (4), an equilibration was formed in such a solution and a change in the concentration of CO₂ would alter the pH value of the solution.

\[
HCO_3^- + H^+ \leftrightarrow H_2O + CO_2.
\]  

(4)

An increased concentration of CO₂ would push the equilibrium to the left making the solution more acidic, while a decreased concentration of CO₂ would cause the solution to be more basic. The two dyes in this indication solution, thymol blue and cresol red, absorbed light differently at different pH values. For example, in an acidic environment, the mixture of the indicator appeared yellow. With an increase of pH, the color of the
indicator changed from orange to red at neutral pH and further to magenta and purple at basic pH (Figure 3-14). This color change can be observed both visually and by UV-vis absorbance measurement (Figure 3-15).

Figure 3-14. Photograph showing the colors of bicarbonate indicator solutions at different pH. From left to right: pH =4, 7, and 10 [107].

As shown in Figure 3-15, the absorbance of indicator solution has two distinctive peaks at 438 nm and 574 nm. At acidic pH, the intensity of peak at 438 nm is higher than the peak at 574 nm. When increasing the pH, the intensity of peak at 438 nm drops while the intensity of peak at 574 nm increases. In this study, the rate of change in the intensity at 547 nm versus time was used as the indication of rate of change in the pH, which was then extended as the indication of CO₂ depletion rate.
The algae/QDs solution was mixed with the indicator solution and the samples were tightly sealed to prevent gas exchange. During photosynthesis, the algae consumed CO\(_2\) over time, causing the pH value of the indicator solution to increase accordingly. The depletion rates of CO\(_2\) were then calculated based on the increase of absorbance values at 574 nm for different sample concentrations. As shown in Figure 3-16, an increased dosage of the QDs resulted in a significant decrease in the CO\(_2\) depletion rate at and above 100 ppm of QD dosage.
Figure 3-16. CO\textsubscript{2} depletion rate comparison. Concentration of QDs above 100 ppm shows significant reduced rate (slope) [107].

3.4.4.2 O\textsubscript{2} evolution rate comparison

To measure the oxygen evolution rate of the algae, an Oxyg32 system (Hansatech Instruments) was used. As shown in figure 3-17, the Oxyg32 system consisted of an electrode disc, an incubation chamber with light source, and software used to control the sensor unit and analyze the results. The oxygen electrode disc consisted of two electrodes immersed in a 50% KCl electrolyte solution. A polarizing voltage of 700 mV ionized the electrolyte and initiated current flow via a series of electrochemical reactions. (Equations
(5) & (6)). Oxygen was consumed during the electrochemistry. The magnitude of the current flow was related to the oxygen concentration of the surrounding media. An $O_2$ permitted membrane was used between the incubation chamber and electrode, which only allowed oxygen molecules to diffuse through.

Figure 3-17. Oxyg32 system [108].

The experiment was conducted with a fixed amount of algal cells treated with various dosages of QDs. Wild type algae and algae/QDs mixtures were placed in the incubation chamber with all measurements taken at room temperature under identical lighting conditions. As shown in Figure 3-18, the oxygen evolution rate was significantly affected by the addition of different QD concentrations to a fixed concentration of algal solution.
Above 5 ppm of QD dosage, the $O_2$ production rate decreased to nearly zero, indicating a significantly reduced photosynthetic activity.

![Graph showing oxygen production rate vs QD dosage](image)

Figure 3-18. Oxygen evolution rate comparison [107].

Both our CO$_2$ depletion and O$_2$ evolution experiments proved that the introduction of QDs to algal growth media affected their photosynthetic activity. Specifically, CO$_2$ depletion was significantly reduced above 100 ppm of the QD dosage, while a decreased O$_2$ evolution rate occurred in the low ppm range of the QD dosage.
3.5 Discussion and summary

In this study, water-soluble CdSe/ZnS QDs were found to have a high affinity for the *Chlamydomonas* sp. algae. The adsorption of the QDs to the algal cell surfaces was from a combined result of nonspecific interactions, as well as possible reactions between the amine groups of the polysaccharides or glycoproteins in the algal cell wall and the carboxyl groups of the MUA ligands coated on the QDs. The porous structure of the algal cell wall also afforded ample binding sites for the QDs. It was shown that the amount of QDs adsorbed onto algae depends logarithmically upon the equilibrium concentration of the QDs. Although QD adsorption on the algae surface was apparent from our SEM imaging, confocal fluorescence imaging showed no clear evidence of QD internalization by algae. This lack of internalization may be explained as a result of the thick algal cell wall, the relatively large size of the QDs, and a lack of capability of the algal cells to perform endocytosis.

Previous studies using ENPs (ZnO, TiO$_2$ and CuO) were mainly focused on the growth inhibition of algae perhaps due to the light shading effect, but neglected the photosynthetic function of the algae. As a primary producer in the food chain, the photosynthetic activity of algae is equally important as its reproduction behavior. As shown in this study, introducing QDs to algal growth media triggered the interactions between the algae and the QDs, which included both adsorption and possible translocation of the QDs within the algal cells. Adsorption of QDs could hinder the CO$_2$
gas flow through algal cells needed for photosynthesis, block the pathways of nutrients uptake, and impede algal mobility via obstruction of their flagella movement. Furthermore, adsorption of QDs could damage algal cell walls to induce pore formation, which facilitated translocation of the QDs. Although not evident in our study, QDs after uptake could also bind to pyrenoids serving as centers for CO$_2$ fixation in algal chloroplasts, or generate reactive oxygen species (ROS) inside algal cells. Both of which would reduce algal bioactivity. Since algae are primary food sources for quatic organisms in natural ecosystems, further studies to decipher the mechanisms and long-term effects of algae-ENP interactions are deemed necessary.
CHAPTER FOUR
NANO-BIOSYSTEM INTERACTION
- CELLULAR LEVEL

The previous chapters have investigated the interactions between nanomaterials and ecological systems from the whole organism level (rice plants) to the cellular level (algae). For rice plants, water uptake provided the driving force for fullerene nanomaterials to move inside plants and further get translocated into the plant tissues and cells. The nanosize enabled fullerenes to further translocate across the cell wall and cell membrane and become stored in the vacuoles of the plants cells; some of the fullerenes even transmitted to the next generation of the rice plants. For algae, the porous structure of their cell wall provided numerous binding sites for the water-soluble QDs to adsorb. Since the size of the QDs aggregates (> 10 nm) exceeded the pore size (~ 5 nm) of the algal cell wall, these nanoparticles were excluded by the algae. The adsorption of QDs onto algae resulted in weakened photosynthetic activities due to light source blockage and inhibition of gas and nutrient transportation.

