Chapter 1: Introduction

Nanomaterials possess unique physicochemical properties such as high reactivity, large surface to volume ratio, exceptional electronic characteristics, quantum effects, etc.[1] Along with the excitement over their distinctive properties, there has been increasing concerns regarding the possible adverse health effects of nanomaterials. A probable downside of these remarkable capabilities is their harmful interactions with biological systems, with the potential to generate toxicity. The widespread use of engineered nanomaterials for a variety of applications, including their use in commercial products such as cosmetics, sunscreens, photovoltaic systems, etc., increases the possibility of these materials to come into contact with humans and the environment. Since various biological processes take place at the nanoscale level, it is likely that engineered nanomaterials may interact with biomolecules as well as cellular activities that are critical to life. It was reported that many nanosystems such as quantum dots (QDs; CdSe, CdTe), carbon nanotubes, metals (Ag, Au, Pt) and metal oxides (ZnO, TiO_2) exhibit characteristic toxic effects towards biological systems.[2] A better understanding of the fundamental interactions of nanoparticles with biological systems is hence critical to better elucidate its applications in the field of biology and medicine.

Metal oxide and carbon nanosystems are being used extensively due to their extra ordinary physico-chemical properties. For instance, TiO_2 and ZnO type materials are extensively studied for several industrial applications.[3] Although the nanotoxicity of TiO_2 is well reported; ZnO nanomaterials have not extensively studied. ZnO is an important II–VI group semiconductor material with direct band-gap of 3.37 eV and large exciton binding energy of ~ 60 meV, making it a promising candidate for many technology applications. Recently, a number of reports have cited interesting toxic characteristics of ZnO NCs. Hanley *et al* found that ~ 8 nm ZnO NCs showed preferential toxicity towards activated T-cells, while being only slightly toxic to primary cells.[4] Ostrovsky *et al.* and Premanathan *et al.* reported that ZnO showed enhanced toxicity towards glioma and leukemic cells, compared to their normal counterparts.[5,6] Both these studies indicated varied toxicity of ZnO towards

different cell types. Another detailed study by Xia *et al.* showed that toxicity of ZnO is related to the generation of reactive oxygen species and release of Zn^{2+} ions within lysosomes.[7] One interesting observation, considering the possible therapeutic use of nanomaterials, was that of ZnO nanocrystals (NCs) exhibiting differential toxicity towards bacteria, normal cells and cancer cells.[8] While the exact mechanisms of ZnO toxicity have not yet been clearly elucidated, suggested mechanisms highlight nanoparticle internalization leading to the generation of reactive oxygen species (ROS) generation, zinc ion release and membrane dysfunction. A systematic study of the influence of size scale, shape and surface chemistry is critical to the understanding of the differential toxicity mechanisms of ZnO NCs.

Amongst various carbon nanostructures, carbon nanotube (CNT) and fullerenes have been extensively explored for both industrial application as well as their toxicological impact.[9] Graphene is a new addition to the family of carbon nanostructures, with Andre Geim and Konstantin Novoselov winning the Nobel Prize for Physics in 2010 for its invention.[10] Graphene, the single-atom-thick two-dimensional (2D) allotrope of carbon, is a fascinating nanomaterial with unique physico-chemical properties that have invoked an enormous impact in material science since its discovery in 2004. Graphene is expected to revolutionize the technological advances in electronics, ultrafast computing, solar-energy harvesting, energy-storage and light-weight displays. Recently graphene has also been proposed for biomedical applications such as drug delivery and anti-cancer therapy.[11,12,13] In effect, these studies imply that surface functionalization of carbon nanostructures has a significant impact on their nano-bio interactions and possible toxicity effects. However, in the case of graphene, the effect of surface functionalization on its interaction with biological systems has not yet been addressed.

II Objectives of the study

More specifically this PhD thesis has the following objectives:

1. Synthesis of ZnO NCs of different size [5nm-1200nm], shape [Spherical, rod] and altered surface chemistry.

2. Study the effects of size, shape, surface chemistry and dosage towards bacteria, human normal primary cells and cancer cells and elucidate the mechanisms of toxicity.
3. Synthesis of pristine graphene and functionalized graphene.
4. Study the *in vitro* cellular and molecular response of graphene towards human primary endothelial cells and human blood components.
5. Evaluate the *in vivo* response of graphene in mice

Hypothesis

In combination with size-scale effects, the chemical composition and surface-chemistry play critical roles in determining the nano-bio interactions and toxicity of nanomaterials.

