9. SUMMARY AND CONCLUSIONS

The work demonstrated in this thesis describe the formulation and development of mesoporous silica nanoparticles as a novel platform for targeted delivery of anticancer agent.

9.1 Introduction:

Cancer remains one of the world's most devastating diseases, with more than 10 million new cases every year. Despite the significant advances in cancer detection, prevention, surgical oncology, chemotherapy and radiation therapy, there is still no common cure for this disease. Conventional chemotherapy relies on the premise that rapidly proliferating tumor cells are more likely to be destroyed by cytotoxic agents than normal cells. In reality, however, these cytotoxic agents have little or no specificity, which leads to systemic toxicity causing undesirable side effects. Accordingly, the development of tumor-specific drug delivery systems for anticancer agents, differentiating the normal and cancer cells or tissues, is an urgent need to dramatically improve the efficacy of cancer chemotherapy. There is a vast range of strategies available for drug delivery in cancer. The current focus in development of cancer therapies is on targeted drug delivery to provide therapeutic concentrations of anticancer agents at the site of action and spare the normal tissues. The increased concentration of a drug in the site of disease, made possible by targeted delivery, can be used to increase efficacy, reduce side effects, or achieve sum of both of these.

Nanoparticles as drug delivery systems enable unique approaches for cancer treatment. Over the last two decades, a large number of nanoparticle delivery systems have been developed for cancer therapy, including organic and inorganic materials. Nanoscale drug delivery vehicles have shown the ability to encapsulate a variety of therapeutic agents such as small molecules (hydrophilic and/or hydrophobic), peptides, protein-based drugs, and nucleic acids. Advances in cancer proteomics and bioinformatics have allowed the development of targeted therapies, which were referred to as a "magic bullet". Nanocarriers may be surface functionalized to attach biomolecules in order to achieve active tumor targeting. Surface ligands include antibodies, aptamers, peptides, or small molecules which recognize tumor-specific or tumor-associated antigens in the tumor microenvironment. In general, ligands such as peptides, sugars, and small molecules are more attractive than antibodies due to higher stability, higher purity, ease of production through synthetic routes, and non-immunogenicity. The active targeting mechanism

takes advantage of highly specific interactions between the targeting ligand and certain tissues or cell surface antigens to increase cellular uptake and increase tumor retention.

These nanocarriers include polymeric nanoparticles, dendrimers, nanoshells, liposomes, inorganic/metallic nanoparticles, hybrid nanoparticles, micelles, and magnetic and bacterial nanoparticles.

Inorganic nanomaterials have special structures and chemophysical properties. Among inorganic nanoparticles, copper oxide (CuO) are cytotoxic depending on their size. CuO NPs are of great interest due to their high redox cycling property and cytotoxic effect on different cells via oxidative stress. CuO nanoparticles are also found to induce apoptosis through mitochondrial pathway. Furthermore, these nanoparticles can easily cross the biological barriers and reach target organs. Among inorganic nanomaterials, mesoporous silica nanoparticles are centre of focus because of their unique properties. Since the first report using MCM-41 type mesoporous silica nanoparticles (MSNs) as drug delivery system in 2001, the last few years have witnessed an exponential increase in research on biomedical application of MSNs. It has been one of the hottest areas in nanobiotechnology and nanomedicine for designing biocompatible MSNs and multifunctional counterparts in disease diagnosis and therapy. Mesoporous silica nanoparticles (MSNs) have some unique advantages including high surface area and large pore volume, tunable particle size (10-1000 nm) and pore diameter (2-30 nm), uniform mesoporosity, flexible morphology, facile surface functionalization, excellent biocompatibility and biodegradation. Textural properties of MSNs provide the possibility to load high amount of drugs within MSNs carriers. On the other hand, there are abundant silanol groups on the surfaces of mesoporous channels and the outer surfaces of MSNs, which facilitate their surface functionalization. As nanocarriers, mesoporous silica nanoparticles with unique mesoporous structure have been explored as effective drug delivery systems for a variety of therapeutic agents to fight against various kinds of diseases including bone/tendon tissue engineering, diabetes, inflammation, and cancer.

