CHAPTER 6

Summary & Future perspectives

In the present PhD thesis, the fundamental interactions of two distinctly different nanomaterials systems, viz., ZnO and graphene, with biological systems were investigated, with special emphasis on the role of size-scale, surface-chemistry, and nature of bio-chemical environment. During the last decade, general notion about nanotoxicology was centered on the effects of size-scale and surface characteristics, while the overwhelming effect of material composition was almost ignored. The initial over-emphasis on the promising biomedical applications of heavy metal containing quantum dots is the best example of this approach, which turned counter-productive with the understanding of their serious toxicity effects. In view of the emerging demands for assessing nanomaterial toxicities, in this thesis, we have specifically investigated the importance of bulk compositional character of nanomaterials by selecting two distinctly different materials, viz., one from metal oxide family (ZnO) and another from carbon family (graphene). In a nutshell, our investigations indicated that, although the nano-size scale, shape and surface-chemistry play important roles in determining the toxicity effects of nanomaterials, the bulk compositional chemistry (chemical stability in biochemical environment, biodegradability, free-radicals release, etc) contributed dominantly to determining their toxicity effects. The results and observations derived from various experiments that lead us to the above conclusion is summarized as below:

- ZnO nanocrystals of well-defined sizes ranging from 5nm to 1.2 μm and three distinct surface chemistries formed by silica, starch or polyethylene glycol coating were synthesized by wet-chemical methods and the specific role of size scal,

surface capping and aspect ratio on their toxicity effects towards bacteria, normal and cancer cells were investigated.

- ZnO nanoparticles showed enhanced antibacterial effect as their size was reduced from micro to nanoscale. The effect was significant at around 5-7 mM concentrations, and more interestingly, gram negative *E.coli* showed more sensitivity towards nano-ZnO compared to the gram positive *S. aureus.* Electron microscopic studies indicated that the toxicity was caused by membrane damage, probably due to the direct interaction of Zn ions or reactive oxygen stress.
- In case of eukaryotic cells, ZnO NCs, irrespective of their size scale difference or surface modifications, exhibited differential toxicity towards primary versus cancer cells. Flow cytometry and confocal microscopy studies revealed that ZnO NCs undergo rapid 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, whereas normal cells having neutral pH remained unaffected at the same concentration. These results clearly indicate how the bulk chemical stability (dissolution) of ZnO under varied physiological conditions of normal and cancer cells influence its toxicity pro.

- Unlike ZnO which undergoes dissolution or degradation, igraphene – an allotrope of carbon, has a distinctly different toxicity profile, with surface chemistry playing a dominant role in its nano-bio interactions. It was found that hydrophobic pristine graphene (*p*-G) accumulated preferentially on the plasma membrane of epithelial cells causing high oxidative stress leading to apoptosis, whereas, carboxyl functionalized hydrophilic graphene (*f*-G) was internalized by the cells without causing much toxicity up to 48 h. However, this effect was found to bein the case of human primary endothelial cells, where irrespective of surface functionalization, both *p*-G and *f*-G induced cellular toxicity through an oxidative stress paradigm. Toxicogenomic studies in HUVEC also revealed that both graphene systems cause altered expressions of various critical genes govern the key cellular functions, whose down regulation leads to DNA damage.

Genotoxicity analysis by comet assay further indicated the possibility of DNA damage by both *p-G* and *f-G* in HUVEC cells.

- In contrast to HUVEC cells, both graphene systems showed better compatibility towards human primary blood components, wherein the membrane integrity of RBC, plasma coagulation, platelet and immune cell functions remained unaltered upon treatment with both *p*-G and *f*-G. In short, these *in vitro* studies clearly demonstrated that, in addition to the hydrophilic or hydrophobic characteristics of graphene, differences in various cell types, including membrane characteristics, endocytotic mechanisms, physiological functions etc, greatly influenced their cellular interaction.

- The *in vitro* findings were further reinforced through *in vivo* studies using Swiss Albino mice model. *In vivo* bio-imaging analysis using radio-labeled graphene (^{99m}Tc-*f*-G) displayed extended retention of both types of graphene in lungs. Furthermore, histological examination revealed that both graphene systems accumulated in all major organs such as lung, liver, spleen, and kidney and induced severe pathological changes, including inflammation, pulmonary edema and granuloma formation at the dosage of 20 mg kg^{-1} body weight. Specifically, *p*-G caused more damages in the bronchial epithelium of lungs, while *f*-G entered in to the alveolae and induced damages in the alveolar epithelium. In liver, *p*-G caused more irritations in the sinusoidal line while *f*-G entered in to the peri-sinusoidal spaces causing damages to the hepatocytes. Blood biochemical analysis revealed elevated levels of biochemical markers, indicating altered liver functions in both the samples. In spleen, *p*-G remained in the red pulp, while *f*-G caused damage to the marginal zone, indicated by the loss of demarcation between white pulp and red pulp. In effect, *p*-G caused more structural damage to these organs, while *f*-G got into the cell and caused cellular damage including cytotoxicity. RT-PCR analysis of serum samples also showed that both graphene systems induced acute immune and inflammatory responses. Confocal Raman spectral mapping of different organ tissues revealed that the vibrational signature of graphene is not altered even after three months, indicating the retention of graphene in these organs, with no sign of biodegradation.

In summary, all the above experimental results indicate that, ZnO NCs and graphene exhibit specific patterns of nano-bio interactions which are determined by multitude of factors including chemical composition, size, shape, surface properties and the micro-environment where the interaction happens. While ZnO NCs with similar size, shape and surface chemistry behave differently to normal cells and cancer cells, likewise graphene with same size and surface chemistry showed differential behaviour to epithelial cells, macrophage cells, endothelial cells and blood cells, indicating that the nature of biological micro-environment is a critical player in determining the nanotoxicity, which was least considered in earlier studies. This demands extensive investigations on each and every nanomaterial with every possible cell systems and using *in vivo* models, for a meaningful understanding of their actual toxicity effects under various scenarios. Thus, no generalization of nano-size or surface chemistry effects can be applied in the toxicity evaluation of nanomaterials.

FUTURE PERSPECTIVES

Our studies indicated a potential application of ZnO as an anti-cancer therapeutic agent, or at least as an adjuvant which may target the tumor micro environment together with other chemotherapeutics. However, this needs to be investigated in detail using tumor xenograft models of various solid tumors where tumor micro-environment is acidic. Regarding nanotoxicity, further studies focusing on the developmental and reproductive toxicity and carcinogenicity of ZnO will be required before implementing its proposed applications.

In case of graphene, or broadly for all other carbon nanosystems, the biggest challenge would be to get it cleared from the body without tissue accumulation; because graphene accumulated in the tissues were found to cause toxicity in all major organs. It appears that biodegradability of carbon nanostrucres is a remote possibility and hence future investigations may focus more on facilitating clearance pathways, probably by reducing the lateral dimensions and optimizing size and dose requirements for various applications and further surface modifications such as PEGylation.