Summary and Conclusion

The major objective of the present thesis was to synthesize different inorganic (GNPs, AgNPs and NdNPs) nanoparticles (NPs) using biological systems and investigating their biomedical applications especially against cancer and infectious diseases. Therefore, following aspects have been covered in this thesis: 1) Isolation of bacteria/fungi from the campus of integral university responsible for the synthesis of inorganic nanoparticles. 2) Molecular identification and characterization of isolated bacteria/fungi by using 16s rRNA/rDNA or 18s rRNA/rDNA analysis. 3) Enzymatic synthesis of gold and silver nanoparticles and their bio conjugation with different drugs. 4) Characterization of biosynthetic NPs by UV-Vis spectroscopy, DLS, SEM and TEM. 5) Cytotoxic effect of above mentioned bioconjugated nanoparticles in normal and cancer cell lines (in vitro) to check the bioavailability and change in the potency of the drug. 6) Cytotoxicity and kinetics of these NPs in SD rats.

First chapter is a brief review of the existing and proven research work in the field of nanotechnology which summarizes its significance in diverse fields of science followed by discussion on various methods of NPs synthesis with focus on characterization, drug conjugation and functionalization techniques. Further, it gives an account of different properties and biomedical applications of NPs. At the end of this chapter, the scope and significance of the present work have been discussed.

Second chapter deals with the synthesis and characterization of silver (AgNPs) and neodymium (NdNPs) NPs by using fungi and bacteria [isolated from the campus of Integral University, Lucknow]. Further, molecular identification of bacteria and fungi was done by using 16S and 18S rRNA/rDNA technique,
respectively. However, their partial 16S and 18S rRNA/rDNA gene sequences were deposited in NCBI GenBank and accession numbers KC967214, KC967215 and KC967216 for Myroides odoratimimus, Acinetobacter lwofii and Bacillus sp., respectively and KF913250 and KF913249 were obtained for Aspergillus fumigatus and Aspergillus sp., respectively. For the synthesis of AgNPs and NdNPs, bacterial strains (Myroides odoratimimus, Acinetobacter lwofii and Bacillus sp.) were inoculated in a freshly prepared sterile nutrient broth at 37°C temperature with 180 rpm rotation for 24 hrs whereas fungal strains (Aspergillus sp., Aspergillus fumigatus) were inoculated in MGYP (malt extract 0.3%, glucose 1.0%, yeast extract 0.3%, and peptone 0.5%) at 27°C temperature with 180 rpm rotation for 96-120 hrs. After the completion of incubation period, the cultures were centrifuged at 5000 rpm for 15 min and the biomass (bacterial and fungal cells) was used for the biosynthesis of nanoparticles. The biomass of each isolated bacterial strain was added in different flasks containing 10^{-3}M either AgNO_3 or Nd(NO_3)_2 at 37°C for 24 hrs on an orbital shaker. Similarly, fungal biomass of each isolated strain was added in different flasks having 10^{-3}M either AgNO_3 or Nd(NO_3)_2 at 27°C for 96-120hrs on an orbital shaker. The formation of the AgNPs and NdNPs was monitored by UV-VIS spectroscopy using Shimadzu (Model No-UV 1800) double beam UV-VIS spectrophotometer. The surface plasmon resonance of AgNPs and NdNPs exhibited an emission band at 416-418nm and 285-287nm, respectively. Moreover, TEM, SEM and DLS characterization of both types of NPs showed them to be spherical and monodispersed with sizes ranging from 9-25 nm. The stability of both the NPs were confirmed by zeta potential analysis, which showed a range of -7mV to -13 mV.
Third chapter deals with the synthesis and characterization of gold nanoparticles (GNPs) using different enzymes *viz.* trypsin, bromelain and nitrate reductase followed by bioconjugation with a drug (Secnidazole). Here, enzymatic synthesis of GNPs-HSA was analyzed by using UV-Vis spectroscopy. It is well known that surface plasmon resonance of GNPs-HSA exhibit ruby red colour and an emission band at 520-533 nm. Interestingly, GNPs synthesized in the present study showed peak close to 520 nm, even after a month or year of reaction indicating its stability at room temperature, which might be due to the presence of capping agent in the reaction mixture. The GNPs-HSA formed were in the size range of 7-20 nm, which was confirmed by the DLS, SEM and TEM.

