Synthesis and Application of Polymer Stabilized, Water Dispersible Copper Based Nanoparticles as Anti-cancer and Diagnostic Agents

A dissertation submitted
to Kent State University in partial
fulfillment of the requirements for the
degree of Doctor of Philosophy

by
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April 2017
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<tbody>
<tr>
<td>AAS</td>
<td>Atomic absorption spectroscopy</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>ATR</td>
<td>Attenuated total reflectance</td>
</tr>
<tr>
<td>ATTM</td>
<td>Ammonium tetrathiomolybdate</td>
</tr>
<tr>
<td>CA</td>
<td>Contrast agent</td>
</tr>
<tr>
<td>CF2</td>
<td>Copper ferrocyanide</td>
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<td>Density functional theory</td>
</tr>
<tr>
<td>DAHP</td>
<td>Diammonium hydrogen phosphate</td>
</tr>
<tr>
<td>DI</td>
<td>Deionized</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<td>Dimethyl sulfoxide</td>
</tr>
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<td>Deoxyribonucleic acid</td>
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<td>Disk scanning unit</td>
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<td>EDC</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
</tr>
<tr>
<td>EDX</td>
<td>Energy dispersive X-ray spectroscopy</td>
</tr>
<tr>
<td>EPA</td>
<td>Environmental Protection Agency of the United States of America</td>
</tr>
<tr>
<td>EPR</td>
<td>Electron paramagnetic resonance</td>
</tr>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>ESR</td>
<td>Electron spin resonance</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>FDA</td>
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<td>FDG</td>
<td>Fluorodeoxyglucose</td>
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<tr>
<td>HT29</td>
<td>Human colorectal cancer cell line</td>
</tr>
<tr>
<td>IC50</td>
<td>Inhibitory concentration</td>
</tr>
<tr>
<td>IPB</td>
<td>Insoluble Prussian blue</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Human breast adenocarcinoma cell line</td>
</tr>
<tr>
<td>MPAC</td>
<td>Metastatic adenocarcinoma of the pancreas</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-di-methylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cutoff</td>
</tr>
<tr>
<td>NIR</td>
<td>Near infrared</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NOX</td>
<td>NADPH oxidase</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticle/Nanoparticulate</td>
</tr>
<tr>
<td>NS</td>
<td>Nanosheet</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
</tr>
<tr>
<td>OER</td>
<td>Oxygen evolution reaction</td>
</tr>
<tr>
<td>PB</td>
<td>Prussian blue</td>
</tr>
<tr>
<td>PBA</td>
<td>Prussian blue analog</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC3</td>
<td>A type of prostate cancer cell line</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PTT</td>
<td>Photothermal therapy</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
</tr>
<tr>
<td>PXRD</td>
<td>Powder X-ray diffraction</td>
</tr>
<tr>
<td>RF</td>
<td>Radio frequency</td>
</tr>
<tr>
<td>RLCF2</td>
<td>Radiolabeled copper ferrocyanide</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SAED</td>
<td>Selected area electron diffraction</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SPB</td>
<td>Soluble Prussian blue</td>
</tr>
<tr>
<td>SPIO</td>
<td>Superparamagnetic iron oxide</td>
</tr>
<tr>
<td>STEM</td>
<td>Scanning transmission electron microscopy</td>
</tr>
<tr>
<td>SWNT</td>
<td>Single-walled carbon nanotube</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TR</td>
<td>Texas red</td>
</tr>
<tr>
<td>XO</td>
<td>Xanthine oxidase</td>
</tr>
<tr>
<td>XRD</td>
<td>X-ray diffraction</td>
</tr>
</tbody>
</table>
Acknowledgments

With the deepest feeling of gratitude, I sincerely thank my research advisor Prof. Songping D. Huang for all his support and encouragement throughout the phase of my research in his lab. Working in Prof. Huang’s group, I certainly understood from him that “out-of-the-box” approach needs to be a ‘must-have’ chemical tool for any chemist regardless of the area in addressing some of the challenges encountered in science. I believe that with the practical experience I gained in his lab, my skills, knowledge and mental exercise in chemistry have expanded tremendously and hopefully this experience drives me to take up future professional endeavors with more confidence, zeal and motivation.

I also thank my former advisor Prof. Scott D. Bunge for his guidance, encouragement and especially for being considerate during the first few years of my graduate research in his lab.

I thank my dissertation committee members for their support and suggestions.

I am grateful to the Department of Chemistry and Biochemistry, Kent State University, for providing me assistantship all through the years of my graduate program. Also, I thank our academic program coordinator Erin Michael-McLaughlin for all the assistance she provided and making graduate life smoother. She is one of the best employees I have ever met.

I’m grateful for the all technical help provided by several people during my research. Special thanks to Dr. Min Gao from Liquid Crystal Institute, for providing help in acquiring high resolution transmission electron microscopic images and diffraction pattern for few of my nanoparticle samples; Prof. Gail C. Fraizer (Department of
Biological Sciences) for training me in cell culture work; Dr. Min-Ho Kim (Department of Biological Sciences) for access to confocal microscope; Dr. Bing Yu from Dr. Kim’s lab for training me in confocal microscopy; Prof. Robert Twieg for access to IR spectrophotometer; Prof. Roger Gregory for access to freeze dryer unit; Dr. Yaorong Zheng for access to flow cytometer; Dr. Michal Marszewski, Andrew Sharits (Ohio State University) and Alexandre Goncalves for obtaining powder XRD data for my bulk samples; Dr. Vindya Perera for relaxivity measurements for my nanoparticle samples. Without their immense help, my dissertation would be meagre.

I sincerely thank Dr. Ren-Gen Xiong, South-east University, China for providing me necessary support during my internship in his lab. I couldn’t forget how amazed I was with the kind of importance and priority given to my work as well as the warm treatment by his group members. I specially thank his group member Mr. Wei-Quio Liao for all the assistance he offered. It’s an unforgettable and enriching experience working in his lab.

My former and current labmates of Prof. Huang’s lab are always friendly, supportive and helpful. Many thanks to Dr. Vindya Perera, Dr. Murthi Kandanapitiye, Don Pryor, Nick Penman, Huda Alamri, Bogdan Benin, Wenjun Sun, Fan Wang, Katharine Greskovich, Madeline Goosmann, Zhongxia Wang and Adam Cooke. I wish them all success and happiness in their life.

Thanks to my friends Jowita Marszewska, Michal Marszewski, Jibin Abrahram Punnoose, Sangeetha Selvam, Santanu De, Muralidhar Palavalasa, Ganapathi Alluri, Partha Choudhury, Alexandre Goncalves, Maria Costa and Luiz Souza, who made my stay in USA memorable and comfortable. I wish them all success and happiness in their life.
My special thanks to my dear friend Sreehari Seerla who always showed his attention, encouraged me throughout my PhD career and wished my best. I wish him and his family members happiness and prosperity.

Finally, but most importantly, I would like to thank my family members in India: my mother, elder and younger sisters, brother-in-law and his parents. Many thanks to my nephew- the new member of our family- who made us happy with his cheerful talk and play. I do not take pleasure when I mention that my mom’s and younger sister’s pain is my gain and to acknowledge this is embarrassing but not exaggerating. Embarking on this doctoral journey would not have been possible without their love, support and well wishes. They have always desired my well-being and success more than I ever desired for myself.

Sriramakrishna Yarabarla
Kent, Ohio, USA
March 12, 2017
happily dedicating this work to the three most important people in my life

**Pratima** (my younger sister)  
*for her resilient spirit...*

**Bhavani** (my elder sister)  
*for her unconditional love...*

and

**Renukadevi Chilamkurthy** (my mother)  
*for everything...*
1. Introduction

1.1.1 Reactive Oxygen Species

The term reactive oxygen species (ROS) is used to describe broadly a variety of oxygen-containing, reactive chemical species, including free radicals, ions and molecules derived from molecular oxygen.

Table 1-1. Common ROS, chemical formulae and Lewis structures.

<table>
<thead>
<tr>
<th>ROS</th>
<th>Chemical formula</th>
<th>Lewis structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxygen</td>
<td>O₂</td>
<td>⋅O⋮O⋅</td>
</tr>
<tr>
<td>Superoxide anion</td>
<td>O₂⁻</td>
<td>⋅O⋮O:</td>
</tr>
<tr>
<td>Peroxide</td>
<td>O₂²⁻</td>
<td>⋅O⋮O:</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>H₂O₂</td>
<td>H:O⋮O: H</td>
</tr>
<tr>
<td>Hydroxyl radical</td>
<td>OH⁻</td>
<td>⋅O⋮H</td>
</tr>
<tr>
<td>Hydroxide ion</td>
<td>OH⁻</td>
<td>⋅O⋮H</td>
</tr>
</tbody>
</table>
The reduction of oxygen in eukaryotic organisms through the addition of electron results in the formation of superoxide ions, the subsequent reduction of which leads to the generation of other ROS. Some of the common examples of the ROS and the structures are given in the table 1-1. ROS can be generally classified into two groups: free radical ROS and non-radical ROS. The former type of ROS contains one or more unpaired electron(s) in their valence shells. Examples include superoxide (O$_2^-$), hydroxyl radical (OH), nitric oxide (NO$^-$), peroxyl (ROO$^-$), alkoxyl radicals (RO$^-$) among many others. The later type of ROS does not possess unpaired electron(s); however, they are also chemically reactive and can be transformed to other ROS. Examples of this type include hydrogen peroxide (H$_2$O$_2$), organic hydroperoxides (ROOH), peroxynitrite (ONO$^-$), etc. In relevance to cancer, superoxide, hydrogen peroxide and hydroxyl radical are the most studied ROS.

1.2 Cellular Generation of Reactive Oxygen Species (ROS)

ROS are important in serving essential biological functions such as in signaling cell growth and differentiation, mediating inflammation, regulation of enzyme activity to modulate their functions, eliminating pathogens and foreign particles by phagocytes, etc. Scheme 1-1 depicts the intracellular generation of ROS. Three major cellular sites of ROS production include the mitochondrial electron transport chain, the endoplasmic reticulum (ER) and the NADPH oxidase (NOX) complex. A variety of ROS are produced in biological systems primarily from the conversion of superoxide anion which is initially generated by the mitochondrial electron transport of aerobic respiration.
A small proportion of oxygen molecules (1-2%) are converted to superoxide anion radical by the electrons that escape from the respiratory chain. Subsequent dismutation of the superoxide anion radical in the presence of super oxide dismutases (SOD1, 2 and 3) results in the generation of H$_2$O$_2$ as the secondary product.$^{12}$ In the peroxisomes, H$_2$O$_2$ can be rapidly converted by catalases to water molecules. Other cellular processes that produce ROS are the metal catalyzed hydroxyl radical (·OH) formation from H$_2$O$_2$ and NO· formation from arginine by nitric oxide synthase (NOS). Of these ROS, ·OH radicals are the more reactive and hence more damaging to the various cellular components such as lipids, proteins and nucleic acids. NO· is a reactive free radical that further converts to
a non-radical species called peroxynitrate (ONOO\textsuperscript{\textordfou} \textordfou) by reacting with superoxide anion. Peroxynitrate is capable of modifying the structure and function of proteins.

ROS are somewhat like a “double-edged sword”. A moderate increase in ROS is beneficial in promoting the cell proliferation and differentiation whereas excessive generation of ROS can lead to the oxidative damage of cellular components such as lipids, proteins and DNA. Therefore, to prevent the deleterious effects caused by ROS, cells regulate the levels of ROS by maintaining a balance between ROS generating and scavenging processes. Excess of ROS can be eliminated by ROS-scavenging enzymes such as gluthathione peroxidase (GPX), glutathione reductase (GR) coupled with the NADPH cycle and xanthane oxidase (XO). The balance between the generation of oxidants and their elimination through the utilization of antioxidant capacity in the cells is referred to as redox homeostasis.

1.3 Cancer and ROS: Biological Basis for Therapeutic Selectivity

Under physiological conditions, normal cells maintain the redox homeostasis owing to their reserved antioxidant capacity. Imbalance in the redox homeostasis can cause an increase in ROS level and lead to a state called ‘oxidative stress’. One fundamental difference between normal and cancer cells is the higher level of endogenous oxidative stress in cancer cells and the activation of adaptive mechanisms to deal with such higher oxidative level and can be understood from the schematic 1-2.

Normal cells in standard physiological conditions exhibit lower level of basal ROS and maintain the redox homeostasis by adjusting the ROS generation and elimination. Normal cells maintain the ROS within the threshold (shown as horizontal dashed line in scheme 1.2) for cell death owing to their reserve antioxidant capacity. This means that, unlike cancer cells, normal cells can better handle the exogenous ROS stress, i.e. any oxidative insults caused by exogenous agents. However, mutations caused by irreparable oxidative DNA damage related to critical genes such as oncogenes or tumor suppressor genes can induce carcinogenesis and tumor progression. Furthermore, the continuous mutations that cancer cells undergo for their survival is accompanied by accumulation of ROS causing redox imbalance. Therefore, cancer cells, in general, exhibit higher basal ROS level. Consequently, these cells upregulate the antioxidant capacity to maintain the subtle balance between the higher ROS level needed for their survival and yet mitigate
the level from rising above the death threshold. This subtle act of redox homeostasis makes cancer cells more vulnerable to exogenous ROS stress. Any oxidative insult caused by exogenous agents that increases the ROS level above the threshold level can overwhelm the antioxidant capacity with the net imbalance in scavenging the excess ROS, thus leading to death of cancer cells. As such, this crucial difference between cancer and normal cells can constitute a biological basis to exploit and design new therapeutic alternatives that selectively work on redox modulation in cancer cells.

1.4 Nanoparticles in Cancer Therapy

Nanomedicine is an emerging interdisciplinary field of nanotechnology that combines the inputs primarily from biology and chemistry to engineer nanosystems with the intended biomedical applications in diagnosis and health management of diseases such as cancer. Traditional cancer therapies that involve the small molecular chemotherapeutic drugs (organic molecules or metallodrugs) have inherent shortcomings including shorter circulation times due to rapid clearance, lack of target specificity, systemic side effects and acquired drug resistance.\textsuperscript{14} Compared to their molecular counterparts, nanomedicine offers distinct advantages of active/passive targeting, higher solubility, longer circulation times, better bioavailability and biocompatibility, controlled and sustained release of active components, multifunctional and multimodal capabilities, reduction/elimination of systemic side effects.\textsuperscript{14} Hence, nanomedicine can overcome many of the aforementioned limitations of molecular drugs.

Nanomedicine encompasses a wide variety of nanosystems ranging from liposomal drugs, polymer drug conjugates, macromolecule-drug conjugates and inorganic based nanoparticles.\textsuperscript{15-18} Several nanoparticles (NPs) of the above systems have entered
practical clinical use after FDA approval. A notable example of liposomal based NP drugs is Doxil (100 nm), approved for the treatment of AIDS-associated Kaposi’s sarcoma, that encapsulates a widely used chemotherapeutic drug called doxorubicin into stealth liposome carriers comprised of hydrogenated soy phosphatidylcholine, cholesterol, and PEGylated phosphoethanolamine. This drug had dramatically improved the circulation half-life of doxorubicin and enhanced the drug deposition in tumor tissue.

Several other liposomal drugs in clinical use include Ambisome (amphotericin B liposomes), DepoCyt (cytarbine liposomes) and Visudyne (verteporfin liposomes).

Among the polymer-drug NP conjugates approved for clinical use, Oncaspar (PEG–L-asparaginase) is a notable example used to treat acute lymphoblastic leukemia. Within the class of macromolecule-drug conjugates that have the hydrodynamic sizes varying from 5-200 nm, Abraxane, a prominent albumin bound paclitaxel with an average size of 130 nm, was approved by FDA in 2005 for second-line treatment of patients with metastatic breast cancer. This drug has been found in clinical studies to double the therapeutic response rate, increase the time of disease progression, and increase overall survival of the patient. Furthermore, the drug has also been in use for first-line treatment of advanced non-small cell lung cancer (NSCLC) in combination with carboplatin and for the first-line treatment of metastatic adenocarcinoma of the pancreas (MPAC), in combination with gemcitabine.

Inorganic based nanomaterials are currently being studied extensively for use in cancer therapy. The wider choice of systems and their robustness in physiological and tumor environments coupled with their inherent advantageous physical attributes place inorganic based nanomaterials at the forefront of research in cancer therapy. The primary
functions of inorganic-based nanomaterials are broad and depend on the intended applications. One of the most intensely studied applications of inorganic NPs is the ability to serve as efficient drug delivery vehicles/carriers to carry the drug payload to the target tumor site of interest. Several inorganic nanoplatforms based on iron oxide, metallic gold, quantum dots, carbon nanotubes (CNTs), graphene, silica etc. have been employed for this purpose. The drug can be conjugated to the nanocarrier either as a prodrug or in its active form by appropriate functionalization on the surface. Dhar et al. in 2009 reported the development of polyvalent oligonucleotide gold nanoparticle conjugates (DNA-Au NPs) as drug delivery vehicles for carrying platinum (IV) prodrugs. The superior cancer cell killing ability of the prodrug containing conjugates (Pt-DNA-Au NPs) was demonstrated in a variety of cell lines. In particular, the IC$_{50}$ of Pt-DNA-Au NPs was found to 0.9 μM in A549 cells (human lung carcinoma) after treatment for 72 hours which is greatly superior to the molecular drug cisplatin with an IC$_{50}$ of 11 μM. IC$_{50}$ can be defined as that concentration of the drug at which the cell viability of cancer cells is reduced by half, i.e. by 50% of the initial cell population is inhibited.

Furthermore, inorganic NP drug delivery systems may be imparted with additional functionalities to include the receptor specific ligands to achieve the targeted delivery to tumor site. Moreover, surface functionalization of NPs with targeting moieties increase the cellular uptake of the NPs, which leads to increased drug efficacy and thereby a reduced therapeutic dose of the active drug. An example is the folic acid moiety-containing Pt(IV) drug tethered to the single-walled carbon nanotubes (SWNT) longboat developed by Dhar et al. This drug delivery system was successfully utilized
for the targeted delivery of platinum anti-cancer agent to the folate receptor positive [FR (+)] cancer cells.

Another way inorganic NPs can serve as drug delivery platforms is by the encapsulation of the drug molecules within the interior pores/void regions of the NPs. Numerous reports on drug loaded iron oxide based NPs for cancer therapy have been published recently by several groups.\textsuperscript{23-24} For example, in a study by Schleich \textit{et al.}, iron oxide NPs encapsulated with chemotherapeutic drugs like doxorubicin or paclitaxel have been found to inhibit CT26 cells (a colorectal cancer cell line) \textit{in vitro} and delayed the tumor growth \textit{in vivo} when tested in CT26 tumor bearing mice.\textsuperscript{25} Prussian blue analogues based NPs are another type of inorganic nanosystems that have been explored for drug delivery recently.\textsuperscript{26}

Apart from applying as drug delivery systems, the inherent physical properties like super paramagnetism and near infra-red absorption also make some inorganic nanomaterials suitable for cancer therapy. The use of such nanomaterials requires the application of external stimulation such as a magnetic field, as in the case of hyperthermia or near IR light with photo thermal therapy (PTT). The super paramagnetic nature of iron oxide NPs has been explored intensively by numerous group worldwide for hyper thermic treatment.\textsuperscript{27-33} Carbon nanotubes (CNTs),\textsuperscript{22,34-37} copper-based\textsuperscript{38-41} and gold-based\textsuperscript{42} nanostructures are some of the inorganic nanomaterials that are actively being studied for their potential use in PTT.
1.5 Anti-Cancer Potential of Inorganic Nanoparticles Based on Elevated ROS Level

Unlike the previously mentioned inorganic nanomaterials that are either used for drug delivery purposes or as anti-cancer agents in the presence of external stimuli, there are several other inorganic-based nanomaterials, particularly metal oxide and chalcogenide nanoparticles, that exhibit anti-proliferation ability towards cancer cells, owing to their intrinsic ability to induce oxidative stress. Research in this area is attractive, since these NPs possess favorable features such as inherent toxicity towards cancer cells while less or nontoxic to normal cells, passive localization of the NPs in the tumor tissue, facile synthesis and non-requirement of the application of external stimuli. While not absolutely required, conjugation of receptor specific ligands via surface functionalization may lead to targeted localization, higher uptake and therefore may further reduce the therapeutic dose. In spite of these advantages, this area is relatively unexplored. In 2008, Hanley et al. reported on the preferential ability of ZnO NPs (8-13 nm) in killing cancerous T cells (about 28-35 times) compared to normal cells. These NPs were found to induce apoptosis apparently due to the involvement of the generation of ROS. Silver NPs stabilized by chitosan (labeled as Ag-CS NC system) are shown to exhibit anti-proliferation effects on HT29 cells (colorectal cancer cell line) by Sanupi et al. Flow cytometry studies revealed that metallic particles caused the changes in the mitochondrial membrane potential as well as elevated ROS production with the consequence of augmentation of apoptotic cell death. Recently, Murugan et al. reported the anti-cancer potential of polydisperse (20-100 nm) spherical titania (TiO$_2$) NPs synthesized by hydrothermal method. These NPs exhibited higher in vitro cytotoxicity
against breast cancer MCF-7 cell line compared with HBL-100 normal breast cell line. Experimental evidence showed that these NPs induced apoptosis by nuclear fragmentation and mitochondrial-mediated pathway in MCF-7 cells. All the aforementioned examples highlight the therapeutic potential of nanoparticles based on the modulation of cellular ROS level.

1.6 Copper complexes as Anti-Cancer Agents and Limitations

Copper is one of the few metals currently being explored in the area of cancer treatment as metallodrugs (mostly encompass metal complexes) and nanoparticulate forms. Interest in copper-based complexes as anti-cancer agents has increased enormously since the late previous decade, as evidenced by the surge in the number of reports published with primary focus on finding alternative strategies to platinum based drugs, particularly in the context of overcoming acquired resistance to platinum therapy. Depending on the ligand system, copper can be stabilized as Cu(I) and Cu(II) complexes. As such, numerous copper complexes based on a vast array of ligand donor sets, such as semicarbazone, thiosemicarbazones, Schiff base systems, quinolone, hydrazones, coumarin (thiocarbohydrazone), 1,10-phen and related diamine, phosphines and their derivatives among many other ligand sets, have been developed and investigated for their anti-cancer properties. Unlike a majority of platinum complexes for which the cancer cell killing ability is attributed to the irreparable covalent DNA intercalation, copper complexes induce cancer cell death by a variety of mechanisms such as oxidative stress by ROS elevation (that can lead to oxidative DNA cleavage), proteasome inhibition and noncovalent DNA interactions (including intercalative, electrostatic and groove binding of complexes along major and minor groove). Although
the IC\textsubscript{50} values of several of the copper complexes range from micromolar to sub-micromolar and even nanomolar values, the practical effectiveness of these complexes in the clinical setting, in line with their \textit{in vivo} capability, is yet to be proved.

1.7 Copper Based Nanoparticulates (NPs) for Cancer Treatment

Copper is an essential trace element required for number of biological functions in majority of aerobic organisms. Free copper is potentially toxic to cells due to ability of copper to catalyze the generation of ROS by ‘Fenton-like chemistry’ and is therefore tightly regulated by copper binding proteins and biomolecules such as glutathione and metallothionein. Intracellular copper is predominantly in reduced form and bound to large molecules and proteins that collectively regulate its transport, influx, intra cellular trafficking and efflux.

Copper-based nanomaterials are attractive in the area of cancer therapy. Only a handful of copper based NPs ie. CuO, Cu\textsubscript{2}O, CuS, CuI, and Cu\textsubscript{3}PO\textsubscript{4} NPs were reported in literature for their anti-cancer ability.\textsuperscript{50-54} These NPs were reported to increase the oxidative stress in the cancer cells. The anti-proliferative effect of the copper based NPs is attributed to the ability of surface copper generating ROS by Fenton-like reaction. As shown in the scheme 1.3, Cu(I) undergoes Fenton-like process, i.e. the reduction of H\textsubscript{2}O\textsubscript{2} by Cu(I), to generate hydroxyl radicals. The oxidized copper is reduced back to Cu(I) by Haber-Weiss reaction completing the catalytic cycle with the net effect of generation of hydroxyl radical, hydroxide ion and molecular oxygen.\textsuperscript{55}
Scheme 1-3. Copper catalyzed ROS production.