Compared with plant cells, no cell wall is present in mammalian cells. The cell membranes of mammalian cells, therefore, serve as the primary barrier for the cells to communicate with the extracellular space. The questions of how the surface charge of nanomaterials may affect cells and how cell membranes respond to nanomaterials are the major foci of this chapter.
4.1 Introduction

A great deal of research has been focused on developing nanomaterials for drug delivery, bioimaging, diagnosis, therapy, etc. For these applications, standard mammalian cell lines are usually used as the model host systems [109]. Translocation of nanomaterials across cell membrane into cytosol usually involves cell membrane attachment/adsorption, ligand signaling, endocytic process, and passive diffusion [110, 111]. Once inside the cells, the optical, thermal, and electrical properties of the nanomaterials facilitate the release and function of the loads that they carry. In the meantime, research in these areas must address the toxic effects introduced by the nanomaterials. Studies have shown that the involvement of nanomaterials caused lipid peroxidation that damaged the structure of the cell membrane [112]. A high concentration of reactive oxygen species (ROS), which is harmful to the cell, has been found to be affiliated with the introduction of certain nanomaterials [74]. Overdose of nanomaterials could cause contraction of the cell due to both physical adsorptions and consumption of lipids by endocytosis of the nanoparticles [76]. To resolve such complex issues, a better understanding on the interactions between nanomaterials and the cell, especially the cell membrane, is needed.

4.1.1 Cell membrane

The cell membrane, comprised mostly of phospholipids and proteins, is one of the most important organelles in the cell. It functions to hold the internal structures of the cell
together while maintain the communications between the intracellular and extracellular space. The semi-permeable cell membrane also directs movements of substances in and out of the cell through ion channels, pumps, and transporters embedded in the lipid bilayer. Due to the asymmetric distribution of the phospholipids in the lipid bilayer, the concentration gradients of ions across the cell membrane, and the peripheral proteins (carrying a net negative charge) associated with the inner bilayer, the entire cell membrane is weakly negatively charged [113].

4.1.1.1 Ion channels and pumps

Ion channels are the pathways for ions to move across the cell membrane driven by their concentration gradients. Pore-forming proteins that are embedded in the cell membrane are the major components of the ion channels. The specificity of ion channels is afforded by the unique architecture or binding ligands of each ion channel. Besides concentration gradient, which serves as the driving force for the movement of ions, a variety of gating mechanisms are also involved in the operation of ion channels to direct the rate of ion flow. In general, ion channels can be classified into voltage-gated, ligand-gated, among others [114].

Unlike ion channels that allow ions to move through passive transport, ion pumps (transporters) move ions across the cell membrane against their concentration gradient. Such movement requires energy, and the sources of energy include adenosine
triphosphate (ATP) and the concentration gradient of a second ion [115]. For example, the plasma membrane Ca\(^{2+}\) ATPase pumps out one Ca\(^{2+}\) ion through the hydrolysis of one ATP molecule. The sodium-calcium pump removes two Ca\(^{2+}\) ions against the Ca\(^{2+}\) concentration gradient while importing three Na\(^{+}\) ions following the Na\(^{+}\) concentration gradient. (Figure 4-1)
Figure 4-1. Schematic of ion channels and ion pumps [116].
4.1.1.2 Membrane potential

As mentioned before that cell membranes are weakly negatively charged. This negative charge is due to the asymmetric distribution of phospholipids, the net flow of cationic ions out of the cells, and the negatively charged peripheral proteins associated with the inner membrane. The resulting voltage difference across the cell membrane is termed as the membrane potential.

The membrane potential has two major functions. First, it allows the cell to function as a battery, providing power to operate a variety of molecular devices embedded in the membrane, such as voltage-gated ion channels. Second, in electrically excitable cells such as neurons, the membrane potential is pivotal for transmitting signals between different parts of the cell [117].

4.1.1.3 Phase transition of cell membrane

The most abundant component of the cell membrane, phospholipids, diffuse laterally and in between layers (flip-flop) constantly [118]. The mobility (fluidity) of the lipid molecules and its response to the change of temperature are known as the phase behavior of the cell membrane [119]. Since diffusion is thermodynamically driven, lipid movement at low temperatures is highly constrained and the cell membrane is in the gel phase. As temperature increases, lipid molecules diffuse more rapidly and the cell
membrane transits to the fluid phase. The area per lipid molecule in the gel phase is relatively smaller than in the fluid phase (Figure 4-2).

At normal body temperature, cell membrane assumes both the fluid phase and the gel phase. These structures remain stable at a fixed temperature. However, when the cell undergoes endocytosis, a portion of the lipid molecules are consumed, and the area per lipid molecule increases accordingly. As a result, the area of the cell in the fluid phase increases. When exocytosis occurs, the cell recycles lipid molecules back into the cell membrane and the area of the cell in the gel phase increases accordingly [120-122].

![Figure 4-2. Illustration of lipid phase. Left: gel phase; right: liquid phase [123].](image)

4.2 Experiments

Both positively and negatively charged nanoparticles (TiO$_2$, Au, and QDs) were introduced in this study to examine the membrane responses to the nanomaterials
carrying various surface charges. Membrane potential, phase transition, and uptake and toxicity of the nanomaterials were studied with several bioassays documented below.

4.2.1 Nanoparticle characterizations

Both positively and negatively charged TiO$_2$, Au (Vive Nano) and QDs (Ocean Nanotech) nanoparticles were purchased and used as received. The concentrations of the nanoparticles applied to the cells were 1 µg/L to 1 g/L, obtained by diluting the stock solutions using Milli-Q water. The hydrodynamic sizes of these nanoparticles were approximately 10 nm in diameter in all cases as measured by dynamic light scattering. The zeta potentials of the positively charged and negatively charged nanoparticles were +65 mV and -60 mV (ZetaSizer Nano), respectively.

4.2.2 Cell membrane potential - FLIPR assay

To evaluate the effects of nanoparticles surface charge on membrane potential, a cell membrane potential evaluation kit FLIPR was used. The FLIPR kit contained a lipophilic dye that fluorescently labeled the cell membrane. The fluorescence intensity of the dye was highly dependent on the membrane potential. An increased intensity indicated a depolarized cell membrane, while a decreased intensity indicated a hyperpolarized cell membrane or loss of membrane integrity (Figure 4-3). A fluorescence microplate reader (BioTek) was used to excite the lipophilic dye at 530/25 nm and collect the fluorescence
intensity at 590/20 nm overtime.

Figure 4-3. Correlation between FLIPR intensity and cell membrane potential [124].