Research Questions

ZnO

1. How does varied nano-size, shape, surface chemistry and dosage of ZnO NCs affect the biological functioning of prokaryotic and eukaryotic cells?
2. Do ZnO NPs having varied size and surface chemistry show any differential toxicity to normal and cancer cells? If yes, what is the mechanism of preferential toxicity?

Graphene

1. What is the effect of altered surface chemistry of graphene in its interaction with cells: Cellular uptake, Cell functions (Viability, ROS stress, and mitochondrial functions), genotoxicity, inflammation and hemocompatibility?
2. How does pristine and functionalized graphene affect the whole genome expression of human primary endothelial cells?
3. What is the biodistribution and toxic effect of *p*-G and *f*-G in *in vivo* mice

Chapter 2: Materials and Methods

2.1 Synthesis of ZnO NCs

The synthesis of spherical ZnO NCs mainly involves the reaction of zinc salt with an alkali hydroxide in alcoholic or aqueous medium. Depending on the size and surface-chemistry properties of NCs required for the present study, we optimized three different methods of synthesis:

a) ***Preparation of ~ 5 nm size NCs****:* An ethoxyethanol route was selected for making fluorescent, 5 nm sized crystalline ZnO quantum dots. In a typical preparation, 50 ml of 0.1 M of zinc acetate dihydrate is made to react with 50 ml of 0.1 M of NaOH in ethoxyethanol medium. The reaction mixture was stirred for 30 mins at ambient temperature. The clear solution thus obtained was found to show bright fluorescence under UV excitation, thereby indicating formation of ZnO NCs. In order to obtain silica capping on the surface of these NCs, a modified Stöber method was followed. Typically, ~ 200 μl of tetra-ethyl-ortho-silicate (TEOS) solution was added to the as prepared ZnO nanocrystal solution with stirring, followed by a step-wise addition of 500 μl water and 200 μl ammonia. ***b) Preparation of 200 nm size ZnO:*** Forced hydrolysis of zinc acetate in diethylene glycol (DEG) at 160°C was done to obtain large sized ZnO nanocrystals. In a typical synthesis, 0.03 M zinc acetate was added to 300 ml DEG. This solution was heated under reflux to 160°C. The particle size and shape were controlled by optimizing the precursor concentration and hydrolysis ratio.

c) ***Starch coated ZnO NCs:*** For starch coated samples, we followed an aqueous phase synthesis as reported by Vigneshwaran *et al.* In a typical preparation, 0.1 M zinc nitrate hexahydrate was dissolved in 500 ml of 0.5% soluble starch solution by stirring. After complete dissolution, 500 ml of 0.2 M sodium hydroxide solution was added drop-wise under constant stirring. The reaction was allowed to proceed for 2 h after the complete addition of sodium hydroxide. The solution was centrifuged and washed with distilled water. After thorough washing, ZnO NCs were dried at 80°C for 3 h.

2.2 Synthesis of graphene nanosystems

To prepare pure graphene (HG), direct current arc discharge of graphite evaporation was carried out in a water-cooled stainless steel chamber filled with a mixture of

hydrogen and helium in different proportions without using any catalyst. The proportion of H_2 and He used in our experiments is H_2(200 torr)/He (500 torr). In a typical experiment, a graphite rod (Alfa Aesar with 99.999% purity, 6 mm in diameter and 50 mm long) was used as the anode and another graphite rod (13 mm in diameter and 60 mm in length) was used as the cathode. The discharge current was 125 A, with an open circuit voltage of 60 V. The arc was maintained by continuously translating the cathode to keep a constant distance of 2 mm from the anode. The deposit formed on the inner walls were collected carefully and examined for further characterization.

***Carboxyl Functionalization**:* As prepared graphene (25 mg) was refluxed with dilute nitric acid (2 M) for 12 h. The product was washed with distilled water repeatedly to remove any traces of acid. Graphene thus obtained, functionalized with hydrophilic groups, could be dispersed in water or physiological media.

Chapter 3: ZnO NCs toxicity towards bacteria, normal and cancer cells

In this chapter, we have investigated the *in vitro* cellular response of ZnO NCs towards bacteria, human normal primary and cancer cells.