In the present study, a novel method was developed for the synthesis of GNPs of different sizes using bromelain as a reducing as well as a capping agent by modulating the concentration of bromelain and incubation temperature. Here, when 0.33 mg/ml concentration of bromelain was used for the synthesis of GNPs, a characteristic ruby red color with fundamental surface plasma resonance at 522 nm was observed. The hydrodynamic diameter of brom-GNPs by DLS was estimated to be 58.65 nm. The topographical studies of brom-GNPs were performed under SEM and the particles were found to be monodisperse and spherical. Whereas, the particle size of brom-GNPs by TEM was found to be 10.75 nm. It is well known that DLS obtain the hydrodynamic radius of the particle while TEM estimates the projected area diameter. In fact, a thin electric dipole layer of the solvent adheres to the surface of a dispersed particle when it moves through a liquid medium. Consequently, the hydrodynamic diameter provides information of the inorganic core along with coating material and the solvent layer attached to the particle. However, when size is estimated by TEM, this hydration layer is absent and information only about the
inorganic core is observed. Therefore, the hydrodynamic diameter observed by DLS always found to be greater than the size estimated by TEM. In agreement with these facts, results also showed that the hydrodynamic diameter of brom-GNPs appears larger in DLS as compared to size estimated by TEM. Further, the stability of nanoemulsion was estimated by zeta potential which was found to be -16.6mV. However, size of GNPs increases with increase in concentration of bromelain. Therefore, diameter of brom-GNPs at concentration 0.66 mg/ml, 1.66 mg/ml and 3.33 mg/ml of bromelain found to the in a range of 11.27-24.68 nm, 22.26-32.26 and 30.42-42.14nm, respectively by TEM. Zeta potential of GNPs synthesized by 0.66mg/ml, 1.66mg/ml and 3.33 mg/ml of bromelain were found to be -9.83mV, -9.67mV and -6.47mV, respectively. Since best size was observed at 0.33mg/ml concentration of bromelain, hence, by keeping bromelain concentration (0.33mg/ml) constant different temperatures such as 50°C, 60°C and 70°C were exploited to get best temperature for the synthesis of GNPs. Then, the characterization of brom-GNPs synthesized at 50°C, 60°C and 70°C was done by UV-Vis spectroscopy, DLS, SEM, Zeta potential and TEM. These brom-GNPs were formed spherical in shape when analysed under SEM with sizes in a range of 22.6-31.1 nm, 36.2-39.5 nm and 50.8-62.1nm at 50°C, 60°C and 70°C temperature respectively. Zeta Potential of brom-GNPs synthesized at 50°C, 60°C and 70°C temperature were found to be -9.56mV, -6.83mV and -6.12mV, respectively. All these results clearly showed that GNPs of different sizes could be produced by modulating the concentration of bromelain and reaction temperature.

In addition, in this chapter, synthesis of Human Serum Albumin (HSA) encapsulated trypsin mediated synthesized GNPs at different concentrations of trypsin has been described. Synthesis of GNPs-HSA has been confirmed by their
representative SPR band as measured by UV–Vis absorption spectroscopy. The GNPs-HSA solution prepared at different concentrations of trypsin [increasing from 0.33 to 1.66 mg/ml] showed an increase in absorbance. The SPR absorption band for GNPs-HSA at 0.33 mg/ml, 0.66 mg/ml and 1.66 mg/ml concentration of trypsin were found to be 522 nm, 527 nm and 539 nm, respectively. The pronounced red shifting of the plasmon has been associated with increased nanoparticle size. Thus, there might be a dose dependent increase in size which was confirmed by TEM analysis.

Here, TEM of GNPs-HSA synthesized by using 0.33 mg/ml concentration of Trypsin revealed that particle size was 8 ± 2 nm; however, the hydrodynamic diameter by DLS showed that particles size was 40 ± 2nm. In addition, TEM and DLS analysis of GNPs-HSA synthesized by using 0.66 mg/ml concentration of trypsin showed that the particle size was 10±3 nm and hydrodynamic diameter was 75.65±4 nm. In contrast, when 1.66 mg/ml concentration of trypsin was used, GNPs-HSA nanoparticle size was found to be in a range of 17-35 nm and hydrodynamic diameter by DLS was estimated to be 110±5nm. Further, SEM micrograph image of all the samples confirmed spherical shape and monodispersity of the GNPs-HSA.