Human colon adenocarcinoma cell line, HT-29, was used as a model mammalian cell line in this study. About 2,000 cells were seeded in each well of a 96-well microplate and allowed to attach overnight at 37 °C with 5% CO₂. The FLIPR dye (10 µL) was then added to each well and allowed to label the cells at room temperature for 30 min. After the fluorescence intensity was stabilized, 50 µL of nanoparticles of varied concentrations were added to each well with 6 repeats. The real time fluorescence intensity was recorded for 20 min.
4.2.3 Cell viability assay - DHL

The cytotoxic effects of the nanoparticles were evaluated by the DHL Cell viability and proliferation assay. This assay contained a fluorescent dye that labels living cells. When excited at 530/60 nm, the fluorescence intensity at 590/60 nm is a direct indicator of the number of living cells (Figure 4–4).

Figure 4–4. Illustration of DHL cell viability and proliferation assay. The dehydrogenases (e.g. LDH) in the living cells will continuously reduce resazurin to the strongly fluorescent resorufin [125].
HT-29 cells were seeded in a 96-well microplate and treated with nanoparticles as described in section 4.2.2. After 20 min in incubation, the cells were washed extensively using PBS buffer three times. Non-viable cells were removed from the well through this washing process. The viability assay was then added to each well to evaluate the number of living cells.

### 4.2.4 Fluorescence imaging and analysis

To visualize the interaction between nanoparticles and the cell, both positively and negatively charged QDs were incubated with HT-29 cells and laser scanning confocal imaging was performed. QDs were incubated with HT-29 cells under the same conditions as described in section 4.2.2 for TiO$_2$ and Au nanoparticles. After incubation, the cells were washed three times with PBS to remove the free QDs in the growth media. Images were captured using a 40X oil immersion objective and the excitation/emission filters were set as 540 nm/580 nm.

### 4.2.5 Laurdan assay

The Laurdan assay evaluates the phase and phase transition of the cell membrane. This assay is based on a lipophilic dye called Laurdan (Figure 4-5). The Laurdan dye has a fluorescence spectrum that is highly sensitive to its environment (Figure 4-6). When residing in the cell membrane, the dye senses the phase transition of cell membrane and
its fluorescence spectrum shifts accordingly. To evaluate the phase transition, the generalized polarization (GP) value is usually calculated based on the fluorescence intensity at different wavelengths [126]. As shown in the equation (1),

\[ GP = \frac{I_B - I_R}{I_B + I_R}, \]  

where \( I_R \) denotes the fluorescence intensity in the red region (490 nm), and \( I_B \) denotes the fluorescence intensity in the blue region (440 nm). As the membrane transits from the gel to the fluid phase, the fluorescence intensity of Laurdan in the blue region decreases and increases in the red region. As a result, the GP value decreases.

Figure 4-5. Illustration of laurdan dye labeling the cell membrane and sensing the phase transition. (black dots: water molecules) [127].
To monitor the phase transition of the cell membrane, 100 µM of Laurdan dye was incubated with HT-29 cells for 1 h. After incubation, the cells were washed using PBS three times to remove the free Laurdan dye. A fluorometer was used to excite the Laurdan dye at 340 nm and collect the spectrum from 400 nm to 500 nm. Same concentrations of both positively and negatively charged Au nanoparticles were added to the cells and their spectra were collected over time. The fluorescence intensities at blue and red region ($I_B$ and $I_R$) were used to calculate the GP value.

**4.3 Results and discussion**
4.3.1 Membrane depolarization by nanoparticles

Figure 4-7 shows the fluorescence intensities of the FLIPR kit over a period of time. For cells incubated with negatively charged TiO₂, the fluorescence intensities increased over time, with higher nanoparticle concentrations caused more rapid increases. Since the fluorescence intensity was closely related to the cell membrane potential, the negatively charged TiO₂ caused depolarization of the cell membrane over time. For positively charged TiO₂ incubated cells, the same trend was found for the concentration at 0.1 ppm (10,000X) and 10 ppm (100X). However, at the highest concentration (1,000 ppm, or 1X), the fluorescence intensity increased during the first 3 min before decreased thereafter.

Similar procedures were applied to cells incubated with Au nanoparticles. Both negatively and positively charged Au nanoparticles caused depolarization in the cell membrane. The highest concentration of the positively charged Au did not induce a dramatic decrease in fluorescence intensity, as observed for the TiO₂ treated cells (Figure 4-8).
Figure 4-7. Fluorescence intensity vs. time (TiO$_2$ treated cells).
As demonstrated by the FLIPR membrane potential measurements, both positively and negatively charged nanoparticles depolarized the cell membrane. However, the underlying mechanisms are believed to be different. For the cells treated by negatively charged nanoparticles, electric repulsion would keep most of the nanoparticles outside the cell membrane, due to the weakly negatively charge of the latter. As a result, a net negative charge induced an electric field that offset the membrane potential (Figure 4-9a). For the cells treated by the positively charged nanoparticles, electric attraction would promote translocation of the nanoparticles across the cell membrane. Once inside the cells, the positively charged nanoparticles would neutralize the negative net charge of the inner membrane, thereby depolarizing the membrane potential (Figure 4-9b).
Figure 4-9. Schematic illustration of cell membrane depolarization due to charged nanoparticles. (left: negatively charged; right: positively charged)
4.3.2 Localization of nanoparticles based on their surface charge

To verify the hypothesis proposed in section 4.3.1, positively and negatively charged QDs were incubated with HT-29 cells to identify localization of the QDs. Figure 4-10 shows two representative images of HT-29 cell incubated with negatively charged (a) and positively charged QDs (b). Most of the negatively charged QDs remained outside and in between the cells, while positively charged QDs were mostly internalized by the cells.

![Confocal fluorescence images showing localization of both (left) positively and (right) negatively charged QDs with HT-29 cells.](image)

For positively charged QDs, electric attraction between the QDs and the weakly negatively charged cell membrane promoted their binding and translocation of the nanoparticles across the cell membrane. For negatively charged QDs, electric repulsion
hindered their binding and translocation of the nanoparticles was far less prevalent as compared to the positively charges QDs.

Since the fluorescence emission of the QDs is proportional to their concentration, a fluorescence microplate reader was used to quantify the amount of oppositely charged QDs associated with HT-29 cells after incubation. As shown in Figure 4-11, the amount of QDs associated with the cells increased with concentration, while more positively charged QDs were associated with the cells than the negatively charged nanoparticles.

![Figure 4-11. Uptake of positively (blue) and negatively (red) charged QDs.](image)

4.3.3 Toxicity of nanoparticles
As shown in Figure 4-7, the dramatic decrease in fluorescence intensity as measured for the cells exposed to the positively charged TiO$_2$ was due to the loss of membrane integrity, as indicated by the confocal images (Figure 4-12).