3.1 Toxicity towards bacteria

The specific role of size scale, surface capping, and aspect ratio of zinc oxide (ZnO) nanoparticles on toxicity toward prokaryotic cells was investigated. ZnO nano and microparticles of controlled size and morphology synthesized by wet chemical methods were used for the toxicity studies. The toxicity towards bacteria was studied using *Escherichia coli* (W3110) and *Staphylococcus aureus* (ATCC 25923). Scanning electron microscopy (SEM) was conducted to characterize any visual features of the biocidal action of ZnO. The results indicate that the toxicity towards *E. coli* increased as the particle size decreased from micro to the nano regime. Clearly, toxicity was observed only above 5 mM concentrations of ZnO, with no apparent toxicity for capping agent PEG. With regard to the mechanism of antibacterial action of PEG-capped ZnO, substantial loss of membrane integrity was observed as changes in cell morphology of bacterial (*E. coli*) surface. The proposed mechanism of action of PEGylated ZnO on *E. coli* is similar to the antibacterial action on Gram positive bacteria, *S. aureus*. However, the effect towards Gram-positive bacteria was less than

that on Gram-negative bacteria at the same concentration. Also, the influence of particle size on antibacterial activity toward *S. aureus* was less than that for *E. coli.* The antibacterial activity of starch-coated ZnO was less than PEG-capped 40 nm size ZnO for equivalent concentrations. Representative results of the bacterial count for various sizes (40 nm–1.2 μm) of PEG-capped ZnO particles for different concentrations (1–7 mM) are shown in Fig. 3. We observed that the antibacterial activity increased with reduction in particle size. Loss of membrane integrity was observed as changes in bacterial morphology (*E. coli*) in the SEM analysis of the bacteria before and after ZnO exposure, as shown in Fig.4.

3.2 Toxicity towards normal versus cancer cells

To investigate differential toxicity of ZnO NCs towards normal human primary cells versus cancer cells, first we have studied the dose dependent effect of ZnO NCs on the metabolic activity of three human primary cells (HDF, lymphocytes and HUVECs) and two cancer cell lines (MCF-7 and KB). The effect of varying particle size on the viability was examined by treating both primary and cancer cells with ~ 5 and 200 nm ZnO NCs. Interestingly, both nanoparticles showed almost same toxicity with no significant difference in the concentration at which the cell function was impaired. We have also studied the effects of varied surface chemistry on cell viability using 5 nm sized ZnO NCs capped with silica, starch or PEG. Same dosage of ZnO (0 - 500 μM) was used for the studies. Surprisingly, all the surface modified samples exhibited almost the same toxic behavior as that of bare ZnO NCs. The effect of ZnO NCs interaction with the plasma membrane permeability was studied using LDH assay. ZnO NCs did not induce any significant LDH leakage up to 12 h and 500 μM. However, after 24 h, cancer cells registered ~ 60 - 70% LDH leakage, whereas primary cells remained intact with no significant leakage of LDH. Another signature of ZnO NCs was the induction of early-stage stress in cancer cells which was evident from the morphological deformation caused by NCs treatment. While primary cells maintained a normal morphology before and after treatment with ZnO, cancer cells showed rounded, stressed morphology (Fig 5).

Further investigations on the oxidative stress regime by studying mitochondrial

superoxide formation and mitochondrial membrane potential gave valuable insight into the observed differential toxicity effect. Under high ROS stress, superoxide produced in mitochondria play an important role in triggering apoptosis. Our study shows that, ~ 63.2% of ZnO NCs treated cancer cells expressed elevated levels of mitochondrial superoxide while HUVECs remain unaffected. Interestingly, our results showed that the mitochondrial potential of cancer cells gets completely depolarized by ~ 200 μM ZnO NCs, while that of primary cells remain unaltered. This result correlated very well with the observation of elevated mitochondrial superoxide formation in ZnO treated cancer cells compared to primary cells. Further, the cell cycle analysis also confirmed preferential cell cycle arrest at S phase in cancer cells while primary cells continued to exhibit normal cell cycle progression even after treating with 300 μM ZnO for 24 h. This confirms the ability of ZnO NCs to stall DNA synthesis in cancer without causing any effect on primary cells at the same or higher concentration. Collectively, all the above results, suggest that ZnO NCs trigger preferential apoptosis in cancer cells by imparting systematic stress regimes including intracellular ROS, mitochondrial superoxide and depolarization of otherwise hyperpolarized mitochondrial membrane potential.