In the end, it could be safely stated that GNPs of different sizes may be produced in a quantitative way by controlling the experimental conditions like concentration of enzymes or reaction temperature.

Second part of the third chapter discusses successful conjugation of an antibacterial drug [secnidazole] with GNPs-HSA. Here, GNPs-HSA have been synthesized by using nitrate reductase as a reducing agent and HSA as a capping protein. Further, the binding of secnidazole with HSA protein capped on GNPs-HSA was studied. The SPR (surface plasma resonance) absorption band for secnidazole bioconjugated Human Serum Albumin encapsulated gold nanoparticles (GNPs-HSA-
Snd) appears at 525 nm, with a significant broadening and a slight decrease in intensity compared to the Plasmon band for GNPs-HSA at 527 nm. The Plasmon resonance is a surface phenomenon which alters after attachment of any ligand at the surface. The ligand binding significantly affects the absorption intensity and full width at half maximum (FWHM) of absorption band. The observed broadening in absorption band is attributed to the attachment of secnidazole drug at the surface of NPs. The absorption spectrum of GNPs-HSA-Snd reveals two peaks at 320 nm and 525 nm corresponding to secnidazole aromatic nitrite transitions and GNPs-HSA respectively, suggesting its binding to GNPs-HSA. It also reveals that the particles are smaller in size and uniformly distributed. The particle size of the GNPs-HSA was estimated to be 7±2 nm under TEM. TEM micrographs of GNPs-HSA-Snd reveal the attachment of secnidazole (and consequent enlargement of the particles) as a bimolecular layer at the surface, resulting in some blurring of the micrograph. The hydrodynamic diameter of GNPs-HSA appears large (18±3nm) in DLS due to the interaction and binding of solvent to the surface of GNPs-HSA. Eventually, GNPs-HSA-Snd appear bigger than non conjugated (GNPs-HSA) one due to subsequent enlargement of nanoparticles. The GNPs-HSA were found to be negatively charged with a zeta potential of -13.3 mV, which can be one of the reasons for their long term stability and the electrostatic repulsive forces between the GNPs-HSA might protect them from getting closer and thereby preventing agglomeration or clumping in aqueous suspension. The zeta potential of GNPs-HSA-Snd was found to be -7.97 mV which is well in the range required for stable emulsion. The decrease in zeta potential may be attributed to the decrease of the free amino groups on the GNPs-HSA-Snd after bioconjugation of the secnidazole drug to these groups. The quantitative estimation of conjugated secnidazole with GNPs-HSA was determined
by RP-HPLC chromatography by using the C-18 column. The amount of bioconjugated secnidazole was found to be 70% indicating efficient binding of secnidazole with GNPs-HSA-Snd.

Unravelling the nature of the interactions of GNP–HSA-Snd would pave the way for the design of nanotherapeutic agents with improved functionality, enriching the field of nanomedicine. Along with this, the antibacterial activity of AgNPs, NdNPs and GNPs-HSA-Snd (synthesized in chapter 2 and 3) against different pathogenic microorganisms was also tested. The bacterial strains *Myroides odoratimimus* (Accession No.KC967214), *Acinetobacter lwoffii* (Accession No. KC967215), *Bacillus sp.* (Accession No. KC967216) and fungal strains *Aspergillus fumigatus* (Accession No. KF913250) and *Aspergillus sp.* (Accession No. KF913249) mediated synthesized AgNPs, NdNPs showed substantial antibacterial potential. The best MIC was observed for *Aspergillus sp.* mediated synthesized AgNPs against *Escherichia coli* (NCIM 2065) and it was found to be 20µM/ml. Similarly, for NdNPs, synthesized by *Aspergillus fumigates* showed best antibacterial activity against *Micrococcus luteus* (NCTC 10240) and it was found to be 11.49µM/ml. It was observed that conjugated secnidazole showed greater potency against *Klebsiella pneumoniae* (NCIM 2957) and *Bacillus cereus* (NCIM 2156) than pure drug and it was found to be 12.2 and 14.11 times respectively. This potent antibacterial activity of the GNPs-HSA might be due to the penetration into bacterial cells, damaging of cell membrane, and releasing of cell contents. A further prospect suggested that AgNPs are highly effective and their potency dependents upon the shape and size of AgNPs or silver ions released in the solution. It might have contributed to the bactericidal properties of AgNPs. Since many pathogenic microbes are attaining resistance against most of the existing antibiotics but resistance against
NPs requires a chain of mutations and systematic evolution of specialized genes which is highly impossible. These NPs might provide an efficient alternative. Thus, the easy and environmentally benign mode for the synthesis of the NPs makes it economical and preeminent alternative to the present antibiotics.