However, all the aforementioned copper-based NPs reported were bare inorganic nanoparticle cores without any surface stabilization. As was mentioned earlier, providing surface coating on NPs increase the circulation time, prevent aggregation and improve the uptake in the tumor. Considering the fact that copper-based NPs are sporadic but are attractive compared to their molecular counterparts, there is a necessity of adding a more diverse set of rationally designed and better performing copper-based NPs.

1.8 Introduction to PET Imaging

Positron Emission Tomography (PET) is a medical imaging/diagnostic technique in which the tissue or organ of interest is visualized by the localization of positron (positively charged electron) emitting radionuclides. Scheme 1.4 depicts the principle of PET. The positron emitted by the radionuclide undergo annihilation with the nearby electron of the surrounding region, the tissue of interest, thereby generate two gamma photons with equal energy of 511 keV in opposite directions.
Scheme 1-4. Principle of positron emission tomography.

In a clinical setting, a radiotracer/radiopharmaceutical consisting of the positron emitting radionuclide is employed for diagnostic purpose. After injecting the radiotracer into the patient that allows for the localization of radiotracer in the tumor region, the patient is moved through a PET scanner comprising of large ring of detectors. The photons emitted as result of the positron annihilation are detected and the signals from the detector are processed to construct the images of tissue. Several radionuclides that found widespread medical research include $^{11}$C ($t_{1/2} = 20$ min), $^{18}$F ($t_{1/2} = 110$ min), $^{44}$Sc ($t_{1/2} = 238$ min), $^{64}$Cu ($t_{1/2} = 762$ min), $^{68}$Ga ($t_{1/2} = 68$ min), $^{89}$Zr ($t_{1/2} = 4710$ min). The main advantage of the PET imaging technique is its high sensitivity which means a low dose requirement of the radiotracers typically in pico-mole quantities. As an example, $^{18}$F-FDG - currently the predominantly used radiotracer for PET imaging - is required in dosages of 370-740 MBq, which corresponds to approximately 6 pmol. Use of radioisotopes with longer half-lives have specific advantages such as ease of kinetic
studies of the slow biological processes and off-site production of the radiopharmaceuticals away from the medical facility.

1.9 Radiolabeled $^{64}$Cu-Chelate Complexes for PET Imaging and Limitations

Owing to its favorable decay characteristics such as longer half-life ($t_{1/2} = 12.7$ h) and ease of production using both medical generators and nuclear reactors, $^{64}$Cu is currently being extensively studied for its application in PET imaging.

Figure 1.1. Open chain and macrocyclic chelates used for radiolabeling of $^{64}$Cu (reproduced from Ref. 56 with permission from The Royal Society of Chemistry).
This area is predominantly comprised of $^{64}\text{Cu}$-chelates based on macrocyclic ligands.\textsuperscript{56} Thus far, the main focus of many such studies has been devoted to the development of suitable multi-dentate chelates (mostly macrocyclic) and their derivatives (figure 1.1). Several studies have confirmed that copper chelates suffer from \textit{in vivo} ligand exchange with biomolecules \textit{via} ‘transchelation’.\textsuperscript{57} More recently bifunctional chelates have emerged to impart better kinetic and thermodynamic stability to the metal-chelate complexes.

\section*{1.10 Copper Based NPs for PET Imaging}

Studies utilizing $^{64}\text{Cu}$ labeled NPs for PET imaging have emerged in the previous decade most of which include the radioisotope that is linked to these NPs through the chelates chemically conjugated to the surface of NPs. These NPs suffer from the drawbacks, namely detachment of the radionuclide-chelates from the NP surface or the displacement of radionuclide \textit{via} transchelation or both.\textsuperscript{40} Overcoming such challenges while benefiting from the nanotechnology has recently led to the development of intrinsically labeled nanoparticles, in which the radionuclide is incorporated within the NPs itself. Zho \textit{et al.} reported CuS NPs as the first chelate-free multifunctional copper-based nanoplatform that is intrinsically labeled with $^{64}\text{Cu}$ prepared in a hot cell.\textsuperscript{40} More recently, Chakravarthy \textit{et al.} reported a facile approach for the industrial-scale synthesis of intrinsically labeled CuS NPs using the $^{64}\text{Cu}$ radionuclide produced at a low specific activity in a research reactor.\textsuperscript{58} It was claimed by the group that using this approach circumvents the need for use of an expensive medical cyclotron facility to produce high specific activity $^{64}\text{Cu}$ and the requirement of elaborate procedures for radiochemical separation of $^{64}\text{Cu}$ from the irradiated target. Apparently, $^{64}\text{Cu}$ based nanoplatforms have
inherent advantage since the radiolabeling strategy employing the use of ‘exogeneous chelates’ would not be suitable to prepare radiolabeling agents with clinically relevant doses of adequate specific activity using the $^{64}$Cu produced at such low specific activity in a research reactor.

1.11 Introduction to Magnetic Resonance Imaging (MRI) and Contrast Agents

MRI is a sophisticated non-invasive medical imaging technique used in clinical settings for the diagnosis of disease and treatment monitoring in patients. Some of the key features of MRI are the use of non-ionizing radiation (radio waves) and the high resolution with which the three-dimensional anatomical and functional information can be obtained. MRI is based on the difference in the proton ($^1$H nuclei) spin densities in different tissues (primarily from water, the major component in the tissues) including the cancerous tumors and how the protons of water molecules of these tissues undergo relaxation after the nuclear spin moments of protons are briefly aligned by a radiofrequency (RF) pulse along the direction of an applied magnetic field.

Each proton ($^1$H nucleus) has an intrinsic spin angular momentum of half integer value (I=1/2). A spinning charged proton can act as a magnetic dipole and interacts with the external magnetic field. In the absence of an applied magnetic field, a collection of protons, like those of water molecules in a tissue, orient randomly, resulting in a net zero magnetization. However, when an external magnetic field ($B_o$) is applied, the individual dipoles align themselves in the direction of $B_o$ (scheme 1.5 a) and undergo precession about the axis parallel to $B_o$ with a characteristic frequency called Larmor frequency ($\omega_o$) (scheme 1.5b)
Scheme 1-5. a) randomly oriented protons (\(^1\)H nuclei) in the absence of external magnetic field (left) align along the direction of the applied magnetic field (B\(_o\)) (right). b) individual protons undergo precession about B\(_o\) with a frequency \(\omega_0\) called Larmor frequency.

The Larmor frequency is directly proportional to the strength of the magnetic field and is related as

\[
\omega_0 = \gamma \cdot B_o
\]

where \(\omega_0\) is the Larmor frequency in megahertz (MHz), B\(_o\) is the magnetic field strength experienced by the proton in tesla (T) and \(\gamma\) is the proportionality constant for each nucleus called as gyromagnetic ratio. Furthermore, all the individual nuclei populate between the two allowable spins states +1/2 and -1/2 that are parallel (spin-up) or anti-parallel (spin-down) to the applied magnetic field with the relative population of these spin states governed by the Boltzmann distribution and produce a bulk magnetization (M\(_o\)).
Scheme 1-6. a) aligned protons contribute to a net longitudinal magnetization ($M_z$) in the z-direction. Application of RF pulse flip the spins to higher state. b) Flipping of nuclear spins contribute to the transverse magnetization ($M_{xy}$). c) Decay of $M_{xy}$ and loss of phase coherence after the RF pulse is turned off. d) complete restoration of $M_z$. e) $T_1$ relaxation profile corresponding to the restoration of $M_z$. f) $T_2$ relaxation corresponding to the decay of $M_{xy}$.

At equilibrium, the longitudinal component ($M_z$) of the bulk magnetization is a net contribution of nuclei aligned towards $B_0$ (scheme 1.6a) whereas the incoherent precession of these protons results in net zero magnitude of transverse component ($M_{xy}$). Application of RF pulse at 90° to the applied magnetic field at a frequency equal to that of Larmor precession ($\omega_0$) perturbs the equilibrium arrangement of the spin states and causes the rotation of $M_o$ to the transverse plane. The absorption of RF energy by the protons causes the spin flipping with a phase-coherent precession of the protons and result in a transverse component ($M_{xy}$) of non-zero magnitude in the transverse plane (xy plane) as depicted in scheme 1.6.b. Following the excitation, once the RF transmitter is
turned off, the protons emit the energy back at a frequency $\omega_0$ and restore the original spins-state equilibrium through the time dependent process called relaxation (scheme 1.6 c). Consequently, the $M_0$ is restored to its original magnitude provided that no RF pulse is applied before the complete relaxation (scheme 1.6 d). A loop of wire (a receiver coil) placed perpendicular to the transverse plane records the MR signal as a voltage induced by the Free Induction Decay (FID) of the transverse magnetization ($M_{xy}$) during its precession. Two independent processes namely spin-lattice and spin-spin interactions cause the longitudinal and transverse relaxation denoted as $T_1$ and $T_2$ respectively. $T_1$, also called as spin-lattice relaxation, is the time required for the longitudinal component ($M_z$) of the magnetization to return to 63% of the initial value (scheme 1.6.e). $T_1$ provides the mechanism by which the protons give up their energy to return to their original orientation. This return follows an exponential relationship and is given as follows,

$$M(\tau) = M_0 \left(1 - e^{-\tau/T_1}\right)$$

Here $\tau$ is the time following the RF pulse. Generally, $T_1$ values in tissues range from milliseconds to several seconds. Spin-lattice relaxation measures the rate of energy transfer from an excited proton to the surrounding environment. The key to this energy transfer is the presence of certain molecular motions (e.g., molecular rotation, tumbling of proteins) of the lattice nearby the excited proton. If the frequency of molecular motion matches with the resonant frequency ($\omega_0$), the energy transfer is efficient and the relaxation is faster (i.e. $T_1$ is shorter) allowing more protons to equilibrate sooner. Spin-spin relaxation refers to the energy transfer that occur between excited proton and an adjacent proton and follows a time dependent exponential decay of the maximum transverse magnetization ($M_{xy}$) given by the following equation
\[ M_{xy}(t) = M_{xy,\text{max}} e^{-t/T_2} \]

where \( T_2 \), the spin-spin relaxation time, also called as transverse relaxation time, is the time required for the transverse component of the net magnetization to decay to 37% of its initial value through the loss of phase coherence (scheme 1.6 f). It is to be noted that, in MR applications, the net effects observed are the representative of the collection of magnetic moments of all the protons rather than the individual protons themselves. Differences in the relaxation times among the tissues gives rise to signal contrast in MRI. In spite of this inherent contrast, it may not be sufficient to distinguish the pathological from normal tissue. The use of contrast agents (CAs) increases the signal difference and hence the contrast between the host tissue from the surrounding regions. CAs employ paramagnetic species such as \( \text{Gd}^{3+}, \text{Mn}^{2+} \) or \( \text{Fe}^{3+} \) that interact with the electrons and protons of the surrounding water molecules in the tissue and decrease the relaxation times. In general, most MRI CAs are referred to as indirect agents, unlike the computed tomography (CT) or PET scanning agents that are referred to as direct agents. This is because with the use of CT and PET agents, the image of the tissue is constructed based on the direct visualization of the agents localized in the tissue. In MRI, a single paramagnetic ion of CA can affect the relaxation process of large number of protons in the nearby tissue, that in turn enhance the signal intensity and hence are indirectly involved in imaging.

MRI CAs are categorized as \( T_1 \)-weighted and \( T_2 \)-weighted agents based on whether the \( T_1 \) relaxation or \( T_2 \) relaxation rate is accentuated by these exogenous agents. The presence of \( T_1 \)-weighted contrast agents in the tissue accentuate the longitudinal relaxation rate (\( 1/T_1 \)) of the protons nearby and produce a high intensity bright spots of
the localized region in the MR image with better signal-to-noise ratio. Examples of T₁ weighted agents include various gadolinium and manganese chelates and nanoparticles of gadolinium and manganese oxides. T₂ agents, on the other hand, accelerates the transverse relaxation rate (1/T₂) of the water protons and produce a dark spot of the localized region. Examples of effective T₂-weighted CAs include super paramagnetic iron oxide nanoparticles (SPIONs).

1.12 Gadolinium and Manganese Based MRI Contrast Agents, Advantages and Disadvantages

Figure 1.2. Selected Gd-chelate MRI contrast agents (Adapted with permission from ACS publications. Reference: Caravan, P., Gadolinium(III) Chelates as MRI Contrast Agents: Structure, Dynamics, and Applications. Chemical reviews 1999, 99 (9), 2293-2352).

Gd³⁺ has a high spin state (S=7/2) due to seven unpaired electrons and long electronic relaxation time, which makes it a primary choice for MRI scans. However, free
Gd$^{3+}$ is highly toxic and was found to accumulate in liver, spleen, and bones in mice with an LD$_{50}$ ~ 0.2 mmol kg$^{-1}$. Clinical MRI scans predominantly employ gadolinium-chelates. Reported in 1981, Magnevist™ is the first Gd-based MRI contrast agent. Several Gd-chelates were approved as T$_1$ CAs for clinical use (figure 1.2).$^{59}$ These chelates were designed to exert kinetic and thermodynamic stability to the complexes and minimize Gd-associated toxicity to a biologically tolerable level.$^{60}$ However, investigations published in 2006, brought into light the association of Gadolinium chelates in high doses (that were required to achieve high signal-to-noise ratio for better imaging of abdominal region) with nephrogenic systemic fibrosis (NSF), a rare fibrosing condition occurring in few patients with severe renal dysfunction or patients undergoing dialysis.$^{61-63}$ These shortcomings of Gd-based CAs has led to the pursuit of alternative T1-weighted CAs and brought a renewed attention to the manganese-based CAs. Possessing a high spin state of S=5/2 (i.e. 5 unpaired electrons), Mn$^{2+}$ is also desirable for MRI applications. Two Mn-based CAs were approved for clinical use: Mn(II)-dipyridoxyl diphosphate (MN-DPDP, commercial name is TESLASCAN®) as liver specific ‘hepatotropic agent’ and an oral CA of liposomal encapsulated MnCl$_2$ (LumenHance®).$^{64}$ The main drawback of use of Mn(II) complexes is their lability towards efficient sequestration of Mn(II) by biological structures in living organisms.$^{65}$ NPs toward MRI applications offer several advantages such as delivery of high pay load of paramagnetic centers, enhancement in the relaxation rate even at lower doses.
1.13 Research Objectives and Design Criteria of NPs

The objectives of this dissertation work are twofold. The first of the two is to evaluate the anti-cancer properties of a series of copper-based NP systems rationally designed and synthesized via aqueous route utilizing simple salt metathesis reactions in the presence of biocompatible polymers. The second objective is to study a portion of these NPs for their suitability as PET scanning agents. Both the applications of copper-based NPs are relatively underexplored compared to the other inorganic NP systems. In chapter 2, copper tetrathiometolybdate NPs are explored both for anti-cancer properties and suitability for PET applications. In chapter 3, synthesis and anti-cancer potential of Libethentie nanosheets are described. Chapter 4 describes two types of copper Prussian blue analog NP systems. Chapter 5 reports the synthesis and evaluation of copper-manganese Prussian blue analog solid solutions for theranostic potential. There is lack of systematic study that shows the behavior of these NPs under the conditions that represent physiological and tumor intracellular and extracellular milieu. Therefore, for the design and synthesis of the copper based NPs in this dissertation, following criteria are considered.

1.13.1 Size of the NPs

In order to maximize the efficient use of the NPs for the desired in vivo function without putting undue burden of long-term accumulation in internal organs, it is necessary to design NPs with proper accumulation and clearance mechanisms. Therefore, size of the NPs is a major factor.
Scheme 1-7. Illustration of passive targeting by enhanced permeability and retention (EPR) effect and nanoparticle uptake by cancer cell via non-receptor mediated endocytosis.

Regarding accumulation, NPs with sizes below 100 nm can enter through the leaky vasculature and spontaneously accumulate in the tumor area because of the large fenestrations between the endothelial cells of the blood vessels produced by angiogenesis in tumors. This phenomenon of tumor specific and passive localization of particles is called enhanced permeability and retention (EPR) effect (scheme 1-7). With regard to clearance, nanoparticles with sizes below 10 nm are preferable, since particles below this size can effectively undergo rapid glomerular filtration in kidneys.67-68

1.13.2 Polymer coatings

The NPs are designed to have surface coating of a biocompatible polymer during the synthesis. The presence of a hydrophilic, biocompatible polymer will provide protection for NPs from serum protein binding, give longer circulation times in the blood
pool and prevent the NP recognition by opsonins of the reticulo endothelial system.\textsuperscript{16, 69} Employing polymers such as PVP and PEG also provides sufficient hydrophobic organic chains that will facilitate the cellular internalization of NPs via non-receptor mediated endocytosis.

1.13.3 Choice of anionic ligands

The anionic counterparts are chosen to prepare stable NP systems. Moreover, these anions are non-toxic. Tetrathiomolybdate is used for the removal of excess copper in patients suffering from Wilson’s disease. Prussian blue and its analogs are known to be safe in biosystems and can be handled in gram quantities \textit{in vivo}. Phosphates constitutes the main component of the physiological buffer system. Therefore, the use of NPs containing the aforementioned anions poses no harm in the event of degradation of NPs \textit{in vivo}. 
2. Synthesis and Evaluation of PVP Coated Copper Tetrathiomolybdate (\(\text{Cu}_2\text{MoS}_4\)) Nanoparticles for Anti-Cancer and PET Applications

2.1 Introduction

The biological applications of copper-based nanoparticles (NPs) started to emerge at the beginning of this decade. These applications include anti-cancer agents and positron emission tomography (PET) scanning agents.\textsuperscript{70} Only few of these NPs were reported for their intrinsic ability to inhibit cancer cells selectively without the use of an external stimuli. For example, Guo et al. demonstrated that crystalline and amorphous CuS NPs induce apoptotic cell death specifically and significantly in human cancer cells (Hep G2 and HL-60 cells) compared to normal cells (V79-4).\textsuperscript{53} Wang et al. demonstrated the differential cytotoxicity of poly-dispersed cuprous oxide NPs (sizes 40 to 110 nm) with cancerous cells (HeLa and melanoma cells) displaying greater sensitivity than normal human (293T) or mouse (MEF) cell lines.\textsuperscript{52} The cytotoxic effects of copper-based NPs can be attributed to their ability to induce oxidative stress in the host cell by elevating the ROS level. Consequences of oxidative stress include damage to cellular components such as DNA, lipid bilayer and proteins, and reduction in mitochondrial membrane potential. These changes can trigger apoptotic pathways.\textsuperscript{54} It is not clear why the anti-proliferative action of copper-based NPs involves the release of copper ions in
some cases but it is the intact surface copper of the same NP system that causes the cell inhibition in other cases as reported by several groups.\textsuperscript{71-75} Regardless of this, the main requirement of the NP drug for its \textit{in vivo} administration is the stability and the preservation of its active component till it gets internalized by tumor cells.

With regard to the PET applications, intrinsically labeled $^{64}$Cu-containing NPs are preferred since they are chelate-free and overcome the limitations of chelate based $^{64}$Cu radiopharmacueticals and NPs.\textsuperscript{40,58} Furthermore, these NPs are incorporated with high loading of radionuclides per particle compared to $^{64}$Cu-chelate conjugated NPs. In order for the efficient delivery of radionuclide payloads to the targeted area, the NPs have to remain stable until they are internalized into cells. Highly stable and nontoxic NPs are useful as PET scanning agents and are required only in nanomolar to picomolar quantities.

Because of the attractive features of copper-based NPs from the application standpoint, further research is needed in exploration of other copper-based NP systems with good stability for the aforementioned applications, which may eventually translate into clinical use. In this project, we explore the synthesis and anti-cancer potential of the ultra-small polymer coated copper tetrathiomolybdate (Cu$_2$MoS$_4$) nanoparticles (abbreviated as CTM@PVP NPs). The nanoparticles are constructed using a highly thiophilic and soft Lewis acidic Cu$^+$ cation and soft donor sulfide containing tetrathiomolybdate (MoS$_4^{2-}$) anion that gives a highly stable NP system under biologically relevant conditions. Tetrathiomolybdate (TM) is nontoxic and exhibits anti-angiogenic, anti-fibrosis and anti-inflammation effects owing to its potent copper removing capability. TM is also used as an effective drug in the initial treatment of
acutely ill Wilson’s disease patients. Furthermore, the suitability of the NPs for intrinsic labeling of $^{64}$Cu radionuclide intended for PET applications is also evaluated, by performing the stability studies.

2.2 Experimental Section

2.2.1 Materials

Unless otherwise stated, all the chemicals were purchased from Sigma-Aldrich and used as received without further purification. Copper standard (1000 ± 10 μg/mL copper in 3% (v/v) HNO$_3$) was purchased from inorganic ventures and Texas Red cadaverine was purchased from molecular probes. Sodium citrate and citric acid are from Spectrum® and Lab Chem Inc respectively. Regenerated cellulose membranes (MWCO 3500 kDa) and (MWCO 12,000-14,000 kDa) were obtained from Membrane Filtration Products, Inc. (Texas, USA). DI water was obtained from an in-house IONPURE Plus 150 deionization unit. The resistivity of the DI water is at least 17 MΩ-cm.

2.2.2 Synthesis of PVP Coated Copper Tetrathiomolybdate (Cu$_2$MoS$_4$) Nanoparticles (CTM@PVP NPs)

Synthesis of CTM@PVP NPs was performed in a mixed solvent system (water and formamide). 0.027 g (0.105 mmol) of (NH$_4$)$_2$MoS$_4$ was dissolved in 80.0 mL of formamide, resulting in a bright red colored solution, to which 0.5 g of polyvinylpyrrolidone (average m wt. 40,000) was added. DI water was added to the above mixture to facilitate faster dissolution of PVP and the solution was kept stirred. The mixture was filtered to remove the traces of insolubles. Separately, aqueous solutions of 0.027 g (0.2 mmol) CuCl$_2$ (3.0 mL) and 0.0352 g (0.2 mmol) of ascorbic acid (2.0 mL) were
were prepared and mixed together which formed a white suspension of CuCl within few seconds. The suspension was then quickly added to the thiomolybdate solution under vigorous stirring to generate a deep red colored NP dispersion. The final ratio of water to formamide is 1:4 in the system.

2.2.3 Synthesis of PVP Coated Copper (I) Sulfide Nanoparticles Cu₂S@PVP NPs

Same procedure as above except that Na₂S was used as the anionic precursor.

2.2.4 Purification of CTM@PVP NPs and Cu₂S@PVP NPs

The NP mixture was transferred to a regenerated cellulose tubular membrane dialysis bag (MWCO 12,000-14,000) and dialyzed against DI water for 24 hours to remove the byproduct and excess reactant. The outside water was replaced with fresh DI water every half an hour for the first 4 hours, followed by every 6 hours for the rest of the dialysis period. The purified NP dispersion was then lyophilized to obtain pure and dry polymer coated NP sample.

2.2.5 Synthesis of CTM and Cu₂S Bulk Samples

Bulk CTM was synthesized in a similar manner as for nanoparticles except in the absence of polymer. Quantities of the precursor are 2.0 g for CuCl₂·2H₂O and 2.75 g for ATTM respectively. For Cu₂S bulk, the above procedure was followed except that stoichiometric excess of Na₂S was used as anionic precursor.

2.2.6 Atomic Absorption Spectrometric (AAS) Analysis of Copper Content in CTM and Cu₂S NPs

A sample of about 50.0 mg of lyophilized PVP coated NPs was decomposed in 2.0 mL concentrated nitric acid (>90%) and digested overnight. The solution was made
up to a final volume of 100.0 mL in a standard volumetric flask and analyzed by AAS fixed with a copper lamp at a wavelength of 324.7 nm. Copper standards in the concentration range of 1.0 to 7.0 ppm were prepared from an initial stock solution of 1000 ppm copper solution. The standard calibration chart was obtained by plotting the data points of absorbance vs. concentration of copper standards. The data points were fit to a straight line and the average concentration of copper in the sample corresponding to the measured absorbance in triplicate was determined from the standard calibration chart.

