Chapter 2

Review of Literature
&
Aims and Objectives

2.1 Nanoparticles in cancer therapy:

In the fields of medicine, diagnosis and pharmaceutical science, Nano-biotechnology has attracted collective scientific interest due to their interesting chemical and physical properties (Schaming and Remita, 2015; Bicer and Sisman, 2010). Synthetic feasibility, low production cost and less toxic synthesis process covers several applicability of metal nanoparticles (Brust and Kiely, 2002; He, Y. 2007; Hussain et al., 2011). Due to unique physicochemical approaches, several synthesis methods have been developed to synthesize diversified types of novel metallic NPs (Khan et al., 2019; Kumar et al., 2012; Park et al., 2016). Globally, cancer constitutes one of the most dangerous health issues in the current century. The unique biochemical properties of nanoparticles are applicable to the approaches of cancer treatment. Nano-scale molecules possess enhanced reactive surface area which helps them to function as a better drug than conventional drugs and pharmaceuticals. The anticancer potential of metal nanoparticles including gold, silver, and copper have gradually being familiar (Jain et al., 2012; Jannathul and Lalitha, 2015; Sanpui et al., 2011; Jose et al., 2011).

However, the low cost of copper compared to that of gold and silver, is more attractive for researchers. The advantages of metal oxide nanoparticles include structural changes, change in electrochemical characteristics, and change in surface properties of the nanoparticles. There are several scientific reports on the anticancer potential of copper oxide NPs (Wang et al., 2012; Siddiqui et al., 2013; Wang et al., 2013; Laha et al. 2014). However inadequate research consequences have been disseminated on the antitumor potency of metallic copper NPs (Chen et al., 2006; Ahmad et al., 2010). Herein, a brief summary of copper oxide NPs as anticancer agents in the applications of the therapeutic field of cancer chemotherapy was presented. Broadly there are two synthesis approaches of NPs namely, chemical and green methods. In the chemically synthesis process toxic hazardous chemicals were used. Generally in the “Green” synthesized method copper NPs are produced through the utilization of phytochemicals that are present in the plant extract. These extracts help to generate nanoparticles with desired shapes and sizes by controlling the reaction temperature, time, and pH and, furthermore, the concentrations of the plant extract and metal salt (Philip, D. 2010; Kumar et al., 2011; Din et al., 2017). Copper NPs possess binding capacity with the biomolecules due to substances such as proteins, enzymes etc. which helps to cleave the DNA of cancer cells. This binding property is mainly due to

changeable surface chemistry of copper NPs (Fang et al., 2010; Thanh and Green, 2010; Wang et al., 2015).

2.2. Mechanism of ROS generation by CuONPs:

Cupric and cuprous ions participate in redox reaction by which ROS generation occurs (Gaetke and Chow, 2003). Ascorbic acid can reduce the Cu^{2+} ion into Cu^{+}. Then the Cu^{+} ion reacts with hydrogen peroxide and forms hydroxyl radicals through the Haber-Weiss reactions (Das et al., 2015).

(1) $O2 \bullet^{-} + Cu2+ \rightarrow O2 + Cu+$

(2) $Cu+ + H2O2 \rightarrow Cu2+ + OH^{-} + OH\bullet$

OH• radical generation on the surface of CuONPs is the main cause of DNA damage (Angelé-Martínez et al., 2017). By the help of two primary mechanisms CuONPs can produce ROS which can destroy the DNA. One is the surface of the nanoparticles which interacts with biomolecules and another mechanism is the solution in which copper dissolved from the nanoparticles surface. The free radical generation during these two processes is very unstable **(**Karlsson et al., 2008; Studer et al., 2010). DNA damage due to ROS generation ultimately leads to cellular toxicity and apoptosis.

2.3. Mechanism of CuONPs in cancer cell apoptosis:

Apoptosis and necrosis are two diverse mechanisms liable for major cell death. Apoptotic cells show alteration of cellular morphology as well as molecular and biochemical properties. On the other hand, necrosis produces a leakage in total cell content (Darzynkiewicz et al., 1997; Tatton and Rideout, 1999). In cancer cell development and progression apoptosis is a pivotal phenomenon. There is a complex mechanism by which cancer cells avoid apoptosis and continue to proliferate with the basic characteristics of cancer (Ashkenazi, A. 2002).