In the fourth chapter, we discussed the results of in-vitro cytotoxicity of AgNPs, NdNPs, pure bromelain and brom-GNPs against the selected human cancer cell lines, such as MCF-7, MDA-MB 231, A549, HeLa and normal cells 3T3-L1. These NPs were found highly effective against prescribed cell lines. The dose-response activity was checked by MTT assay and IC$_{50}$ was calculated. Aspergillus sp. mediated synthesized AgNPs showed maximum activity against lung cancer cells (A549) and IC$_{50}$ for the same was found to be 133 µM/ml. Similarly, Aspergillus fumigates mediated synthesized NdNPs showed substantially greater activity against HeLa cancer cell line and IC$_{50}$ for the same was found to be 428 µM/ml. The changes in potency of bromelain after capping with GNPs and pure bromelain were studied by MTT assay against 3T3-L1, MCF-7 and MDA-MB 231. IC$_{50}$ for brom-GNPs were found to be 24.54 µg/ml, 12.12 µg/ml and 7.70 µg/ml against 3T3-L1, MCF-7 and MDA-MB 231 respectively, and IC$_{50}$ for pure bromelain were found to be 10.7 µg/ml, 14 µg/ml, and 8.71 µg/ml against 3T3-L1 and, MCF-7 and MDA-MB 231 respectively. The fungi synthesized AgNPs induce apoptosis in the selected human cancer cells as shown by the ultra-structural changes observed in the form of cell shrinkage, formation of membrane blebs and apoptotic nuclei which was analysed by the ROS (reactive oxygen species) and DAPI (4',6-diamidino-2-phenylindole) nucleic acid staining method.
Fifth chapter deals with the development of an animal model of primary lung cancer for investigating the disease mechanism and for the development of therapeutic strategies. Further, LD$_{50}$ of brom-GNPs on mice model was also calculated. A reproducible mice model for lung carcinoma was successfully developed by using oral dosage of N-nitroso di ethylamine (NDEA). Here, swiss albino mice were randomly assigned into three groups treated differently. Mice in group two were given 100ppm NDEA in drinking water; mice in group three were given 150ppm NDEA; while mice in group one was given drinking water only as a control. After 15 days of oral administration of NDEA (100ppm to 150ppm), the tumor was promoted with 500ppm dosage of Phenobarbitone (PB) along with set concentration of NDEA for another 15 days. Tumors were detected in 58.33% of mice provided with 100ppm NDEA along with 500ppm of PB. While, 75.00% of mice were observed to developed tumor provided with 150ppm NDEA along with 500ppm PB in drinking water. All mice were sacrificed 11 weeks after the first NDEA dosage and lung tissues were collected for the diagnosis of cancer. Characteristic features of these lung tumors, were: (I) Appearance of tumors within a short period of time, (ii) dose-related progression of the lung carcinogenesis was noticed, (iii) Targeted lung carcinogenesis was observed with no effect on other organs and (iv) No specific strain of mice was required (as in the present study lung cancer is induced in Swiss albino mice). Further, the mechanism of NDEA induced lung carcinogenesis by investigating the inhibitory effect of NDEA on Dual specificity phosphatase-1 (DUSP-1) using molecular docking approach was investigated. Free energy of binding for ‘NDEA- DUSP1catalytic domain-interaction’ was found to be -3.99 kcal/mol after that the investigation of inhibitory
effect of NDEA on DUSP-1, we have estimated LD$_{50}$ of bromelain encapsulated NPs, 6 doses were given intraperitonealy to 6 groups of mice, 5 mice in each group.