The cell viability test based on the DHL assay also indicated cytotoxicity of the TiO$_2$ nanoparticles (Figure 4-13). Keep in mind that the fluorescence intensity was proportional to the number of living cells after nanoparticles treatment. For cells treated with negatively charged TiO$_2$, the number of viable cells decreased with the increase of the nanoparticle concentration. At the highest concentration, the cell viability was approximately 70% as compared to the control cells without exposed to any nanoparticles. For cells treated with positively charged TiO$_2$, the cell viability was only 10% as compared with the control, indicating severe cell damage caused by the positively charged TiO$_2$. For cell treated with Au nanoparticles, the cell viability remained the same when compared with the control, indicating that Au nanoparticles were largely biocompatible.
Figure 4-12. Confocal images showing the control cells (left) and cells incubated with the highest concentration of positively charged TiO\textsubscript{2} nanoparticles (right).

Figure 4-13. Cell viability vs. concentration of the nanoparticles.
Results from the cell viability assay and the membrane potential assay (in section 4.3.1) are consistent in terms of the cell behaviors in the presence of the TiO$_2$ and Au nanoparticles. These assays strongly confirm that the surface chemistry of nanoparticles plays a significant role in their induced cell responses.

4.3.4 Phase transition caused by nanoparticles

As Figure 4-13 shows, the GP value decreased over time for the cells treated with positively charge Au nanoparticles, while that for the cells treated with negatively charged Au nanoparticles remained relatively stable. Such different phase transitions can be explained from the following two aspects.
Firstly, nanoparticles of different surface charges interacted differently with the lipid molecules in the cell membrane. The most abundant phospholipids in the cell membrane have a positively charged head group and a negatively charged neck. When interacting with positively charged nanoparticles, the electric repulsion pushed the head group down to cause a lateral expansion of the lipid molecule. As a result, the area per lipid molecule increased and the cell membrane became fluidized. In contrast, negatively charged nanoparticles acted differently on lipid molecules in the cell membrane: the electric...
attraction between a lipid and a nanoparticle pulled the head group of the lipid molecule straight, such a conformational change of the lipid molecule resulted in a decreased area per lipid molecule.

Secondly, cells undergo endocytosis when exposed to nanoparticles. The process of endocytosis consumes lipids as the latter are required for forming endosomes to internalize the nanoparticles. As the number of lipid molecules in the cell membrane decreased, the area per lipid molecule increased and the cell membrane became fluidized. For cells exposed to the positively charged nanoparticles, the two effects above combined to greatly fluidize the cell membrane. For cells that were exposed to the negatively charged nanoparticles, these two effects counteracted each another, thus slowing down the fluidic transition of the cell membrane.

4.3.5 Surface charge and surface chemistry

The studies as documented in this chapter were mostly focused on the effects of the surface charge of nanoparticles, while one must be aware that other surface chemistry factors of the nanoparticles may be equally important. As indicated by both the membrane potential and viability assays, the positively charged TiO$_2$ nanoparticles caused more membrane damage than the positively charged Au nanoparticles. Compared to the relatively inert surface of the Au, the TiO$_2$ nanoparticles had a greater ability in absorbing light, especially in the UV. The capabilities of TiO$_2$ to oxidize the lipids and
create ROS in cytosol have been reported in literature, part of the reasons for the dramatically decreased fluorescence intensity in the FLIPR assay and low cell viability of the cells exposed to the positively charged TiO$_2$ nanoparticles [74, 112].
CHAPTER FIVE
PARRALLEL COMPARISON OF NANO-BIOLOGY
VS. NANO-ECOLOGY INTERACTION

In chapters two and three I described the interactions between nanomaterials (carbon-based and quantum dots) and ecological systems (rice plant and algae). In chapter four I studied the surface charge effects of nanomaterials (metals, metal oxides and quantum dots) on the mammalian cell. From these chapters I concluded that the structure and function of the cell, the building block of any biological systems, and the physiochemistry properties of the nanomaterials play essential roles on the fate of nanomaterials in biological and ecological systems.

As a necessary extension to the aforementioned studies, chapter five aims at providing a parallel comparison on the interactions between nanomaterials and both plant and mammalian cells.

5.1 Introduction

As introduced earlier, carbon-based nanomaterials (CBNMs) represent one of the most commonly used and studied class of nanomaterials. With surface modifications CBNMs can be suspended and readily enter biological and ecological systems. Such surface modifications include both chemical functionalizations and physical adsorption.
Chemical functionalization requires breaking the sp² bonds on the surfaces of the CBNMs, which would result in defects of the structure and further compromise the optical, electrical, or mechanical properties of the CBNMs. Physical adsorption, in comparison, does not alter the structure or the properties of the CBNMs. However, physical adsorption is less stable and the CBNMs tend to aggregate or agglomerate overtime.

Cells are the fundamental units for both biological and ecological systems. Mammalian and plant cells share a lot of similar organelles such as the cell membrane, mitochondria, and endoplasmic reticulum, etc. There are also some unique features which are only present in plant cells such as the cell wall, chloroplasts, and vacuoles. Because of these latter organelles, plant cells are less vulnerable to forensic materials and can produce most of the energy and nutrients required on its own. Since the function of the cell is strongly correlated with its structure, mammalian and plant cells show distinctive behaviors and responses to CBNMs.

In this chapter, two fullerene-based nanomaterials are reintroduced, fullerol C₆₀(OH)₂₀ and C₇₀-NOM (Figure 5-1). C₆₀(OH)₂₀ is a derivative of fullerene C₆₀. The surface of a C₆₀(OH)₂₀ molecule is covalently functionalized with 20 hydroxyl groups (-OH) to render its water solubility or suspendability. C₇₀-NOM, as described in chapter two, is a supramolecular complex formed by fullerene C₇₀ and natural organic matter (NOM). The physical adsorption of the amphiphilic NOM onto a small cluster of hydrophobic C₇₀
molecules renders the entire complex water suspendability.

Figure 5-1. Structures of $C_{70}$-NOM (a) and $C_{60}(OH)_{20}$ (b) [128].

HT-29 - the human colon adenocarcinoma cancer cell line introduced in chapter four is again used in the current study as a model mammalian cell line. For plant cells, *Allium cepa* (onion) cells are introduced for the following three reasons. First, a single layer of *Allium cepa* epidermal cells can be obtained easily and consistently by peeling off the first layer of the leaf tissue of *Allium cepa*. Secondly, the structure of the epidermal cells is distributed very uniformly throughout the whole layer. Lastly, the epidermal cells have little chlorophyll and hence produce minimal autofluorescence.