In comparison with other metal oxide NPs (e.g., ZnO, TiO2, Fe2O3), copper oxide nanoparticles (CuONPs) showed a considerably high level of toxicity than other metal oxide nanoparticles (Karlsson et al., 2008). The antitumor activity of CuONPs has been established against various types of cancers such as cancers of the liver, lungs, nasopharynx, breasts, cervix and pancreas (Sankar et al., 2014). Several reports on cytotoxic and genotoxic effects of CuONPs have been studied. These studies indicated that CuONPs was able to provoke high pro-inflammatory

responses *in vitro* (Lee et al., 2007; Gnanavel et al., 2017; Shafagh et al.,2015; Siddiqui et al., 2013; Joshi et al., 2016) and *in vivo* system (Midander et al., 2009; Chen et al., 2006).

The mechanism of apoptosis consists of several genes. The p53 protein acts as a caretaker of the cells. The p53 protein is associated with maintaining genomic constancy through the activation of cell cycle checkpoints, DNA repair and apoptosis (Liu and Martin, 2001). The anti-apoptotic protein bcl-2 inhibits cell death whereas the bax is a pro-apoptotic protein which stimulates apoptosis (Reed, JC. 2006). The ratio of bax/bcl-2 protein drives the fate of the cell. If the ratio is increased there is a decrease in the cellular confrontation to apoptotic stimuli, leading to apoptosis (Chougule et al., 2011; Gao and Wang, 2009). Due to apoptotic stimuli, disruption of the mitochondrial integrity leads to activation of Caspases which ultimately lead to apoptosis (Youle and Strasser, 2008). Although there is a significant lack of knowledge about the mechanisms of CuONPs, several studies indicate that the probable mechanism of apoptosis induced by CuONPs in cancer cells is through ROS generation via mitochondrial mediated apoptosis pathway. Due to ROS generation inside the cancer cells a cascade of caspases became activated during the apoptosis. Cleaved Caspase-3 is the key regulatory protein for DNA fragmentation and morphological changes related to apoptosis (Jänicke et al., 1998). When p53 is up regulated, it helps to activate the member of bcl-2 family that is Bax protein. Bax protein encourages permeabilization of the outer mitochondrial membrane. Due to permeabilization of membrane, soluble proteins stimulate Caspase activation following their release from the intermembrane space into the cytosol (Fuentes-Prior and Salvesen, 2004). Among the soluble proteins cytochrome C is well known. Cytochrome C facilitates the formation of apoptosome complex after it binds with apoptosis protease activating factor-1 (Apaf-1). Auto activation was observed when Apoptosome binds with procaspase-9. Pro-caspase-9 changes its own conformation and activates itself. Activated Caspase-9 can stimulate caspase-3 which cleaves substrates on aspartate residues. The proteolytic activity is associated with apoptosis phenomenon (Youle and Strasser, 2008).

2.4. Toxic effect of Copper Oxide Nanoparticles:

Copper has been considered an essential trace element which is very vital for our growth and development. *In vitro* toxicity of CuONPs depends on the penetration of NPs directly inside the cells and simultaneously dissolution and distribution of toxic Cu^{2+} ions into the cytoplasm are

responsible for toxicity (Midander et al., 2009; Studer et al., 2010). Accumulation of the NPs inside the cell or tissue influences lysosomal disruption and permeabilization of lysosomal membranes (Cho et al., 2011; Jin et al., 2008) which promotes ROS generation and ultimately cellular apoptosis. The negatively charged cell plasma membrane effortlessly welcomes cationic NPs whereas neutral and anionic charged NPs are less internalized than a positive one (Banquy et al., 2009).

ROS generation due to copper ions showed several toxic impacts such as oxidation of DNA and DNA strand breaks (Kawanishi et al., 1989). Copper ions inflict damage of mitochondrial respiration through lipid peroxidation of cell membrane (Gaetke et al., 2003). It is proved by Heinlaan et al., 2008 and Karlsson et al., 2008 that CuONPs are extremely toxic compared to bulk Cu and as well as other metal oxide nanoparticles also. Although toxicity depends on several physical and chemical properties of the NPs such as size, shape, state of a dosage form, chemical composition, surface area and surface chemistry (Powers et al., 2006). ROS is responsible for hepatotoxicity and nephrotoxicity mentioned by Chibber and Shanker (2017). Elevated level of pro-inflammatory cytokines indicates oxidative stress inside the system as a result amendment of physiology was observed. Histopathological studies showed toxic impact on liver, kidney and brain tissues (Lee et al., 2016; Roda et al., 2017). Another study also showed toxic effect on kidney tissue due to oxidative stress exposure in mice by CuONPs during a period of 3 days (Sarkar et al., 2011). There are very few studies about the immunotoxic effects of CuONPs. Lymphocytes are vital participants in the immune system of our body. CuONPs showed toxicity on human lymphocytes through oxidative stress by altering the glutathione enzyme level (Assadian et al., 2018).

