8.1. Summary.

8.1.1. Introduction.

Brain tumours or gliomas are deadly forms of brain tumours which originate in the central nervous system. Glioblastoma multiforme is classified as Grade IV type of glioma which has high proliferative and invasive nature which makes it difficult to treat. Lack of early and accurate diagnosis contributes to its lethality. The classical approaches for the treatment of gliomas include use of chemotherapeutic drug such as temozolamide, cis platin, carmustine, etc.., but the adverse drug effects associated with non targeted delivery give poor treatment outcomes. The radiotherapy and surgical approaches often have greater adverse effects on the health and safety of the patient.

Novel drugs such as lenalidomide which is a vascular endothelial growth factor (VEGF) inhibitor has greater and specific activity as compared to previous generation of drugs which act non-specifically. The VEGF inhibitors act on the VEGF receptors which promote the proliferation and neovascularization of tumours and are often overexpressed in cancerous cells including gliomas. The challenge with these novel anti-cancer moieties is their low solubility resulting into poor bioavailability which hampers their delivery to the site of action. The nanotechnology approach which includes use of nanocarriers has gained attention for the delivery of the therapeutics using nanocarriers by the means of active or passive targeting,

In case of cancerous cells, it has been found that many organelles play a vital role in the oncogenesis. Mitochondria are one of the major organelles which have a key role in glioma by promoting cell proliferation, resistance to cytotoxic drugs and apoptosis. Thus targeting of the mitochondria can open new paradigms in treatment of cancers including glioma.

The concept of theranostics came into existence since the year 2006 which has been a topic of interest for researchers due to its advantage over the conventional nanocarriers by simultaneous delivery of diagnostic as well as therapeutic agenst from a single platform thereby improving the diagnosis and treatment outcomes which can reduce the adverse effects associated with use of toxic diagnostic agents and radiation in the conventional diagnostic techniques. The active targeting of the theranostic nanocarriers can be achieved by conjugation with conjugation of various targeting moieties.

Polyethyleneimine is a customisable polymer having branched or linear structure with different molecular weights. The presence of terminal amine groups make it easy for its modification with targeting ligands containing carboxylic groups. The modified PEI can be coated on the nanoparticles for active targeting purpose. Iron oxide nanoparticles are inorganic nanoparticles which have MRI contrast due to their magnetic nature which can be enhanced when doped with lanthanides. They are relatively stable, biocompatible and biodegradable as compared to the conventional polymeric nanoparticles. They also possess specific characteristics such as hyperthermia in presence of alternating magnetic fields which make them useful in treatment of cancer.

8.1.2. Fabrication and characterisation of magnetic metallic core.

For the fabrication of magnetic metallic core iron and gadolinium were selected as doping of gadolinium on iron oxide shows dual contrast in presence of MRI. The available methods were screened to select the most feasible method for the synthesis. Polyol method was selected for the synthesis of gadolinium doped iron oxide nanparticles which gave smallest size of 99.82±3.60 nm by dynamic light scattering method at an optimised temperature and reaction time of 250°C and 2 hours respectively. Although TEM analysis was done to confirm the exact particle size which was less than 10 nm with spherical morphology.

The elemental analysis was carried out by EDAX technique which gave the presence of gadolinium and iron in the metallic core at the atomic composition of 2.06 and 97.96 % respectively which shows the doping of the lanthanide. Further investigation was done by XRD which confirmed the doping of gadolinium on iron oxide by the analysis of the spectra and % crystallinity. Dual MRI contrast was observed with the Gd: Fe ratio of 1:5. The vibrating sample magnetometry showed that the Gd doped iron oxide nanoparticles had preferred paramagnetic behaviour. The metallic core showed hyperthermia in presence of alternating magnetic field at 744 kHz in a short period of 1.83 minutes with low concentration of 5mg/ml.

For the improvement of aqueous dispersibility the carboxylation of metallic core was done which was confirmed with change in zeta potential from 31.56±4.53mV to -22.3±3.85mV due to anchoring of carboxylic groups on the surface.

8.1.3. Modification of PEI.

In order to accomplish active targeting of theranostic nanoparticles for targeting brain tumours, the modification of polyethylenimine was performed with folic acid (PEI-FA) , triphenyl phosphonium (PEI-TPP) and its combination(PEI-FA-TPP). The modification was done using carbodiimide chemistry and modified PEI was characterised by florescamine assay and infrared spectroscopy which confirmed the alteration. PEI-FA and PEI-TPP were synthesized to check the feasibility of reaction while PEI-FA-TPP was used in fabrication of theranostic nanoparticles due to the presence of dual targeting moieties.