5.2 Experiments
5.2.1 Materials synthesis and characterizations

- C$_{70}$-NOM

The supramolecular complexes of C$_{70}$-NOM (Figure 5-1a) were synthesized as described in Chapter two. Different concentrations of C$_{70}$-NOM (from 10 to 110 mg/L) were prepared by diluting the stock C$_{70}$-NOM suspension (1 mg/mL) using Milli-Q water. Due to the aggregation effect of C$_{70}$-NOM, UV-vis absorbance measurements were conducted to quantify the exact amount of C$_{70}$-NOM exposed to the cell lines. As shown in Figure 5-2, the absorbance value at 400 nm of the C$_{70}$-NOM suspension was measured right after sonication (blue dots). The suspensions were then placed at room temperature overnight. The absorbance at the same wavelength was then measured to quantify the amount of C$_{70}$-NOM remained in the aqueous solution (green dots). The difference of the absorbance values obtained from these two measurements denotes the amount of aggregated C$_{70}$-NOM.
The size distribution of C\textsubscript{70}-NOM complexes was measured by dynamic light scattering (Nanosizer S90). As shown in Figure 5-3, at lower concentration (10 mg/L), the hydrodynamic size of C\textsubscript{70}-NOM complexes was centered at 20 nm (ranging from 18.17 \textendash 43.82 nm). With increasing concentration, due to the aggregation effect, the center of hydrodynamic size shifted to 35 nm (ranging from 27.36 to 100 nm).
Figure 5-3. Hydrodynamic size of C$_{70}$-NOM at 10 mg/L (a) and 110 mg/L (b).
• $C_{60}(OH)_{20}$

Fullerol $C_{60}(OH)_{20}$ (Figure 5-1b) was purchased from BuckyUSA and dissolved directly in Milli-Q water. The solubility of the suspensions was characterized by UV-vis absorbance measurements. Since the surface of $C_{60}(OH)_{20}$ is covalently bonded with hydroxyl groups, the suspension of $C_{60}(OH)_{20}$ was remarkably stable overtime. To test the stability of such suspensions, ultracentrifugation was applied to all $C_{60}(OH)_{20}$ suspensions (10~110 mg/L). As shown in Figure 5-4, the blue dots stand for the absorbance value at 252 nm of each concentration before ultrasonication (10,000 g RCF, 5 min). The red dots stand for the absorbance value at the same wavelength after ultrasonication. The difference in the absorbance values denotes the amount of $C_{60}(OH)_{20}$ being spun down and separated from the aqueous suspension.
The size distributions of C$_{60}$(OH)$_{20}$ was measured using dynamic light scattering. As shown in Figure 5-5, the hydrodynamic size of C$_{60}$(OH)$_{20}$ at 10 mg/L was centered around 1.5 nm (ranging from 1.12 to 1.74 nm), indicating the presence of individual C$_{60}$(OH)$_{20}$ molecules. At a higher concentration (110 mg/L), the center of hydrodynamic size increased to 20 nm (ranging from 15.69 to 24.36 nm). The larger size complexes were formed through hydrogen bonding between hydroxyl groups and hydrophobic interactions.
Figure 5-5. Hydrodynamic size of \( C_{60}(OH)_{20} \) at 10 mg/L (a) and 110 mg/L (b).

5.2.2 Cell lines

- HT-29 human colonic adenocarcinoma cells
HT-29 cells were cultured in DMEM with 1% penicillin streptomycin, 1% sodium pyruvate, and 10% fetal bovine serum. Approximately 5,000 HT-29 cells were seeded in each well (200 µL) of an eight-chamber glass well and allowed to attach overnight at 37°C with 5% CO₂. After the cells reached a 60% confluence, C₇₀-NOM and C₆₀(OH)₂₀ were added in each chamber glass well to obtain final concentrations of 10, 30, 50, 70, 90 and 110 mg/L. After 9 h incubation, the cells were thoroughly rinsed three times using PBS buffer to remove dead cells and free nanoparticles.

- *Allium cepa* cells

*Allium cepa* samples were obtained from produce quality onion bulbs. Storage leaves of area 1 cm² were removed, and the sample was peeled from the inside layers of the plant leaf tissue. Samples were immersed in C₆₀(OH)₂₀ and C₇₀-NOM suspensions to obtain final concentrations of 10-110 mg/L in MS buffer.

5.2.3 Confocal imaging

To evaluate the effects of nanomaterials on the *Allium cepa* cells, a plant cell viability kit (Sigma) was used. The plant viability kit contains two fluorescent dyes, propidium iodide (PI) and fluorescein diacetate (FD). The PI dyes emit in red by diffusing into the nonviable cells and intercalating with their DNA. The FD dye fluoresces in green after diffusing into viable cells and hydrolyzed by the enzymes therein. After 9 h incubation,
the cells were washed in MS buffer prior to addition of the plant cell viability assay, and
examined under a fluorescence microscope (Zeiss A1). Fluorescence images were viewed
from the FITC (for FD emission) and Rhodamine (for PI emission) channels. Damaged
cells showed orange fluorescence (peak at 620 nm) in the nuclear region when viewed
under the Rhodamine channel.

The treated HT-29 cells were directly examined under a laser scanning confocal
microscope (LSM510, Zeiss). An Argon laser of 488 nm was used as an excitation source
for confocal imaging. For each sample condition, 10 images (900×900 µm) were
acquired using a 10× oil immersion objective. The images were then analyzed and the
living cells of each sample were counted using LSM Image Browser.

5.2.4 Transmission electron microscopy (TEM) imaging of *Allium cepa*

For TEM imaging, thin layers of Allium cepa cells were fixed in 3.5% glutaraldehyde
overnight and dehydrated in a graded series of ethanol. The dehydrated samples were
then embedded in LR white resin overnight at 40°C and sectioned into ~200 nm thick
thin films using an Ultracut E Microtome. No osmium tetroxide was added to prevent the
introduction of artifacts. TEM images were acquired using a Hitachi H7600 microscope
operated at 80 and 100 kV. The lattice structures of C$_{70}$-NOM and C$_{60}$$(\text{OH})_{20}$ were
captured using a Hitachi H9500 microscope operated at 150 kV. The lattice spacing of
the nanoparticles were analyzed by performing Fast Fourier Transform (FFT) of the TEM
images, using the software “Diffractogram”.