Additionally toxic chemicals during the synthesis of NPs attributes towards organ toxicity. To avoid this synthesis of CuONPs using bio inspired components has attracted increased attention (Sharma et al., 2015) (Umer et al., 2014) (Shah et al., 2015).

2.5. Surface coated CuONPs:

Surfactants or polymer gives protection to Cu nanoparticles (Huang et al., 1997) (Khanna et al., 2007). The nanoparticles and stabilizer or functional groups of the stabilizer interact with the metal atoms by hydrophilic interaction. NPs are always in motion. So, the stabilizer always is not able to prevent oxidation and aggregation.

Engineered nanocarriers allowed the release of the drugs to the specific targeted site. Poly β-amino ester, a biocompatible cationic polymer which is pH sensitive undergoes rapid dissolution and discharges all its content in acidic environment and acts as a good vehicle to deliver anticancer drug (Shenoy et al., 2005). Biocompatible and biodegradable polymers can be used as a surface coating because they are easily dispersed, encapsulated, or adsorbed on the surface.

Several polymers are being used today for the sustained release of drugs. Among them, poly lactic-co-glycolic acid (PLGA), polyglycolic acid (PGA) and polylactic acid (PLA) are well known (Makadia and Siegel, 2011; Torchilin, VP. 2000; Tancini et al., 2015).

Nanoparticles coated with polymers like poly ethylene glycol (PEG) and poly ethylene oxide (PEO) avoids its uptake in the liver, spleen and lymph nodes via mononuclear phagocytic system. In this way surface coated NPs improves their accumulation in the tumor site (Moghimi et al., 2001; Nicolazzi et al., 2003). The surface modification of the NPs mainly helps to accumulate the particles in target tissues or cells with targeting moieties.

2.6. Immunostimulatory role of Copper Oxide nanoparticles:

"Immune surveillance" is the major mechanism by which our immune system protects against several diseases. Viruses, bacteria, and cancer cells can be quickly identified as foreign antigens and eradicated by immune cells. However, the major concern is that successful pathogens have modified enough themselves by inhibiting phagocytosis, blocking antigen presentation, or directly killing immune cells (Ernst et al., 1999). Cancer cells antigens have the ability to convert the tumor microenvironment (TME) into immunosuppressive state by employing immunosuppressive immune cells. With the help of this mechanism, immunosuppressive immune cells released inhibitory cytokines and enzymes that will helps in tumor immune evasion (Quail and Joyce, 2013).

Aluminium is a commonly used adjuvant to induce cell mediated immunity (Guy, B. 2007; Harandi et al., 2010). Now NPs have been used as a delivery vehicle for antigen by antigen proteins or suitable coated materials. Furthermore, immune response can be developed by vaccine-induced immunity through the delivery of antigen to the dendritic cell. It has been reported that when NPs act as an antigen delivery vehicle, it is able to increase the immune