2.2.7 Powder X-ray Diffraction Measurements of CTM and Cu$_2$S Bulk Powders

Powder X-ray diffraction patterns (XRD) of the bulk powders of CTM and Cu$_2$S were obtained at room temperature on a X'Pert PRO powder X-ray diffractometer manufactured by PANalytical Inc. (Westborough, MA, USA). The experimental setup used Bragg-Brentano geometry in θ-θ configuration, copper as a radiation source (Cu K$_\alpha$ radiation), and diffracted beam curved crystal monochromator to eliminate Cu K$_\alpha$. The pattern was collected in a range of 2θ values from 15.00° to 80.00° with a step size 0.05°.

2.2.8 TEM and EDX Measurements

A small amount of lyophilized NP sample was dispersed in 95% ethanol and sonicated for 10 minutes. TEM specimen was then prepared by placing a droplet of sonicated NP solution on to a carbon coated copper TEM grid (400 mesh) and left to air-dry before use. Particle images and energy dispersive X-ray (EDX) spectra were acquired using a 200 kV FEI Technai F20 Transmission Electron Microscope (TEM) equipped with a field emission gun and an attached EDAX spectrometer in STEM mode. The operating voltage and current of the instrument were 4000 V and 55 μamp respectively.
2.2.9 Copper Release Studies in Citrate and PBS Buffers

The stability of CTM NPs was studied in a 0.5 M citrate buffer at pH values of 4.8 and 6.0 along with PBS (pH 7.2). The initial pH of the prepared citrate buffer was 4.4 and was adjusted to the desired final pH using concentrated NaOH solution. PBS was employed for the study at pH 7.2. Typically, 50 mg of pure and dry CTM NPs were transferred to a pre-processed regenerated cellulose membrane dialysis bag (MWCO 3,500-4,000) that was sealed on one end. 5.0 mL of the buffer solution was added to the bag and the other end of the bag was sealed. The bag was then suspended in 95.0 mL of the buffer solution under mild stirring. Starting at \( t = 0 \) minutes, aliquots in 2.0 mL were drawn from the external buffer every 20 minutes for the first two hours followed by an interval of one hour for the next 8 hours. A final sample was drawn after a period of 24 hours. All the samples were analyzed for the released copper using AAS. Volume correction for each successive sample was applied and the values were reported in ppm.

2.2.10 Synthesis of Texas Red Cadaverine C5 Dye Conjugated CTM and Cu2S NPs

Dye labeling was performed post synthesis of the nanoparticles. 100 \( \mu \)L of 1.0 mM citric acid and sodium citrate solution was added to 500 \( \mu \)L of 0.32 mM CTM NPs and stirred for an hour. Meanwhile, mixtures of 0.5 mg of Texas Red C5 dye in 100 \( \mu \)L DI water and 0.5 mg of N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDAC) in 100 \( \mu \)L DI water were homogenized together by sonication for 5 minutes to generate EDC coupled dye. The mixture was then added to the citric acid coated NP dispersion and continued to stir overnight to obtain dye labeled CTM NPs. The unreacted dye was separated from the dye labeled NP solution by dialysis of the reaction mixture against DI water for 24 hours. During the whole preparation and purification, the
solutions were stirred in the dark to avoid the degradation of the light sensitive dye. An aliquot of the dialyzed NP dispersion was subjected to fluorescence spectrophotometry to confirm the conjugation of dye to the CTM NPs. Dye labeled Cu$_2$S NPs were also prepared following the above procedure.

2.2.11 Cell Culture and Anti-Proliferation Studies in Cancer Cell Lines

Human colorectal adenocarcinoma (HT29) cell line was used for the anti-proliferation activity studies. HT29 cells were cultured in a complete growth medium prepared from McCoy’s 5A medium by supplementing with 10 % Fetal Bovine Serum (FBS) and 1 % antibodies. Cells were incubated at 37 °C in water-saturated air supplemented with 5 % CO$_2$. Spent medium from the flasks was replaced every three days with fresh complete growth medium and the cells were passaged at 90 % confluence. Cell counting was performed by trypan blue exclusion method using a hemocytometer. The anti-proliferation activity of the CTM@PVP and Cu$_2$S@PVP NPs was evaluated by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay. Typically, cells were seeded in a 96 well-plate at a cell density of 15,000 cells/well and incubated at 37 °C in water-saturated air supplemented with 5 % CO$_2$ for 24 hours. Drug solutions of desired concentrations were prepared by diluting the NP aqueous dispersions in the complete growth medium. Cells in each well were then treated with the 100 $\mu$L of drug solution of appropriate concentration of NPs and incubated at 37 °C in water-saturated air supplemented with 5 % CO$_2$. The experiment was performed in triplicate of wells for each concentration of the NPs. Control cells were also grown in the same well-plate without the treatment of drug. After 24 hours, 20 $\mu$L of MTT dye was added to each of the well and incubated further for 4 hours. Finally, the spent medium
was aspirated out followed by the addition of 150 μL of DMSO to each well to dissolve the purple formazan crystals. The absorbance of the solution in each well was measured at 560 nm using a microplate reader. Cell viability at each concentration was calculated from the ratio of the absorbance of the drug treated sample to that of control and expressed as percentage along with the deviation.

2.2.12 Cellular Uptake Studies of Dye Labeled CTM and Cu2S NPs Using Confocal Microscopy

PC3 cells were seeded at a density of 100,000 cells/mL onto a coverslip placed in 6 well-plate and incubated for 24 hours at 37 °C in water-saturated air supplemented with 5% CO2. The coverslips were pre-coated with poly-L lysine before use to facilitate better cell adherence. The spent medium was then aspirated off, followed by addition of different dilutions of dye labeled NPs starting from 100 μM dispersed in growth medium. The control comprised of cells without the treatment of the dye labeled NPs. After incubation of the cells for 4 hours, the medium containing the NPs was removed and the cells were washed with PBS twice to remove the traces NPs. The coverslips were then glued on a microscope glass slide. Confocal images were obtained using an Olympus IX81 microscope with 40X oil objective in the z-scanning mode. The microscope was equipped with an Olympus DSU (disk scanning unit).

2.2.13 Quantification of Intracellular Reactive Oxygen Species (ROS) Elevation by CTM NPs Using DCFDA Assay

2′,7′-dichlorofluorescein diacetate (DCFDA) assay is widely used for the study of intracellular ROS production. DCFDA is a cell permeable non-fluorescent precursor of fluorescent dye dichlorofluorescein (DCF). DCF is cell-impermeable. Upon entering the
cells, DCFDA is hydrolyzed by esterase that remove the DA group. The resultant H$_2$DCF is then converted by cellular oxidants to DCF, the fluorescence of which is therefore a measure of intracellular ROS level. In this study, HT29 cells were seeded in a 96 well-plate at a cell density of 5X10$^5$ cells per well and incubated for 24 hours at 37 °C in humidified air supplemented with 5% CO$_2$ to facilitate the cell attachment. Sample cells were then treated with 100 $\mu$L of McCoy’s complete growth medium containing NPs of varying concentrations ranging from 400 $\mu$M to 50 $\mu$M and incubated for 4 hours. This step was followed by removal of medium and washing the cells with Hank’s balance salt solution (HBSS) twice. Cells in each well were then exposed to growth medium containing 40 $\mu$M DCFDA for 30 minutes followed by removal of medium and washing twice with HBSS. Control1 consisted of cells untreated with NPs and DCFDA. Control 2 consisted of cells treated with DCF-DA but not with NPs. It is to be noted that instead of using standard aspiration techniques to remove the medium/washings from well-plate, the medium/washings was expelled from the wells by sudden inverting of the well-plate at once while holding the well-plate firmly. It was ensured that no residual medium or wash solution is left before proceeding to the next step. Also, at each step, no detachment of the cells from the wells observed. Finally, 200 $\mu$L of HBSS was added to each well and the initial fluorescence readings were taken immediately using a microplate reader. Successive readings were taken at 0.5, 1.0, 2.0, 4.0 and 5.0 hours respectively from the initial reading. During the whole procedure, the well-plate was kept out of light to minimize the fading of the fluoroprobe. The experiment was run in quadruplicates of wells. The ROS production at the above time intervals was quantified as percentage increase in fluorescence relative to that of the average initial fluorescence of control 1.
Statistical analysis was made by comparisons of data for cells treated with both NPs and DCFDA against control 2 using a two-tailed unpaired Student’s t-test.

2.3 Results and Discussion

2.3.1 Synthesis and PXRD Characterization of CTM Bulk

As reported by a few groups in the last few years, the synthesis of Cu$_2$MoS$_4$ is generally performed in a mixture of organic solvents in solvothermal or in reflux conditions under nitrogen atmosphere. For example, Crossland et al. reported the first synthesis of Cu$_2$MoS$_4$. Their procedure involved the reaction of a Cu(I) salt namely Cu(CH$_3$CN)$_4$BF$_4$ and (NH$_4$)$_2$MoS$_4$ in a 1:1 mixture of butyronitrile and N,N-dimethylformamide in an autoclave between 110 and 220 °C.\textsuperscript{77} Tran et al. reported the synthesis of highly crystalline Cu$_2$MoS$_4$ adapting the procedure used by Pruss et al. They obtained the crystalline product by refluxing a solution containing (NH$_4$)$_2$MoS$_4$ with 2 equivalents of [Cu(CH$_3$CN)](BF$_4$) in DMF-acetonitrile or DMF-isobutynitrile solvent mixtures for 2 hours at 135 °C and under a nitrogen atmosphere.\textsuperscript{78} In a recent article, Zhang et al. reported the use of different precursors and solvent system. They obtained the highly crystalline product under solvothermal conditions by reacting mixture of Na$_2$MoO$_4$, thioacetamide (C$_2$H$_5$NS) and Cu$_2$O in ethylene glycol at 160 °C for 12 hours.\textsuperscript{79} Also, recently Zhang et al. reported the synthesis of nanobelts of Cu$_2$MoS$_4$ via hydrothermal method using sacrificial Cu$_2$O and (NH$_4$)$_2$MoS$_4$ precursor at 160 °C.

Contrary to the above synthetic procedures, we attempted to prepare CTM bulk at room temperature by a simple salt metathesis reaction between Cu$^+$Cl and (NH$_4$)$_2$MoS$_4$ in mixed solvent conditions. The synthesis was accomplished in a facile two step reaction as depicted in scheme 2.1. In the first step, Cu$^{2+}$ salt was reduced in the presence of ascorbic
acid (L-AscH) in DI water to form suspension of CuI\textsubscript{Cl}. In the second step, treatment of the aqueous suspension of sparingly soluble CuI\textsubscript{Cl} with MoS\textsubscript{4}\textsuperscript{2-} in 4:1 mixture of formamide and water yielded the deep reddish brown precipitate of the product Cu\textsubscript{2}MoS\textsubscript{4} instantly.

**Scheme 2-1. Two-step synthesis of CTM bulk.**

Powder XRD diffraction data obtained for the purified bulk CTM is shown in figure 2.1. The peak positions match well with that reported by Crossland *et al.*\textsuperscript{77} All the peaks were identified and the corresponding Miller indices were assigned based on the published PXRD pattern by Zhang *et al.* The broad peaks of the diffractogram reflect the poorly crystalline nature of the product obtained by the precipitation reaction at room temperature. Since, the crystallinity of the NPs is not a prerequisite for the intended applications as anti-cancer agent and diagnostic agent, no attempts were made to further improve the crystallinity. A study conducted by Guo *et al.* regarding the effect of crystallinity on anti-proliferation effects of CuS NPs showed that amorphous CuS NPs have “significantly stronger anti-proliferation and apoptosis inducing activities” than the nanocrystals of the same material.\textsuperscript{53}
2.3.2 Synthesis and Characterization of CTM@PVP NPs

The NPs were synthesized following the similar procedure for the preparation of bulk but in the presence of the biocompatible polymer called polyvinylpyrrolidone (PVP) (see experimental section for details). To slow down the decomposition of MoS$_4^{2-}$ precursor in aqueous solution, the synthesis was performed in a mixed solvent system of 4:1 formamide and water. After the completion of reaction, the solvent (formamide) along with the excess, unbound polymer was removed by extensive dialysis against DI water. The NPs are extremely stable in water when re-dispersed with no visible aggregation observed even after several months.

2.3.3 Mechanism of CTM@PVP NP formation

The following unverified mechanism is proposed to illustrate the formation and growth of NP from the two-step reaction starting from CuCl$_2$ (scheme 2-2).
Proposed mechanism for the formation of CTM NPs.

In the first step, reduction of CuCl₂ by ascorbic acid produces the suspension of sparingly soluble cuprous chloride (CuCl) particles (depicted in grey). The CuCl particles are in equilibrium with the dissociated Cu⁺ and Cl⁻ ions present in extremely low concentrations. In the next step, the highly thiophilic and soft Lewis acid Cu⁺ ions strongly interact with MoS₄²⁻ ligand and give rise to nuclei of the insoluble product Cu₂MoS₄ (depicted in deep red). The consumption of the dissociated Cu⁺ ions in the presence of MoS₄²⁻ serves as a driving factor for the further dissociation of CuCl particles into Cu⁺ and Cl⁻ ions to maintain the equilibrium. As the dissociation progress, the size of the CuCl particles continues to decrease with the concomitant growth of the of CTM nuclei into larger particles. The process continues till the entire CuCl solid is consumed. The polymer PVP present in the solution effectively coats the surface of CTM NPs and prevents them from aggregation.
Figures 2.2 display the TEM image of CTM@PVP NPs (top) and the particle size distribution (bottom). The NPs are poly-dispersed with irregular shapes and have an average size of 5.5 ± 1.8 nm (based on the longest dimension of the particles). EDX spectral analysis of the NPs confirmed the presence Mo and S of the CTM@PVP NPs.
and O and C of the polymer (figure 2.3). The presence and amount of copper in the redispersed NP sample solution was independently confirmed by AAS.

![EDX spectrum of CTM NPs](image)

**Figure 2.3. EDX spectrum of CTM NPs.**

The presence of polymer coating was confirmed by FTIR spectroscopy. The IR spectra of ammonium tetrathiomolybdate (ATTM), bulk CTM, PVP and CTM@PVP NPs are displayed in figure 2.4. A comparison of IR spectra of NPs and pure polymer (PVP) reveals that all the notable bands due to the polymer are also present in the spectrum of NP sample. In particular, the C=O stretching vibration of the PVP amide unit at 1650 cm\(^{-1}\) for pure polymer can be observed at 1652 cm\(^{-1}\) for CTM@PVP NPs. No peaks from that of the CTM were found distinctly in the spectra for NPs.
Figure 2.4. IR spectra of ammonium tetrathiomolybdate (orange), PVP polymer (blue), CTM@PVP NPs (magenta) and CTM bulk (black).

2.3.4 Synthesis of Cu$_2$S Bulk and Cu$_2$S@PVP NPs

Synthesis of Cu$_2$S bulk was performed by following the method used for the preparation of CTM Bulk.

**Step 1:** \[ 2 \text{CuCl}_2(\text{aq}) \xrightarrow{\text{L-AscH, RT}} 2 \text{CuCl}(s) \]

**Step 2:** \[ 2 \text{CuCl}(s) + \text{Na}_2S(\text{aq}) \xrightarrow{4:1 \text{formamide-water, RT}} \text{Cu}_2S(s) \]

Scheme 2-3. Two-step synthesis of Cu$_2$S bulk.

The facile two-step reaction involved the reduction of Cu(II) salt using L-ascorbic acid to form insoluble Cu$^1$Cl microparticles as the first step and the subsequent metathesis reaction of the microparticle suspension with Na$_2$S in 4:1 formamide-water solvent mixture as the second step yielded the Cu$_2$S bulk precipitate (reaction scheme 2.3). Since
the sulfide precursor used was highly hygroscopic, it was used in stoichiometric excess quantity to ensure the complete reaction of the CuCl. The synthesis of Cu$_2$S@PVP NPs was carried out in the presence of polymer PVP (avg. m wt. 40,000). After the reaction was complete, the excess reactant along with the unbound polymer was separated by extensive dialysis.

Figure 2.5. TEM image of Cu$_2$S@PVP NPs (top) and particle size distribution (bottom).
A TEM image obtained for the dialyzed product of the Cu$_2$S@PVP NPs is displayed in figure 2.5. The particles are poly-dispersed with irregular shapes and have an average size of 5.6 ± 2.5 nm. The average particle size of Cu$_2$S@PVP NPs was found to be same as CTM@PVP NPs (5.5 ± 1.8 nm) within their standard deviations. It is to be noted that except for the sulfide precursors i.e. MoS$_4^{2-}$ for CTM and S$^{2-}$ for Cu$_2$S NPs, all other reaction conditions for the respective syntheses were identical. Therefore, the mechanism of NP particle formation is possibly similar to that for CTM@PVP NPs. Cu$_2$MoS$_4$ and Cu$_2$S belong to the metal chalcogenide family and the similar sized NPs of which allowed us to compare the anti-proliferation activity of both CTM and Cu$_2$S against a particular cancerous cell line.

**Figure 2.6. EDX spectrum of Cu$_2$S@PVP NPs.**

EDX spectrum showed the signals of elements S from the NPs and C, N and O of the polymer (figure 2.6). The presence of copper was verified separately by AAS. Powder
XRD pattern obtained for the bulk sample could not be matched to a single phase Cu$_2$S (figure 2.7).

**Figure 2.7. PXRD pattern of Cu$_2$S bulk.**

Since there are large numbers of non-stoichiometric copper (I) sulfide phases reported in powder XRD database, Cu$_2$S bulk is likely a mixture of two or more phases of copper (I) sulfide. It is to be noted that Cu$_2$S@PVP NPs are intended for comparison purposes only, and because the bulk and NPs are synthesized following the respective identical conditions as for that of the bulk and NP of CTM, no attempts to further optimize the phase purity of the product were made, which otherwise would deviate the synthesis conditions from the original. The basis for our argument that the bulk and NP belong to copper (I) sulfide (i.e. Cu$_2$S) stems from the empirical observations made regarding the stability of CuS and Cu$_2$S NPs. Cu$^{II}$S@PVP NPs, prepared directly by treating the CuCl$_2$ salt with MoS$_4^{2-}$ ions, resemble the olive green colored Cu$_2$S@PVP NP.
dispersion but gradually disintegrated with time, eventually changing its color to pale blue. On the other hand, the color of Cu$_2$S@PVP NPs remains intact as olive green. Such an observation can be attributed to the difference in the Lewis acid character of Cu(II) and Cu(I) ions and the consequent thiophilicity. Due to its soft Lewis acid character, Cu(I) is more thiophilic compared to Cu(II) and forms stronger interactions with S$^{2-}$-ion, forming a stable nanodispersion. Cu(II) is a borderline Lewis acid and therefore the initially formed CuS dispersion decomposed gradually due to the hydrolysis.

2.3.5 Stability of CTM NPs in PBS and Citrate Buffers

The stability of CTM@PVP NPs was assessed at different pH values relevant to physiological and tumor conditions. PBS was employed for study at pH value of 7.4 and represents physiological pH and ionic strength. 0.5 M citrate buffer was used for studies at pH values of 4.8 and 6.2. These pH values represent the acidic tumor site and endosomal proton concentrations respectively.\textsuperscript{80-81} Experimentally, the NP dispersion sealed in dialysis bag was soaked in the buffer solution of desired pH value. The samples of the buffers were analyzed for copper content at regular intervals over a period of 8 hours. As shown in the figure 2.8, at pH values of 4.8, 6.2, and 7.4, the copper released was below the detectable level of the instrument. Further analysis of the buffer samples after 24 hours shows no signs of copper release. Therefore, the studies confirm the extreme stability of CTM@PVP NPs against copper leaching.
Figure 2.8. Copper release profile of CTM@PVP NPs at biological relevant pH values 4.8 (red circles), 6.0 (blue squares) and 7.4 (black triangles). Theoretical maximum release is shown in dotted line.

2.3.6 Cellular Uptake of CTM NPs

Confocal microscopy imaging was performed to determine the cellular internalization of the CTM@PVP NPs. Since, the NPs are non-fluorescent, the NPs were surface conjugated with the fluorescent Texas Red Cadaverine C5 (TRC5) dye. This was achieved by first coating the NPs with citric acid molecules on the surface followed by EDC coupling of the dye with the activated carboxyl groups. The dye labeled CTM NPs were separated from residual unbound dye by extensive dialysis against DI water. Figure 2.9 displays the emission spectrum of the CTM NPs@PVP-TRC5. The emission at $\lambda = 608$ nm is typical of the Texas Red dye and confirms the surface conjugation of the dye.
Figure 2.9. Fluorescence spectrum of Texas Red C5 dye labeled CTM NPs.

Figure 2.10 displays the bright field and confocal fluorescence images of the PC3 cells acquired in z-scanning mode of the instrument settings.

Figure 2.10. a) and b) are the bright field and confocal images of control PC3 cells respectively. c) and d) are the bright field and confocal images of PC3 cells treated with Texas Red C5 dye labeled CTM NPs respectively.
The bright field images of the control (untreated) and NP treated cells are shown in the left panel respectively. Confocal images (right panel) of control cells (untreated) did not exhibit any fluorescence. In contrast, red fluorescence signals in the perinuclear area for the PC3 cells treated with dye labeled CTM NPs for 3 hours were observed in confocal mode and confirm the uptake of NPs. The NPs were likely internalized and distributed in the cytoplasmic region of the cells.

2.3.7 Anti-proliferation Studies of CTM@PVP NPs and Cu$_2$S@PVP NPs

The anti-proliferation effects of CTM@PVP and Cu$_2$S@PVP NPs was studied in HT29 (human colorectal carcinoma) cancer cell line using MTT colorimetric assay.

![Cytotoxicity profile of CTM@PVP NPs in HT29 cells (human colorectal carcinoma) and HEK293 cells (normal kidney cells).](image)

**Figure 2.11.** Cytotoxicity profile of CTM@PVP NPs in HT29 cells (human colorectal carcinoma) and HEK293 cells (normal kidney cells).
Furthermore, the cytotoxicity effect of CTM@PVP NPs was also assessed in normal kidney cells (HEK 293). Clearly, in the same concentration range of the NP drug studied, the anti-proliferation effect of the CTM@PVP NPs is more pronounced in cancer cells than in normal cells after 24 hours of treatment (figure 2.11).

Cu$_2$S@PVP NPs also exhibited similar cytotoxicity profile in HT29 cells. (figure 2.12) The IC$_{50}$ value is defined as that concentration of the drug at which 50% of the cells are inhibited (hence 50% cell survival) with respect to the control.

![Cytotoxicity profile of Cu$_2$S@PVP NPs in HT29 cells (human colorectal carcinoma).](image)

Using sigmoidal curve fit (not shown), the IC$_{50}$ values for both the NPs were estimated from the dose-response curves of each cell line. The IC$_{50}$ values of CTM@PVP and Cu$_2$S@PVP NPs observed for HT29 cell line were 24.1 ± 7.1 and 27.9 ± 5.9 μg mL$^{-1}$,
respectively. The corresponding values in μM are 375 ± 112 and 439 ± 93, respectively. Since, the surface copper ions are responsible for the anti-proliferation activity, the similar IC$_{50}$ values (within the deviation) of both type of NPs can be attributed to the same size of the NPs and the possible similarity in the surface copper environment of the NPs.

Table 2-1. Comparison of IC$_{50}$ values of CTM and Cu$_2$S@PVP NPs with CuS NPs from literature.

<table>
<thead>
<tr>
<th>Nano system</th>
<th>Size (nm)</th>
<th>IC$_{50}$ (μM)</th>
<th>Cell line</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTM@PVP NPs</td>
<td>5.5 ± 1.8</td>
<td>375 ± 112</td>
<td>HT29</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6282 ± 118</td>
<td>HEK 293</td>
<td></td>
</tr>
<tr>
<td>Cu$_2$S@PVP NPs</td>
<td>5.6 ± 2.5</td>
<td>439 ± 93</td>
<td>HT29</td>
<td>This work</td>
</tr>
<tr>
<td>CuS ANPs</td>
<td>50</td>
<td>292</td>
<td>HL-60</td>
<td>Guo et al. $^{53}$</td>
</tr>
<tr>
<td></td>
<td>53</td>
<td>18.53</td>
<td>Hep G2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.39</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CuS NCs</td>
<td>60</td>
<td>460</td>
<td>HL-60</td>
<td>Guo et al. $^{53}$</td>
</tr>
<tr>
<td></td>
<td>181</td>
<td>29.26</td>
<td>Hep G2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.95</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A further comparison was made with the IC$_{50}$ values of CuS NPs (another chalcogenide system) reported by Guo et al. also shown in table 2.1. The authors demonstrated in two different cancer cell lines that the anti-proliferation effects of CuS amorphous nanoparticles (ANPs) were significantly stronger than the CuS nanocrystals (NCs) of
roughly similar size. It appears from the comparison that Cu(II) chalcogenides, irrespective of crystallinity, exhibit better anti-proliferation effect than Cu(I) chalcogenides.

2.3.8 Catalytic ROS generation of CTM@PVP NPs

![Graph showing ROS generation](image)

**Figure 2.13.** Quantification of intracellular ROS elevation by CTM NPs after 4 hours of incubation in HT29 cell line using DCFDA assay. *** indicates the statistically significant difference between NP treated cells and control (p<0.001) at 95% confidence interval (CI); ### indicates the statistically significant difference between cells treated with 25 μM CTM NPs and 100 μM CTM NPs (p<0.001) at 95% CI; ^^^ indicates the statistically significant difference between cells treated with 100 μM CTM NPs and 400 μM CTM NPs (p<0.001) at 95% CI.

It is reported that copper based nanoparticles exhibit cytotoxicity toward cancer cells by increasing the intracellular ROS level.\textsuperscript{50, 54, 82} To test whether surface copper of CTM@PVP NPs can cause the elevation of intracellular ROS level, a widely used DCFDA assay was employed. 2',7'-dichlorofluorescein diacetate (DCFDA) is a cell permeable non-fluorescent precursor which is oxidized by cellular ROS to produce cell
impermeable 2’,7’-dichlorofluorescein (DCF) dye with an emission wavelength at 525 nm. The emission intensity is therefore a measure of cellular ROS level. The assay was conducted in a dose dependent manner. After treatment of HT29 cells with the NPs for 4 hours followed by a brief exposure to the dye precursor DCFDA, the fluorescence intensities at 525 nm were measured and compared to the untreated cells. As shown in figure 2.13, CTM@PVP NPs caused a significant increase in emission intensity w.r.t. the control cells in a dose dependent manner. The results suggest that upon cellular uptake NPs can cause increased oxidative stress by elevation of ROS level in cancerous cell lines. This can be understood by the catalytic redox cycle that involves the Fenton-like redox reaction mediated by surface copper Cu⁺ of CTM@PVP NPs in the presence of cellular H₂O₂ and Haber-Weiss reaction for reduction of the formed Cu²⁺ ion by superoxide back to Cu⁺ with a net generation of hydroxide radicals in the overall catalytic cycle. The increase in oxidative stress due to the elevation of ROS level might cause the irreparable damage of cellular components and lead to apoptosis.
Since the catalytic cycle for the elevation of ROS involves the redox changes of Cu (I) to Cu (II) and back to Cu (I), it was predicted that the catalytic cycle would be more efficient in the presence of a reducing agent such as vitamin c (L-ascorbic acid) that can promote the Cu(II) reduction to Cu (I) and improves the catalytic efficiency of the ROS elevation. This would in turn lower the IC\textsubscript{50} of the NP drug. To test this possibility, \textit{in vitro} cell viability study involving the co-treatment of NP drug together with excess of vitamin C (5 and 10 times) in HT29 cells was performed and the cell viability after 24 hours of treatment was evaluated by MTT assay. Figure 2.14 displays the results of the MTT assay for co-treatment. Contrary to our prediction, in the presence of vitamin C the cell viability was observed to be same as that of untreated control cells within the standard deviations. Vitamin C is a known antioxidant capable of scavenging ROS in
living systems. Therefore, it appears from the combinatorial study that the ROS scavenging effect of vitamin C is more dominant than the promotive effect on redox catalytic cycling. From a different perspective, this observation is a confirmation that cancer cells are more vulnerable to the elevation of oxidative stress induced by copper based NPs. Our observation is consistent with that formerly reported by Fahmy et al. using CuO NPs. They studied the effect of co-treatment of CuO NPs and antioxidant resveratrol on cell viability of human laryngeal epithelial cells (HEp-2 cells) and found higher cell viability in the presence of resveratrol (an antioxidant) compared to the CuO treatment.\textsuperscript{71} This altered cell viability was attributed to the decreased oxidation of phospholipids of HEp2 cells by CuO NPs in the presence of resveratrol. It may also be inferred from the present study that a more effective strategy to inhibit cancer cells proliferation would be a combinatorial treatment that involves both the simultaneous elevation of cellular ROS level and depletion of cellular antioxidant reservoir inside the cancer cells.

2.3.9 Evaluation of CTM NPs for PET applications

The high stability of CTM@PVP NPs at different pH values prompted us to evaluate its suitability towards PET scanning agents. For this purpose, synthesis of CTM NPs was modified to include surface coating with citric acid along with PVP to render the NPs biocompatible at concentrations below 100 $\mu$M. The resultant NPs were labeled as RLCTM@PVP NPs. The biocompatibility of the NPs was evaluated in HeLa cell line. As shown in figure 2.15, average cell viability results of NP treated HeLa cell after 24 hours determined by MTT assay from three independent trials, confirm the NPs are biocompatible even at the highest concentration (80 $\mu$M of copper).
Figure 2.15. Viability of He La cells after incubation with RLCTM@PVP-Cit NPs for 24 hours determined by MTT assay.

This highest concentration studied is at least three orders of magnitude higher than the concentrations required for clinical PET applications.
The stability of NPs towards copper leaching was studied in several media. RLCTM NP dispersions of total copper concentration of 3.0 mM sealed in dialysis bags were soaked in DI water, PBS, citrate buffer (pH values of 4.8 and 6.2) and $M^{2+}$ solution of 100 ppm concentration (where $M^{2+} = Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}$ and $Zn^{2+}$) at 37 °C. The media were analyzed for copper content by AAS after 4 hours and 24 hours. The copper released expressed as the percent of copper w.r.t to the theoretical maximum at both time points for all the media is shown in figure 2.16. The highest percent copper release is only 0.4 % (in $Ca^{2+}$ solution) which is considered negligible. Therefore, copper leaching studies confirmed the high stability of RLCF2@PVP-Cit NPs indicating its suitability for the preparation of intrinsically labeled NPs.

The cellular uptake of RLCTM@PVP-Cit NPs was confirmed by confocal microscopy using Texas Red cadaverine C5 labeled NPs in HeLa cells. The bright red fluorescence was observed from the perinuclear region of the cells treated with dye labeled NPs (figure
2.17, bottom right) after 4 hours of incubation which indicates the localization of NPs in the cytoplasmic region upon internalization. In contrast, control cells did not exhibit any fluorescent signals.

Figure 2.17. Top images: Bright field (left) and confocal images (right) of control HeLa cells. Bottom: Bright field (left) and confocal images (right) of HeLa cells treated with Texas Red C5 dye labeled RLCTM@PVP-Cit NPs.

2.4 Conclusion

In conclusion, PVP coated nanoparticles of CTM were successfully synthesized at room temperature in a mixed solvent system of water and formamide. The poorly crystalline CTM NPs exhibit anti-proliferation effect in HT29 (human colorectal carcinoma) cancer cell line by inducing elevated oxidative stress but is less toxic to the normal kidney cells. Furthermore, the surface coating of CTM NPs was modified to render them biocompatible. The biocompatibility and high stability makes these surface
modified NPs suitable for preparing intrinsically labeled $^{64}\text{Cu}$ containing CTM NPs for PET scanning agent.
3. Synthesis, Characterization and Evaluation of Anti-Cancer Potential of Libethenite (Cu$_2$(OH)PO$_4$) Nanosheets

3.1 Introduction

First discovered in 1823 the mineral Libethenite, which is copper hydroxy phosphate (Cu$_2$(OH)PO$_4$, abbreviated as CHP), belongs to an orthorhombic crystal system with unit cell parameters of $a = 8.062$, $b = 8.384$ and $c = 5.881$ Å. Owing to its special crystal structure, this compound has interesting optical, magnetic and catalytic properties and therefore has been studied for a variety of applications in the recent times. In particular, numerous catalytic and photocatalytic applications of CHP have been reported over the last decade. For example, copper hydroxy phosphate (CHP) has been found to exhibit promising activity as a catalyst for water oxidation with turnover frequency similar to that of several common oxygen evolution reaction (OER) catalysts. Photocatalytic applications of Libethenite include degradation of dyes under UV-visible and near-infrared (NIR) light. Other catalytic applications are the oxidation of olefins, alcohols and azo dyes in the presence of molecular oxygen and oxidizing agents like H$_2$O$_2$. Several of these oxidation reactions involved the generation of hydroxyl radicals by CHP which acts as a catalyst that increases the conversion of the substrates. Xiao et al. reported that hydroxyl radicals resulting from the interaction of Cu-OH of the CHP catalyst with H$_2$O$_2$ are important intermediates.
responsible for high activity of the catalyst towards the hydroxylation of phenol, benzene and oxidation of naphthol and benzene.\textsuperscript{92} Based on the electron spin resonance (ESR) investigations, it was found that catalytic conversion increased with the increased concentration of hydroxyl radicals. Such catalytic activity was not observed with the use of a widely popular zeolitic catalyst TS-1. It was suggested that Cu\textsubscript{2}(OH)PO\textsubscript{4} decomposed H\textsubscript{2}O\textsubscript{2} to generate a high concentration of hydroxyl radicals, which may be responsible for the high catalytic activity. Similar observations were also reported by others later. Using IR and ESR techniques, Meng \textit{et al.} characterized the catalytic oxidation of olefins and alcohols by molecular oxygen over CHP catalyst.\textsuperscript{91} The results confirmed the formation of intermediate hydroxyl radicals over the surface of CHP that may be responsible for the high catalytic activity of CHP. In contrast, TS-1 catalyst was found to be inactive for the above reaction.

Since our research objective is to develop new copper based nanoparticulate systems capable of modulating the intracellular ROS level in cancerous cells, the aforementioned observations reported by several groups regarding the possible formation of intermediate ROS at the active catalytic sites on the surface of CHP that promote heterogeneous Fenton-like oxidation reactions seems to be favorable to explore the NP form of CHP as potential anti-cancer agent. We propose that CHP in its nanoparticulate form is an effective anti-cancer agent due to its ability to generate ROS. Unlike several metal-based NP systems where biological clearance is an issue, CHP could be a safe alternative because in the event of degradation of CHP either by the macrophages or by lysosomes, the degradation products i.e. Cu\textsuperscript{2+} ions and phosphate ions can easily be handled by the body without leaving any toxic residues. This is possible since excess
cellular copper can be pumped out by the copper transporter systems and the higher copper level in the blood are reduced by the copper metabolic function of liver to restore the copper homeostasis. This project explores the synthesis of colloidal CHP stabilized by a biocompatible polymer and its anti-cancer applications. To the best of our knowledge neither colloidal CHP nor the biological applications of CHP were reported previously in literature.

3.2 Experimental Section

3.2.1 Materials

Unless otherwise stated, all the chemicals were purchased from Sigma-Aldrich and used without further purification. Copper standard (1000 ± 10 μg/mL copper in 3% (v/v) HNO₃) was purchased from inorganic ventures, Texas Red cadaverine was purchased from molecular probes, dextran T10 was a generous gift from China. Sodium citrate and citric acid were purchased from Spectrum® and Lab Chem Inc respectively. Regenerated cellulose membranes (MWCO: 3500 kDa) and (MWCO 12,000-14,000 kDa) were obtained from Membrane Filtration Products, Inc. (Texas, USA). DI water was obtained from an in-house IONPURE Plus 150 deionization unit. The resistivity of the DI water is at least 17 MΩ-cm.

3.2.2 Synthesis of CHP@CMRDT10 Nanosheets (NSs)

Carboxymethyl reduced dextran T10 (CMRDT10) polymer used for nanosheet (NS) stabilization was synthesized following a procedure from a patent. For the synthesis of the NSs, a 5.0 mL aqueous solution of 0.5 g of CMRDT10 polymer was added to an aqueous solution of CuCl₂·2H₂O (0.017g; 0.1mmol in 95.0 mL of DI water)
and stirred for 10 minutes. The pH of the solution changed from 5.02 to 5.98 upon the addition of polymer solution and the color changed to pale blue. Then 1.0 mL of aqueous solution containing 0.05 mmol of dihydrogen ammonium phosphate (NH$_4$)$_2$HPO$_4$, abbreviated as DAHP) was added dropwise to the above solution with vigorous stirring. The resulting pale bluish suspension was stirred additionally for half an hour and lyophilized. The dry product was re-dispersed in 10 mL of DI water and dialyzed for 6 hours to remove the ionic byproduct and unbound polymer. This was followed by lyophilization to obtain pure and dry polymer coated CHP NSs. The NSs are labeled as CHP@CMRDT10 NSs. The same procedure was followed for the synthesis of CHP@CMRDT20 NPs except that the polymer used was CMRDT20. The NSs were analyzed for copper by AAS. Presence of phosphorous was confirmed by qualitative phosphomolybdate test.

### 3.2.3 Synthesis of small CHP@CMRDT10/PVP NSs

Small CHP NSs of the average width 5.14 nm were synthesized by the modification of the above synthesis. 0.5 g sodium carboxymethyl cellulose (average MW= 90,000) and 0.5 g of PVP (average MW= 8,000) were dissolved in water by heating. Upon cooling the mixture to room temperature, the pH of the mixture was adjusted from 7.24 to 4.00 using 1.0 M HCl. Then 1.0 mL of 0.1 mmol of CuCl$_2$ in water was added dropwise to the polymer mixture and stirred for 10 min. The resulting mixture was pale blue. This was followed by the dropwise addition of 1.0 mL of DAHP (0.05 mmol) in DI water under vigorous stirring. The pale blue dispersion of NSs was stirred for additional 20 minutes, dialyzed for 4 hours and lyophilized to obtain pure and dry polymer coated NSs.
### 3.2.4 Synthesis of Libethenite Single Crystals by Hydrothermal Method

CuCl$_2$.2H$_2$O (0.35 g, 2.0 mmol) and K$_2$HPO$_4$ (0.38 g) were mixed in DI water (8 mL). The pH of the mixture was adjusted from 5.75 to 2.00 using concentrated HCl. The mixture was then transferred to a 23 mL Teflon container, sealed in an autoclave and heated in an oven at 120 °C for a week. Block like greenish crystals obtained were separated from supernatant, washed twice with DI water, 95% ethanol and acetone successively. Crystals are left to dry in ambient conditions. A sample crystal was analyzed by single crystal XRD for unit cell verification.

### 3.2.5 Synthesis of Bulk CHP Sample

Synthesis of bulk CHP was performed following the procedure from literature.$^{89}$ Briefly, an aqueous solution of CuCl$_2$.2H$_2$O (20 mmol, 5.12 g) was combined with an aqueous solution of stoichiometric excess (NH$_4$)$_2$HPO$_4$. The mixture was stirred at 60 °C overnight followed by filtration of the solid product. The product was washed with DI water twice followed by acetone twice. The wet solid product was dried in an oven heated at 60 °C before it was used for characterization.

### 3.2.6 Atomic Absorption Analysis (AAS) of Copper Content in CHP NSs

A sample of 44.0 mg of lyophilized NSs was decomposed in 3.0 mL concentrated nitric acid (>90 %) and gradually heated to dryness over a hot plate. The residue was then heated in a furnace at 615 °C for 4 hours. Carbon free residue was then dissolved in 1.0 mL HNO$_3$ and the solution was made up to a final volume of 100.0 mL in a volumetric flask and analyzed by AAS fixed with copper lamp at a wavelength of 324.7 nm. Copper standards in the concentration range 1.0 to 5.0 ppm were prepared from an initial stock solution of commercially purchased 1000 ppm copper standard. Standard calibration
chart was obtained by plotting the data points of absorbance vs concentration of copper standards. The data points were fit to a straight line and the average concentration of copper in the sample corresponding to the measured absorbance in triplicate was determined from the standard calibration chart.

3.2.7 Synthesis of Texas Red C5 Dye Conjugated CHP NSs

To 4.0 mL of a 3.0 mg/mL CHP@CMRDT10 NSs was added 0.5 mg of Texas Red C5 dye in 100 μL DMSO and 0.5 mg of N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDAC) in 100 μL DI water. The mixture was continued to stir overnight to obtain dye labeled CHP NSs. The unbound dye was separated from the dye labeled NS dispersion by dialysis of the reaction mixture against DI water for 48 hours. An aliquot of the dialyzed NS dispersion was analyzed by fluorescence spectroscopy to confirm the conjugation of the dye to the NSs.

3.2.8 Powder XRD Characterization of Bulk CHP

Powder X-ray diffraction pattern (PXRD) was obtained at RT on a X'Pert PRO powder X-ray diffractometer (PANalytical Inc., Westborough, MA, USA). The experimental set up used Bragg-Brentano geometry in θ-θ configuration, copper as a radiation source (Cu Kα radiation), and diffracted beam curved crystal monochromator to eliminate Cu Kα. The pattern was collected in a range of 20 values from 10.00° to 70.00° with a step size 0.05°.

3.2.9 TEM and EDX Measurements

A small amount of lyophilized NS sample was dispersed in DI water and sonicated for 5 minutes. An equal amount of acetone was added to precipitate the excess of polymer that formed a top floating layer which was separated by centrifugation. The
supernatant was further treated with an equal amount of acetone and centrifuged again. TEM specimen was then prepared by placing a droplet of sonicated NP solution on to a carbon coated copper TEM grid (400-mesh) and allowing to air-dry overnight before use. Particle images and EDX spectrum were acquired using a 200 kV FEI Technai F20 Transmission Electron Microscope (TEM) equipped with a field emission gun and an attached EDAX spectrometer. The operating voltage and current of the instrument were 4000 V and 55 μamp respectively.

3.2.10 Copper Release Studies in Citrate and PBS Buffers

The potential for release of copper by CHP was studied at three different pH values 4.8, 6.2 and 7.4. PBS (1X) was employed for the study at pH value 7.4. Citrate buffer was used for studies at pH values of 4.8 and 6.2. The pH of the prepared citrate buffer was adjusted to the desired final pH value of 4.8 or 6.2 using concentrated NaOH solution. Typically, in an experiment, 50 mg of pure and dry CHP@CMRDT10 NSs were transferred to a pre-processed dialysis bag (regenerated cellulose membrane; MWCO = 3,500-4,000) sealed on one end. 5.0 ml of the buffer solution was added to the bag and the other end of the bag was sealed. The bag was then suspended in 95.0 mL of the buffer solution under mild stirring. Starting at t = 0 minute, aliquots of 2.0 mL were drawn from the external buffer every 20 minutes for the first two hours followed by an interval of 1 hour for the next 12 hours. Additional samples were drawn at 24, 30, 33 and 48 hours respectively. All the samples were digested with known volume of concentrated HNO₃ overnight. The samples were then analyzed for the released copper by AAS. Volume correction for each successive sample was applied and the values were reported as percent release w.r.t theoretical maximum. The kinetic parameters of copper release at
pH values 4.8 and 6.2 were obtained by fitting the release profiles to pseudo first order equations.

3.2.11 Cell Culture and Cytotoxicity Studies

Cytotoxicity studies were performed on different cell lines. Prostate Cancer (PC3) cells were cultured in DMEM supplemented with 10% Fetal Bovine Serum (FBS). Human colorectal adenocarcinoma (HT29) cells were cultured in a complete growth medium prepared from McCoy’s 5A medium by supplementing with 10% FBS. HEK293 (normal kidney cells) were cultured in EMEM supplemented with 10% FBS. Cells were incubated at 37 °C in water-saturated air supplemented with 5% CO₂. Spent medium from the flasks was removed and fresh complete growth medium was added to the flask every three days. Cells were passaged at 90% confluence. Cell counting was performed by trypan blue exclusion method using a hemocytometer. The cytotoxicity of the CHP NSs was performed by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay. Typically, cells were seeded in a 96 well-plate at a cell density of 10,000 cells/well and incubated at 37 °C in water-saturated air supplemented with 5% CO₂ for 48 hours. Drug solutions of required copper concentrations were prepared by diluting the NS aqueous dispersions in the complete growth medium. After 24 hours of incubation, cells in each well were then treated with the 100 µL of drug solutions of appropriate concentration and incubated at 37 °C in water-saturated air supplemented with 5% CO₂. The experiment was performed in triplicate of wells for each concentration of the NSs. Control cells were also grown in the same well-plate without the treatment of drug. After 24 hours, the medium was removed from each well followed by addition of 100 µL of fresh medium and 25 µL of MTT dye. After 6 hours of incubation with the dye, the spent
medium was aspirated out followed by the addition of 100 μL of DMSO to each well to dissolve the purple formazan crystals. After 15 minutes, the absorbance of the solution in each well was measured at 560 nm using a Spectramax M4 microplate reader. Cell viability at each concentration was calculated from the ratio of the absorbance of the drug treated sample to that of control and expressed as percentage along with the deviation. The average IC$_{50}$ value was obtained from three independent trials.

3.2.12 Confocal Microscopy Imaging of PC3 Cells Treated Using Texas Red Cadaverine Dye Conjugated CHP NSs

PC3 cells at a cell density of 4 $\times$ 10$^5$ cells/mL were seeded in a 6 well-plate containing microscope glass cover slips and incubated for 24 hours at 37 °C in water-saturated air supplemented with 5% CO$_2$ to facilitate cell attachment onto the glass cover slips. After the removal of the spent medium, cells were treated with fresh DMEM medium containing the dye labeled NSs and incubated for 4hours. The cover slips were then rinsed with medium once followed by 1X PBS twice, transferred onto glass slides and glued. Confocal images were obtained using an Olympus IX81 microscope equipped with an Olympus DSU (disk scanning unit) at 40X magnification in the z-scanning mode.

3.2.13 Quantification of Acellular and Intracellular Reactive Oxygen Species (ROS) Elevation by CHP NSs Using DCFDA Assay

Firstly, the ability of CHP NSs to induce ROS generation from hydrogen peroxide under acellular conditions was evaluated by DCFDA assay. In a 96 well-plate, 50 μL of 4 μM CHP NSs, 100 μL of 40 μM DCFDA & 50 μL of 200 μM H$_2$O$_2$ were placed in the same order in each well of a row. Similarly, other rows contain 40 and 160 μM CHP NSs such that final concentrations of the NSs are 1, 10 and 40 μM in the respective rows upon
the addition of all reagents. The final concentrations of DCFDA and H$_2$O$_2$ in all the rows were 20 and 100 $\mu$M respectively. Control wells contain H$_2$O$_2$ and DCFDA only without CHP NSs. The well-plate was maintained at 37 °C in an incubator. Fluorescence measurements at 525 nm were recorded at t = 0, 1, 2 and 3 hours at an excitation wavelength of 488 nm using a Spectramax M4 plate reader. Data was expressed as fold increase in fluorescence of NSs treated solutions with respect to the initial reading of control.

For cellular ROS assay, PC3 cells were seeded in a 96 well-plate at a concentration of 15,000 cells per well by placing 100 $\mu$L of cell suspension in each well. The cells were incubated for 24 hours to facilitate the cell attachment. Next day, cells were treated with appropriate concentration of CHP NSs peptized in complete growth medium and incubated for 4 hours. After removal of the medium from wells and subsequent washing of the cells with HBSS twice, the cells were treated with 40 $\mu$M of DCFDA solution and incubated for 30 min. The cells were then washed with HBSS twice, and 100 $\mu$L fresh HBSS was added per each well. Fluorescence readings at 525 nm were taken at t = 0, 0.5 1, 2 and 3 hours at an excitation wavelength of 488 nm using Spectramax M4 plate reader. Data was expressed as fold increase in fluorescence of NSs treated solutions with respect to the initial reading of control.

3.3 Results and Discussion

3.3.1 Synthesis and Characterization of the Copper Hydroxy Phosphate (CHP) i.e. Cu$_2$(OH)PO$_4$ (Libethenite) Bulk Sample

The original goal was to synthesize and explore the utility of the polymer stabilized copper phosphate nanoparticulate system for anti-cancer applications. A test
reaction was first carried to verify the plausibility of the synthesis of Cu₃(PO₄)₂ bulk. Hydrothermal conditions were employed for this test reaction to obtain highly crystalline bulk product. Briefly, aqueous solutions of sodium triphosphate and copper chloride were mixed together and the resultant slurry was transferred into a Teflon lined autoclave, sealed and heated at 120 °C. for one week. Interestingly, as confirmed from the data of unit cell parameters acquired using single crystal X-ray ray diffraction, the deep greenish crystalline product turned out to be the mineral Libethenite which is copper hydroxy phosphate (Cu₂(OH)PO₄). The product may also be obtained by the hydrothermal treatment of reaction mixture at 105 °C for 24 hours. Furthermore, the same product was obtained in bulk, albeit with traces of impurities, under vigorous boiling conditions suggesting the possible success of the synthesis of synthetic Libethenite.
Figure 3.1. PXRD patterns of Libethenite mineral (top) and CHP bulk (bottom).

Figure 3.1 shows the powder XRD spectra of mineral Libethenite (top) and CHP bulk product (bottom). Both patterns agree well. At least thirteen different structures of copper phosphates or hydroxyphosphates other than CHP have been discovered. Peaks highlighted in blue ovals at 2θ of 15.4 and 33.6 ° respectively were possibly due to the traces of impurity, likely belong to one of these phases. However, with only two such diffraction peaks, it is highly difficult to identify the phase. Nevertheless, PXRD results
determined the successful synthesis of the bulk CHP. Subsequent literature search revealed that CHP can be prepared over a wide pH range both under hydrothermal and non-hydrothermal conditions.

### 3.3.2 Synthesis, Characterization, and Proposed Mechanism of Formation of CHP@CMRDT10 NSs

The synthesis of Libethenite as microstructures via the aqueous and hydrothermal routes was reported by others in the literature. However, these particles were in powder form without any polymer coating to stabilize them in colloidal form. Furthermore, the use of such microparticles was limited to optical and catalytic applications. To the best of our knowledge colloidal Libethenite stabilized by a surface coating agent is not reported previously in the literature. In addition, no biological applications of therapeutic relevance are found for the Libethenite based NP systems. We, therefore, modified our goal to explore this unique opportunity i.e. to synthesize the polymer stabilized Libethenite aqueous colloid and evaluate its potential anti-cancer properties.

Applying the successful synthesis of polymer stabilized CHP NSs required the appropriate choice of both the surface coating agent and hydrogen phosphate (HPO$_4^{2-}$) precursor. The initial attempt to synthesize the CHP nanoparticulate in the presence of PVP (avg. mol. wt. 8000 g/mol) resulted in the immediate formation of the bulk precipitate upon the slow and dropwise addition of an aqueous solution of (NH$_4$)$_2$HPO$_4$ to the cupric solution (2:1 molar ratio) containing the polymer. Further attempts, using PVP of higher molecular weight (average m. wt. 40,000 g/mol) also failed to produce polymer stabilized nanosized product despite varying the relative amounts of polymer and the
metal precursor. Similarly, use of another biocompatible polymer called polyethylene glycol (PEG) was also unsuccessful. Finally, after the above unsuccessful trials using the neutral polymers like PVP and PEG, CHP nanocolloid can only be prepared, albeit with short term stability of a few weeks, with the use of a cellulose based biocompatible ionic polymer called carboxyl methyl reduced dextran T10 (CMRDT10). The synthesis was shown in the reaction scheme 3.1 below.

\[
2 \text{CuCl}_2(\text{aq}) + (\text{NH}_4)_2\text{HPO}_4(\text{aq}) + 2 \text{H}_2\text{O}(\text{l}) \xrightarrow{\text{CMRDT10, stirr RT, 20 min}} 2 \text{Cu(OH)PO}_4@\text{CMRDT10(Ns)} + 2 \text{NH}_4\text{Cl(ng)} + 2 \text{HCl(ng)}
\]

**Scheme 3-1. Reaction scheme for the synthesis of CHP@CMRDT10 NSs.**

It was observed that the choice of dihydrogen phosphate precursor also effects the colloidal formation. Whereas the use of \((\text{NH}_4)_2\text{HPO}_4\) as the starting material resulted in the colloidal CHP in the presence of CMRDT10, the use of \(\text{Na}_2\text{HPO}_4\) under the similar conditions did not yield the stable dispersion suggesting the possible role of ammonium ions in influencing the particle growth and aggregation process.
Figure 3.2. a) and b) TEM images of CHP@CMRDT10 NSs. c) Size distribution histogram of CHP@CMRDT10 NSs. d) Selected area electron diffraction (SAED) pattern shown by CHP@CMRDT10 NS.

Figure 3.2 shows the TEM images obtained for a sample of NSs on a carbon coated copper grid. The sheets have flat rectangular like morphology (fig. 3.2.b). Hence forth, the product is referred to as CHP NSs. The NSs are poly-dispersed w.r.t to the width and length and have an average width of 97 ± 27 nm. Figure 3.2.d shows the SAED diffraction rings of the NSs. The corresponding Miller indices of the rings were assigned and the calculated d-spacings agreed closely with that of the mineral Libethenite. The data is listed in the table 3.1. SAED results unambiguously confirmed the chemical identity of the synthesized NSs as Cu$_2$(OH)PO$_4$. 
Table 3-1. Comparison of d-spacings of CHP NSs determined from SAED pattern with those of Libethenite determined by XRD.

<table>
<thead>
<tr>
<th>d-spacing (CHP NS SAED), Å</th>
<th>d-spacing (Libethenite XRD), Å</th>
<th>2θ (degrees)</th>
<th>(hkl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.7494</td>
<td>4.7649</td>
<td>18.62</td>
<td>(011)</td>
</tr>
<tr>
<td>4.0258</td>
<td>4.0400</td>
<td>22.00</td>
<td>(020)</td>
</tr>
<tr>
<td>2.9108</td>
<td>2.9166</td>
<td>30.65</td>
<td>(220)</td>
</tr>
<tr>
<td>2.4771</td>
<td>2.4501</td>
<td>36.68</td>
<td>(031)</td>
</tr>
</tbody>
</table>

Energy dispersive X-ray (EDX) spectroscopic analysis of the NS reveals the characteristic signals of carbon, oxygen, phosphorous and chlorine (figure 3.3). The presence of phosphorus was further confirmed independently by phosphomolybdate test.
Figure 3.3. EDX spectrum of CHP@CMRDT10 NSs.

Since the EDX signals of copper are also due to the copper grid itself, the presence of copper was rather confirmed by AAS. It is to be noted that EDX spectrum also showed the chlorine peak. The presence of chlorine is possibly due to the electrostatic interaction of the byproduct HCl on the surface of the NSs that cannot be removed easily by dialysis.

The process of formation of polymer-stabilized nanodispersion may be explained based on surface charge arguments. The stringent requirement of the presence of both the ionic polymer and ammonium ions in facilitating the colloidal CHP possibly indicates that controlled interaction and minimization of the surface charge during the particle growth are the critical factors that affect the kinetics of the growth, aggregation and
surface coating of nanosheets. Thus, to account for the above observations a mechanism as shown in scheme 3.2 is proposed.

Scheme 3-2. Proposed mechanism for the formation of CHP@CMRDT10 NSs.

In the absence of the ionic polymer, the dissolution of cupric ions hydrolyze the water molecules and form \([-\text{Cu}^{\text{III}}\text{-OH}]^+\) species in solution (step I). In step II, deprotonation of \(\text{HPO}_4^{2-}\) in the presence of two equivalents of such copper-hydroxyl...
species occurs which results in the loss of an equivalent of water molecule with concomitant ligation of the two Cu(II) ions with the resultant phosphate anion and formation of neutral Cu$_2$(OH)PO$_4$ nuclei. The ligation also results in substitution of two labile ligand water molecules.

With the repeated steps of hydrolysis by Cu(II) ions, deprotonation of HPO$_4^{2-}$ and ligation of the resultant phosphate in the presence of Cu(II)-hydroxyl species, a network of Cu(OH)PO$_4$ grows and give rise to smaller. Further growth of the smaller particles into a well-defined larger particle is effected by the availability of surface hydroxyl groups. It may be proposed that the presence of carboxymethyl reduced dextran lowers the kinetics of the above process in two ways by (i) coordinating with the Cu(II) ions, the hydrolysis of Cu(II) ions is suppressed. As a result, the surface charge is lowered, (ii) hindering the ligation by the HPO$_4^{2-}$ to the hydrolyzed species of Cu(II), the rate of network formation is lowered which in turn limits the availability of the surface hydroxyl groups. Neutral polymers like PVP and PEG, on the other hand, have less influence on the minimization of surface stabilization. Therefore, even in their presence the smaller particles grow uncontrollably and leads to the formation of bulk precipitate. Cho et al. reported the role of OH$^-$ ions in influencing the morphology of Cu$_2$(OH)PO$_4$ nanoparticles.$^{89}$ Their work demonstrated that limiting the availability of surface hydroxyl ions, by careful adjustment of pH of the reaction mixture, strongly directs the anisotropic growth of the CHP nanoparticles.

In step III, the ammoniums interact strongly with the surface hydroxyl groups of the smaller particles and assist in self-assembled aggregation of the smaller particles to form the larger nanosheets that are then effectively capped by the anionic polymer. Such role
of ammonium ions introduced from raw materials in the self-assembly and aggregation of 
La2(MoO4)3 nanoflakes, formed in homogeneous solution, into highly porous pompon 
shaped microarchitectures was previously reported by Bu et al.98 It was mentioned by Bu et al. that the ammonium ions strongly interact with the surface -OH groups on the 
La2(MoO4)3 particles and effect the crystallization and morphology. This may also 
explain why the use of sodium dihydrogen phosphate precursor did not lead to colloidal 
dispersion of CHP. Unlike the ammonium ions, sodium ions may not effectively assist in 
self-assembly of the smaller particles of CHP and as a result, the smaller particles grow 
uncontrolled into larger ones that eventually precipitate out as bulk.

The size of the nanosheets can further be reduced or optimized by employing 
polymers of higher molecular weights namely CMRDT20 (avg. M. wt. 20,000 g/mol) and 
sodium carboxyl methyl cellulose (average m. wt. 90,000 g/mol). The TEM images of the 
samples prepared using these polymers are displayed in figure 3.4.
Figure 3.4. a) and b) are the TEM image and size distribution of CHP@CMRDT20 NSs respectively. c) and d) are the TEM image and size distribution of CHP@CMC/PVP NSs respectively.
Attenuated total reflectance (ATR) IR spectra confirmed the presence of the polymer coating. The IR spectrum of CHP bulk is displayed in figure 3.5.a and the spectra of pure polymer (red) and the polymer coated CHP NSs (blue) are displayed in figure 3.5.b respectively. For the lyophilized polymer coated CHP NSs, the IR spectrum exhibited bands predominantly by those of polymer confirming the surface coating of NSs by CMRDT10. The broad \(-\text{OH}\) vibrations were observed at 3317 cm\(^{-1}\) for pure
polymer and 3365 cm$^{-1}$ for polymer coated CHP respectively. The peaks at 2919, 1412 and 1339 cm$^{-1}$ for the pure polymer are distinctive of -CH$_2$ and -CH bonds. The same bands were observed at 2925, 1412, 1339 cm$^{-1}$ for surface coated polymer on CHP NSs. The observed bands at 1591 and 1595 cm$^{-1}$ for the pure polymer and the surface coated polymer respectively can be attributed to the anti-symmetric -COO$^-$ stretching of carboxylic salts of the polymer.$^{99}$ However, no bands due to CHP were distinctly observed for the NSs. Atomic absorption analysis of the lyophilized CHP@CMRDT10 NS samples revealed that CHP was present in 1.5% by mass indicating a very low fraction of the NSs. The high polymeric content relative to the CHP apparently masked the stretching and bending modes of CHP. Therefore, these bands were difficult to be distinguished in the spectrum.

3.3.3 Copper Release Studies of CHP Bulk in Biologically Relevant Media

The stability of CHP towards copper release at various pH values relevant to physiological, tumor and intracellular environments were studied. Phosphate buffer saline (PBS) was employed to represent the physiological pH value of 7.4. The buildup of lactic acid in the extracellular milieu of the tumor lowers the pH to 6.0 to 6.5.$^{80}$ On the other hand, intracellular lysosomal pH values is between 4.5-5.0.$^{81}$ Therefore, citrate buffer was employed for copper release studies at pH values of 6.2 and 4.8 to represent acidic tumor and lysosomal pH values respectively. Bulk CHP placed in a dialysis bag was incubated in buffer of desired pH value (PBS for pH 7.4, 0.1 M citrate buffer for pH 4.8 or 6.2). Samples drawn from the buffer at regular intervals over a period of 48 hours were analyzed for copper by AAS. The copper release profiles at pH 4.8, 6.2 and 7.4 over a time-period of 48 hours are shown in figure 3.6. At pH value of 7.4, copper released is
below the detectable level over the 48-hour time-period indicating that CHP is non-degradable at physiological conditions. Such high physiological stability allows for the longer circulation time of CHP NSs in the blood pool and subsequent accumulation at the tumor site through EPR effect. At the pH values of 4.8 and 6.2 maintained by citrate buffer, CHP exhibited a first order kinetic release profiles. The copper released after 4 hours of incubation was 16.5 and 11 % at pH values of 4.8 and 6.2 respectively. After 8 hours, the extent of copper release was 30 and 23 % respectively.

Figure 3.6. Copper release profiles of CHP bulk at biologically relevant pH values of 4.8 (red), 6.2 (blue) and 7.4 (black).

It is to be noted that the CHP can be synthesized over a wide pH range including highly acidic conditions. The instability of bulk CHP towards copper release in citrate buffers at pH values of 6.2 and 4.8 is likely due to the presence of a large excess of citrate ligands of the buffer which bind to and detach the copper from the surface of CHP.
However, for the NSs the carboxyl groups of the polymer coating effectively bind to the surface copper and reduce such ligand exchange. Therefore, the presence of polymer coating is likely to lower the metal release from CHP both in the endosomes and lysosomes inside the cells assuming the cellular uptake of the NSs follows the endocytic pathway.

### 3.3.4 Cytotoxicity of CHP NSs Toward HT29 and PC3 Cell Lines

The cytotoxicity of the NSs was evaluated *in vitro* on two different cancerous cell lines by MTT assay. Cells were treated with different concentrations of NSs for 24 hours with the highest concentration set at 500 μM of copper.

**Figure 3.7. Cytotoxicity profiles of CHP@CMRDT10 NSs in a) HT29 cells (human colorectal carcinoma cells) and b) PC3 cell line (prostate cancer cells.)**
Figures 3.7 a) and b) display the cell viability profiles of HT29 and PC3 cells respectively treated for 24 hours with different concentrations of CHP@CMRDT10 NSs. CHP NSs exhibited similar toxicity profile for both the cell lines with the IC$_{50}$ values of 50 ± 2 and 47 ± 3 μM respectively as determined from the average of three independent trials. The corresponding values in μg/mL are 3.2 ± 0.1 and 3.0 ± 0.2 respectively. It is to be noted that the above concentrations are that of total copper and include both the surface and interior copper of the NSs. The cytotoxicity data for different copper based nanosystems including those reported earlier by other group in literature, are shown in table 3.2

<table>
<thead>
<tr>
<th>Nano system</th>
<th>Size (nm)</th>
<th>IC$_{50}$ (μM)</th>
<th>IC$_{50}$ (μg/mL)</th>
<th>Cell line</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHP@CMRDT10 NSs</td>
<td>97 ± 27 (avg. width)</td>
<td>50 ± 2</td>
<td>3.2 ± 0.1</td>
<td>PC3</td>
<td>This work</td>
</tr>
<tr>
<td>CuI NPs</td>
<td>35 ± 4.2 (avg. dia.)</td>
<td>39</td>
<td>2.5</td>
<td>MCF7</td>
<td>Laha et al.$^{54}$</td>
</tr>
<tr>
<td>Cu$_3$(PO$_4$)$_2$ NPs</td>
<td>67 ± 6.3 (avg dia.)</td>
<td>157</td>
<td>10</td>
<td>MCF7</td>
<td>-do-</td>
</tr>
</tbody>
</table>

A comparison of the IC$_{50}$ values of CHP NSs with those reported by Laha et al. shows that, although larger in size, CHP@CMRDT10 NSs exhibit comparable cytotoxicity as that of CuI NPs. Furthermore, CHP@CMRDT10 NSs showed better cytotoxicity profile than for Cu$_3$(PO$_4$)$_2$ NPs.$^{54}$ It is assumed that the different cell lines noted above have similar dose response for each NP system. The better toxicity of CHP...
NSs may be explained by the presence of polymeric coating on the surface of the NS that renders the efficient cellular uptake of the NSs possibly by non-receptor mediated endocytosis. In contrast, the reported NPs are bare and do not have any surface coating.

3.3.5 Dye Labeling and Cellular Uptake Studies

The cellular uptake of CHP NS was assessed by confocal microscopy imaging of the cells treated with fluorescent dye conjugated NS. Texas Red cadaverine, a red-fluorescent dye with an emission wavelength of 608 nm was employed for dye labeling purpose. The primary amine of the dye can be chemically conjugated to form peptide bonds with carboxylic groups of the proteins and water-soluble biopolymers in aqueous solutions using the water-soluble carbodiimides. CMRDT10 coating on the surface of NSs has residual free carboxylic acid groups that are available for further functionalization. Dye labelling on CHP NSs was performed post-synthesis of the NSs as shown in the scheme 3-3.

Scheme 3-3. EDC coupling scheme for the conjugation of Texas Red C5 dye to CHP@CMRDT10 NSs.
The formation of peptide bond between the primary NH$_2$ group of the dye and the carboxyl (-COOH) was facilitated by EDC coupling. After the unbound dye was separated from the product by extensive dialysis, the presence of surface conjugated dye on CHP NSs was confirmed by fluorescence spectroscopy.

Figure 3.8. Fluorescence spectra of CHP@CMRDT10 NSs (black) and the Texas Red C5 dye-labeled CHP@CMRDT10@TRC5 NSs (red).

Figure 3.8 shows the emission spectrum of unlabeled CHP@CMRDT10 NSs (black) and the Texas Red C5 dye-labeled CHP@CMRDT10@TRC5 NSs (red). The emission at 613 nm can be attributed to presence of the dye conjugated to the surface coated polymer on the CHP NSs. The cellular uptake of the NSs was then assessed by confocal fluorescence microscopy. Figure 3.9 shows the fluorescence images of the PC3 cells acquired by confocal microscopy. Images 3.9.a and 3.9.c are the bright field images of the untreated and CHP@CMRDT10@ TRC5 NS treated cells respectively. Images on
the right 3.9.b and 3.9.d are the fluorescent confocal images of the same. The red fluorescence signal observed around the dark nuclear region of the cells, upon incubation of cells for 4 hours with the CHP NS@CMRDT10@TRC5, indicates the cytoplasmic localization of the NSs confirming its uptake by the cells. In contrast, no fluorescence was observed for untreated cells (control).

Figure 3.9. a) bright field image of control PC3 cells. b) confocal images of the same. c) bright field image of CHP@CMRDT10@TRC5 NS treated PC3 cells. b) confocal images of the same.

3.3.6 Acellular and Cellular ROS Elevation by CHP@CMRDT10 NSs

It is reported that copper based nanoparticles exhibit cytotoxicity toward cancer cells by increasing the intracellular ROS level.\textsuperscript{50, 54, 82} To test whether surface copper on CHP NSs can lead to the generation of ROS, an acellular ROS assay involving the oxidation of DCFDA by the free radicals generated from the Fenton-like reaction between H\textsubscript{2}O\textsubscript{2} and the surface copper was performed first. The results are shown in the
The increase in fluorescence intensity of the NS treated \( \text{H}_2\text{O}_2 \) solutions containing the dye precursor was observed at different time periods in a dose dependent manner. Such increase in intensity is caused by the oxidation of DCFDA to the fluorescent DCF by free radicals generated from the decomposition of \( \text{H}_2\text{O}_2 \) catalyzed by CHP NSs. On the other hand, the increase in the fluorescence of the control can be attributed to the auto oxidation of the dye precursor. Solutions containing NSs of copper concentrations 10 and 40 \( \mu \text{M} \) respectively exhibited significant fold increase of fluorescence intensity compared to that of control indicating the potential of CHP to produce ROS by heterogeneous Fenton-like reaction.

Figure 3.10. Acellular ROS generation profile of CHP NSs as determined using DCFDA assay.

Furthermore, the intracellular ROS generation catalyzed by CHP NSs was also confirmed by DCFDA assay. After incubation of PC3 cells with CHP NSs of 20 \( \mu \text{M} \) concentration of copper for 4 hours followed by brief treatment with dye precursor, the
first fluorescence measurements were made. From the plot, there is 63-fold increase in the fluorescence with respect to that of initial control reading. Furthermore, there was ~ 96 and 105-fold increase in fluorescence after 30 minutes and 1 hour respectively. The fluorescence reached plateau after one hour suggesting the maximum intra cellular conversion of the DCFDA to DCF. Thus, both acellular and cellular ROS generation studies indicate that CHP NSs may cause the cancer cell death likely by elevating ROS level.

![Figure 3.11. Elevation of cellular ROS level by CHP@CMRDT10 NSs in PC3 cell line as determined by DCFDA assay.](image)

3.3.7 Potential for Integration of Multiple Treatment Strategies

CHP strongly absorbs in the near infrared (NIR) region (λ > 800 nm). Wang et al. demonstrated that CHP is an effective photocatalyst toward the decomposition of 2,4-dichlorophenol (2,4-DCP) in aqueous solutions under NIR light. The photocatalytic property was aroused due to the generation and effective separation of electron-hole pair
when CHP was excited by NIR light. The crystal structure of the Libethenite consists of two different types of Cu(II) centers that differ in their coordination environments. The first type of Cu(II) center has an octahedral coordination and the second type has a trigonal pyramidal environment. As depicted in the figure 3.12, the axially elongated CuO$_4$(OH)$_2$ octahedron shares the corner oxygen of the bridging hydroxyl group with the axially compressed CuO$_4$(OH) trigonal bipyramid.

Figure 3.12. Portion of crystal structure of CHP depicting the CuO$_4$(OH)$_2$ octahedral and CuO$_4$(OH) trigonal bi-pyramidal environments of two different types of copper centers.

Density Functional Theory (DFT) studies carried out by the same group to determine the electronic band structure revealed the difference in the density of states at
copper sites of the octahedron and that of the trigonal bipyramid. The observed photocatalytic activity was attributed to a favorable electronic band structure with varied degrees of contributions of the density of states of Cu(II) (3d) and O 2p to the highest occupied and lowest unoccupied bands that transfers and effectively separates the electron from the CuO$_4$(OH) trigonal bipyramid to the CuO$_4$(OH)$_2$ octahedron upon photoexcitation in the presence of NIR.

In view of its strong NIR absorption property, it would be interesting to explore the photo thermal therapy (PTT) potential of CHP. Additionally, integrating multiple treatment strategies i.e. the inherent ROS inducing capability of CHP and the NIR-PTT to multiply the cytotoxic effect on cancer cells will further reduce its therapeutic dose and possibly serve as an efficient therapeutic treatment method. However, such an exploration is beyond the scope of this work.

3.4 Conclusion

In conclusion, in this project synthetic Libethenite (CHP) NSs were successfully developed. With the appropriate choice of the polymer the CHP was stabilized in colloidal form as nano-sized sheets in aqueous media using an anionic polymer. Moreover, it was demonstrated that the size and morphology of nano CHP can be drastically reduced using the similar polymers of higher molecular weight. In vitro studies demonstrated that CHP has the potential to inhibit cancer cells likely due to the elevation of ROS level causing oxidative insult in the host cells. To the best of our knowledge, neither the synthesis of colloidal Libethenite nor the bio-application of synthetic Libethenite was reported previously. The favorable NIR absorption property of CHP in separating electron-hole pair upon photoexcitation is an attractive feature that
may also be explored to study its potential in photo thermal therapy (PTT) and if found successful may lead to an effective combinatorial treatment platform that will further reduce its therapeutic dose.
4. Copper Prussian Blue Analogs for Anti-Cancer and PET Applications

4.1 Introduction

First discovered accidentally in 1752 by Pierre Joseph Macquer, Prussian Blue (PB) is the first synthetic inorganic coordination polymer ever known. This coordination polymer and the Prussian Blue analogs (PBAs) belong to a general class of metal hexacyanometallates $A_x M_y [M'(CN)_6]_z$ where (A is a monovalent cation, M and M’ are the di or trivalent transition metal ions or lanthanides). Two types of PB exist 1) the so called “soluble” Prussian blue (SPB) which is $A' Fe^{III}[Fe^{II}(CN)_6]$ where $A'$ is a monovalent cation such as $K^+$, $Na^+$ and $NH_4^+$ and 2) the insoluble Prussian blue (IPB) which is $Fe_4^{III}[Fe^{II}(CN)_6]_3$. Both forms are insoluble in water. Due to the finely dispersed colloidal particles in water, the SPB gives an appearance of a solution and therefore the term “Soluble Prussian blue” is a misnomer. Owing to its intense deep blue color, PB has been applied as pigment in paintings since its discovery in the eighteenth century. However, the uses of PB and PBAs have expanded to many other areas due to extensive studies of the physical and chemical properties arising from their interesting framework structures. Figure 4.1 illustrates the general structure of the coordination framework of PB and analog its analogs (PBAs).
Figure 4.1. General structure of Prussian Blue and PB analogs with Fm$\overline{3}$m space group symmetry. In a) M can be Cr, Mn, Co, Ni, Cu, Zn, Cd or Os for PBAs. b) Vacant $[\text{Fe(CN)}_6]^{4-}$ sites occupied by coordinated water molecules. c) monovalent ion centers (generally alkali ions) shown in yellow for soluble PBA type. (Picture adapted with permission from ACS publications, Reference: Ojwang et al. *Inorg Chem* 2016, 55 (12), 5924-5934).

The crystal structure comprises of M(NC)$_6$ and M'(CN)$_6$ octahedral units that are linked together by the bridging CN groups. The linked octahedra form a three-dimensional network by the linear repeating -NC-M’-CN-M-NC- units. In case of PB, M and M’=Fe$^{II}$/Fe$^{III}$. For PBA, M= Cu, Ni, Co, Mn, Cr, Zn etc.$^{101-109}$ To maintain the overall charge neutrality of the framework, some of the $[\text{Fe(CN)}_6]^{3/4-}$ sites are inherently vacant resulting in a porous network of high surface area. In the case Fe$^{III}$[$\text{Fe}^{II}$(CN)$_6$]$_3$, 25 % of the $[\text{Fe}^{II}$(CN)$_6$]$^{3-}$ sites are vacant. The cavities and vacant sites arise in the framework structure can accommodate water molecules (trapped as zeolitic water) and coordinated water molecules respectively.

PB is found to be exceptionally robust under both highly acidic and physiological conditions, non-toxic even when consumed in gram quantities and is suitable for its biological applications.$^{110-111}$ In 2003, FDA approved the use of Prussian blue for the safe and effective removal of radioactive cesium, radioactive thallium and non-
radioactive thallium.\textsuperscript{112} PB can trap the radionuclide cesium and thallium in the intestines and keeps them from being reabsorbed by the body, speeds up their excretion from the body and thus reduces their biological half-life. Similarly, PB and its analogs (PBAs) also have been explored for a variety of interesting applications in the areas of gas storage, catalysis, sensors, batteries and electrochromic displays.\textsuperscript{113-123}

Whereas PB and its analogs (PBAs), in bulk and nanoparticulate (NP) forms, have been evaluated for the above-mentioned applications for a relatively longer time, the potential biological applications of the NPs started emerging only since the beginning of this decade. Ever since the first study on PBNPs as a new generation T1 weighted MRI contrast agent was reported by Shokouhimehr \textit{et al.}\textsuperscript{124}, increased research attention has been paid towards the utility of both PB and PBAs as nanoprobebs for magnetic resonance imaging (MRI)\textsuperscript{125}, computed tomography (CT)\textsuperscript{126}, positron emission tomography (PET) imaging\textsuperscript{127}, nanocarriers for drug delivery,\textsuperscript{26} and nanotherapeutic agents in photodynamic therapy (PTT) for the treatment of cancer.\textsuperscript{128} This easy to synthesize coordination polymeric network allows for the incorporation of various di- and tri-valent metal ions of biomedical relevance making it a versatile platform for a wide variety of biological applications.

All the aforementioned biological applications rely on the non-toxic nature of the PBNPs and PBANPs. Copper based NPs, in general, are known to be detrimental to cancer cells due to the ability of surface copper in performing heterogeneous Fenton-like chemistry to elevate cellular ROS level.\textsuperscript{50,53-54} Such ability may further be enhanced by increasing the surface area to volume possessed by NPs that provides large number of catalytically active copper sites per unit area than compared to bulk or nanoparticles of
higher sizes. Owing to the highly porous nature of the PBA systems, attributable to the intrinsic vacancies in their frameworks, it would be of interest to synthesize the NPs of copper PBAs and evaluate its anti-cancer potential. Provided the NPs are extremely small, a combination of intrinsic defects of PBAs and the incorporated redox active copper centers shall reduce the therapeutic doses and lead to more effective cancer therapeutics. Although, previously used as a drug delivery platform\textsuperscript{26}, to the best of our knowledge, no copper based PBA NPs were evaluated as such for their intrinsic anti-cancer ability. Therefore, it is of our interest to synthesize copper PBA NPs of both types of hexacyanoferrates and evaluate their \textit{in vitro} cytotoxic potential towards cancer cells.

In this project, we synthesized two types of copper based PBA NPs namely copper hexacyanoferrate (II) (CF\textsubscript{2}(1:1)@PVP NPs) and copper hexacyanoferrate (III) (CF\textsubscript{3}(1:1)@PVP NPs) stabilized in solution in the presence of a biocompatible polymer. The stability of both the NPs towards copper and free cyanide release, their cellular uptake and the intracellular ROS elevation were studied. The anti-cancer effects of the two types of NPs were evaluated in PC3 cell line (prostate cancer). Since the surface area varies drastically with the change in size of the NPs, the size dependent cytotoxicity of CF\textsubscript{2}(1:1)@PVP NPs was also studied.

Furthermore, the favorable characteristics such as the small size and high stability of the CF\textsubscript{2}(1:1)@PVP NPs against copper leaching in the presence of various biologically relevant divalent ions prompted us to explore its potential as PET scanning agent. With the appropriate choice of the surface coating, these NPs can be rendered non-toxic as demonstrated in HeLa cells and make them suitable for diagnostic application by incorporating \textsuperscript{64}Cu, a radionuclide for PET imaging, into CF2@PVP/Cit NPs.
4.2 Experimental Section

4.2.1 Materials

Unless otherwise stated, all the chemicals were purchased from Sigma-Aldrich and used as such without further purification. Copper standard (1000 ± 10 μg/mL copper in 3% (v/v) HNO₃) was purchased from inorganic ventures, Texas Red cadaverine was purchased from molecular probes, dextran T10 was a generous gift from China. Sodium citrate and citric acid are from Spectrum® and Lab Chem Inc respectively. Regenerated cellulose membranes (MWCO 3500 kDa) and (MWCO 12,000-14,000 kDa were obtained from Membrane Filtration Products, Inc. (Texas, USA). DI water was obtained from an in-house IONPURE Plus 150 deionization unit. The resistivity of the DI water is at least 17 MΩ-cm.

4.2.2 Synthesis of CF₂(1:1)@PVP NPs

An aqueous solution of CuCl₂ (50 mL, 1.0 mM) was added to 50 mL of aqueous solution containing K₄[Fe(CN)₆] (1.0 mM) and 0.625 g of PVP (average m. wt. 40,000 g/mol) while stirring rapidly. The mixture instantaneously turned deep maroon brown. The mixture was stirred for an additional 15 minutes at RT before subjected to lyophilization. The lyophilized solid mixture was peptized in 40 mL DI water and dialyzed against DI water for 24 hours with frequent replacement of dialyzing water with fresh DI water.

4.2.3 Synthesis of CF₃(1:1)@PVP NPs

Same procedure as that for CF₂(1:1)@PVP NPs as above except that ferricyanide was used in place of ferrocyanide.
4.2.4 Synthesis of CF2 Bulk Powder

Synthesis of the bulk was performed at room temperature. Briefly, an aqueous solution of CuCl$_2$ (300 mM, 20.0 mL) was added slowly to an aqueous solution of K$_4$[Fe(CN)$_6$] (200 mM, 20.0 mL) while stirring. The molar ratio of Cu to Fe is 3:2. Maroon colored precipitate was formed instantaneously. The mixture was centrifuged at 4000 rpm for 10 minutes followed by washing with DI water and centrifugation twice before being finally washed twice with acetone. The sample was used for characterization by PXRD and AAS.

4.2.5 Synthesis of CF3 Bulk Powder

The procedure was same as above except ferricyanide was used in place of ferrocyanide. The resulting precipitate was olive green in color.

4.2.6 Conductometric Titration for the Determination of Stoichiometry of the Bulk Products CF2 and CF3

The stoichiometry was determined by conductometric titration using method of continuous variation. Briefly, 1.0 mM CuCl$_2$ solutions ranging from 14.0 mL to 1.0 mL, decremented by 1.0 mL for each successive solution, were titrated against incremented volumes of 1.0 mM Fe(CN)$_6^{4-}$ for CF2 or Fe(CN)$_6^{3-}$ for CF3 so that the total volume of the reactant solutions mixed was 15.0 mL for each titration. Conductivity of each reaction mixture was then recorded using QAKLON PC700 pH/conductivity meter. Job’s plots were drawn for mole fraction of CuCl$_2$ vs conductivity ($\mu$S/cm). The stoichiometry of the reaction corresponds to the one at which minimum conductivity is shown by the reaction mixture.
4.2.7 Atomic Absorption Analysis of the Bulk and NPs

A small amount of weighed sample was treated with concentrated HNO$_3$ in a crucible and heated to dryness. The residue was then heated in a furnace at 630 °C for 4 hours. The inorganic residue was then digested with mixture of concentrated HNO$_3$ and HCl. The final volume was made up to 100 mL and analyzed by AAS with Cu absorption wavelength at 324.7 nm. Appropriate dilution factor was applied wherever required.

4.2.8 Transmission Electron Microscopy (TEM) Imaging and EDX Measurements of NP samples

A small amount of lyophilized NP sample (CF$_2$(1:1) @PVP NPs or CF$_3$(1:1) @PVP NPs was re-dispersed in 750 μL of DI water followed by addition of an equal quantity of acetone. The mixture was centrifuged and the pellet was redispersed in 500 μL DI water followed by equal amount of acetone. The centrifugation step was repeated and the pellet of the NPs was finally dispersed in 500 μL DI water. Small droplet of this NP dispersion was placed on the TEM grid and air dried overnight. Particle images and EDX spectrum were acquired using a 200 kV FEI Technai F20 Transmission Electron Microscope (TEM) equipped with a field emission gun and an attached EDAX spectrometer in STEM mode. The operating voltage and current of the instrument were 4000 V and 55 μamp respectively.

4.2.9 Stability Against Metal Release of CF$_2$(1:1)@PVP NPs and CF$_3$(1:1)@PVP NPs in Various Media

The stability of both NPs towards metal leaching was evaluated using a variety of media. Briefly 5.0 mL samples of NP dispersions of copper concentration of 3.0 mM were sealed in dialysis bags (MWCO 3,500) and incubated in each of 100 mL of HCl
solution of pH value 1.0, 0.1 M citrate buffers of pH values 4.8 and 6.2, PBS (pH value 7.4) and various M(II)Cl$_2$ (where M(II) is Mg(II), Ca(II), Fe(II) and Zn(II)) salt solutions of 100 ppm concentration of M(II) ion at 37 °C. After incubation for 24 hours, the sample of medium was analyzed for Cu using AAS. The copper release was expressed as the percentage of the theoretical concentration of total copper.

4.2.10 Free Cyanide Release Test

Free cyanide release in various biologically relevant media was experimentally verified by a fluorometric method using the commercially available cyanide test kit developed by LaMotte Co. (Chestertown, Maryland; Code 7387-01). Manufacturers protocol was followed for analysis.

4.2.11 Cell Viability Studies

Cytotoxicity studies were performed on Prostate Cancer (PC3) cell line. Cells were cultured in Minimum Essential Medium supplemented with 10% FBS. Cells were incubated at 37 °C in water-saturated air supplemented with 5% CO$_2$. Spent medium from the flasks was removed and fresh complete growth medium was added to the flask every three days. Cells were passaged at 85% confluence. Cell counting was performed by trypan blue exclusion method using a hemocytometer. The cytotoxicity of the CHP NSs was evaluated by 3-[4, 5-dimethylthialzol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay. Typically, cells were seeded in a 96 well-plate at a cell density of 10,000 cells/well and incubated at 37 °C in water-saturated air supplemented with 5% CO$_2$ for 24 hours to allow for cell attachment. Drug solutions of required copper concentrations were prepared by serial half dilution from a stock solution of aqueous dispersion of NPs in the complete growth medium. Cells in each well were then treated with the 100 μL of
NP drug solutions prepared by serial half dilutions from the initial NP stock solution and incubated at 37 °C in water-saturated air supplemented with 5% CO₂. The experiment was performed in triplicate of wells for each concentration of the NPs. Control cells were also grown in the same well-plate without the treatment of drug. After 24 hours, the medium was removed from each well followed by addition of 100 μL of fresh medium and 25 μL of MTT dye. After 4 hours of incubation with the dye, the spent medium was aspirated out followed by the addition of 100 μL of DMSO to each well to dissolve the purple formazan crystals. After 15 minutes, the absorbance of the solution in each well was measured at 560 nm using a microplate reader. Cell viability at each concentration was calculated from the ratio of the absorbance of the drug treated sample and the average absorbance of control and expressed as percentage along with the deviation. The average IC₅₀ value was obtained from three independent trials involving at least two different batches of synthesized NPs.

4.2.12 Quantification of Intracellular ROS Elevation by DCFDA Assay

Intracellular ROS elevation by CF2 and CF3 NPs was quantified using DCFDA assay. PC3 cells were seeded in a 96 well-plate at a cell density of 2.5 X10⁵ cells per well and incubated for 24 hours at 37 °C in humidified air supplemented with 5% CO₂ to facilitate the cell attachment. Sample cells were then treated with 100 μL of complete growth medium containing NPs of varying concentrations ranging from 10 μM to 60 μM and incubated for 4 hours. Control cells are untreated with NPs. This step was followed by removal of medium and washing the cells with HBSS twice. The cells in each well were then exposed to growth medium containing 40 μM DCFDA for 30 minutes followed by removal of medium and washing twice with HBSS. Fluorescence reading were taken
immediately using a Spectramax M4 microplate reader. During the whole procedure, the well-plate was kept out of light to minimize the interferences with the dye. The experiment was run in quadruplicates of wells. The fluorescence contribution by the DCFDA treated cells without the treatment of NPs was eliminated from the total fluorescence intensities of the dye treated cells and the reduced intensities obtained were used for calculations. The ROS elevation was quantified as fold increase in fluorescence relative to that of the average initial fluorescence of control. Statistical analysis was made by comparisons of data for control cells and NP treated cells using a two-tailed unpaired Student’s t-test.

4.2.13 Surface Conjugation of CF2_{(1:1)}@PVP NPs and CF3_{(1:1)}@PVP NPs Using Texas Red Cadaverine Dye

For CF3_{(1:1)}@PVP NPs, dye labeling was performed post-synthesis of the NPs. 100 μL of 1.0 mM citric acid and sodium citrate solution was added to 500 μL of 0.32 mM CF3_{(1:1)}@PVP NPs and stirred for an hour. Meanwhile, mixtures of 0.5 mg of Texas Red C5 dye in 100 μL DI water and 0.5 mg of N-(3-dimethylaminopropyl)-N-ethylcarboadiimide hydrochloride (EDAC) in 100 μL DI water were homogenized together by sonication for 5 minutes to generate EDC coupled dye. The mixture was then added to the citric acid coated NP dispersion and continued to stir overnight to obtain dye labeled CF3_{(1:1)}@PVP NPs. The unreacted dye was separated from the dye labeled NP solution by dialysis of the reaction mixture against DI water for 24 hours. During the whole preparation and purification, the solutions were stirred in the dark to avoid the degradation of the light sensitive dye. An aliquot of the dialyzed NP dispersion was
subjected to fluorescence spectrophotometry to confirm the conjugation of dye to the NP surface.

For CF3(1:1)@PVP NPs, the above procedure did not work. Instead, the NP dispersion was directly treated with the dye solution, kept stirred overnight and dialyzed to remove the unbound dye.

4.2.14 Confocal Fluorescence Microscopy Imaging

PC3 cells at a cell density of 4 x 10^5 cells/mL were seeded in a 6 well-plate containing microscope glass cover slips and incubated for 24 hours at 37 °C in water-saturated air supplemented with 5% CO₂ to facilitate cell adherence onto the glass cover slips. After the removal of the spent medium, cells were treated with fresh complete growth medium containing the dye labeled NPs and incubated for 4 hours. The slides were then rinsed with medium once followed by 1X PBS twice, transferred and glued onto a glass slide. Confocal images were obtained using an Olympus IX81 microscope equipped with an Olympus DSU (disk scanning unit) at 40X magnification in the z-scanning mode.

4.3 Results and Discussion

4.3.1 Synthesis and Characterization of CF2 and CF3 Bulk

Synthesis was accomplished by a simple salt metathesis reaction. The bulk compounds were prepared by addition of an aqueous solution of copper salt to an aqueous solution of the hexacyanoferrate (II) (for CF2) or hexacyanoferrate (III) (for CF3) in the absence of polymer. In order to determine the exact composition of the
product, conductometric titrations using method of continuous variations were performed.

**Figure 4.2.** Job’s plots for the conductometric titration of a) 1.0 mM CuCl₂ vs 1.0 mM K₄[Fe(CN)₆] for CF2 (top) and b) 1.0 mM CuCl₂ vs 1.0 mM K₃[Fe(CN)₆] for CF3 (bottom) respectively. χ is the mole fraction of CuCl₂.

Figure 4.2.a and 4.2.b show the Job’s plots obtained by the method of continuous variation for the conductometric titration of copper chloride with K₄[Fe(CN)₆] and K₃[Fe(CN)₆] respectively. The extreme left data points on the plots 4.1 a) and b) correspond to the ionic conductivity of pure 1.0 mM [Fe(CN)₆]⁴⁻ and [Fe(CN)₆]³⁻.
respectively. On the other hand, the data points at the extreme right correspond to the conductivity of 1.0 mM copper chloride solution. Between the extremes, the ionic reactants were reacted together in several relative proportions such that the total number of moles of the reactants is kept constant for each titration. It is clear that the mole fraction of copper chloride at which the conductivity is the least corresponds to the stoichiometry of the reaction since maximum yield of the solid product is obtained at this stoichiometry. From the Job’s plots, the mole fraction of copper chloride in both the cases was determined to be 0.60. This suggests the stoichiometric ratio of copper and anionic precursors as 3:2 and therefore the possible reactions for the formation of bulk products are shown below.

\[
3\text{CuCl}_2(\text{aq}) + 2K_2[\text{Fe}^{II}(\text{CN})_6]_2(\text{aq}) \rightarrow K_2\text{Cu}_3[\text{Fe}^{II}(\text{CN})_6]_2(\text{s}) + 6\text{KCl(\text{aq})}
\]

\[
3\text{CuCl}_2(\text{aq}) + 2K_3[\text{Fe}^{III}(\text{CN})_6]_2(\text{aq}) \rightarrow \text{Cu}_3[\text{Fe}^{III}(\text{CN})_6]_2(\text{s}) + 6\text{KCl(\text{aq})}
\]

Scheme 4-1. Reaction schemes for the synthesis of CF2 (K2Cu3[FeII(CN)6]2) and CF3 (Cu3[FeIII(CN)6]2) bulk.

The identity of the bulk products was confirmed by powder X-ray diffraction data. Figures 4.3 a) and b) are the PXRD patterns of bulk CF2 (top, shown in red) and CF3 (bottom, shown blue) respectively.
Figure 4.3. PXRD patterns of CF2 bulk (top, red) and CF3 bulk (bottom, blue). Also, shown in the bottom, in black spikes, is the PXRD pattern of Cu₃[Fe³⁺(CN)₆]₂·1.33H₂O found in database (JCPDS 70-2702).

Both exhibit similar patterns as they belong to the same space group and have similar coordination frame work. The pattern for CF3 matches well with that of Cu₃[Fe³⁺(CN)₆]₂·1.33H₂O (shown as black spikes, JCPDS 70-2702) and corroborates the composition determined by conductometric titration. For CF2 bulk, although the peaks are slightly broader, the peak positions agree well with that of K₂Cu₃[Fe²⁺(CN)₆]₂ reported
recently by Ojwang et al.\textsuperscript{129} It is noteworthy that a comparison of the relative intensities of the peaks (200) and (220) for CF2 and CF3 bulk patterns shows a reversal of the relative intensities albeit on an arbitrary scale. Furthermore, all the relevant peaks for CF2 appear at higher angles compared to CF3. The respective peak positions for CF2 and CF3 and their differences are listed in Table 4.1.

Table 4-1. Peak positions (2θ) and difference for the indexed peaks in PXRD of CF2 and CF3 bulk powders.

<table>
<thead>
<tr>
<th>Peak index</th>
<th>2θ for CF2</th>
<th>2θ for CF3</th>
<th>Δ (2θ\textsubscript{CF2}-2θ\textsubscript{CF3})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(220)</td>
<td>17.755</td>
<td>17.410</td>
<td>0.345</td>
</tr>
<tr>
<td>(200)</td>
<td>24.910</td>
<td>24.775</td>
<td>0.135</td>
</tr>
<tr>
<td>(400)</td>
<td>35.605</td>
<td>35.395</td>
<td>0.210</td>
</tr>
<tr>
<td>(422)</td>
<td>44.005</td>
<td>43.825</td>
<td>0.180</td>
</tr>
</tbody>
</table>

Both the observations are consistent with those reported by Ojwang et al. Based on the combined information from PXRD and conductometric titrations, the probable chemical compositions for CF2 and CF3 bulk were thus assigned as $\text{K}_2\text{Cu}_3[\text{Fe}^{\text{II}}(\text{CN})_6]_2$ and $\text{Cu}_3[\text{Fe}^{\text{III}}(\text{CN})_6]_2\cdot x\text{H}_2\text{O}$ ($x$ is undetermined considering it as irrelevant) respectively.

4.3.2 Synthesis and Characterization of CF2(1:1)@PVP NPs and CF3(1:1)@PVP NPs

A simple one-step salt metathesis reaction of the aqueous solutions of Cu(II) precursor and the anionic precursor was employed for the NPs synthesis at very low concentrations of the precursors. The precursors were used in a 1:1 molar ratio. Synthesis was performed in the presence of biocompatible polymer PVP (average mol wt. 40,000 g mol\textsuperscript{-1}) to facilitate the surface coating of CF2(1:1)@PVP and CF3(1:1)@PVP NPs during the
NP growth. The colloidal dispersions of the products were dialyzed and subjected to lyophilization to obtain pure and dry PVP coated NPs.

Figure 4.4. a) and c) are the TEM images of CF2(1:1)@PVP NPs and CF3(1:1)@PVP NPs respectively. b) and d) are the particle size histograms of the same respectively.

The TEM images and particle size distributions for CF2(1:1)@PVP and CF3(1:1)@PVP NPs are displayed in figure 4.4. The particles are roughly spherical and poly-dispersed and have an average size of 6.5 and 15.3 nm respectively. Synthesized under similar conditions, CF3(1:1)@PVP NPs have average particle size twice larger than NPs CF2(1:1)@PVP NPs.
Figure 4.5. EDX spectra of CF$_2$(1:1)@PVP NPs (top) and CF$_3$(1:1)@PVP NPs (bottom) respectively.

The energy dispersive x-ray (EDX) spectroscopic analysis of the NP confirms the presence of the elements Cu, Fe, C, N and O in both the NPs (figure 4.5). Furthermore, the presence of Cu, Fe and K were determined independently by AAS.
Figure 4.6. IR spectra of a) PVP40K (black), CF2 bulk powder (brown) and CF2_{(1:1)}@PVP NPs (red) (top) and b) PVP40K (black), CF3 bulk powder (green) and CF3_{(1:1)}@PVP NPs (blue).