The biggest challenge in application of MSN for cancer treatment is to obtain "zero premature drug release". Variety of biocompatible, biodegradable polymers such as polyethylene glycol (PEG), poly (acrylic acid), natural polymer like gelatin etc can be used for surface capping of mesoporous silica based nanosystems. These polymer end cappers possessing significant

diffusion barrier properties can act as a gatekeeper to provide the intracellular drug release from mesoporous silica. Moreover, some of these polymeric materials are capable to respond to a some stimulus due to their intrinsic ability to alter their physical or chemical properties and using such polymers, nanoparticulate drug delivery systems can be engineered in such a way as to selectively change their properties/functions (for example, facilitate drug release or cellular uptake) in response to specific internal or external stimuli/triggering mechanisms, i.e. behave as smart stimuli-sensitive preparations. Such nanopreparations are designed to behave dynamically in response to various internal cues in the microenvironment of the pathological area or to certain external stimuli. Internal stimuli that are characteristic for the pathological areas, such as tumors, infarcts, sites of infection, etc., include local changes (compared to normal physiological values) in pH, temperature (local hyperthermia that accompanies inflammation), redox conditions (such as high intracellular glutathione levels), and the expression of certain molecules, including those with enzymatic activity. External stimuli or stimuli that could be artificially applied from outside of the body include heat, magnetic fields, light, and ultrasound, and can be employed to facilitate "on-demand" changes of certain functions of nanomedicines.

Among the different endogenous and exogenous stimuli, redox potential has recently appeared as the most unique, fascinating, promising and clinically applicable trigger for ''active'' intra-cellular drug and gene release. As compared to various stimuli such as light and magnetic field that are applied externally and require sophisticated devices, redox is a ubiquitous internal stimulus existing naturally in tumor tissues as well as in cancer cells. The design rationale of reduction-sensitive nanosystems usually involves incorporation of disulfide linkage(s) in the polymer main chain, at the polymer side chain or in the cross-linker. The disulfide bonds while stable under an oxidative conditions are rapidly cleaved, at a time scale from minutes to hours, under a reductive environment through thiol-disulfide exchange reactions. Furhtermore, pH sensitive activation is also of particular interest, as delivery can be autonomously activated in vitro and in vivo. When the nanoparticles are taken up into cells, they enter cells by endocytosis and will encounter endosomal/lysosomal environments where low pH condition is prevalent. In addition, tumor interior has low pH environment due to hypoxic conditions. This feature provides an advantage that the drug release is more restricted to cancer.

Chitosan (CS) is a non-toxic biodegradable polycation with a high number of primary amino groups. These amine functional groups render cationic character to the polymer and are

responsible for a range of significant features including in situ gelation, mucoadhesion, efflux pump inhibition, high cellular permeability as well as bioavailability for oral administration of drugs which make CS as an outstanding candidate in drug delivery systems. CS can be swelled in cancerous tissues due to their acidic media and this property endows the polymer with the ability of discrimination between normal and cancerous cells for controlled drug release.

Current drug delivery systems, however, do not have the ability to guide themselves to a target. They reach the target area as a result of blood circulation and extravasation followed by intratumoral retention and distribution. So the active targeting is required to guide the drug/drug carriers to a target site. Number of targeting moieties such as Folic acid, Lectins has been used for effectively targeting the tumor cells, out of which, folic acid as an inexpensive, water soluble and stable vitamin without adverse effect on normal cells and low immunogenic response has attracted a great deal of attention for active targeting. The over expression of the FA receptor in epithelial malignancies, such as colorectal, ovarian, and breast cancer cells in comparison with most normal cells make FA conjugates as facile and infallible strategy to promote the receptor-mediated endocytosis of nanoparticles. The vesicular trafficking of FA conjugates makes them able to move through many organelles and release efficiently their cargo into the cell cytoplasm.