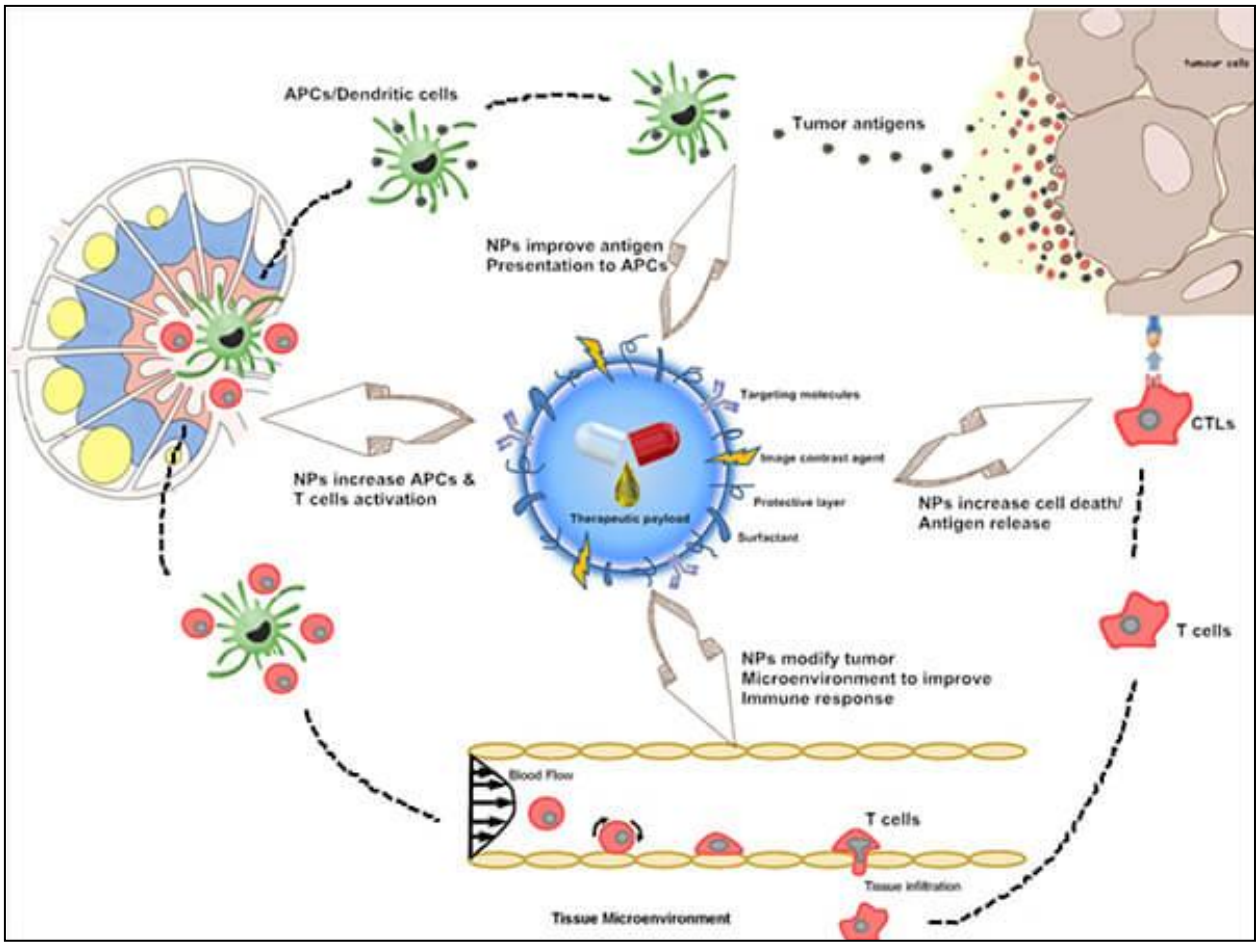

Fig 2.1: Depiction of the complex pathway involved in cancer immunotherapy.
Source: https://www.cancer.gov/nano/cancer-nanotechnology/treatment

response with several folds compared to the soluble antigen alone (Akagi et al., 2011; Uto et al., 2011).

Recently, the nanomaterials have proved a surge for the activation and maturation of APCs. Delivery of nanoparticles to APCs plays a vital role towards the CD8+ T cell response. Nanoparticles are ornamented with targeted ligand or Abs on to the surface to deliver in the specific desired site (Kwon et al., 2005). Metal oxide NPs can be used as a nanovacccine scaffolds as they are able to easily penetrate several types of cell and suitable for delivery of antigen to APCs (Kostarelos et al., 2007; Konduru et al., 2009). Several studies demonstrated that nanoparticles can alter the immune response against the specific antigen and proficiently target APCs to assist the appropriate processing and presentation (Prasad et al., 2011; Villa et al., 2011).These nanoplatforms play a pivotal role in targeted delivery and controlled release of

antigens, adjuvants and immunoregulatory agents. PLGA modified NPs were able to capture antigen which successfully captured the tumor-specific neoantigens. Simultaneously histone proteins and alarmin proteins galvanize host defense through stimulation of strong antitumor immune response (Oppenheim and Yang, 2005).

2.7. Folic acid mediated targeted delivery of NPs:

Bare nanoparticles face so many complications for targeted cancer therapy. Firstly non-coated NPs can be easily identified by MPS (mononuclear phagocyte system) which is present in the liver, bone marrow and lungs. The next problem is hydrophobicity which can influence the adsorption of bio-components of blood onto the surface of NPs. Accumulation of ions from NPs to the tumoral site enhances the EPR (Enhance permeability and retention) effect. Circulation of the NPs in the bloodstream for a long time is required to reach the target site but this long time exposure of NPs in the bloodstream is the major concern regarding toxicity (Ulbrich et al., 2016).