The presence of PVP polymer coating on the surface of both NPs was confirmed by FTIR spectra for the lyophilized samples. Shown in the figure 4.6.a, at the top are the attenuated total reflectance (ATR) FTIR spectra of PVP 40K, bulk CF2, and
CF$_2(1:1)@$PVP. Figure 4.6.b at the bottom displays the ATR FTIR of PVP40k, bulk CF3 and CF$_3(1:1)@$PVP NPs. One distinct feature of the IR spectra of the PB and its analogs is the appearance of intense $\nu(C=\!\!\!N)$ stretching vibration band in the 2000-2150 cm$^{-1}$ region.$^{126-127, 130}$ For CF2 and CF3 bulk, this band appears at 2079 and 2101 cm$^{-1}$ respectively. Despite the small amount of NPs present in the polymer coated NPs of CF2 and CF3, this $\nu(C=\!\!\!N)$ stretching is distinctly identified (as pointed by arrows in the spectra) at 2101 and 2105 cm$^{-1}$ respectively. A comparison of IR spectra of NPs with the spectrum of pure polymer PVP reveals all the notable bands due to the polymer also present in those of NP samples. In particular, the C=O stretching vibration of the PVP amide unit at 1660 cm$^{-1}$ for pure polymer can be observed at 1649 and 1658 cm$^{-1}$ for CF$_2(1:1)@$PVP NPs and CF$_3(1:1)@$PVP NPs respectively. The slight red shift of the C=O stretching frequencies of the NP samples suggest the weakening of the bond C=O bond strength and is attributable to the binding of the polymer to the surface of NPs.

4.3.3 Stability Studies of Copper and Free Cyanide Release from CF$_2(1:1)@$PVP NPs and CF$_3(1:1)@$PVP NPs in Various Biologically Relevant Media

The polymeric coating on the surface imparts hydrophobicity to the NPs, allows for a longer circulation time in the blood pool and facilitates the non-receptor mediated endocytosis of the NPs into the cancerous cells in a disguised manner. However, an essential prerequisite for this to happen is that the NP by itself must remain stable in vivo from the point of its intake till it gets localized inside the cells of the intended tumor site. This means that CF2 and CF3 NPs must encounter varied environments under both the physiological conditions and the acidic tumor environment during its course of journey, that could pose a challenge to their structural integrity in terms of framework degradation.
and copper leaching. Any loss of the copper from the CF2 and CF3 NPs under such conditions, either due to trans-chelation by biomolecules like proteins and/or substitution by biologically relevant metal ions devoid these NPs of their purpose. Moreover, it is of paramount importance that the NPs should resist the framework degradation, \textit{in vivo}, which otherwise might result in the potential release of highly toxic free cyanide ions and serve the contrary to the original purpose of anti-cancer application. Therefore, the stability of the CF2 and CF3 NPs was studied in several biologically relevant media. PBS was employed for studies at physiological pH value of 7.4. Citrate buffer was used to maintain the acidic pH values of 6.2 and 4.8 respectively relevant to acidic tumor environment and cellular lysosomes respectively. Experimentally, 5.0 mL aqueous dispersions of the NPs of 3.0 mM total concentration of copper sealed in a dialysis bag were incubated in 50.0 mL of appropriate media for 24 hours at 37 °C; samples of the media were then analyzed for the copper content by AAS.

![Copper Leaching Results](image)

\textbf{Figure 4.7.} Copper leaching results under different conditions.
Figure 4.7 displays the extent of copper release for CF$_2$(1:1)@PVP NPs (red bars) and CF$_3$(1:1)@PVP NPs (blue bars) in a variety of media. The data shows that CF$_2$(1:1)@PVP NPs released no copper above the minimum detectable limit at physiological pH value of 7.4. Also, CF$_2$ NPs are immune to copper leaching even at highly acidic pH value of 1.0 suggesting its extreme stability against copper release. Furthermore, except in Zn$^{2+}$ containing media, no copper release beyond detectable level was observed in the presence of divalent metal ions. In the presence of Zn$^{2+}$ ions there is a 11% release of Cu$^{2+}$. Typical concentration of zinc in human blood, serum and urine is 800 ± 200, 109 to 130, and < 500 μg/dL serum. The corresponding values in ‘ppm’ are 8 ± 2, 1.09 to 1.30 and < 5 respectively. The concentration of Zn$^{2+}$ ions used for the study is 100 ppm which is approximately twelve times higher than the normal concentration of zinc in human blood. Therefore, the in vivo ion exchange of Cu$^{2+}$ in the presence of Zn$^{2+}$ for CF NPs may likely be minimal. At pH values of 4.8 and 6.2 maintained by 0.5 M citrate buffer, the Cu$^{2+}$ release was 25.5 and 46.4% respectively. The higher copper release at pH value of 6.2 can be attributed to the presence of citrate in di-anionic form that chelates the copper in a bidentate fashion. At pH value of 4.8, citrate exists as mono-anion and has no chelating ability which explains why the copper release was less compared to that at pH value of 6.2. CF$_3$(1:1)@PVP NPs on the other hand showed significant release of Cu$^{2+}$ in all the metal ion solutions as well as in PBS and citrate buffers. This is possibly due to the less stable copper coordination in the CF$_3$ framework which makes the surface Cu$^{2+}$ susceptible to ion exchange or leaching.

The potential release of cyanide ion in vivo in the event of degradation of the coordination network of PB could be a major hindrance for its bio-applications in view of
high toxicity of free cyanide ion. Hence, the stability of NPs towards free cyanide release in various biologically relevant media was experimentally verified by a fluorometric method using the commercially available cyanide test kit developed by LaMotte Co. (Chestertown, Maryland; Code 7387-01). As shown in the figure 4.8, even after incubation of NPs for 48 hours, free cyanide levels are still less than 0.2 ppm. Both the NPs are found to be extremely stable. These levels are lower than the “maximum contaminant level” limit for free cyanide in drinking water (0.2 ppm) enforceable under National Primary Drinking Water Regulations set by United States Environment Protection Agency (EPA). Only under extremely acidic pH value of 1.0, CF3 NPs showed a release of 0.4 ppm of free cyanide after 48 hours. The free cyanide test results confirm the inertness of the framework structure against degradation.

Figure 4.8. Free cyanide release in different media.

Therefore, it is clear that from the above results that CF2(1:1)@PVP NPs have an inert framework structure with strongly bound Cu$^{2+}$ and is highly stable under
physiological conditions. On the other hand, it is reasonable to assume that CF3\textsubscript{(1:1)}@PVP NPs has highly inert framework towards degradation but with less tightly bound Cu\textsuperscript{2+} which make it less stable towards copper leaching under the same conditions.

4.3.4 Cellular ROS Generation by CF2\textsubscript{(1:1)}@PVP NPs and CF3\textsubscript{(1:1)}@PVP NPs

To test whether surface copper of CF2\textsubscript{(1:1)}@PVP NPs and CF3\textsubscript{(1:1)}@PVP NPs can cause the elevation of intracellular ROS level, a widely used DCFDA assay was employed. 2',7'-dichlorofluorescein diacetate (DCFDA) is a cell permeable non-fluorescent precursor which gets oxidized by cellular ROS to produce cell impermeable 2’,7’- dichlorofluorescein (DCF) dye that has an emission wavelength at 525 nm.
Dose dependent ROS elevation by CF$_2$

\((1:1)\)@PVP NPs (red, top) and CF$_3$

\((1:1)\)@PVP NPs (bottom, blue) in PC3 cells. Control refers to untreated PC3 cells. The emission intensity is therefore a measure of cellular ROS level. The assay was conducted in a dose dependent manner. After treatment of PC3 cells with the NPs for
4 hours, followed by a brief exposure to the dye precursor DCFDA, the fluorescence intensities at 525 nm were measured and compared to the untreated cells. As shown in the figure 4.9.a and b, both CF2(1:1)@PVP NPs and CF3(1:1)@PVP NPs caused a significant increase in emission intensity w.r.t the control cells in a dose dependent manner. The results suggest that upon cellular uptake, both the NPs can cause increased oxidative stress by elevation of ROS level in cancerous cell lines. This can be understood by the Fenton-like redox reaction mediated by surface copper Cu\(^{2+}\) and Cu\(^{+}\) in the presence of cellular H\(_2\)O\(_2\).

4.3.5 Cytotoxicity of CF2(1:1)@PVP NPs and CF3(1:1)@PVP NPs

The ability of CF2(1:1)@PVP NPs and CF3(1:1)@PVP NPs to induce cell death was studied in PC3 (prostate cancer) cell line. The cell viability was determined by MTT assay after treatment of PC3 cells with NPs for 24 hours. Figures 4.10.a and b display the PC3 cell viability profiles for CF2 and CF3 NPs respectively. The average cell viability at each concentration determined from three independent trials was plotted against the log of copper concentration. From the sigmoidal curve fitting of the dose response profiles, the average IC\(_{50}\) values of CF2(1:1)@PVP NPs and CF3(1:1)@PVP NPs were determined to be 55 ± 5 μM and 75 ± 8 μM respectively. The corresponding values in μg/mL are 3.5 ± 0.3 and 4.8 ± 0.5 respectively. The difference in the IC\(_{50}\) values may be attributed to the difference in the size of the NPs of CF2 (6.5 nm) and CF3 (15.3 nm).
Figure 4.10. Cytotoxic effects of CF2(1:1)@PVP and CF3(1:1)@PVP NPs on prostate cancer (PC3) cell line.

Furthermore, for CF2(1:1)@PVP NPs the size dependent cytotoxicity on PC3 cells was demonstrated. Two different higher sized CF2 NPs namely 5CF2(1:1)@PVP NPs and 10CF2(1:1)@PVP NPs were synthesized starting with 5 and 10 times the concentrations of the copper and ferro cyanide precursor solutions used to prepare the original CF2(1:1)@PVP NPs while using the same amount of polymer (see the experimental
The sizes for 5CF2 and 10CF2 NPs are yet to be determined. In general, increasing the amount of precursors tend to increase the size of the NPs in the synthesis. Hence, it is presumed that the sizes are progressively higher for 5 CF2 and 10CF2 NPs compared to original CF2(1:1)@PVP NPs.

**Figure 4.11. Size dependent cytotoxic effect of CF2(1:1)@PVP NPs on PC3 cell line.**

PC3 cell viability studies of the three NPs were compared. As shown in the figure 4.11, CF2(1:1)@PVP NPs of the smallest size exhibited the highest cytotoxicity with an IC50 value of 55 ± 5 μM. On the other hand, 5CF2(1:1)@PVP NPs and 10CF2(1:1)@PVP NPs were not significantly toxic. Even at the highest dose of 500 μM of copper, the cell viability is as high as 80% for these NPs. The IC50 values for the later were undetermined since they could be at least ten times higher than that of CF2(1:1)@PVP NPs and beyond.
the range of copper concentrations studied for the original NPs. Such a drastic difference in cell viability profiles may be attributed to the differences in the sizes of the NPs. Since, CF2 NPs have the smallest size, they possess the highest surface area to volume ratio. Consequently, the number of active copper sites per unit surface area of the NPs is also the highest for CF2\textsubscript{(1:1)}@PVP NPs making them highly toxic for cancerous cells.

The non-toxicity of higher sized CF2 NPs allows for the other biological applications, for example as a drug delivery agent. One such glaring application was reported recently by Mukherjee et al.\textsuperscript{26} In their studies they showed that CF3 NPs, which they referred as CPB\textsubscript{(1:1)} NPs, with an average size ~65 nm are biocompatible even at a higher dose of 4.370 \( \mu \)g/\( \mu \)L of copper (equivalent to 68.8 mM of copper) as determined by MTT assay. The biocompatibility led to the evaluation of CPB\textsubscript{(1:1)} NPs as drug delivery agent for the slow and sustained release of Doxorubicin (a drug approved by FDA for chemotherapy) in cancer therapy.

4.3.6 Dye Labeling and Cellular Uptake of CF2\textsubscript{(1:1)}@PVP and CF3\textsubscript{(1:1)}@PVP NPs

The cellular internalization of CF2 and CF3 NPs was assessed by confocal microscopy using the Texas Red dye labeled NPs. Texas Red cadaverine C5 is a red fluorescent dye with an emission wavelength of 602 nm when excited at 582 nm. CF3 NPs were chemically conjugated to Texas Red C5 dye on their surface by EDC coupling. For CF2 NPs, attempts to conjugate the dye with CF2 NPs surface coated the citric acid/citrate groups \textit{via} EDC coupling led to the aggregation of the particles during the coupling reaction. Hence for CF2, the NPs were directly treated with dye to facilitate the attachment of the dye through electrostatic interactions. The presence of the dye on the
NP surface was confirmed by fluorescence spectroscopy after extensive dialysis of the reaction mixture to remove the unbound dye.

Figure 4.12 displays the confocal images of PC3 cells treated with dye labeled CF3\(_{(1:1)}\)@PVP NP for 3 hours. The red fluorescence observed around the dark area of the nuclei of the PC3 cells, using the z-scanning mode of the microscope, indicates the localization of the NPs in the cytoplasmic region possibly due to the non-receptor mediated endocytosis. In contrast, no fluorescence was observed for control cells (untreated cells).

4.3.7 Synthesis and Characterization of RLCF2@PVP-Cit NPs

The other part of the project focused on studying the suitability of CF2 NPs as PET imaging agent. \(^{64}\)Cu is a positron emitting radioisotope and owing to its favorable decay characteristics has been extensively studied for its use as PET imaging
applications. This area is predominantly comprised of $^{64}\text{Cu}$ copper-chelates based on macrocyclic ligands. The main focus in this area is devoted to the development of suitable macrocyclic chelates, more recently as bifunctional chelates, to impart kinetic and thermodynamic stability to the metal-chelates. Numerous studies have shown that these copper-chelates suffer from copper exchange with biomolecules \textit{in vivo via} ‘transchelation’.

Although studies utilizing $^{64}\text{Cu}$ labeled NPs have begun just less than a decade, the radioisotope is linked to these NPs through the chelates that are chemically conjugated to the surface of NPs. These NPs also suffer from the drawbacks namely the displacement of radionuclide \textit{via} “transchelation” and / or the detachment of the radionuclide-chelates from the NP surface. Alternatively, the high stability of CF2 NPs (as shown in the previous section of this chapter) under the physiologically relevant conditions may provide kinetic and thermodynamic stability and thus eliminate the aforementioned limitations associated with chelate systems. Additionally, if intrinsically labeled, the NPs possess high loading of specific activity and can act as better and sensitive imaging agents. To the best of our knowledge only copper based NPs ie. CuS NPs that were incorporated with labeled $^{64}\text{Cu}$ within the NPs have been reported to date.\textsuperscript{40,58} In order to apply CF2 NPs for this purpose, the NPs have to be nontoxic in addition to being stable. It is well known that surface coating of NPs with citrate ligands passivates the NPs and renders them less toxic. Therefore, we have modified the synthesis of CF2\textsubscript{(1:1)}@PVP NPs to include the citrate coating on the surface of NPs in addition to the biocompatible polymer PVP. The resulting NPs are labeled as RLCF2@PVP-Cit NPs.
Figure 4.13. TEM image of RLCF2@PVP-Cit NPs (top) and particle size distribution (bottom).
TEM imaging revealed that NPs are roughly spherical in shape with an average diameter of 6.06 ± 2.32 nm (figure 4.13). The average size is similar to that of CF2(1:1)@PVP NPs.

![Figure 4.14. IR spectra of citric acid (green), PVP40K (black), RLCF2 bulk (blue), and RLCF2@PVP-Cit NPs (red).](image)

The presence of polymer coating on the surface of NPs was confirmed by FTIR spectroscopy. One distinct feature of the IR spectra of the PB and its analogs is the appearance of intense ν(C≡N) stretching vibration band in the 2000-2150 cm⁻¹ region. This characteristic band also appeared at 2015 cm⁻¹ in the spectrum for the lyophilized sample of RLCF2@PVP-Cit NPs figure 4.14). A comparison of IR spectra of NPs with the spectrum of pure polymer PVP 40K reveals all the notable bands due to the polymer were also present in those of NP samples. In particular, the C=O stretching vibration of the PVP amide unit at 1660 cm⁻¹ for pure polymer can be observed at 1657 cm⁻¹. However,
no peaks related to the citric acid could be assigned for the spectrum of RLCF2@PVP-Cit NPs based on the spectrum for pure citric acid; therefore, the presence of citrate coating could not be confirmed unambiguously. This is probably due to the large excess of polymer that effectively masked the IR bands of the citrate coating.

4.3.8 Stability of RLCF2@PVP-Cit NPs

![Copper leaching results of RLCF2@PVP-Cit NPs under different conditions.](image)

Figure 4.15. Copper leaching results of RLCF2@PVP-Cit NPs under different conditions.

One of the major concerns of the use of $^{64}$Cu-chelate complexes is the transchelation or the detachment of the radionuclide Cu from the copper chelates. Copper leaching is also a possibility in case of NPs in the presence of other biologically relevant ions. Hence, the stability of NPs towards copper leaching was studied in several media. NPs dispersions of total copper concentration of 3.0 mM sealed in dialysis bags were soaked in DI water, PBS and $M^{2+}$ solution of 100 ppm concentration (where $M= Mg^{2+},$
Ca$^{2+}$, Mn$^{2+}$, Fe$^{2+}$ and Zn$^{2+}$) at 37 °C. The media were analyzed for copper content by AAS after 4 hours and 24 hours. The copper released expressed as the percent of copper w.r.t to the theoretical maximum at both time points for all the media is shown in figure 4.15. The copper release is below the detectable level of the instrument indicating the high stability of RLCF2@PVP-Cit NPs. Furthermore, the copper release in Fe$^{2+}$ containing medium was only 0.7% after 24 hours.

4.3.9 Cell viability of RLCF2@PVP-Cit NPs

The cytotoxicity of RLCF2@PVP-Cit NPs on HeLa cell line was evaluated by three independent trials using MTT assay. The average cell viability of the three trials was plotted at each concentration of total copper of NP solution.

![Graph showing cell viability](image)

Figure 4.16. Viability of He La cells after incubation with RLCF2@PVP-Cit NPs for 24 hours determined by MTT assay.
After 24 hours of incubation of He La cells with the NP containing medium, the cell viability was found to be above 90% even at the highest concentration of copper tested i.e. at 80 μM (figure 4.16). In comparison, the IC\textsubscript{50} value of CF2\textsubscript{(1:1)}@PVP NPs for PC3 cell line was 55 μM. The strongly bound citrate coating on the surface of the NPs passivates and makes the NPs less or non-toxic. Usually for PET application, the radiotracers are required below nanomolar quantities. The highest concentration of copper in NP solution tested for cell viability was roughly of four orders of magnitude higher than the amount required for practical applications. Therefore, it may be concluded that RLCF2@PVP-Cit NPs are safe for practical uses.

4.3.10 Cellular Internalization of RLCF2@PVP NPs

For cellular uptake studies, dye labelling of NPs using Texas Red Cadaverine C5 was performed following a similar procedure for CF2 NPs. Figure 4.17 display the confocal images of HeLa cells treated with dye labeled RLCF2@PVP-TC5 for 3 hours. The red fluorescence observed around the dark area of the nuclei in the NP treated HeLa cells, using the z-scanning mode of the microscope, indicates the localization of the NPs in the cytoplasmic region possibly due to the non-receptor mediated endocytosis. In contrast, no fluorescence was observed for control cells (untreated cells).
4.4 Conclusions

In this project, we demonstrated for the first time that copper PBAs of both hexacyanoferrates have the anti-cancer effect when the sizes are 15 nm or below. DCFDA assays results reveal that the NPs can induce oxidative stress in cancer cells by catalytically elevating the ROS level. The cytotoxic affects can be tailored by tuning the size of the CF2 NPs. Furthermore, with the appropriate choice of the surface coating agent, the surface properties of the CuPBA NPs can be modified to impart toxicity or non-toxicity and accordingly applied for anti-cancer or diagnostic applications.
5. Synthesis and Evaluation of $K_{2}Cu_{x}Mn_{3-x}[Fe(CN)_{6}]_{2}$ Solid Solution Prussian Blue Analog Nanoparticles as Single Agent for Theranostic Potential

5.1 Introduction

Nanotheranostics is currently an intensely pursued field of nanotechnology that encompasses the design and utility of a variety of novel as well as well-established nanosystems empowered with therapeutic and diagnostic capabilities intrinsically or by external means. Virtually, most of the well-known inorganic nanosystems including gold NPs, quantum dots, metal oxides like super paramagnetic iron oxide (SPION), silica, carbon materials, Prussian blue are being explored for the development of effective theranostic agents. The choices of NP system, diagnostic imaging modality (MR, optical scattering, fluorescence, PET, SPECT, CT, ultrasonography) and therapeutic approach (conjugated drugs, intrinsic NIR absorption, magnetic heating, radiotherapy, gene therapy, photoacoustic) offer a vast array of combinations to explore and bring forth effective multifunctional systems for an intended theranostic purpose. As a consequence, this field has been rapidly expanding and holds promise for some of the theranostic agents finding their way to clinical use.

Magnetic Resonance Imaging (MRI) is one of the widely used non-invasive imaging modality that provides real-time, high resolution images with excellent soft
tissue contrast, greater anatomical and functional details. A large number of nanotheranostics with MR imaging modality are based on superparamagnetic iron oxide (SPIO) nanoparticles. SPIO particles are T2 weighted contrast agents and are FDA approved for clinical imaging of liver (Feridex®-USA; Endorem®-Europe). However, the negative contrast provided by T2 agent is reflected as a dark domain with low signal intensity in the image and cannot be distinguished from hypointense regions such as hemorrhage and blood clots that are common in many lesions. In addition, the high superparamagnetic susceptibility of SPIO particles can induce localized magnetic field perturbations in the surrounding tissue and cause susceptibility artifacts also called as ‘blooming effect’. This effect results in obscure images that demolishes the background around the lesions. On the other hand, due to their signal-enhancing positive contrasting ability, T1-weighted contrast agents are much preferable. Although most of T1 contrast agents are predominantly Gd-chelates, the association of a Gd-chelates with nephrogenic systemic fibrosis (NSF) in some of the patients raised safety concerns regarding their usage. This puts impetus on non-Gd substitutes for developing T1 agents with better safety profile. Paramagnetic Mn(II) ion is the natural choice since its contains five unpaired electrons. Both Mn based NPs and chelates are being reported with improved $r_1$ relaxivity needed for an efficient T1 agent.

In the context of theranostic applications, manganese based theranostics are relatively sporadic. Moreover, the construction of theranostic agents generally involves complex multistep chemical procedures to impart the functionalities. It is therefore, interesting to develop through facile approaches a single NP agent that has an intrinsic
capability of exhibiting both MR imaging modality and anti-cancer activity. Such systems are rarely reported.

In this chapter, a single step synthesis of homogeneous solid solution of copper-manganese Prussian blue analog nanoparticles with general formula $K_{2Cu^xMn^{3-x}}[Fe(CN)_6]_2$ labeled as, CxM@PVP PBA NPs is described. The relative proportions of copper and manganese precursors can be varied to synthesize series of solid solutions. For a representative sample C60M@PVP NPs with Cu to Mn ratio of 3:2, the anti-cancer and magnetic relaxivity properties are studied and reported.

5.2 Experimental Section

5.2.1 Materials

Unless otherwise stated, all the chemicals were purchased from Sigma-Aldrich and used as such without further purification. Copper standard (1000 ± 10 $\mu$g/mL copper in 3% (v/v) HNO$_3$ was purchased from inorganic ventures. Regenerated cellulose membranes (MWCO: 3500 kDa and MWCO 12,000-14,000 kDa) were obtained from Membrane Filtration Products, Inc. (Texas, USA). DI water was obtained from an in-house IONPURE Plus 150 deionization unit. The resistivity of the DI water is at least 17 M$\Omega$-cm.

5.2.2 Synthesis of CxM PBA Bulk Powders

(where $x$ is in % copper 0, 20, 40, 60, 80 and 100; letters C and M refer to copper and manganese):

Synthesis of the bulk solids was performed at room temperature. Briefly, to an aqueous solution of $K_4[Fe(CN)_6]$ (100 mM, 50.0 mL) while stirring, an aqueous solution
containing CuCl₂ and MnCl₂ in the desired ratio, such that the molar ratio of (Cu+Mn) to Fe(CN₆)⁴⁻ for all the preparations was 3:2, was added. The colors of the mixture varied from white for x = 0 (i.e. no Cu(II)), pale claret for x = 20 to deep claret for x = 100 (i.e. no Mn(II)). The mixtures were stirred for an additional hour at RT and then dialyzed against DI water overnight to remove the ionic byproduct. The dialyzed mixtures were lyophilized to obtain pure and dry bulk products.

