CHAPTER 6

summary & future scope of work
Summary

The present work involves studies on assessment of biocompatibility of gold and silver nanoparticles in cell culture for tissue engineering applications. As the field of Nanotechnology continues to develop, the studies on the cellular uptake of nanoparticles, with respect to their size and shape, are required in order to advance nanotechnology for biomedical applications. Similarly it would be of interest to assess nanoparticle toxicity so as to use them for intracellular applications. Detailed studies on uptake kinetics of nanoparticles by cells have not been well characterized and quantified as a function of their size and shape. As nanoparticles become more common and widely produced, the chances of unplanned events leading to their dissemination and accumulation in the environment increase, and could lead to unforeseen changes to biological systems. So far, most cytotoxicity studies on nanomaterials have been focused on aerosols and involve particle uptake by the lungs. The toxicity of nanoparticles inside the biological system has always been an issue of concern. We specifically selected gold and silver nanoparticles because of their well-known applications in traditional Ayurvedic & Unani medicine, amenability of synthesizing these nanoparticles of various sizes and shapes, easy characterization by UV-Vis spectrophotometry and Transmission Electron Microscopy (TEM). Furthermore, the reports on gold nanoparticles for cell imaging, targeted drug delivery, cancer diagnostics and therapeutic applications indicate their potential in biology and medicine.

For the treatment of cells with nanoparticles, it is essential that the nanoparticles are prepared in aqueous solution and are stable in the treatment medium. Almost all metal nanoparticles synthesized by general wet chemical methods are prone to agglomeration in salt solutions. We observed that serum in culture medium provides stability to nanoparticles against salt induced agglomeration. The phenomenon of protection against agglomeration is instantaneous and advantageous from an in vivo application point of view.

During the study we first checked the dose and time dependent nanoparticle cytotoxicity on different types of cells including fibroblasts, epithelial, endothelial, macrophages, and cancerous cells. To examine the effect of gold nanoparticles on cell proliferation, metabolism and cell viability: trypan blue dye exclusion test and 3-
(4,5-dimethylazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay were performed. Our results indicate that Au(0) nanoparticles do not show detectable cytotoxicity up to 200 μM concentration of gold for 72 h exposure. Reactive oxygen species (ROS) and nitrite species (RNS) play an important role in toxicity. Hence, in response to gold nanoparticles, release of ROS and RNS species in a dose and time dependent manner was studied in RAW264.7 macrophage cells. We found no change in the levels as compare to untreated cells. There was no induction in the stress induced cytokines viz. TNF and IL-1 beta levels in response to increasing concentration of gold nanoparticles at RNA as well as protein level. We also didn't observe any change in total protein profile of the cells treated with nanoparticles. Taken together, these data clearly indicate the biocompatible nature of gold nanoparticles at the concentrations tested.

Subsequently, cellular uptake kinetics of gold nanoparticles was studied using various microscopic tools. The macrophage cells were exposed to gold nanoparticles and early events of Endocytosis of bare gold were studied employing atomic force microscope. Contact mode height and friction images of the cells treated with gold nanoparticles for 5 min exhibited a well defined nucleus as a dome-like structure with a few nanopits (depth 25-70 nm). The nucleus was surrounded by a large number of micropits (depth 150-300 nm) on the rest of the cell surface and the gold nanoparticle aggregates present in the micropits were found to be ~70-85 nm in size. The shape and size of the micropits suggested gold nanoparticle uptake via pinocytosis. Later, we synthesized fluorescent gold nanoparticles using lysine & poly-l-lysine as spacers and FITC as a fluorophore. The fluorescent gold nanoparticles were physically as well biologically characterized for cell culture applications. Fate of fluorescent gold nanoparticles inside the cellular compartment was assessed using confocal microscope. The gold nanoparticles were found accumulated in lysosomes in perinuclear fashion. They do not enter inside the nucleus. These findings were further confirmed by intracellular transmission electron microscopy. We went one step ahead to see the mode of internalization of gold nanoparticles. By using specific phagocytotic and macropinocytosis inhibitor cytochalasin ‘B’, it could be successfully demonstrated that gold nanoparticles do not enter the cells solely via phagocytosis and macropinocytosis.
We attempted to fabricate nanoparticle coated alginate hydrogels for further tissue engineering applications and found that coated hydrogels possess excellent properties for cellular adhesion thus can serve as micro carrier system for large scale culture of adherent cells and cellular products.