Doxorubicin (DOX) is an established anticancer drug that belongs to the class Anthracycline. Anthracyclines are reported to have multiple damaging effects on cellular components, and these are assumed to be the causative factors for the anti-cancer effects of DOX. Mechanisms of action reported include inhibition of DNA and RNA polymerases, alkylation of DNA, intercalation with DNA and intercalation with topoisomerase II. Other mechanisms reported are disruption of the Ca^{2+} homeostasis and generation of reactive oxygen species via quinine redox recycling. Despite its broad-spectrum antineoplastic activity, adverse events, particularly cardiotoxicity, has limited the use of conventional doxorubicin in clinical practice. This was especially so in patients with advanced disease requiring dose escalation. The therapy-limiting toxicity for this drug is cardiomyopathy, which may lead to CHF and death. An approach to ameliorating doxorubicin-related toxicity is to use drug carriers, which engender a change in the pharmacological distribution of the drug, resulting in reduced drug levels in the heart.

9.2 Aims and Objectives:

This work was aimed to develop mesoporous silica nanoparticles as a novel platform for controlled as well as targeted delivery of anticancer agent "Doxorubicin" for improved breast cancer therapy. The research work included synthesis of mesoporous silica nanoparticles using sodium silicate as an economic silica precursor and cetyltrimethyammonium bromide as a surfactant. $CuCl_2$ was used as a copper source to produce CuO loaded MSN. The synthesised nanoparticles were further taken for surface modification using various surface modifying agents to facilitate drug loading and conjugation of selected polymer in order to provide stimuli-responsive drug release within cancer cell by active targeting.

The research work was carried out to achieve following objectives:

- Synthesis of MSN with required characteristics such as surface area, pore size and pore volume.
- Synthesis of CuO loaded MSN with desired characteristics like surface area, pore size and pore volume.
- Functionalization over MSN surface for improved drug loading and easy polymer conjugation. The functionalization included, MSN-NH_2, MSN-COOH, MSN-SS-NH_2, CuO-MSN-NH_2, CuO-MSN-COOH and CuO-MSN-SS-NH_2.
- Loading of Dox into functionalised MSN.
- Synthesis of polymer and targeting ligand conjugate.
- Attachment of polymer-targeting ligand conjugate over MSN surface to achieve active targeting to tumor tissue with dual (pH and redox) responsive intracellular drug release and minimum premature drug release.

9.3 Synthesis of MSNs and MO-MSNs:

Two different type of mesoporous silica nanoparticles (MCM-41 and SBA-16) were synthesized using industrial-grade sodium silicate (Na_2SiO_3) as an economic silica source with a view to reduce the synthesis cost. MCM-41 type of MSNs were synthesized under alkaline conditions using hexadecyltrimethyl-ammonium bromide (CTAB) as a template agent and ethyl acetate as pH regulator while SBA-16 type of MSNs were synthesized under acidic conditions using poloxamer 407 as a template and hydrochloric acid as pH regulator. The major advantage of employing Na_2SiO_3 as the silica source is its cost as well as wide availability. In order to enhance the effectiveness of the treatment, Metal oxide (MO) such as CuO and ZnO loaded mesoporous silica nanoparticles were synthesized using similar method applied for the synthesis of ordered MSN with use of metal chloride ($CuCl_2$ and $ZnCl_2$) as a metal source. The synthesized nanoparticles were characterized for physicochemical properties and using different techniques such as dynamic light scattering (DLS), zeta potential and Brunauer–Emmett–Teller (BET) analysis. MCM-41 was found to have smaller particle size and higher surface area as compared to SBA-16 and hence MCM-41 type of MSNs were selected for further studies. Atomic absorption spectroscopic (AAS) method was utilized to quantify the amount of metal in MO-MSN. CuO was found to be loaded to more extent than ZnO. Furthermore, chorioallantoic membrane (CAM) assay was also performed to measure the anti-angiogenic potency of MO-MSNs and CuO-MSNs were found to show better anti-angiogenic activity. Hence, among ZnO-MSNs and CuO-MSNs, CuO-MSNs were selected for further studies. The selected nanoparticles were further characterized using Transmission Electron Microscopy (TEM), Field Emission Scanning Electron Microscopy (FE-SEM), Energy Dispersive X-ray Spectroscopy (EDX) and Small Angle X-ray Scattering (SAXS) and Wide Angle X-ray Scattering (WAXS) analysis method. The synthesized MSN had sufficiently large surface area (850 m^2/g) with pore size of about 2.5nm while CuO-MSN had surface area of about 568 m^2/g with 2.7nm pore size. Both the particles were found to have particle size less than 150nm. TEM images of MSNs showed presence of hexagonal pores while CuO-MSNs showed presence of incorporated CuO nanoparticles too. The XRD studies proved that the nnaoparticles were amorphous in nature with four distinctive peaks in SAXS corresponding to MCM-41 type of MSNs.

9.4 In vitro degradation of MSNs and CuO-MSNs:

Degradation of silica is always a question. Hence, a laboratory scale method was developed to measure the silicic acid (a dissolved fraction of silica or degraded water soluble silica) in form of molybdosilicic acid (blue coloured compound, detected by UV spectroscopy), in vitro which proved that mesoporous silica nanoparticles get completely degraded within 5-6 days.

9.5 Toxicity profiling and in vivo degradation of MSNs and CuO-MSNs:

Any new synthesised drug delivery carrier must be safe and not toxic. Hence, both MSN and CuO-MSN have been evaluated for toxicity profile. Complete toxicity study was performed including acute (single dose), sub-acute and chronic toxicity (multiple dose) in mice. Any changes in weight, visible and/or palpable dermal infection, presence of ascites, and grooming or impaired mobility were closely monitored every day. At the end of the study, various hematological parameters (complete blood count) and biochemical parameters were assessed. In case of sub-acute and chronic toxicity study, mice were sacrificed and histopathology of 7 major organs was observed to check any changes in cellular structure and toxicity. The synthesized MSN as well as CuO-MSN were found safe to be used at the dose up to approx. 40mg/kg. Simultaneously in vivo biodegradation study was also performed. The in vivo biodegradation was measured by calculating the amount of silicic acid in urine. MSN and CuO-MSN were found biodegradable in nature and both were completely excreted out of body within 3-4 days.

9.6 Surface functionalizations, Drug loading and characterizations:

Different surface modification of MSN as well as CuO-MSN were done using different surface modifying agents. 3-Aminopropyltriethoxysilane (APTES) is commonly used to prepare cationic amine groups on nanoparticles. The successful grafting of APTES over nanopaticles surfce was evidenced by corresponding peak in FTIR spectrum. It was further confirmed by change in zeta potential from negative to positive and ninhydrin test (test to confirm presence of primary amine groups). Post modification of MSN-NH_2 and CuO-MSN-NH_2 was done to achieve COOH group on the surface. It was done by reacting the surface-bound amine groups of MSN-NH_2 and CuO-MSN-NH_2 with succinic anhydride to form MSN-COOH and CuO-MSN-COOH nanoparticles. The successful modification of amine group was evidenced and qualitatively monitored by FTIR and change in zeta potential.

The selected anticancer drug, doxorubicin (DOX), was loaded in to amino functionalized as well as carboxyl functionalized to find the effect of surface functionalization on drug loading. The amount of loaded drug was calculated by measuring the amount of free drug using spectrofluorimetry. MSN-COOH and CuO-MSN-COOH were found to have higher drug loading as compared to their amino functionalized analogues because DOX contain positive charge. DSC analysis of drug loaded nanoparticles was performed to detect the presence of any surface adsorbed drug and the drug was found completely loaded within the pores.