5.3 Results and Discussions

5.3.1 Cell damage - microscopy images

5.3.1.1 Allium cepa cells

Figure 5-6 shows the optical images of Allium cepa cells incubated with different concentrations of C$_{60}$(OH)$_{20}$ and C$_{70}$-NOM. The bright field images (a, d and g) show cells that are incubated with nanomaterials have damaged morphology. The damage was further confirmed from the fluorescence images (b, e and h). The red spots show the location of PI dyes, and indicate that the cell structure is compromised or the cells are no longer viable. The images (c, f and i) shows the fluorescence in the green channel. Due to the autofluorescence from the Allium cepa cells, the signals interfere with the fluorescence of FD. Comparing the images from 30 mg/L C$_{60}$(OH)$_{20}$ incubated cells (a, b and c) and 70 mg/L C$_{60}$(OH)$_{20}$ incubated cells (d, e and f), more orange fluorescent spots can be seen, indicating increased cell damage with increased C$_{60}$(OH)$_{20}$ concentration.

The appearances of contagious nonviable cells (regions denoted by red arrow in Figure 5-6 c and orange spots in Figure 5-6 e) further suggest that upon C$_{60}$(OH)$_{20}$ uptake cells underwent necrosis, which is often invoked by abnormal environmental conditions and viruses.

Figure 5-6 (g, h and i) show the cells incubated with 50 mg/L C$_{70}$-NOM. Less cell
damage were found in these cells. The reasons are mostly due to the larger hydrodynamic size of C$_{70}$-NOM complexes and their hydrophobic surface. These cells were further treated with mannitol (0.8 M) for 15 min. The mannitol gradient across the cell surfaces induced an osmotic pressure, which in turn split plant cell walls from their underlining plasma membranes. C$_{70}$ aggregates were revealed by the osmosis assay as mostly adsorbed on or trapped within the hydrophobic cellulose matrices of the plant cell walls (Figure 5-6 i).
Figure 5-6. Optical imaging of *Allium cepa* cells incubated with C\textsubscript{70}-NOM and C\textsubscript{60}(OH\textsubscript{20}). a-c, Plant cells incubated with C\textsubscript{60}(OH\textsubscript{20}) of 30 mg/L. d-f, Plant cells incubated with C\textsubscript{60}(OH\textsubscript{20}) of 70 mg/L. g-i, Plant cells incubated with C\textsubscript{70}-NOM of 50 mg/L. b,e,h, The orange fluorescence indicates staining of nucleic acids by PI due to loss of cell viability. c,f,i, The bright green fluorescence indicates hydrolysis of FD by intracellular esterases of viable cells. a,c, Examples of non-viable (red arrows) and viable cells (black arrow). g-i, Osmosis procedures were applied to split plasma cell membranes (pink arrow in i) from plant cell walls (white arrow in i). Aggregation of C\textsubscript{70} particles is exemplified by the blue arrow in (i). Scale bar: 50 µm [128].
5.3.1.2 HT-29 cells

After 9 h incubation and a thorough washing to remove dead cells and unbound nanoparticles, the number/density of viable HT-29 cells decreased continuously with increased C$_{70}$-NOM concentration up to 70 mg/L, and then leveled off at higher concentrations due to nanoparticle aggregation (Figure 5-7 e). The cell morphology also changed from the healthy elongated form to the less viable, more spherical shapes at higher C$_{70}$-NOM concentrations, showing abundant nanoparticle aggregates bound to and imbedded within the cell membranes (Figure 5-7, a-d). Cell lysis was rare but visible, likely due to exhaustive endocytosis (Figure 5-7 d), and necrosis in the damaged cells.
Figure 5-7. Mammalian cell damage in the presence of C$_{70}$-NOM of various concentrations. a, HT-29 cell control. b, HT-29 cells incubated with C$_{70}$-NOM of 30 mg/L. c, HT-29 cells incubated with C$_{70}$-NOM of 110 mg/L. d, Cell lysis (indicated by red arrow) in the presence of C$_{70}$-NOM of 110 mg/L. The aggregation of C$_{70}$ particles is evident in (b-d). e, HT-29 cell count in the presence of C$_{70}$-NOM of various concentrations. The asterisks indicate data which are statistically different from the control (p<0.01). NOM: positive control [128].
In contrast to the $C_{70}$-NOM treated cells, no cell damage was found for HT-29 cells exposed to $C_{60}(OH)_{20}$ of all concentrations used (Fig. 5-8), confirming the low affinity of $C_{60}(OH)_{20}$ for cell membranes. These divergent results in the damage induced by $C_{70}$-NOM and $C_{60}(OH)_{20}$ to HT-29 cells are in good agreement with the in vitro study by Sayes et al. and the simulations by Qiao et al. on the cytotoxicities of pristine fullerene $C_{60}$ and fullerol $C_{60}(OH)_{24}/C_{60}(OH)_{20}$ respectively.
Figure 5-8. Mammalian cell damage in the presence of $\text{C}_{60}(\text{OH})_{20}$ of various concentrations. a, HT-29 cell control. b, HT-29 cells incubated with $\text{C}_{60}(\text{OH})_{20}$ of 30 mg/L. c, HT-29 cells incubated with $\text{C}_{60}(\text{OH})_{20}$ of 110 mg/L. d, HT-29 cell count in the presence of $\text{C}_{60}(\text{OH})_{20}$ of various concentrations [128].
5.3.2 Localization of nanomaterials in plant cells - TEM images

Figure 5-9 shows the localization of C$_{60}$(OH)$_{20}$ and C$_{70}$-NOM with *Allium cepa* cells observed by TEM. For cells incubated with C$_{60}$(OH)$_{20}$, due to their small size and good solubility, C$_{60}$(OH)$_{20}$ readily permeated through the plant cell wall driven by a concentration gradient, and were mostly excluded by the plasma membrane due to their hydrophilicity. Under capillary and van der Waals forces these nanoparticles were confined between the cell wall and the plasma membrane (Figure 5-9 e), and grew to protrude the plasma membrane. Since fullerols—unlike pristine fullerenes—have been shown to be inactive in creating reactive oxygen species, the loss of membrane integrity is therefore inferred as a result of mechanical damage exerted by C$_{60}$(OH)$_{20}$ aggregation. Such mechanical damage would impinge on membrane fluidity and the transport of nutrients and ions between the plant cell and its extracellular space, further stressing the physiological state of the cell and its neighboring cells. C$_{60}$(OH)$_{20}$ clusters occasionally appeared near the plasma membrane within the cytoplasm (Figure 5-9 f and g), likely due to a low-level steady state endocytosis in the plant cell and to membrane damage. Furthermore, clustering of C$_{60}$(OH)$_{20}$ between adjacent epidermal cell walls also implies that transport of C$_{60}$(OH)$_{20}$ in the plant tissue was partially conveyed through the apoplastic pathway, whose blockage could also have an impact on cell viability.
Figure 5-9. TEM imaging of carbon nanoparticle uptake by *Allium cepa* cells. a, Control showing plant cell wall and plasma membrane. The cell wall typically bends towards its intracellular space. b-d, Plant cell walls entrapped with C$_{70}$-NOM clusters of 50~400 nm. C$_{70}$-NOM concentration: 50 mg/L. d, Magnified view of a C$_{70}$-NOM cluster in (a). e-g, Translocation of C$_{60}$(OH)$_{20}$ across plant cell walls. C$_{60}$(OH)$_{20}$ clusters can be seen (e) at the interface between the plant cell wall and the plasma membrane and (f, g) in intracellular space. C$_{60}$(OH)$_{20}$ concentration: 50 mg/L. g, Magnified view of the C$_{60}$(OH)$_{20}$ clusters in (f) [128].
5.3.3 Plant cell damage comparison