8.1.4. Fabrication and characterization of theranostic nanoparticles.

The coating of drug lenalidomide was done on the carboxylated metallic core using nanoprecipitation technique. The aqueous phase consisted of metallic core dispersed in 0.8% solution of polyvinyl alcohol while the organic phase consisted of PLGA and drug dissolved in acetone. The organic phase was added to aqueous phase under constant stirring after which the evaporation of the organic phase would yield the drug coated core which was incubated with the PEI-FA-TPP solution to get theranostic nanoparticles.

To confirm whether the drug has been adsorbed or encapsulated DSC studies were performed for the lyophilised theranostic nanoparticles which showed no peak of drug indicating that the drug has been encapsulated which was confirmed by determining the %entrapment efficiency which was 97.49.82±0.42 for the optimised formulation while the particle size and zeta potential were found to be 122.26±2.17 nm with PDI of 0.161±0.009 and 12.3±0.52 mV respectively.

The TEM analysis showed that the true particle size was below 100 nm with spherical nanocluster morphology. The *in vitro* drug release study was performed using dialysis sac method with methanolic PBS to maintain sink conditions. The drug suspension showed 96.28±2.43% release in 2 hours while the metallic core and theranostic nanoparticles showed release of 97.54±2.01% and 92.428±4.52% in 8 hours respectively due to the thin polymeric coat on the metallic core. The stability studies were performed for the theranostic nanoparticulate dispersion at room temperature and refrigerated conditions (2-8°C) in which the particle size was elevated at RT while at refrigerated conditions no significant change was observed. The drug content was unaltered at both of the storage conditions.

8.1.5. Cell line and toxicity studies.

The cytotoxicity of the theranostic nanoparticles was evaluated using U87MG glioblastoma cell lines. The MTT studies showed the cytotoxic potential of the theranostic nanoparticles as compared to other components of the formulation. PI staining study showed higher cell death induced by theranostic nanoparticles as compared to free drug. Scratch assay revealed the ability of the theranostic nanoparticles to arrest cell migration or metastasis.

To evaluate the mitochondrial targeting studies such as assessment of mitochondrial membrane potential and ATP levels were performed which showed that the theranostic nanoparticles had disrupted the mitochondrial function. The confocal microscopy confirmed the localization of FITC tagged nanoparticles into the mitochondria.

The toxicity of the theranostic nanoparticles was evaluated qualitatively by mucosal toxicity study while the quantative estimation was done by % hemolysis. The mucosal toxicity study showed that the theranostic nanoparticles were non-toxic as compared to the nasal mucosa treated with positive control (isopropyl alcohol). The mild haemolytic potential of theranostic nanoparticles was seen for the highest concentration of 500µg/ml of drug in theranostic nanoparticles which was under acceptable limits as per the ASTM guidelines.

8.1.6. *In vivo* biodistribution.

The radiolabelling of the theranostic nanoparticles was done using I^{131} using chloramine T oxidation method. The radiolabelled theranostic nanoparticles were administered to Wistar rats by intransal and intravenous routes. At time intervals of 1,4,24 and 48 hrs, animals were sacrificed and the radioactivity was measured in all major organs. The blood and the brain samples were isolated to determine the drug in the samples. It was found the brain/blood ratio of % internalised dose was higher in case of intranasal route at the time period of 1 hour with enhancement ratio of 1.26 as compared to intravenous route.These evidences indicated that the theranostic nanoparticles were capable of targeting the brain.

8.2. Conclusion.

From all the experiments performed in the present work it can be concluded that the developed theranostic nanoparticles served the purpose of diagnosis and therapy. The metallic core used as a template for the theranostic nanoparticles showed dual contrast in MRI imaging due to the gadolinium doping. The paramagnetic behaviour aided the MRI contrast ability and hyperthermia effect which can enhance the treatment outcomes. The high drug loading is suitable for targeting the brain tumours with active targeting offered by the modified PEI coat which also provides mitochondrial targeting for amplification of the cytotoxicity against glioma. The theranostic nanoparticles exhibited low toxicity profile and good stability which is prerequisite for any nanoformulation. *In vivo* biodistribution studies indicated the superior brain targeting by intranasal route as compared to intravenous route.