We next synthesized silver as well as gold structures applying green chemistry approach using curcumin as reducing as well as capping agent. This investigation demonstrate for the first time that curcumin, an active principle of Curcuma longa has a potential of synthesizing silver and gold nanoparticles from AgNO₃ and HAuCl₄ precursors respectively by acting as reducing as well as capping agent. Moreover, this process of formation of silver and gold nanoparticles occurs at room temperature and that too with high pace unlike the chemical methods which require high temperature. This is probably the first instance wherein the reducing agent curcumin undergoes complete degradation under alkaline conditions during the course of nanoparticle synthesis.

The UV-Vis-NIR data reveals that synthesized gold nanoparticles exhibit polydispersity and the concentration of curcumin appears to be a decisive factor in determining shape of gold nanoparticles as lower concentration leads to formation of triangular/hexagonal particles. The spectrum of gold nanoparticle synthesized by lowest concentration of curcumin shows almost monotonous absorption in NIR region with no discernible transverse SPR band. The optical signature of curcumin reduced gold nanoparticles can be better explained in terms of the distribution of sizes and shapes observed in the TEM. The TEM data reveals that the distribution of shapes in case of gold nanoparticles is broad and a significant number of nanoparticles are triangular and hexagonal along with spherical morphologies. Selected area electron diffraction (SAED) pattern of these nanoparticles reveals that the particles are monocrystalline in nature and the points could be indexed based on the face-centered cubic (fcc) structure of gold. The presence of nanoparticles with hexagonal and triangular cross-sections is responsible for the absorption at longer wavelengths.

For the synthesis of silver nanoparticles, silver nitrate solution was reduced by aqueous solution of curcumin under alkaline conditions. Unreacted curcumin was removed by repeated washings of the nanoparticles. The synthesized silver nanoparticles were characterized by UV-Vis-spectrophotometry. A strong absorption
in UV absorption spectra at ~416 nm corresponds to excitation of surface plasmon vibrations in the silver nanoparticles. The peaks at ~346 and ~262 corresponds to free curcumin. While the peak at ~346 was absent in as prepared silver nanoparticles as well as nanoparticle pellet the ~262 peak was present throughout the synthesis protocol. From the TEM images, the particle size distribution was measured and the mean size of the particles was measured to be 11 nm with a standard deviation of 1.64 nm. Selected area electron diffraction (SAED) pattern of these nanoparticles reveals that the particles are polycrystalline in nature and the rings could be indexed based on the face-centered cubic (fcc) structure of silver. There is negligible aggregation of particles in culture medium without FCS supplementation suggesting curcumin degradation products provide adequate stability to silver nanoparticles against salt insults. The curcumin-reduced silver nanoparticles are stable in solution over a period of several months. Yet another important aspect of curcumin reduced silver nanoparticles is their nontoxic nature making them biocompatible for cell culture applications. They do not exhibit antimicrobial activities even at higher concentrations. Inhibition of NF-κB translocation from cytoplasm to nucleus in HUVEC cells by lower concentration (25 µM) of silver nanoparticles indicates that the cells remain unstressed and higher concentration (50 µM) certainly lead to NF-κB translocation from cytoplasm to nucleus, however this phase is transient and neither affects cell morphology nor cell viability, thus maintaining the healthy state of HUVEC. Moreover, treatment with sub lethal concentrations of AgNP makes HUVEC cells in active state of migration which is highly desirable for wound healing and regenerative process to maintain cellular homeostasis during regular cell turnover and situations of wear and tear. This is further confirmed by wound healing experiment.