The drug loaded nanoparticles were further reacted with cysteine dihydrochloride to form MSN-SS-NH_2 and CuO-MSN-SS-NH_2. This reaction helps to achieve disulfide linkage on the surface of nanoparticles which is supposed to cleave under redox environment of tumor cells and release drug. The polymer (chitosan) was conjugated with targeting moiety (Folic acid) by carbodiimide reaction. The chitosan-folate conjugate was further reacted with drug loaded DOX-MSN, DOX-CuO-MSN, DOX-MSN-SS-NH_2 and DOX-CuO-MSN-SS-NH_2 to form DOX-MSN-CH-FA, DOX-CuO-MSN-CH-FA, DOX-MSN-SS-CH-FA and DOX-CuO-MSN-SS-CH-FA. Folic acid enhances folate receptor (over expressed in cancer cells) mediated cellular uptake while chitosan helps to reduce pre-mature drug release and increased intracelluar drug release under acidic environment of cancer cell. The synthesized nanoparticles were characterized for particle size analysis, zeta potential measurement, FT-IR, TEM, BET analysis.

The dynamic light scattering analysis showed that the hydrodynamic diameter of synthesized nanoparticles was less than 200nm. The particle size distribution pattern indicated absence of aggregates and confirmed uniform dispersion of nanoparticles. Surface modification and conjugation of targeting moiety was responsible for increase in particle size. The zeta potential indicated stable dispersion. The transmission electron microscopy revealed uniform size and morphology. It also revealed that the particles were nearly spherical to oval shaped with hexagonal pores.

The stimuli triggered drug release was assessed for redox triggered drug release and pH triggered drug release. The results obtained with stimuli triggered drug release confirmed the stability of conjugation and release mechanism which will be useful for controlling drug release as well as reducing unwanted toxicity to healthy cells.

As the formulation was intended for intravenous administration, hemolysis study was performed in order to check the effect of formulation on red blood cells (RBCs). The drug loaded DOX-

MSN and DOX-CuO-MSNs showed lesser percent hemolysis as compared to DOX alone. The capping with CH-FA significantly reduced the hemolysis and found to be biocompatible and safe for intravenous administration.

9.7 Cell line studies:

The cytotoxicity of MSNs and CuO-MSNs was studied against MCF-7 (Human breast cancer) cell line using MTT assay. Plain MSN did not show any toxicity upto 100µg/ml concentration while the CuO-MSN was found to show dose dependant toxicity.

The cytotoxicity of different concentrations of DOX alone, DOX-MSN, DOX-MSN-SS-CH-FA, DOX-CuO-MSN and DOX-CuO-MSN-SS-CH-FA was studies against MCF-7 and MDA-MB-231 cell line for 24, 48 and 72 hours. All the formulations were found to exhibit dose dependent cytotoxicity. DOX-MSN-SS-CH-FA and DOX-CuO-MSN-SS-CH-FA showed less cytotoxicity within 24 h which might be due to lesser release of DOX from CH-FA capped nanoparticles. The cytotoxicity of these nanoparticles was greatly increased after 48 hours. CuO loaded formulations (DOX-CuO-MSN and DOX-CuO-MSN-SS-CH-FA) showed better cytotoxicity as compared to MSNs formulations (DOX-MSN and DOX-MSN-SS-CH-FA).

The generation of reactive oxygen species (ROS) by the nanoparticles was measured by using DCFHDA dye. All the formulations were found to produce ROS but DOX-CuO-MSN and DOX-CuO-MSN-SS-CH-FA showed very high fluorescent which might be attributed to the ROS producing capability of additional CuO incorporated within the MSNs framework.

The change in the mitochondrial membrane potential (MMP) upon addition of DOX alone, DOX-MSN, DOX-MSN-SS-CH-FA, DOX-CuO-MSN and DOX-CuO-MSN-SS-CH-FA was investigated using rhodamine-123. The results showed that DOX-CuO-MSN-SS-CH-FA formulations damaged the mitochondria to high extent as the mitochondrial membrane potential was greatly reduced.

Scratch assay was performed with a view to detect the effect of DOX alone, DOX-MSN, DOX-MSN-SS-CH-FA, DOX-CuO-MSN and DOX-CuO-MSN-SS-CH-FA on the migration of MCF-7 cancer cells. All the formulations restricted the migration of cancer cells, DOX-CuO-MSN-SS-CH-FA being the most effective among all.