To overcome these difficulties of bare NPs, surface coating or modifications with appropriate molecules have been using today to target the cancer cells. At this point two major tumor targeting strategies have been used. One is active targeting and another is passive (Mohamed and van der Walle, 2008). Pathophysiological feature of tumor tissues helps the drug delivery system to be accumulated in the tumor region by passive targeting. At the same time, passive targeting helps to kill specifically tumor cells by NPs through the receptor mediated internalization (Danhier et al., 2010).

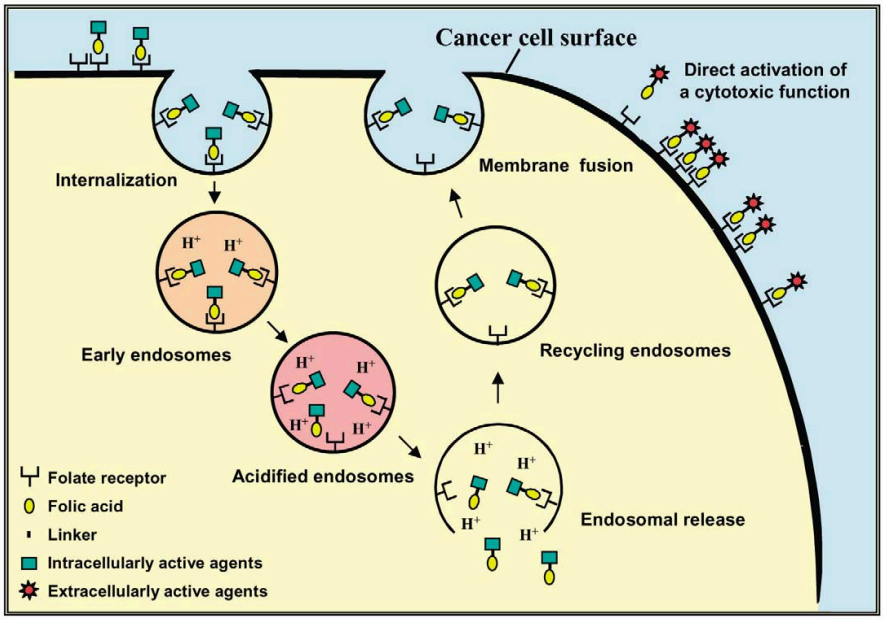

Fig 2.2: Folate-mediated delivery of therapeutic agents to folate receptor-positive cancer cells.
Source: https://doi.org/10.1016/j.addr.2012.09.020

Folic acid is a popular ligand for targeting of folate receptors of cancer cells. Unlike normal tissues, solid tumors in lungs, breasts, ovary, uterus, head and neck have overexpression of folate receptors. This overexpression of folate receptors acts as an extra advantage in distinguishing between normal and tumor cells (Narayanan et al., 2010). Compared to other folate derivatives, folic acid shows 30 times higher affinity for folate receptors. These folate conjugated nanoparticles, enter the cell via receptor mediated endocytosis for targeted intracellular drug delivery.

2.8. Research Challenge:

Chemically and green synthesized CuONPs have several reports on anticancer efficacy but very less is known about their mode of action and toxicity in *in vitro* and *in vivo* model.

2.9. Aims and Objectives:

The current study was intended to evaluate the toxicity effects and as well as minimize toxicity by surface coating. To fulfill these points, we intend to develop multifunctional particles in a step by step process with detailed biological activities. The ultimate objective is to develop multifunctional smart NPs which can be used as an effective tool for targeted cancer therapy and to stimulate immune competent cells for successful elimination of cancer cells.

- Synthesis & Characterization of CuONPs from chemical and green source.
- Selection of best CuONPs based on anticancer and cytotoxicity assay.
- Determination of anti cancer activity selected copper oxide NPs against MCF-7 and HeLa. cancer cells and in *in vivo* solid tumor (4T1) model and cytotoxicity of the metal NPs towards the healthy cells.
- To search out the anticancer ability of surface modified CuONPs followed by conjugation with folic acid for targeted NPs delivery to cancer cells.
- To examine the immunomodulatory effect of surface functionalized NPs.

2.10. Experimental Design: