The work presented in this thesis describes the design and fabrication of polyethylenimine based theranostic nanoparticles for targeting brain tumor via intranasal route.

9.1. Introduction

Brain tumor remains one of the most poorly prognosed cancer; despite advances in technologies, diagnosis and treatment measures. Chemotherapy in the brain tumor detected patients is an adjuvant to radiation and surgery. However, poor passage of the biological actives through the blood brain barrier causes failure and inefficient prognosis of the condition. Nanoparticulate based drug delivery systems have opened new avenues for treatment of brain tumor because of their small size, tunable properties, passage through blood brain barrier due to nano size and specific targeting/ recognition by the site molecules.

Current modalities for treatment of brain tumor involve disruption of the Blood Brain Barrier. However, nanoparticulate based drug delivery systems can deliver the active agent at the site without any disruption of BBB. This thesis precisely focuses on mechanism of action of nanoparticles and their brain tumor targeting potential via active and passive transportation of the particles. Cumulated information and data suggested that drug loaded nanoparticles can successfully act as carriers for potential treatment and thus combating brain tumors. Nanoparticles as a drug delivery system have many advantages due to their versatile nature. The advantages include particle size and surface characteristics of nanoparticles can be easily manipulated to achieve both passive and active drug targeting. Site-specific targeting can be achieved by attaching targeting ligands to surface of the particles or by using magnetic guidance. This will result in concomitant reduction in quantity of the drug required and dosage toxicity. It also enables the safe delivery of toxic therapeutic drugs and protection of non-targeted tissues and cells from severe side effects. This ultimately will results in an increase in the therapeutic index. Relatively high drug loading can be achieved which is very important. Controlled release can be obtained and particle degradation characteristics can be easily regulated by the choice of matrix constituents. The system can be used for various routes of administration including oral, nasal, parenteral, intra-ocular etc. Nanoparticles help in improving bioavailability of various drugs by enhancing aqueous solubility, increasing half-life for clearance or increasing specificity for its cognate receptors. In the recent past various nanocarrier based system have been developed for multimodal therapy and diagnosis of tumor which provide both therapeutic and

diagnostic ability to nanoparticles. Such nanoparticles have been termed as theranostic nanoparticles. The term theranostics encompasses two distinct definitions as defined by the combination of therapeutic and diagnostic agents on a single platform. This interesting approach aims to develop multimodal theranostics systems that use co-encapsulation of multiple different diagnostic modalities and therapeutic in targeting nanomedicines platform. Superparamagnetic iron based nanoparticles (SPIOs) have been widely investigated and is the choice of magnetic material in biomedicine. Although magnetite has been approved by the Food and Drugs Administration, its use in drug delivery and biomedical field is limited due to low saturation magnetization (Ms) of around 400-500 $emu.cm^{-3}$ Hence enhancement of the magnetic moment of magnetic MNP and higher magnetocrystalline anisotropy energy than that of SPIOs in addition to narrow size distribution is the key modification required for widespread use of MNP in biomedical field. To date, the most successful application of magnetic nanoparticles is for magnetic hyperthermia. A remarkable example of this involves SPIO nanoparticles, which are in a phase II study for patients with recurrent GBM in Europe. Preclinical studies indicate that magnetic hyperthermia achieved by SPIO nanoparticles can effectively promote glioma cell death and increase survival. The safety and efficacy of intratumoral hyperthermia using SPIOs coated with aminosilane under a 2.5–18 kA/m and 100 kHz alternating magnetic field in conjunction with radiotherapy have been investigated in patients with recurrent GBM. Experimental results for magnetic fluid hyperthermia (MFH) with iron oxide particles have been promising. The pilot clinical studies regarding magnetic fluid hyperthermia in prostate carcinoma were reported earlier. A patient with prostate cancer received direct tumor injection of aminosilane-coated iron oxide nanoparticles. Thermal treatments of 60 minutes once a week were delivered to the patient for 6 weeks. CT scans indicated that the nanoparticles were retained in the prostate for the entire 6-week period. No systemic toxicity was detected at a median follow-up after one year. FePt nanoparticles have several advantages over iron nanoparticles as discussed above and hence will be therapeutically more effective in comparison to iron nanoparticles. Apart from hyperthermia generation, various nanoparticle constructs containing magnetic elements such as iron, gadolinium, and manganese are in development or have already made their way to a clinical setting for use as MRI contrast agents in the imaging of brain tumors. These nanoparticles have been shown to increase signal enhancement for a long period of time and enhance visualization of the tumor border. Iron oxide nanoparticles have been

extensively studied as T2/T2* contrast agents for brain tumor imaging. Ferumoxytol, an ultra-small SPIO coated with polyglucose sorbitol carboxymethyl ether, has been used as the MRI contrast agent together with a standard gadolinium chelate for patients with recurrent high grade glioma receiving chemotherapy in phase I clinical trials.

Recent research on MNP has led to development of bimetallic or alloy nanoparticles such as FePt, CoPt, FeCo and SmCo5 which possess better magnetic property and chemical stability as compared to SPIOs. FePt nanoparticles have Ms of about 1000 emu.cm^{-3}. With higher MS, MNP experience higher driving forces under a magnetic field, and thus, the efficacy of drug delivery or magnetic separation will be greatly improved. FePt NPs have high Curie temperature, high saturation magnetization and high chemical stability. Due to high value of Ms, FePt nanoparticles also hold great potential for being used as multifunctional MNP for simultaneous drug delivery and hyperthermia therapy. In case of FePt, the magnetocrystalline anisotropy energy is 206 kJ.m^3, which is much higher than SPIOs (5-10 kJ.m3). The hyperthermia generated by FePt can further be modulated by optimizing the size of nanoparticles as an increase in magnetocrystalline anisotropy energy is observed with decrease in particle size of MNP. Owing to its unique magnetic and chemical properties, FePt nanoparticles are being investigated for tumor targeting and treatment. The use of FePt nanoparticles for tumor targeting further depends on chemical composition and colloidal stability of these nanoparticles. Xu et al. reported that FePt nanoparticles undergo leaching in acidic environment to release Fe which acts as cytotoxic agent by catalyzing decomposition of hydrogen peroxide (H_2O_2) into reactive oxygen species (ROS) inside cell which ultimately leads to cellular damage. Hence variation in composition of FePt nanoparticles will elucidate variation in cellular cytotoxicity.

9.2. Aims and Objectives

The aim of the present research work was design and fabrication of polyethylenimine based theranostic nanoparticles for targeting brain tumor. The proposed study was planned to achieve an effective and selective brain tumor targeting using theranostic nanoparticles in order to serve the dual purpose of diagnosis and therapy of tumors using a single nanoparticulate system that will help inhibit the growth of tumor by targeting therapeutic moiety to tumor (sub cellular compartment) so as to prevent metastasis and growth of tumor. The fabricated nanoparticles would further possess an additive effect of hyperthermia for effective tumor

therapy. The targeting will help to reduce the toxicity associated with anticancer therapeutic moieties and will explore metal based novel theranostic nano-platform for targeting brain tumors.

9.3. Synthesis of FePt/ Fe_2O_3@FePt Nanoparticles

FePt nanoparticles were prepared using Solvothermal method. The synthesized nanoparticles were optimized using QbD approach for various process and formulation parameters including the molar ratio of $Pt(acac)_2$ and $Fe(acac)_2$, oleic acid:oleylamine, 1,2-hexadecanediol, reflux temperature and reflux time. Two responses were studied, particle size and atomic composition. Surface modification of optimized FePt nanoparticles was done using different functionalizing agents. The obtained nanoparticles demonstrated small size and stability after various surface modifications. Similarly, Fe_2O_3@FePt core shell nanoparticles were synthesized and method was optimized to get best results for desired response and reproducibility. Various ligands were conjugated to Fe_2O_3@FePt nanoparticles in step wise manner to prepare multifunctional Fe_2O_3@FePt nanoparticles for theranostics of brain tumor. The synthesized FePt and Fe_2O_3@FePt nanoparticles were was further characterized using various characterization techniques such as FT-IR, DSC, TEM, Zeta sizer, Raman Spectroscopy, XPS, AFM, ESR, TGA, EDX, XRD and VSM. TEM showed uniform sized nanoparticles while EDX confirmed presence of both Fe and Pt in nanoparticles. The AFM images show that particle size of FePt nanoparticles was around 6nm while that of Fe_2O_3@FePt nanoparticles was 9.4 nm which is similar to that obtained by particle size analyzer and TEM analysis. AFM under scanning mode shows smooth spherically shaped Fe_2O_3@FePt nanoparticles which confirm uniform layering of Fe_2O_3 over FePt nanoparticles also visible in STEM of Fe_2O_3@FePt nanoparticles. The magnetic behaviour of FePt nanoparticles and Fe_2O_3@FePt nanoparticles was studied using VSM (vibrating sample magnetometer). The FePt nanoparticles exhibited a superparamagnetic state with low remnant magnetization at room temperature along with low value of magnetic saturation (Hs).The magnetic behavior obtained in case of Fe_2O_3@FePt was better than FePt nanoparticles which are due to presence of iron oxide layer. The chemical states of Fe and Pt elements in FePt nanoparticles were detected using XPS. The signal for both Pt and Fe was split into two spin orbit doublet. Results of Raman spectroscopy also confirmed presence of both elements in nanoparticles. The ESR plot demonstrated ROS generating ability of synthesized

FePt and Fe_2O_3@FePt nanoparticles. X-ray diffraction analysis showed the strongest peaks of the (111) and the (200) facet of a face-centered cubic (FCC) structure of FePt and Fe_2O_3@FePt nanoparticles.

9.4. Surface Modification of Fe_2O_3@FePt Nanoparticles

Various ligands were used for modifying surface of synthesized Fe_2O_3@FePt nanoparticles such as SH, COOH, NH_2, HA, TPP, LND, FITC, DOTA-NH_2, DOTA-NCS and Ctx. The surface modification of Fe_2O_3@FePt nanoparticles was done with the aim of imparting tumor targeting ability and cellular co-localization specificity to nanoparticles for efficient treatment of tumor. The particle size of surface modified Fe_2O_3@FePt nanoparticles was more or less similar without any significant difference. Non-significant change in particle size of Fe_2O_3@FePt, Fe_2O_3@FePt-SH, Fe_2O_3@FePt-COOH, Fe_2O_3@FePt-NH_2, Fe_2O_3@FePt-NH_2-FITC and Fe_2O_3@FePt-NH_2/COOH nanoparticles is due to the fact that the surface modifying functional group hardly varies the size of Fe_2O_3@FePt nanoparticles. In case of other surface modified Fe_2O_3@FePt nanoparticles, significant increase in size was observed in comparison to unmodified Fe_2O_3@FePt nanoparticles as the surface modifying agents were having atomic size large enough to vary the particle size of nanoparticles. There was variation in observed zeta potential which can be attributed to presence of different functional groups on surface of nanoparticles.

The various modifications were done for improving the targeting ability of nanoparticles to tumor cell as well as cellular co-localization of nanoparticles after cellular uptake. The modifications were done with specific aim. The conjugation of Ctx was done so as to provide the nanoparticles ability to target hypoxic tumor cells while TPP modification was done to impart mitochondrial targeting ability to Fe_2O_3@FePt nanoparticles. The surface modification with DOTA was done with the aim of radiolabeling Fe_2O_3@FePt nanoparticles for radiotherapy as well as imaging purpose. The conjugation of FITC was done for studying cellular uptake and cellular co-localization in cell lines along with and distribution of Fe_2O_3@FePt nanoparticles after *in vivo* administration. Based on the desired quality attributes of Fe_2O_3@FePt nanoparticles, few amongst the various surface modified Fe_2O_3@FePt nanoparticles were selected for further study viz. Fe_2O_3@FePt-NH_2/COOH-TPP-Drug-HA-Ctx, Fe_2O_3@FePt-NH_2-TPP-COOH-Drug, Fe_2O_3@FePt-NH_2-COOH-Drug and Fe_2O_3@FePt-NH_2-DOTA-NCS nanoparticles.

9.5. Synthesis of pH sensitive HA-LND Conjugate

Synthesis of HA-LND conjugate was done in two steps. The conjugation efficiency was optimized to get maximum yield. The formed conjugates were characterized using FT-IR, DSC and NMR. The obtained IR spectrum of CMHA–LND Conjugates showed all characteristic peaks confirming formation of CMHA-LND Conjugate. The obtained NMR spectrum of CMHA-LND Conjugate showed the presence of a signal in the ^{1}H NMR spectra in the region of 8-15 ppm gives evidence of the presence of an ArNH fragment and confirms the hydrazone structure which makes CMHA-LND conjugate pH sensitive. ESR curves for Fe_2O_3@FePt Nanoparticles and Fe_2O_3@FePt-CMHA-LND nanoparticles demonstrated that the intensity of the curve for Fe_2O_3@FePt Nanoparticles was more that of Fe_2O_3@FePt-CMHA-LND nanoparticles. This can be attributed to the fact that conjugation of HA enhances biocompatibility of Fe_2O_3@FePt nanoparticles by increasing steric hindrance for biomolecules to interact freely with Fe_2O_3@FePt nanoparticles which is responsible for production of ROS .After cellular uptake of Fe_2O_3@FePt-CMHA-LND, the HA layer will be removed, causing enhanced release Fe ions from Fe_2O_3@FePt nanoparticles leading to production of ROS and death of cancer cells ensuring less harm to non-cancerous cells.

9.6. Characterization of Synthesized Nanoparticles

The characterization of both FePt and Fe_2O_3@FePt nanoparticles was done using various techniques. Particle size and zeta potential was analyzed using Malvern zetasizer X-ray diffraction analysis of FePt and Fe_2O_3@FePt nanoparticles showed the strongest peaks of the (111) and the (200) facet of a face-centered cubic (FCC) structure. Broader XRD patterns obtained for FePt nanoparticles suggest smaller particle size which was in accordance with particle size obtained in TEM analysis and zetasizer. The EDAX analysis of the FePt and Fe_2O_3@FePt revealed the presence of Fe, Pt, nitrogen, and carbon. The chemical states of Fe and Pt elements in FePt nanoparticles were detected using XPS. The signal for both Pt and Fe was split into two spin orbit doublet. The Pt 4f signal for Pt element split into 4f7/2 and 4f5/2 with maxima at around 71.0-71.5 eV and 74.0-75.0 eV respectively. The Fe 2p signal also split into 2p3/2 and 2p1/2 with maxima observed at around 710.5-711.5 eV and 724-725 eV due to spin-orbit coupling. Similarly, for Fe_2O_3@FePt, same signal was obtained for Pt and Fe along with

another signal at 710 eV which is attributed to presence of Fe^{+2}over FePt core.AFM images show spherical shape of FePt and Fe_2O_3@FePt nanoparticles with smooth surface. The AFM images show that particle size of FePt nanoparticles was around 6nm while that of Fe_2O_3@FePt nanoparticleswas 9.4 nm which is similar to that obtained by particle size analyzer and TEM analysis. AFM under scanning mode shows smooth spherically shaped Fe_2O_3@FePt nanoparticles which confirm uniform layering of Fe_2O_3 over FePt nanoparticles. The magnetic behaviour of FePt nanoparticles and Fe_2O_3@FePt nanoparticles was studied using VSM (vibrating sample magnetometer). The FePt nanoparticles exhibited a superparamagnetic state with low remnant magnetization at room temperature along with low value of magnetic saturation (Hs).The magnetic behavior obtained in case of Fe_2O_3@FePt was better than FePt nanoparticles which are due to presence of iron oxide layer. FePt and Fe_2O_3@FePt nanoparticles were also characterized using Raman spectroscopy. The Raman spectra clearly showed (Figure 6.23) presence of carboneous material on surface of FePt and Fe_2O_3@FePt nanoparticles. Two bands centered around 1400 cm^{-1} and 1600 cm^{-1} for all the annealed samples. These bands have been identified as the D band and G band, respectively, which occur for graphitic carbon which can be attributed to formation of carbon layer due to pyrolysis of oleic acid at higher temperature.

9.7. *In vitro* Studies

In vitro Magnetic Hyperthermia study revealed the potential of FePt and Fe_2O_3@FePt nanoparticles for hyperthermia therapy of brain tumor. *In vitro* dual heating of FePt and Fe_2O_3@FePt nanoparticles was studied in PBS suspension and U87MG (glioblastomas) cells. The obtained results showed no significant variation in SAR of FePt and Fe_2O_3@FePt nanoparticles in PBS and 5% agarose gel. The obtained experimental results confirm that the hyperthermia generated by FePt and Fe_2O_3@FePt nanoparticles in PBS outside body will be similar inside tumor having highly viscous surrounding. In case of NIR triggered hyperthermia, the temperature increase recorded at 1064 nm was higher than 808 nm due to higher absorbance in the NIR second region resulting from Fe^{2+}-Fe^{3+} transition in FePt and Fe_2O_3@FePt nanoparticles. To mimic tumor mass, about 15 million FePt and Fe_2O_3@FePt nanoparticles labelled cells were concentrated in a small volume in an Eppendorf (150µL). The variation in SLP for magnetic hyperthermia and laser irradiation in cell line and suspension can be attributed

to the fact that thermal energy provided by laser irradiation restores the Brownian motion of FePt and Fe_2O_3@FePt nanoparticles in the cellular environment. The nanoparticles on laser irradiation exhibits plasmonic resonance towards the NIR region of spectra and hence could penetrate biological tissue such as tumor mass. This was demonstrated by mimicking in vitro tumor mass. To mimic tumor mass, about 15 million FePt and Fe_2O_3@FePt nanoparticles labelled cells were concentrated in a small volume in an Eppendorf (150µL). The tumor cells loaded with FePt and Fe_2O_3@FePt nanoparticles (25mg) were then subjected to above mentioned heating protocol. For heating protocol 1, the observed hyperthermia generated was less than that observed in suspension which may be due to confinement of FePt and Fe_2O_3@FePt nanoparticles in endosomes which was not in case of heating protocol involving laser irradiation. The dual heating mode was synergistic and more efficient compared to lone hyperthermia or laser irradiation. The heating power (specific loss of power (SLP)) of FePt and Fe_2O_3@FePt nanoparticles in suspension for magnetic hyperthermia (~300-500 W/g)alone was less than that obtained for laser irradiation (~800-1800 W/g) and dual heating mode (~3000-4500 W/g), while in cell line magnetic hyperthermia (~180-250 W/g) alone was much less than that obtained for laser irradiation (~600-1500 W/g) and dual heating mode (~2500-3500 W/g).

The results of pH responsive drug release study from drug loaded Fe_2O_3@FePt demonstrated an enhanced drug release in acidic pH while very less release was observed in pH 7.4 and pH 6.5. The increased drug release in acidic conditions was due to lysis of pH labile hydrazone bonding between drug and nanoparticles. The Fe release from Fe_2O_3@FePt was very less in the first 6 h in both pH 7.4 (1.3%±0.7%) and 4.8 (5.2%±1.7%). A sudden increase in Fe release was observed after 8 h in pH 4.8 (12.3%±1.4%) while in pH 7.4 (1.3%±0.7%) the increase in Fe release was insignificant. The slow release of Fe demonstrates that FePt in Fe_2O_3@FePt acts as a reservoir of Fe which is not the case with iron oxide nanoparticles.

The obtained results for mucus penetration study demonstrated efficient transfer of Fe_2O_3@FePt nanoparticles across the mucus membrane. The results obtained from the interaction of surface modified Fe_2O_3@FePt in presence of mucin, ECM and plasma demonstrated absence of significant interaction except for nanoparticles modified with Ctx. This can be attributed to the fact that Ctx being a peptide attracts biomolecules toward it owing to hydrophobic interaction between its peptide chain and other biomolecules. Results of

hemocompatibility study of Fe_2O_3@FePt nanoparticles confirm no significant biological interaction with any of blood component leading to potential effect on them.

9.3.2. *In vitro* Cell line studies

The cellular toxicity of FePt nanoparticles, LND and SPANs (surface modified pH sensitive Fe_2O_3@FePt alloy nanoconjugates (Fe_2O_3@FePt-NH_2/COOH-Drug-HA-Ctx)) were evaluated using U87MG cells. The cell viability at 1 µg/mL FePt nanoparticles after incubation for 24h was almost 100% while after 48 h it decreased to 97.2%±3.7%. With increase in concentration of FePt concentration, cell viability decreased linearly. SPANs demonstrated better suppression of U87MG cells. The enhanced suppression of cell viability in case of SPANs can be attributed to the synergistic activity of FePt and LND.

In photothermal therapy, placebo SPANs were used for studying suppression of cell viability in U87MG cells. Although no significant decrease in cell viability was observed at dose of 5µg/mL and 10µg/mL, a 38.3%±4.1% and 51.7%±3.9% decrease in cell viability was observed at the highest studies dose of 50µg/mL after 24h and 48h respectively. An enhancement in suppression of cell viability was observed in case of chemo-photothermal killing of cancer cells owing to cell killing ability of LND. The decrease in cell viability observed after 24h and 48h respectively at dose of 25µg/mL was at half the dose of that required in case of photothermal therapy. MTT assay was performed to assess cell viability in presence of surface various surface modified Fe_2O_3@FePt nanoparticles (Fe_2O_3@FePt, Fe_2O_3@FePt-NH_2-HA, Fe_2O_3@FePt-NH_2/COOH, Fe_2O_3@FePt-NH_2-TPPbr, Fe_2O_3@FePt-NH_2-TPP-COOH, Fe_2O_3@FePt-NH_2-TPP-COOH-Ctx, Fe_2O_3@FePt-NH_2/COOH-Drug,Fe_2O_3@FePt-NH_2-TPP-COOH-Drug, Fe_2O_3@FePt-COOH-DOTA-NH_2, and Fe2O3@FePt-NH_2-DOTA-NCS).

The transport ratio of SPANs across *in vitro* BBB culture was higher than pure LND through the co-culture model at all the tested time points. Apart from co-culture method, mono layer model constructed using U-373MG cells and MDCK cells were also included in study to understand the role of tight junction of BBB in permeation of molecules. In case of monoculture, the permeation of pure LND and SPANs was more or less similar. This can be attributed to the formation of improper junctions between the cells. In the absence of magnetic field, the permeation of LND, Fe_2O_3@FePt and SPANs was time and concentration dependent while in

presence of magnetic field, enhancement in transport ratio was observed even at lower time interval.

An increase in ROS generation was obtained with increase in concentration of SPANs which may be attributed to the fact that increase in SPANs concentration enhances Fe released after particular time interval leading to increase in ROS generation. The results further demonstrated that increase in SPANs will cause decrease in cell viability. Apart from intracellular ROS generation, surface generation of ROS by SPANs in presence of magnetic field was studied using methylene blue degradation assay.

For colonogenic assay, the numbers of colonies for untreated, FePt treated, Fe_2O_3@FePt treated ad SPANs treated cells were found to be 295.00± 20.5, 189 ± 55, 103±44 and 45±17.5 at concentrations of 25 μg/mL respectively (P<0.05). These results indicate that the cells lose the ability to replicate in the presence of nanoparticles. The qualitative and quantitative studies on cellular internalization of SPANs in U87MG cancer cells were performed by Prussian blue staining and ferrozine based assay respectively. The prolonged incubation time enables to reach higher intracellular loading of SPANs which is required for suppressing U87MG cell viability in a dose dependent and time dependent manner.

Mitochondria targeting ability was assessed for M-SPANs (Fe_2O_3@FePt-NH_2/COOH-TPP-Drug-HA-Ctx) and SPANs (Fe_2O_3@FePt-NH_2/COOH-Drug-HA-Ctx). Orange to yellow fluorescence in merged images indicates the co-coloration of green and red fluorescence, demonstrating the successful localization of M-SPANs-FITC in mitochondria as well as targeting ability of M-SPANs. Contrary to M-SPANs, the localization of SPANs-FITC in mitochondria was low as demonstrated by less orange to yellow fluorescence in merged images. Higher fluorescent intensity was observed in M-SPANs-FITC (63.7±3.9) compared with SPANs-FITC) (24.4±3.2) in isolated mitochondria study suggesting positive effect of mitochondria targeting TPP in facilitating mitochondrial localization. These results further confirm mitochondria targeting ability of M-SPANs.

To investigate change in mitochondrial membrane potential due to SPANs and M-SPANs, tetramethylrhodamine methyl ester (TMRE) fluorescence assay was performed in quench mode in U87MG cells keeping FCCP (carbonylcyanide 4-(trifluoromethoxy) phenylhydrazone) as positive control. The results suggest that mitochondria were severely

damaged by M-SPANs than SPANs. Flow cytometry analysis after treating U87MG cells keeping FCCP a positive control further supports above conclusion.

In order to investigate the interaction between mtDNA and nanoparticles, the detection of intracellular ATP levels was done. These results confirm that the M-SPANs do affect the mitochondrial ATP production which is quite beneficial for effective tumor suppression.

Effect of SPANs and M-SPANs on enzymatic activity of individual mitochondrial respiratory chain complexes was measured in U87MG cells. M-SPANs exhibited inhibitory effect on all four respiratory complexes i.e. complex I (37.1±3.9), complex II (43.8±5.7), complex III (31.6±5.1) and complex IV (19.5±3.8). Complex III (and IV appeared to be the most sensitive respiratory chain complex. SPANs inhibited the respiratory complex less significantly (79.2±6.3, 78.4±7.1, 84.1±7.9, 48.6±5.8) than M-SPANs. A plausible explanation of these observations may be correlated to incorporation of TPP molecule in the inner mitochondrial membrane.

9.3.3. *In vivo* Animal Study

The results showed that SPANs and M-SPANs greatly altered the pharmacokinetic profile of LND when compared to plain drug suspension. Study indicated that the AUC was higher for SPANs and M-SPANs as compared to plain drug suspension. This may be attributed to drug-HA conjugates present in both SPANs and M-SPANs. Because, this conjugates could provide better passive targeting by enhanced permeation and retention (EPR) effect.

When M-SPANs after intranasal and intravenous administration were compared, higher C_{max} and AUC were observed with intranasal administration in brain. The drug targeting percentage (DTP %) represents the percentage of drug directly transported to the brain via the olfactory pathway. The M-SPANs showed the DTE% (64.11±4.68) and DTP% (74.34 ±3.76) suggesting that M-SPANs possessed better drug targeting efficiency.

The result of nasal penetration study demonstrated enhanced olfactory uptake of M-SPANs. The enhanced fluorescence reveals significant accumulation of M-SPANs which can significantly increase accumulation of drug in brain and tumor because Ctx has been also demonstrated to be overexpressed in glioma cells. The result demonstrates significant accumulation of M-SPANs in brain after 60 min which confirm efficient nose to brain transfer of SPANs via olfactory neurons.

The histopathological condition of nasal mucosa after treatment with isopropyl alcohol as positive control (a), PBS (pH 6.4) as negative control (b), LND drug solution (c) and M-SPANs (d) was observed to confirm the safety of M-SPANs for nasal administration. Neither cell necrosis nor removal of the cilia from the nasal mucosa was observed after treating with M-SPANs. These observations indicate that the M-SPANs did not cause any deleterious response and adverse effect on nasal mucosa.

Biodistribution result demonstrated that almost 50% of administered activity from nasal cavity transfers into brain. The overall result suggest that M-SPANs can be potential brain targeted theranostic agent as the **^{177}Lu** possess both therapeutic ability against tumor as well as imaging ability which can be utilized for gamma scintigraphy counting as well as radio-luminescence imaging. From the result of biodistribution study performed using ^{177}M-SPANs, we can observe that the presence of ^{177}M-SPANs was dominant in brain in the first 3h (22.63%±0.25%) after administration followed by stomach (47.76%±2.6%) which is due to drainage of ^{177}M-SPANs into stomach via nasal drainage passage. After 1 day (1D) of ^{177}M-SPANs administration, 94.29±0.04% of injected activity was observed in intestine while injected activity decreased drastically in both brain and stomach. Maximum injected activity after 3 days (3D) was observed in urine/stool (82.55±0.05%) followed by intestine (16.56±0.1%). The obtained result demonstrated clearance of M-SPANs after 3 days. An important finding was the minimum accumulation of M-SPANs in liver. Maximum injected activity was observed after 1h (2.59±0.73%). After 1 day no significant injected activity was observed in liver which confirms minimum chances of liver toxicity which happens due to accumulation of nanoparticles in liver.

Mitochondrial accumulation of M-SPANs in brain cells was determined with respect to effect of SPANs and M-SPANs on enzymatic activity of individual mitochondrial respiratory chain complexes in brain homogenate. Cryosectioning of brain was done and visualized under fluorescence microscope prior to homogenization to confirm brain accumulation of M-SPANs and SPANs. Significant inhibitory effect of M-SPANs on all four respiratory complexes i.e. complex I (53.1±2.7), complex II (56.8±4.3), complex III (49.7±4.8) and complex IV (34.5±3.9). Complex III and IV appeared to be the most sensitive respiratory chain complex. SPANs inhibited the respiratory complex less significantly (79.2±4.3, 83.1±4.7, 84.9±4.9, 52.7±3.8) than M-SPANs. A plausible explanation of these observations may be correlated to incorporation of TPP molecule in the inner mitochondrial membrane. Since the respiratory complexes are known

to be sensitive to their lipid environment and require phospholipid molecule for their activity, a high proportion of TPP molecule in the membrane could impair insulating property of membrane allowing protons to leak back into the matrix and the membrane structure required for functioning of the protein complexes.

9.4. Conclusions

In the present investigation, we have reported synthesis of pH sensitive drug-alloy nanoconjugates for multimodal therapy with enhanced brain accumulation via intranasal route. The pH responsive drug release will help overcome drug and metal toxicity to normal cells. The ability of SPANs to deliver drug to tumor along with synergistic chemo-magnetophotothermal therapy makes it a novel multimodal therapeutic platform. The multimodal therapeutic approach will help overcoming intrinsic drug resistance through the evasion of P-gp. The multimodal therapeutic approach demonstrates the remote activation of alloy nanoparticles with external magnetic field in combination with infrared laser will be useful for generating clinically effective hyperthermia. The synergistic suppression of cell viability paves the path for use of these nanoparticles for multimodal intranasal therapy of brain tumor. The inherent ability of FePt nanoparticles of behaving as MRI contrast agents opens new avenue for development of SPANs as theranostic platform for simultaneous imaging and multimodal therapy of brain tumor with minimal collateral damage to normal tissue. The excellent mitochondria targeting ability of M-SPANs opens new avenues for development of mitochondria targeted nanocarriers. Our research group is currently investigating the development of SPANs as multimodal therapeutic as well as imaging platform which will further help developing externally guided imaging and therapy of tumor.