Chapter 4: *In vitro* cellular response of graphene nanosystems

4.1 Monkey Kidney Epithelial cells (Vero)

In this chapter we studied the effect of carboxyl functionalization of graphene in pacifying its strong hydrophobic interaction with cells and associated toxic effects. In order to comprehend the influence of hydro-phobic/philic character of pristine graphene (*p*-G) and functionalized graphene (*f*-G) on their biological interactions, kidney epithelial cells (Vero) were treated with 25 µg/ml of the samples for 24 h at 37° C. In Fig. 5, the upper panel (a1 and a2) represents confocal images of *p*-G treated cells clearly depicting outsized aggregation of graphene on the cell membrane, literally masking them completely. Probably, the strong hydrophobic interactions of p-G with the cell membrane lipids might have resulted in this accumulation, which eventually led to the deformation of the cell membrane. The cytoskeletal re-arrangement associated with graphene treatment studied by F-actin specific staining

showed significant destabilization of F-actin alignment in p-G treated cells (Fig. 2a3). In sharp contrast, f-G treated samples in the lower panel (Fig. 2b1 and b2) showed no signs of any accumulation of graphene on plasma membrane, instead f-G flakes were found to be internalized by the cells. Interestingly, the internalized f-G flakes were found to traffic through intracellular barriers and concentrate near the perinuclear region (Fig. 2b2). Most of the examined cells exhibited similar uptake and localization patterns. More interestingly, such an uptake of graphene did not affect the cytoskeletal arrangement of cells as seen. Collectively, all the above results indicated that pristine graphene was found to accumulate on the cell membrane causing high oxidative stress leading to apoptosis, whereas carboxyl functionalized hydrophilic graphene was internalized by the cells without causing any toxicity.

Chapter 5: Molecular Response of Graphene nanosystems towards human primary endothelial cells and in vitro interaction with human blood components

5.1 Gene expression

Molecular mechanisms of cytotoxicity of graphene nanosystems are not yet fully elucidated. To address this question, we have performed whole genome expression array analysis. When gene expression of untreated control cells is compared to gene expression of *p*-G treated cells, a total of 553 genes showed 1.5 fold changes in expression. Significant pathways that were found to be affected by treating HUVEC with *p*-G were: 1) Androgen receptor pathway 2) IL-4 3) IL-6 4) TGFBR and, 5) TNFα/NF-kB. Androgen receptor pathway has significant roles especially in prostate cancer which is activated by IL-6. IL-4 is concerned with the activation and differentiation of both B and T lymphocytes. IL-6 pathway is responsible for activation of acute phase reactions and inflammatory response. TGF-β pathway is involved in many cellular processes like growth, metabolism, differentiation and apoptosis of cells. TNF-α is also responsible in producing acute phase immune response and can also induce apoptosis of cells. NF-kB is another important pathway involved in inflammatory process induced by stress and cytokines. When gene expression of control cells is compared to that of f-G treated cells, a total of 755 genes showed 1.5 fold changes in expression Significant pathways that were

affected by treating HUVEC with f-G were ID pathway and TGFBR pathway. ID pathway can be stimulated by various ligands such as the Vascular Endothelial Growth Factor (VEGF), TGF beta and the T cell receptor. Biological functions affected in case of p-G are cell cycle control, DNA repair, lipid metabolism, cellular adhesion, inflammatory response, Phosphate transport, nucleosome assembly, DNA replication and MAPK signaling. Biological functions affected by *f*-G are DNA repair, DNA replication, cell cycle inflammatory response, lipid metabolism and transport, angiogenesis, cell growth and metabolism, ubiquitin cycle, cellular adhesion, microtubule based movement and nucleosome assembly.

5.3 Interaction of graphene nanosystems with human blood

A fundamental understanding of the interaction of intravenously administered nano systems with blood components is very important for its successful application for various biomedical applications. In this chapter we compared the *in vitro* exposure of human whole blood towards pristine and carboxyl functionalized graphene nanosystems. Studies included hemolysis, platelet activation and aggregation, pro-inflammatory responses and coagulation time analysis. Blood samples were taken from healthy donors with the approval from the Institutional Ethics Committee.