The present study has limitations in giving information about the association of different oncogene products with early stages of carcinogenesis. However, it can be safely stated that this study provides a technique to develop a lung cancer model using Swiss albino mice.

Second part of this last chapter was related to estimation of LD$_{50}$ dosage of brom-GNPs. For this, 6 doses were given intraperitonealy to 6 groups of mice containing 5 mice in each group. The animals were observed for first 2 hours and then at 6$^{th}$ and 24$^{th}$ hour for any toxic symptoms. After 24 hours, the number of dead rats was counted in each group and percentage of mortality calculated. From the results a graph was plotted between prohibit and log-dose which was further used to calculate the LD$_{50}$ dose of brom-GNPs i.e., 25.7 mg/kg body weight. We hope that these findings could be further exploited in future to develop potent anti-lung cancer nano-formulations.

**Scope and Importance**

One of the important areas of nanotechnology is the development of reliable processes for the synthesis of nanomaterials over a range of sizes, shapes and chemical composition. Hence, current research has been directed towards the development of different experimental protocols for the synthesis of nanomaterials of variable sizes and shapes. In order to avoid the use of hazardous chemicals or expensive machinery, environmentally benign approaches are required. This has encouraged several researchers to look for biological systems for nano-material synthesis. Recently, the utilization of prokaryotic cells (such as bacteria, algae) has
emerged as novel methods for the synthesis of nanomaterials. Several reports of NPs synthesis by microbes have shown that the exposure of different inorganic salts to prokaryotic cells resulted in the synthesis of NPs intra-cellularly. However, such biotransformation based NPs synthesis strategies would have greater commercial viability if the NPs could be synthesized extra cellularly. To accomplish this objective, the thesis was focused on the extracellular synthesis of AgNPs and NdNPs using the bacteria and fungus. In these cellular systems, however many proteins, carbohydrates and biomembranes contribute to the overall biological reaction. Better control over NPs size, shape, characteristics and the separation of biological materials are some of the inherent problems associated with biological systems. Therefore, it is desirable to develop more simple enzymatic routes for the synthesis of nanomaterials. Study presented here demonstrated the synthesis of metal NPs of desired sizes using enzymes like trypsin and bromelain. The expected benefits by following these methods are that, analogous to the biological synthesis of metal NPs using microorganisms, efficient synthesis can still be achieved in an environmentally benign process. It is likely to be more cost effective with a scope for large-scale production and the synthesis can be achieved in vitro.

**Future Prospectives**

The ability of microorganisms to reduce inorganic metals has opened up a new exciting green chemistry approach towards the development of natural ‘nanofactories’. However, a number of issues have to be addressed from the nanotechnology and microbiology point of view, before such a biosynthesis approach can compete with the existing physical and chemical methods. The elucidation of biochemical pathways leading to metal ion reduction and formation of NPs is
essential in order to develop a rational bio-based nanoparticle synthesis procedure. Similarly, an understanding of the surface chemistry of the biogenic NPs (i.e., the nature of reductases/capping peptides) is equally important. This would then lead to the possibility of genetically engineered fungi and bacteria to over express specific reducing molecules and capping agents, thereby controlling not only the size of the NPs but also their shape. The rational use of enzymes to modulate nanoparticle size and shape is an exciting possibility. The range of chemical compositions of NPs currently accessible by bio-based methods is currently extremely limited and confined to metals, metal sulfides and iron oxide. Extension of the protocols to enable reliable synthesis of nanomaterials of other oxides (TiO₂, ZrO₂, etc.) and nitrides, carbides etc. using enzymes could make the synthesis protocol, developed during this study, a commercially viable proposition. The enzyme mediated approach towards the synthesis of NPs has many advantages such as ease with which the process can be scaled up, economic viability, control of shape, size, separation of nonmaterials etc.

**Significance of Work**

Synthesis of inorganic NPs by using green machinery is the step to develop the environmentally friendly methods. Bio conjugation of therapeutically important drugs which have low bioavailability will increase the target efficiency heavily.

Our study will be in the direction to develop new improved and highly accurate drug delivery system with enhanced bioavailability of the drugs.