In contrast to the observations made above for the mammalian cells, plant cells showed distinctly different responses to the two types of CBNMs. The damage of plant cells was calculated by determining the percentage of nonviable cells in the PI channel, while the FD channel was used as a reference due to its susceptibility to cell autofluorescence. As shown in the top panel of Figure 5-10, C\textsubscript{70}-NOM caused a mere 0.8% more plant cell damage than the control at 90 mg/L and 110 mg/L, and no damage at lower concentrations. This phenomenon is attributed to the large size and hydrophobicity of the C\textsubscript{70}-NOM, which tended to block the porous plant cell wall and form clusters therein through hydrophobic interactions. For C\textsubscript{60}(OH)\textsubscript{20} incubated cells, the number of damaged cells increased with the concentration. The ease of cell damage at 90 mg/L and 110 mg/L (Figure 5-10, lower panel) is attributed to the gradual aggregation of C\textsubscript{60}(OH)\textsubscript{20} at these concentrations.
Figure 5-10. Percent of *Allium cepa* plant cell damage in the presence of C$_{70}$-NOM and C$_{60}$(OH)$_{20}$ of various concentrations. The asterisks indicate data which are statistically different from the control (p<0.01). NOM: positive control [128].

5.4 Conclusions

The distinctly different plant and mammalian cell responses to nanoparticles can be understood as a combined result of nanoparticle filtration by the porous plant cell wall,
confinement on nanoparticle mobility by the hydrophobic, presence of a thick (a few to tens of micrometers) and rigid plant cell wall and the amphiphilic, thin (~7 nm), and fluidic plasma membrane, and the physiochemical properties of the nanoparticles. The filtration by the plant cell wall favors the uptake of smaller and more hydrophilic nanoparticles. After translocation, these small and hydrophilic nanoparticles are confined at the interface between the plant cell wall and the plasma membrane, and self-assembled to initiate mechanical damage to the plasma membrane. Larger and more hydrophobic nanoparticles of low concentrations exert little damage on the plant cell. However, at high concentrations, adsorption of hydrophobic nanoparticles onto the plant cell wall and their retention within the plant cell wall would still have an impact on the physiological state of the plant cell, as implied by the emergence of cell damage with C_{70}-NOM of 90 mg/L and 110 mg/L. The absence of a cell wall in mammalian cells is favorable for minimizing the adverse effect of hydrophilic nanoparticles, but encourages membrane partitioning by hydrophobic and/or noncovalently-functionalized nanoparticles to induce cell damage. This fundamental study on the biological and environmental responses to nanoparticles clarifies the intensive debates on nanotoxicity, which should prove beneficial for guiding the design of biocompatible and environmentally sustainable nanotechnologies.
CHAPTER SIX

CONCLUSION AND FUTURE WORK

Much knowledge has been obtained from this dissertation research on the biological and environmental implications of nanomaterials. There is much more yet to be learned. This texture of this dissertation is organized into these three complimentary aspects, namely, Nano-Eco interaction on the whole organism level, Nano-Eco and Nano-Bio interactions on the cellular level, and a parallel and first comparison between Nano-Bio and Nano-Eco interactions on the cellular level.

6.1 Nano-Eco interaction - whole organism level

My studies used rice plants as the model system to represent ecological systems on the whole organism level. I have shown that carbon-based nanomaterials (CBNMs), which are usually not considered as water contaminants, can be suspended and transported in natural water sources due to their nonspecific interactions with the natural organic matter (NOM). As the most abundant naturally occurring substances in all waters and soils, NOM possesses both hydrophobic and hydrophilic moieties that enable them to alter the surface properties of the CBNMs.

Specifically, C_{70}-NOM, a supramolecular complex (1-5 nm in hydrodynamic size) formed by C_{70} and NOM through hydrophobic interaction and pi-pi stacking, was
introduced to rice plant seedlings and traces of the C\textsubscript{70} were identified inside rice plant compartments and tissues. Transpiration of water, concentration gradient of the nanoparticles from the roots to the leaves of the plants, as well as the hydrophobicity of the nanoparticles were attributed as the major driving forces for the biodistribution of the C\textsubscript{70}-NOM. Generational transfer of carbon nanoparticles through the progeny of the plants was observed for the first time by optical microscopy, electron microscopy, FT-Raman, and FTIR spectroscopies. The supramolecular complexes of MWNT-NOM, in contrast, were mostly blocked by the rice plants since the average pore size of the plant cell is only approximately 5 nm in diameter, significantly smaller than the hydrodynamic size (~200 nm) of the MWNT-NOM complexes. This study has demonstrated that both the physiochemical properties (hydrodynamic size and surface properties) of the nanomaterials and the plant physiology could play significant roles on the fate of nanomaterials in the plant systems.

6.2 Nano-Eco interaction - cellular level

To understand the cellular level interactions between nanomaterials and ecological systems, single-celled green algae \textit{Chlamydomonas} were used as a model system and fluorescent quantum dots (QDs) were used as a model for engineered nanomaterials (ENPs). The strong adsorption of QDs onto the external surfaces of algae were imaged by confocal fluorescence microscopy and quantified by UV-vis absorbance spectroscopy. The adsorption isotherm was fed to the Freundlich equation and the calculated
association constants indicated a favorable binding between the QDs and the algae, likely due to the nonspecific interactions between the surface functional groups of QDs (MUA) and the cell wall of *Chlamydomonas*. By evaluating the photosynthetic activities of the algae, I have quantified the rates of O$_2$ evolution and CO$_2$ depletion upon their exposure to the QDs. The hindered photosynthetic activities of the algae at or above 50 ppm were attributed to the blockage of light source and hindrance of gas and nutrient transfer by the adsorbed QDs. The physical chemistry and photochemistry used in this study are expected to be applicable to examining aquatic plant and animal species exposed to nanomaterials. Since algae are situated at the bottom of the food chain, their photosynthetic activities could have a profound impact on the entire ecological systems.

6.3 Nano-Bio interaction - cellular level

The plasma membrane of both mammalian and plant cells serve as a barrier to the extracellular space. The state of the cell membrane is strongly correlated with the well being of the cell. In my recent PhD research, membrane depolarization was observed on cells incubated with both positively and negatively charged nanomaterials (TiO$_2$ and Au). However, the underlying mechanisms were believed to be drastically different. Specifically, the positively charged nanoparticles translocated across the cell membrane and neutralized the negative charge on the inner membrane, therefore depolarized the cell membrane. The negatively charged ones were excluded by the cells due to their mutual electric repulsion; such an arrangement induced an electric field that offset the membrane
potential. This hypothesis was substantiated by confocal fluorescence microscopy on cells treated by both positively and negatively charged QDs.

The fluidity of the cell membrane was also found to respond differently to the charged nanomaterials. Endocytosis of the nanomaterials consumed lipid molecules and consequently fluidized the cell membrane. Positively charge nanomateirals, which increased the area per lipid molecule through electrostatic interaction, acted in conjunction with endocytosis to further fluidize cell membrane. Negatively charged nanomaerials, in comparison, decreased the area per lipid molecule and counter-balanced the effect of endocytosis to resist the fluidic transition of the cell membrane.

6.4 Parallel comparison of Nano-Eco and Nano-Bio interactions

The toxicity of nanomaterials is believed to be a result of both the phsiochemical properties of the nanomaterials and the physiochemistry and biology of the host systems. The study in Chapter 5 provided a first parallel comparison on two fullerene-based nanoparticles in both biological and ecological systems. C_{60}(OH)_{20}, with its surface covalently modified with hydroxyl groups, was well dispersed in the aqueous solution and had a hydrodynamic size of ~1.5 nm in diameter. C_{70}-NOM, the supramolecular complex, displayed a hydrodynamic size of ~20 nm in diameter and aggregated overtime.

An increased cell damage rate was found with increased concentration of C_{60}(OH)_{20} for
plant *Allium cepa* cells. This cell damage was attributed to the penetration of the hydrophilic \( \text{C}_{60}(\text{OH})_{20} \) through the hydrophobic plant cell wall and the subsequent mechanical protrusion of the accumulated nanoparticles on the plant cell membrane. In contrast, little damage was observed for \( \text{C}_{70} \)-NOM treated plant cells, mostly due to the aggregation of the molecular complexes and their hydrophobic interaction with the plant cell wall.

Compared to the observations for the plant cells, opposite trends were found for HT-29 mammalian cells incubated with these two types of nanoparticles. An increased cell damage rate was found with the increased concentration of \( \text{C}_{70} \)-NOM, while \( \text{C}_{60}(\text{OH})_{20} \) had little effect on the mammalian cells. This is because the cell membrane had a higher affinity for the hydrophobic \( \text{C}_{70} \)-NOM over the more hydrophilic \( \text{C}_{60}(\text{OH})_{20} \). As a result, exposure of HT-29 cells to \( \text{C}_{70} \)-NOM resulted in a loss of cell membrane integrity due to both the physical adsorption of the \( \text{C}_{70} \)-NOM and endocytosis. This parallel cell study offers a much needed insight for understanding nanomaterials in both biological and ecological systems. This study will also prove beneficial for guiding the design of nanomedicine and environmental sustainable nanotechnologies.

**6.5 Future work**

Future work based on the knowledge obtained from this PhD research can be categorized into two areas.
Firstly, I intend to pursue further research on the fundamental understanding of nanomaterials interacting with biological and ecological systems. While endocytosis of nanomaterials has been extensively studied, little is known about the discharge of nanomaterials by the cell. One on-going project in our lab aims to elucidate the mechanism of exocytosis of nanoparticles in mammalian cells. As exocytosis occurs, lipid molecules are recovered in the cell membrane. The recovery of lipid molecules would result in a decreased area per lipid molecule and induce a transition in the cell membrane from the fluidic to the gel phase. By monitoring the fluorescence signal of a laurdan dye (introduced in Chapter 4), one can obtain information on the discharge rate of nanomaterials by the cell, and the various parameters that govern exocytosis such as temperature of the environment, ionic strength of the cell, and concentration of the nanomaterials, etc.

Secondly, I plan to explore applications of nanomaterials. One of the understandings obtained from this dissertation is that CBNMs, due to their vast hydrophobic surface area, has the potential to absorb and trap water contaminants such as NOM, humic acid, and phenanthrene. Nanomaterials used for water purification have to be well constrained within the application unit. Previously, sodium alginate, extracted from brown algae cell wall, has been found to form biocompatible hydrogels when exposed to Ca$^{2+}$ ions. Such systems can be further developed to constrain CBNMs in the hydrogel and utilized as a water purification unit. By dipping the mixture of CBNMs and sodium alginate into
CaCl$_2$ solution, millimeter-sized CBNMs hydrogels can be easily and repeatedly formed. The size of such hydrogels would make the release and recovery of the CBNMs easy to control. Another advantage of the hydrogel system is its reusability; by immersing the hydrogels in a NaCl solution, the gel is liquefied and the CBNMs can be recovered, cleaned and reused to form hydrogels.
# APPENDIX

## A: Glossary of Terms

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<th>Symbol</th>
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<tr>
<td>CBNM</td>
<td>carbon-based nanomaterials</td>
</tr>
<tr>
<td>QDs</td>
<td>quantum dots</td>
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<tr>
<td>AuNPs</td>
<td>gold nanoparticles</td>
</tr>
<tr>
<td>ENPs</td>
<td>engineered nanoparticles</td>
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<td>SERS</td>
<td>surface-enhanced Raman scattering</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<td>SWNT</td>
<td>single-wall carbon nanotube</td>
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<tr>
<td>MWNT</td>
<td>multiwall carbon nanotube</td>
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<tr>
<td>NOM</td>
<td>natural organic matter</td>
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<td>SEM</td>
<td>scanning electron microscope</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscope</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared</td>
</tr>
<tr>
<td>MUA</td>
<td>mercaptoundecanoic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
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<td>fluorescein diacetate</td>
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<tr>
<td>FITC</td>
<td>fluorescence isothiocyanate</td>
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REFERENCES


26. <http://cnx.org/content/m22963/latest/>


69. M. Khodakovskaya, E. Dervishi, M. Mahmood, Y. Xu, Z. Li, F. Watanabe, and A. S. Biris. Carbon nanotubes are able to penetrate plant seed coat and


93. L. Yang, and D. J. Watts. Particle surface characteristics may play an important role in phytotoxicity of alumina nanoparticles. Toxicology Lett., 158:122, 2005.


104. <http://thiol.org/taxonomy/term/7,18>


123. <http://employees.csbsju.edu/hjakubowski/classes/ch331/lipidstruct/oldynamicves.html>