Hemolysis was performed to evaluate the compatibility of both pristine and functionalised graphene systems towards red blood cells by analyzing the RBC membrane integrity after treatment. Effect of both the graphene systems on platelet function was investigated by platelet activation and aggregation analysis, which showed that the graphene systems do not interfere with the platelet function. Coagulation studies proved that both graphene nanosystems did not interfere with either the intrinsic or extrinsic pathway of coagulation. Immune response analysis done by studying the proliferation of peripheral blood derived mononuclear cells showed that both pristine and functionalized graphene does not interfere with PBMC proliferation under *in vitro* conditions.

5.4 Inflammatory response

Inflammatory response studied by cytokine induction analysis proved that the graphene nanosystems do not induce production of any major pro-inflammatory

cytokines such as IL-1β, IL-6, IL-8, IL-10, TNF and IFNγ. Although these conclusions need to be confirmed by *in vivo* studies, the present work gives an insight into the compatibility of both pristine and functionalized graphene towards different blood components.

Chapter 6: In vivo toxicological evaluation graphene in mice

The *in vitro* findings were further reinforced through *in vivo* studies using Swiss Albino mice model.

6.1 Biodistribution analysis

After intravenous injection of graphene through lateral tail vein, animals were housed for stipulated time periods before they were sacrificed. The injected material was found to reach heart and subsequently get distributed to various organs and as evidenced by histopathological analysis caused inflammation in various organs. Uptake of graphene by reticulo-endothelial system and inflammation are seen in various organs. *In vivo* studies in Swiss albino mice have shown the graphene nanosystems to induce toxicity to major organs like liver, spleen, kidney and lung, which ranged from mild congestion to necrosis and fibrosis of organs. RT-PCR studies on mouse peripheral blood mononuclear cells showed increased expression of inflammation related genes. Functionalized graphene was found to be more toxic than pristine graphene as evidenced from gene expression and histopathological results. In effect, our result shows that graphene irrespective of its surface chemistry, induced serious toxic effects at a concentration of 20mg/kg bodyweight to mice which may question its use for biological applications.

Summary and conclusion

In this PhD thesis the fundamental understanding of the interaction of ZnO and graphene nanostructures towards biological systems and its mechanism has been investigated in detail. The specific role of size scale, surface capping, and aspect ratio of zinc oxide (ZnO) particles on its toxicity towards bacteria, normal and cancer cells was investigated.

Monocrystalline ZnO NCs of size ranging from 5 to 1.2 μm, with three distinct surface chemistries (silica, starch or polyethylene glycol coated) were

synthesized and used for this study. The results showed that toxicity towards bacteria increased with reduction in particle size. Flow cytometry and confocal microscopy studies revealed that ZnO NCs undergo rapid preferential dissolution in acidic (pH ~ 5-6) cancer microenvironment causing elevated ROS stress, mitochondrial superoxide formation, depolarization of mitochondrial membrane, and cell cycle arrest at S/G2 phase leading to apoptosis.

In the case of nanostructured graphene's interaction with kidney epithelial cells, pristine graphene (*p*-G) was found to accumulate on the cell membrane causing high oxidative stress leading to apoptosis, whereas carboxyl functionalized hydrophilic graphene (*f*-G) was internalized by the cells without causing any toxicity towards kidney epithelial cells. Further, we compared the toxicity of both graphene systems towards human umbilical vein endothelial cells (HUVEC) employing toxicity and cell function assays. It is demonstrated that irrespective of surface functionalization, both graphene systems exert a time and dose dependent toxicity. Detailed mechanistic study revealed that graphene treated cells undergo cell death due to cytoskeleton re-arrangements, high intracellular ROS levels, mitochondrial superoxide generation, reduced glutathione, lipid peroxidation and oxidative DNA damage leading to apoptosis.

Our *in vivo* results showed that both graphene systems exhibited a similar bio distribution and accumulation. It was found that graphene accumulated in lung, liver, spleen, kidney, and showed longer retention times. Histopathology studies revealed that both graphene systems induce significant pathological changes, including inflammation, pulmonary edema and granuloma formation at the dosage of 20 mg/kg body weight.

Thus our studies on two different nanomaterial systems clearly illustrate that, irrespective of the surface chemistry, size or shape, nanomaterials have a capacity to interact with biological systems at the intracellular level. This interaction between cells and nanomaterials, both *in vitro* as well as *in vivo* is a critical issue that would determine any future biological applications of such nanostructures. Hence, there is an increasing demand for a detailed toxicological evaluation of nanomaterials before they are put to pertinent uses in biology or medicine.

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