Introduction:

Cancer remains one of the world's most devastating diseases, with more than 10 million new cases every year.[1] Despite the significant advances in cancer detection, prevention, surgical oncology, chemotherapy and radiation therapy, there is still no common cure for this disease.[2,3] 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.[2,4] 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.[5] 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.[6]

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.[7,8]

Inorganic nanomaterials have special structures and chemophysical properties. Among inorganic nanoparticles, copper oxide (CuO) are cytotoxic depending on their size.[9] CuO NPs are of great interest due to their high redox cycling property and cytotoxic effect on different cells via oxidative stress.[10] CuO nanoparticles are also found to induce apoptosis through mitochondrial pathway.[11] Furthermore, these nanoparticles can easily cross the biological barriers and reach target organs.[12] 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.[13] 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.[14-17] 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.[18] 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,[19-21] diabetes,[22] inflammation,[19] and cancer.[24-32]

The biggest challenge in application of MSN for cancer treatment is to obtain "zero premature drug release".[33] Variety of biocompatible, biodegradable polymers such as polyethylene glycol (PEG)[34], poly (acrylic acid)[35], natural polymer like gelatin[36] 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.[37-40] 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.[41,42] 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,[43,44] temperature (local hyperthermia that accompanies inflammation),[45-47] redox conditions (such as high intracellular glutathione levels),[48,49] and the expression of certain molecules, including those with enzymatic activity.[50,51] 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.[39,42]

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.[52] 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.[53,54] 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.[55]

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.[56]

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.[57] Number of targeting moieties such as Folic acid[58], Lectins[59] 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.[60]

Doxorubicin (DOX) is an established anticancer drug that belongs to the class Anthracycline.[61] 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.[61] Mechanisms of action reported include inhibition of DNA and RNA polymerases[62], alkylation of DNA[63], intercalation with DNA[61] and intercalation with topoisomerase II.[64] Other mechanisms reported are disruption of the Ca^{2+} homeostasis[65-67] and generation of reactive oxygen species via quinine redox recycling.[68,69] 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.[70]

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.

Summary:

The chosen drug, Doxorubicin Hydrochloride (DOX), which was provided as a gift sample by Sun Pharmaceuticals Ltd., was analyzed by Physical examination, IR spectroscopy, DSC analyzer and UV-visible spectrophotometry to authenticate the drug. UV-visible spectrophotometry and spectrofluorimetry were selected as analytical methods and calibration curves of drug were obtained in various solvents such as water, phasphate buffer (pH 7.4 and 5.5) using these methods.

Ordered mesoporous silica nanoparticles (MCM-41) were prepared by hydrothermal synthesis using industrial-grade sodium silicate (Na2SiO3) as silica source, hexadecyltrimethyl-ammonium bromide (CTAB) as template agent and ethyl acetate as pH regulator. The major advantage of employing Na2SiO3 as the silica source is its cost as well as wide availability. In order to enhance the effectiveness of the treatment, CuO loaded mesoporous silica nanoparticles were synthesized using similar method applied for the synthesis of ordered MSN with use of copper chloride ($CuCl_2$) as a copper source. The synthesized nanoparticles were chatracterised using different techniques such as Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM), Brunauer–Emmett–Teller (BET) analysis and Small Angle X-ray Scattering (SAXS) analysis method. The synthesised MSN had sufficiently large surface area (850 m^2/g) with pore size of about 5nm while CuO-MSN had surface area of about 532 m^2/g with 5.5nm pore size. Both the particles were found to have particle size less than 150nm. Atomic absorption spectroscopic (AAS) method was utilised to quantify the amount of copper into CuO-MSN.

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.

Drug loading was done to prepare drug loaded MSN-COOH and CuO-MSN-COOH. The amount of loaded drug was calculated by measuring the amount of free drug using spectrofluorimetry. 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 MSN-COOH, CuO-MSN-COOH-CH-FA, MSN-SS-NH_2 and CuO-MSN-SS-NH_2 to form MSN-COOH-CH-FA, CuO-MSN-COOH-CH-FA, MSN-SS-CH-FA and CuO-MSN-SS-CH-FA. Folic acid enhances folate receptor (over expressed in cancer cells) mediated cellular uptake which chitosan helps to reduce pre-mature drug release and increased intracelluar drug release under acidic environment of cancer cell. The synthesized nanoparticles were characterised for particle size analysis, zeta potential measurement, FT-IR, TEM, DSC, FESEM-EDAX, SAXS, BET analysis.

The dynamic light scattering analysis showed that the hydrodynamic diameter of synthesized nanoparticles was less than 150nm. 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.

Degradation of silica is always a question. Hence, a loboratory 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.

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.

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 af 7 major organs was observed to check any changes in cellular structure and toxicity. The synthesised MSN as well as CuO-MSN were found safe to be used at the dose upto 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 4-5 days.

The cytotoxicity of various selected nanoparticles was studied 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.

Ongoing Studies:

1. Cell line studies.
2. In vivo tumor regression studies.

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 4-5 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. The cytotoxicity studies revealed that the CuO loading improved the anticancer activity against MCF-7 (Breast cancer cell line). The CuO-MSN showed fluorescence near 646nm wavelength which can be useful for in vivo imaging of the nanoparticles.

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.

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