The colonogenic assay revealed that the cells lost their ability to replicate in the presence of nanoparticles. As the synthesized nanoparticles were found to generate ROS and disrupt

mitochondrial membrane potential, apoptosis and necrosis induced by these nanoparticles were studied. DOX-MSN-SS-CH-FA and DOX-CuO-MSN-SS-CH-FA showed very high proportion of necrotic cells as compared to others. This clearly indicate that these two formulations were highly toxic to the MCF-7 cells causing death of the cancer cells.

9.8 In vivo anticancer activity:

The in vivo anticancer activity of various formulations was checked against ehrlich ascites carcinoma (EAC) induced tumor in Balb C mice and compared with the control groups: Normal control (no treatment, no tumor), model control (no treatment but tumor), standard control (tumor + DOX). A significant change ($p<0.0001$) in the weight of different treatment group mice was observed as compared to model control. DOX-MSN, DOX-CuO-MSN, DOX-MSN-SS-CH-FA and DOX-CuO-MSN-SS-CH-FA were found to control the weight loss observed in mice treated with DOX alone. All the formulations were found to dissolve the tumor at the end of the treatment. Drug loaded CuO-MSN formulations were found to dissolve the tumor much faster as compared to drug loaded MSN formulations and DOX alone. DOX-MSN-SS-CH-FA, DOX-CuO-MSN and DOX-CuO-MSN-SS-CH-FA showed significant increase in the mean survival time (MST) ($p<0.05$) with 100% survival of all mice till the end of the treatment. All the treatment groups such as DOX-MSN (32.31 % ILS, $p<0.05$), DOX-MSN-SS-CH-FA (38.46 % ILS, $p<0.01$), DOX-CuO-MSN and DOX-CuO-MSN-SS-CH-FA ((38.46 % ILS, $p<0.01$ for both) showed considerable increase in the life span as compared to model control group. All the nanoparticles significantly reduced the hematological and serological toxicities associated with DOX treatment. Mice treated with DOX-CuO-MSN-SS-CH-FA were found to show significant difference ($p<0.05$) in various hematological and biochemical parameters and the difference was comparable to the normal control group. The histopathalogy showed that the tumor was spread to liver and kidneys in model control groups. Histopathology of all organs of mice treated with DOX, DOX-MSN, DOX-MSN-SS-CH-FA, DOX-CuO-MSN and DOX-CuO-MSN-SS-CH-FA was also found comparable with the normal control group without any signs of abnormality. This clearly shows that the treatment not only treated the tumor but also restricted the metastasis also.

Conclusions:

Ordered mesoporous silica nanoparticles as well as CuO loaded mesoporous silica nanoparticles with acceptable surface area and suitable size were successfully synthesized using one of the most economic source of silica, sodium silicate. The synthesized nanoparticles were found safe upto 40mg/kg in mice and did not show any toxicity. Furthermore, both in vitro and in vivo studies revealed that the synthesized mesoporous silica nanoparticles were biodegradable in nanture and got degraded completely within 3-4 days. Various surface functionalisations were carried out for increased drug loading as well as easy polymer conjugation. In vitro drug release studies proved that cysteine and chitosan were successfully conjugated over functioalised MSN surface due to which increased drug release under redox and acidic environment was observed. Furthermore the CH-FA capping was found to improve the biocompatibility of drug loaded nanoparticles significantly. The cytotoxicity studies revealed that the CuO loading improved the anticancer activity against MCF-7 and MDA-MB-231 (Breast cancer cell line). Excellent tumor suppressing activity was demonstrated by nanoparticles against EAC induced breast cancer model which not only treated tumor locally but also restricted the metastasis of cancer. Moreover, the nanoparticles also significantly reduced the DOX associated side effect such as weight loss as well as changes in hematological and serological parameters.

Thus based on the obtained results it can be said that the formulated mesoporous silica nanoparticles were capable of showing dual responsive intracellular drug release which may help to enhance the efficacy of anticancer treatment and reduce undesirable side effects.