In nutshell, present thesis focuses on synthesis and physical characterization of gold and silver nanoparticles using conventional and green chemistry approach and assessment of their biocompatibility for cell proliferation and tissue engineering as well as biotechnological applications, in addition to tracing the intracellular fate of bare gold nanoparticles.
**Salient Features of the study**

1. We have presented detailed studies on the interaction of gold and silver nanoparticles with cells in vitro and have demonstrated the biocompatible properties of gold and silver nanoparticles such as nontoxicity, nonimmunogenicity, and high tissue permeability without hampering cell functionality. In addition, we have shown the antioxidant effect of gold nanoparticles and the path of their eventual internalization in perinuclearly arranged lysosomes. These findings have implications in the design of effective targeted drug/gene delivery systems.

2. The biocompatible fluorescent system employed as beacons for the nanoparticles in the present study is promising for combining cancer imaging and tumor-targeted drug delivery in cancer therapy also can be utilized as endosome/lysosome markers that function without activating or disrupting cellular functions.

3. In this investigation AFM was used to study the mammalian cell interaction with metal nanoparticles. We found that it serves as an excellent tool for study of initial events of endocytosis of metal nanoparticles in mammalian cells.

4. Nanoparticle internalization in the presence of phagocytosis inhibitor Cytochalasin B confirmed that gold nanoparticles do not enter the cells solely via phagocytosis and macropinocytosis. However, the size of nanopits and gold nanoaggregates supports the pinocytic mode of nanoparticle internalization. Therefore, endocytosis of various nanoparticles can not be generalized.

5. Synthesis of silver as well as gold nanoparticles applying green chemistry approach using curcumin as reducing as well as capping agent appears to be promising in terms of therapeutic potential of silver as well as curcumin.

**Scope for Future Work**

There is a strong likelihood that biological activity of nanoparticles will depend on physicochemical parameters not routinely considered in toxicity screening studies. Physicochemical properties that may be important in understanding the toxic effects of test materials include particle size and size distribution,
agglomeration state, shape, crystal structure, chemical composition, surface area, surface chemistry, surface charge, and porosity. *In vitro* techniques allow specific biological and mechanistic pathways to be isolated and tested under controlled conditions, in ways that are not feasible with *in vivo* tests.

In this context the present thesis shed light on the following prospective features to be taken into account to test the biocompatibility of nanomaterials synthesized for medical applications.

1. A large number of new nanomaterials are being synthesized everyday. However, there are very few studies involving biocompatibility assessment of such materials for medical applications employing *in vitro* techniques. This thesis forms a basis to provide elementary screening strategies for injectable drug delivery applications with reference to gold and silver nanoparticles.

2. The PLL modified gold coated alginate beads prepared in the present studies could form a novel micro-carrier for mass cultivation of anchorage dependent cells to get large scale production of biologicals.

3. The synthesis of fluorescent gold nanoparticles will be valuable as cell trackers like quantum dots overcoming the toxicity of later as well as *in vivo* imaging for drug delivery applications.

4. Nanoparticle internalization in the presence of Cytochalasin B confirmed that gold nanoparticles do not enter the cells solely via phagocytosis and macropinocytosis. However, the size of nanopits and gold nanoaggregates supports the pinocytic mode of nanoparticle internalization. It would be interesting to use specific inhibitors for clathrin and caveolin mediated pathways to delineate the exact mode of internalization.

5. The thesis illustrates the synthesis of metal nanostructures using curcumin as a reducing as well as capping agent and the potential application of curcumin reduced silver nanoparticles in cell migration. The results of present work show promise towards the potential of these nanoparticles in vasculogenesis using HUVEC cultures. However this could not be pursued during the tenure of the thesis. It would be indeed interesting to look into the downstream cellular signaling in response to silver nanoparticles. Further, it would be interesting to study in detail the concentration dependent effect of shape and size regulation of curcumin reduced gold nanoparticles.