5.2.3 Synthesis of CₓM@PVP PBA NPs (where x = 20, 40, 60 and 80)

50 mL of an aqueous mixture containing 0.35 g of PVP (avg m.wt. 40,000 g/mol) and varied concentrations of CuCl₂ and MnCl₂ (as indicated by the copper percent for the corresponding NPs) such that the total concentration of the salts is always 1.0 mM were prepared and added rapidly to 50 mL of an aqueous solutions of 1.0 mM K₄[Fe(CN)₆] containing 0.40 g of PVP while stirring. The mixture was continued to stir for 30 minutes and then dialyzed against DI water to remove the ionic byproducts. After an overnight dialysis, the NP dispersion was lyophilized to obtain pure and dry polymer coated NPs. The series of products were labeled as CₓM@PVP PBA NPs, where CₓM represents the homogeneous solid solutions of copper-manganese ferrocyanide PB analogs (PBAs) with varied percentages represented by ‘x’ of copper. Here x = 0 represents pure manganese ferrocyanide PBA K₂Mn₃[Fe(CN)₆]₂ and x = 100 represents pure copper ferrocyanide PBA K₂Cu₃[Fe(CN)₆]₂. The x = 20, 40, 60 and 80 represent the percent copper of the copper-manganese precursor mixture for which the corresponding theoretical compositions of the products are K₂Cu₀.₆₀Mn₂.₄₀[Fe(CN)₆]₂, K₂Cu₂.₂₀Mn₁.₈₀[Fe(CN)₆]₂, K₂Cu₁.₈₀Mn₁.₂₀[Fe(CN)₆]₂ and K₂Cu₂.₄₀Mn₀.₆₀[Fe(CN)₆]₂ respectively.
5.2.4 PXRD of CxM Bulk Powders

Powder X-ray diffraction pattern (PXRD) was obtained at RT on a Rigaku Geigerflex X-ray powder diffractometer operated at 45 kV and 35 mA. The experimental set up used copper as a radiation source (Cu K$_\alpha$ radiation). The pattern was collected in a range of 2θ values from 10.00° to 70.00° with a step size 0.02°.

5.2.5 Atomic Absorption Analysis of the CxM Bulk and NPs

A small amount of weighed sample was treated with con. HNO$_3$ in a crucible and heated to dryness. The residue was then heated in a furnace at 630 °C for 4 hours. The inorganic residue was then dissolved with con. HNO$_3$ and con. HCl. The final volume was made up to 100 mL and analyzed by AAS for Cu and Mn set at absorption wavelengths of 324.7 nm and 279.5 nm respectively. Appropriate dilution factor was applied wherever required.

5.2.6 Transmission Electron Microscopy (TEM) Imaging and EDX Measurements of NP samples

A small amount of lyophilized NP sample was re-dispersed in 750 $\mu$L of DI water followed by addition of an equal quantity of acetone. The mixture was centrifuged and the pellet was re-dispersed in 500 $\mu$L DI water followed by equal amount of acetone. The centrifugation step was repeated and the pellet of the NPs was finally dispersed in 500 $\mu$L DI water. Small droplet of this NP dispersion was placed on the TEM grid and let air dried overnight. Particle images and EDX spectrum were acquired using a 200 kV FEI Technai F20 Transmission Electron Microscope (TEM) equipped with a field emission gun and an attached EDAX spectrometer in STEM mode. The operating voltage and current of the instrument were 4000 V and 55 $\mu$amp respectively.
5.2.7 Stability Towards Metal Release of C60M@PVP PBA NPs in Various Media

The stability of the C60M@PVP PBA NPs towards metal leaching was evaluated in a variety of media. Briefly 5.0 mL samples of NP dispersions of total copper concentration of 3.0 mM were sealed in dialysis bags (MWCO 3,500) and incubated at 37 °C in each of 100 mL of HCl solution of pH value 1.0, 0.1 M citrate buffers of pH values 4.8 and 6.2, PBS (pH value of 7.4) and various M(II)Cl₂ salt solutions of 100 ppm concentration of M(II) ion (where M(II) is Mg(II), Ca(II), Fe(II) and Zn(II)). The media were analyzed for the leached manganese and copper content at time periods of 5 and 24 hours using AAS. The metal ion release was expressed as the percentage of the theoretical total concentration of the corresponding metal ion (ppm).

5.2.8 Free Cyanide Release Test

Free cyanide release in the samples from stability test in various biologically relevant media was also experimentally verified after 48 hours of incubation by a colorimetric method using the commercially available cyanide test reagent strip™ kit obtained from Industrial Test Systems, Inc. South Carolina, USA. Manufacturers protocol was followed for analysis of the samples.

5.2.9 Cell Culture

Prostate cancer (PC3) cells were cultured in Minimum Essential Medium (MEM) supplemented with 10% FBS. Cells were incubated at 37 °C in water-saturated air supplemented with 5% CO₂. Spent medium from the flasks was removed and fresh complete growth medium was added to the flask every three days. Cells were passaged at 85% confluence. Cell counting was performed by trypan blue exclusion method using a hemocytometer.
5.2.10  Cell Viability Studies

The cytotoxicity of the CHP NSs was evaluated by 3-[4, 5-dimethylthiazol-2-y1]-2, 5-diphenyltetrazolium bromide (MTT) assay. Typically, cells were seeded in a 96 well-plate at a cell density of 10,000 cells/well and incubated at 37 °C in water-saturated air supplemented with 5% CO₂ for 24 hours to allow for cell attachment. Drug solutions of required copper concentrations were prepared by serial half dilution from a stock solution of aqueous NSs dispersion in the complete growth medium. Cells in each well were then treated with the 100 μL of NP drug solutions prepared by serial half dilutions from the initial NP stock solution and incubated at 37 °C in water-saturated air supplemented with 5% CO₂. The experiment was performed in triplicate of wells for each concentration of the NPs. Control cells were also grown in the same well-plate without the treatment of drug. After 24 hours, the medium was removed from each well followed by addition of 100 μL of fresh medium and 25 μL of MTT dye. After 4 hours of incubation with the dye, the spent medium was aspirated out followed by the addition of 100 μL of DMSO to each well to dissolve the purple formazan crystals. After 15 minutes, the absorbance of the solution in each well was measured at 560 nm using a microplate reader. Cell viability at each concentration was calculated from the ratio of the absorbance of the drug treated sample and the average absorbance of control and expressed as percentage along with the deviation. The average IC₅₀ value was obtained from three independent trials involving at least two different batches of synthesized NPs.

5.2.11  Magnetic Relaxivity Measurements

Longitudinal (T₁) and transverse (T₂) relaxation measurements for the NP samples were obtained at 60 MHz and 1.4 T magnetic field strength using a Bruker Minispec
mq60 (Bruker, Billerica, MA). The colloidal samples of C$_{60}$M@PVP were prepared by dissolving appropriate amount of lyophilized NPs in DI water, adjusted to have an initial manganese concentration of 3.0 mM and further diluted serially to have successive samples of concentrations half of their previous. The longitudinal ($r_1$) and transverse ($r_2$) relaxivity values were determined as the slopes from the linear fit for the plots of $1/T_1$ or $1/T_2$ Vs manganese concentration respectively.

5.3 Results and Discussion

5.3.1 Synthesis and Characterization of CxM Bulk and CxM@PVP NPs

Synthesis of the series of bulk solid solutions CxM (x refers to the percent of copper of total copper and manganese in the precursor solution), were accomplished at room temperature by simple salt metathesis reaction between the ferrocyanide anionic precursor and different aqueous mixtures containing varied proportions of copper and manganese salts. Nanoparticles synthesis was performed successfully in the similar way but in the presence of the biocompatible polymer PVP 40,000.

In the previous chapter, based on the conductometric titration studies and PXRD characterization, it was established that the stoichiometric ratio for the reaction between Cu$^{2+}$ precursor and ferrocyanide is 3:2. The same stoichiometric ratio was experimentally determined by a previous colleague for the reaction between manganese (II) salt and potassium ferrocyanide.$^{150}$ Therefore, taking both these cases into account, the possible reactions for the formation of bulk products are represented by a generalized reaction scheme shown below.
Scheme 5-1. Synthesis of a) CxM bulk and b) CxM@PVP NPs.

The general formula of the solid solutions can be given as $K_2Cu_xMn_{3-x}[Fe(CN)_6]_2$ (here ‘x’ is the stoichiometric coefficient of copper). As shown in the table 5.1 and 5.2, the chemical compositions of four different solid solutions for bulk and nanoparticles determined by AAS analysis of the copper and manganese contents agree well with the expected compositions. Moreover, the fixed ratio of Cu to Mn in the product closer to the theoretical ratio suggests that the products are likely homogenous solid solutions where in the Cu and Mn are homogeneously distributed in the framework. The expected compositions were calculated based on the mole percent of copper from the total moles of copper and manganese of the precursor salts used in the staring mixture for reaction with ferrocyanide.
### Table 5-1. Composition of CxM bulk powders.

<table>
<thead>
<tr>
<th>% Cu in the starting mixture and label</th>
<th>Cu:Mn (calculated)</th>
<th>Cu:Mn (bulk; AAS)</th>
<th>Expected composition of Bulk CxM</th>
<th>Bulk CxM composition (AAS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 C20M</td>
<td>0.25</td>
<td>0.29</td>
<td>$K_2Cu_{0.60}Mn_{2.40}[Fe(CN)_6]_2$</td>
<td>$K_2Cu_{0.68}Mn_{2.32}[Fe(CN)_6]_2$</td>
</tr>
<tr>
<td>40 C40M</td>
<td>0.67</td>
<td>0.69</td>
<td>$K_2Cu_{1.20}Mn_{1.80}[Fe(CN)_6]_2$</td>
<td>$K_2Cu_{1.20}Mn_{1.80}[Fe(CN)_6]_2$</td>
</tr>
<tr>
<td>60 C60M</td>
<td>1.50</td>
<td>1.49</td>
<td>$K_2Cu_{1.80}Mn_{1.20}[Fe(CN)_6]_2$</td>
<td>$K_2Cu_{1.80}Mn_{1.20}[Fe(CN)_6]_2$</td>
</tr>
<tr>
<td>80 C80M</td>
<td>4.00</td>
<td>4.26</td>
<td>$K_2Cu_{2.40}Mn_{0.60}[Fe(CN)_6]_2$</td>
<td>$K_2Cu_{2.43}Mn_{0.57}[Fe(CN)_6]_2$</td>
</tr>
</tbody>
</table>

### Table 5-2. Composition of CxM@PVP NPs.

<table>
<thead>
<tr>
<th>% Cu in the starting mixture and label</th>
<th>Cu:Mn (calculated)</th>
<th>Cu:Mn (NPs; AAS)</th>
<th>Expected Composition of CxM@PVP NPs</th>
<th>CxM@PVP NPs composition (AAS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 C20M NPs</td>
<td>0.25</td>
<td>0.29</td>
<td>$K_2Cu_{0.60}Mn_{2.40}[Fe(CN)_6]_2$</td>
<td>$K_2Cu_{0.68}Mn_{2.32}[Fe(CN)_6]_2$</td>
</tr>
<tr>
<td>40 C40M NPs</td>
<td>0.67</td>
<td>0.74</td>
<td>$K_2Cu_{1.20}Mn_{1.80}[Fe(CN)_6]_2$</td>
<td>$K_2Cu_{1.28}Mn_{1.72}[Fe(CN)_6]_2$</td>
</tr>
<tr>
<td>60 C60M NPs</td>
<td>1.50</td>
<td>1.65</td>
<td>$K_2Cu_{1.80}Mn_{1.20}[Fe(CN)_6]_2$</td>
<td>$K_2Cu_{1.87}Mn_{1.13}[Fe(CN)_6]_2$</td>
</tr>
<tr>
<td>80 C80M NPs</td>
<td>4.00</td>
<td>6.08</td>
<td>$K_2Cu_{2.40}Mn_{0.60}[Fe(CN)_6]_2$</td>
<td>$K_2Cu_{2.58}Mn_{0.42}[Fe(CN)_6]_2$</td>
</tr>
</tbody>
</table>
5.3.2 PXRD Characterization

The PXRD patterns of CxM PBA powders, K$_2$Cu$_3$[Fe(CN)$_6$]$_2$ and K$_2$Mn$_3$[Fe(CN)$_6$]$_2$ are provided in appendix A. All the patterns were similar as expected. Pure K$_2$Cu$_3$[Fe(CN)$_6$]$_2$ and K$_2$Mn$_3$[Fe(CN)$_6$]$_2$ are isostructural PBAs and hence the incorporation of one transition metal ion into the lattice of another with the substitution of the later can, in theory, lead to homogeneous solid solution with random distribution of both the metal ions. Figure 5.1 shows the variation in the relative intensities of (220) and (200) peaks with copper content. Barring the outlier at the copper fraction of 0.225, the relative intensities followed a linear trend with change in copper content which is an indication of homogenous distribution of copper and manganese ions in the framework. It is not clear why the outlier at lower percentage of copper deviated from the linear relationship. In spite of repetition using the sample prepared in a different batch, the results obtained were the same for lower percent of copper.

![Figure 5.1. Variation of relative intensities with Cu content in CxM PBA.](image_url)
5.3.3 TEM Characterization of C60M@PVP PBA NPs solid solution

As a representative sample, the TEM image of C60M@PVP NPs is shown in figure 5.2 a.

![TEM image and particle size distribution](image)

**Figure 5.2. a) TEM image and b) particle size distribution of Cu60M@PVP NPs.**

The particles are highly poly-dispersed in nature and have an average size of 19 nm (figure 5.2.b). The morphology of the particles is varied and dependent on size. A closer observation at higher magnification (image not included) revealed that smaller NPs tend to have roughly spherical morphology and as the particles get bigger they tend to acquire the imperfect cubic morphology with softer corners. The energy dispersive x-ray (EDX) spectroscopic analysis of the C60M@PVP NPs confirmed the presence of the elements K, Cu, Mn, Fe, C, N and O in the NPs (figure 5.3). The presence of Cl peak is presumably due to the trapped byproduct, KCl, bound strongly on the surface of NP by electrostatic interactions. The presence of Cu, Fe and K were independently confirmed by AAS.
Furthermore, the presence of surface coated polymer in the NPs was confirmed by FTIR spectra for the lyophilized NP samples (figure 5.4). A characteristic feature of the IR spectra of the PB and its analogs is the appearance of intense $\nu(C≡N)$ stretching vibration band in the 2000-2150 cm$^{-1}$ region. For C60M bulk, this band appears at 2064 cm$^{-1}$ respectively. Despite the small amount of NP content present in the polymer coated NPs, the $\nu(C≡N)$ stretching is distinctly identified (as pointed by arrow) at 2075 cm$^{-1}$ respectively. Moreover, a comparison of IR spectra of NPs and pure polymer PVP 40K reveals all the notable bands due to the polymer were also present in those of NP samples. In particular, the C=O stretching vibration of the PVP amide unit at 1660 cm$^{-1}$ for pure polymer can be observed at 1658 cm$^{-1}$ for C60M@PVP PBA NPs.

**Figure 5.3. EDX spectrum of C60M@PVP NPs.**
Figure 5.4. IR spectra of a) PVP40K, b) C60M bulk powder and c) C60M@PVP NPs.
The slight shift of the C=O stretching frequency of the NP samples suggests the weakening of the bond C=O bond strength and is attributable to the binding of the polymer to the surface of NPs.

5.3.4 Stability Studies of Metal and Free Cyanide Release of C60M@PVP PBA NPs in Various Biologically Relevant Media

An essential prerequisite for the NPs is the in vivo stability from the point of their intake until localized inside the cells of the tumor site. This means that C60M@PVP NPs must encounter varied environments under both the physiological conditions and the acidic tumor environment during its course of journey that could pose challenge to their structural integrity in terms of framework degradation and metal leaching. Any loss of the copper or manganese or both from the NPs under such conditions, either due to trans-chelation by biomolecules and/or substitution by biologically relevant metal ions devoid these NPs of this purpose. Furthermore, it is of paramount importance that the PBA NPs should resist the framework degradation in vivo, which otherwise might release toxic free cyanide ions and serve the contrary to the original purpose of theranostic application. Therefore, the stability of the C60M@PVP NPs was studied in several biologically relevant media. PBS was employed for studies at physiological pH and ionic strength of the blood. Citrate buffer was used to maintain the acidic pH values of 6.2 and 4.8 respectively relevant to acidic tumor environment and cellular lysosomes respectively. Experimentally, 5.0 mL aqueous dispersions of the NPs of 3.0 mM total concentration of copper sealed in dialysis bags were incubated in 50 mL of appropriate media for 24 hours at 37 °C; samples of the media were then analyzed for the copper and manganese content by AAS.
Figure 5.5. Copper leaching results under different conditions.

Figure 5.5 displays the extent of copper release for C60M@PVP NPs after 5 hours (blue bars) and 24 hours (green bars) of incubation in a variety of media. The data shows that C60M@PVP NPs released no copper above the minimal detectable limit at physiological pH value of 7.4 even after 24 hours. Also, the NPs are inert towards copper leaching at acidic pH value of 4.8 suggesting its extreme stability towards copper release in endosomes once internalized in the cells. At pH value of 6.2 maintained by 0.5 M citrate buffer, the Cu$^{2+}$ release was 4.9 and 17.0 % respectively after 5 and 24 hours. The higher copper release at pH value of 6.2 can be attributed to the presence of citrate in di-anionic form that chelates the copper in a bidentate fashion. At pH value of 4.8, citrate exists as mono-anion and has no chelating ability that explains why the copper release was less compared to that at pH value of 6.2. Furthermore, copper release in Mg(II) and Ca(II) solutions was below the detectable level (shown as 0 %) after 5 hours. In the presence of Zn(II) ions, even after 24 hours, there was only a 1.7 % release of Cu$^{2+}$. 
Typical concentration of zinc in human blood, serum and urine is 800 ± 200, 109 to 130, and < 500 μg/dL serum. The corresponding values in ppm are 8 ± 2, 1.09 to 1.30 and < 5 respectively. The concentration of Zn$^{2+}$ ions used for the study is 100 ppm which is approximately twelve times higher than the normal concentration of zinc in human blood. Therefore, the in vivo ion exchange of Cu$^{2+}$ in the presence of Zn$^{2+}$ may likely be minimal.

Figure 5.6. Manganese leaching results under different conditions.

Figure 5.6 displays the results for the manganese leaching under the same conditions. There is considerable release of Mn(II) ions from the NPs in the presence of Zn(II) ions (16.2 %) and Fe(II) ions (11.6 %) after 5 hours of incubation. In comparison Cu(II) exhibited negligible release in the same solutions. The stability difference between Cu(II) and Mn(II) is consistent with the empirical rule known as the ‘Irving-Williams series’. According to this empirical rule, the stability exhibited by homologous divalent 3d metal complexes follow the order Cr$^{2+}$<Mn$^{2+}$<Fe$^{2+}$<Co$^{2+}$<Ni$^{2+}$<Cu$^{2+}$>Zn$^{2+}$
irrespective of the nature of the coordinated ligand or of the number of ligand groups involved.151

The potential release of cyanide ions \textit{in vivo} due to degradation of the coordination network of PB could be a major concern in view of high toxicity of free cyanide ion. Hence, the stability of NPs towards free cyanide release in various biologically relevant media was experimentally verified by a fluorometric method using the commercially available cyanide test kit for water samples.

\textbf{Table 5-3. Free cyanide release results after 48 hours of incubation under different conditions.}

<table>
<thead>
<tr>
<th>medium</th>
<th>free cyanide release (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DI water</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>pH 4.8 (0.1 M citrate buffer)</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>pH 6.2 (0.1 M citrate buffer)</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>pH 7.4 (PBS)</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Mg\textsuperscript{2+}</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+}</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Zn\textsuperscript{2+}</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Fe\textsuperscript{2+}</td>
<td>0.05</td>
</tr>
</tbody>
</table>

As shown in the table 5.3, after incubation of NPs for 24 hours, free cyanide levels in all the media are still less than 0.1 ppm. These levels are lower than the “maximum contaminant level” limit for free cyanide in drinking water (0.2 ppm) enforceable under National Primary Drinking Water Regulations set by United Sates
Environment Protection Agency (EPA).\textsuperscript{152} The free cyanide test results confirm the inertness of the framework structure towards degradation. Therefore, it is clear from the above stability studies that C60M@PVP NPs have an inert framework structure with tightly bound Cu\textsuperscript{2+}. On the other hand, the manganese ions are susceptible towards leaching in the presence of Fe(II) and Zn(II) ions.

5.3.5 Cytotoxicity of C60M@PVP PBA NPs

The ability of C60M@PVP PBA NPs to inhibit the cancer cell proliferation was evaluated in PC3 cells (prostate cancer cell line). The cell viability was determined by MTT assay after treatment of PC3 cells with NPs for 24 hours.

![Graph showing cell viability vs. log[Cu(μM)]](image.png)

**Figure 5.7. Cytotoxicity profile of C60M@PVP NPs in PC3 cells.**

Figure 5.7 displays the PC3 cell viability profile for C60M@PVP PBA NPs. The average cell viability at each concentration, determined from three independent trials, was plotted against the log of copper concentration. From the sigmoidal curve fitting of the dose response profiles, the average IC\textsubscript{50} value of C60M@PVP NPs was determined to be 638 ± 104 μM. The corresponding values in μg/mL are 40 ± 6.6. This higher IC\textsubscript{50}
value in comparison to that of CF2\textsubscript{(1:1)}@PVP NPs (from the previous chapter) may be attributed to the net effect of two influencing factors: 1) only 60\% of surface sites are occupied by copper in C60M solid solution compared to pure CF2 PBA and 2) the decrease in the surface area per unit volume of the C60M NPs due to the bigger particle size of C\textsubscript{60}M@PVP NPs (19 nm) compared to pure CF2\textsubscript{(1:1)}@PVP NPs (6 nm).

It may be argued that the overall anti-proliferation effect of C\textsubscript{60}M@PVP NPs on cancer cells is due to the presence of both surface copper and manganese ions instead of only copper ions. Based on the experimentally determined copper to manganese ratio, the equivalent concentration of manganese corresponding to the IC\textsubscript{50} value of C\textsubscript{60}M@PVP NPs is 387 μM which is not an insignificant amount. Therefore, the above argument cannot be ruled out and needed verification. In order to verify the effect of surface manganese on PC3 cell viability, a control study was performed independently. For this, we synthesized pure K\textsubscript{2}Mn\textsubscript{3}[Fe(CN)\textsubscript{6}]\textsubscript{2} NPs (referred to as MnPB@PVP NPs) under the same conditions as those for the C60M@PVP NPs and tested the cell viability in PC3 cancer cells. A single trial cell viability profile MnPB@PVP NPs was determined by MTT assay.
Figure 5.8. Cytotoxicity of MnPB@PVP NPs in PC3 cells.

As displayed in figure 5.8, even at the highest concentration of 500 μM of manganese, the cell viability was 88%. This result is similar to that obtained by our previous colleague using the same NP system on He La cells (Ref. # 150, page # 106 there in). The higher cell viability therefore suggests that surface manganese has a negligible effect. This may be attributed to the inability of manganese, unlike copper, to perform catalytic Fenton-like reaction for the generation of ROS. Hence, it may be concluded that only the surface copper is responsible for the anti-proliferation effect of C60M@PVP NPs.

5.3.6 Relaxivity Studies

Since our goal is to explore the theranostic potential of C60M@PVP NPs for both cancer treatment and MRI application, we further performed relaxivity measurements to assess the MR contrast ability of C60M@PVP NPs due to the presence of Mn(II)
paramagnetic centers. The efficiency of a contrast agent to induce faster relaxation of neighboring protons is given by ‘relaxivity’ which is simply the change in the relaxation rate with the change in the concentration of metal ion of CA. For C60M@PVP NPs, the change in the relaxation rates $1/T_1$ and $1/T_2$ with increasing concentration of Mn (II) measured at a magnetic field strength of 1.4 T are shown in figure 5.9.

![Figure 5.9. Plots of $1/T_1$ (i=1,2) vs the Mn$^{2+}$ concentration at a magnetic field strength of 1.4 tesla for C60M@PVP NPs.](image)
From the plots, it was clear that the change in the proton relaxation rates varied linearly with manganese concentration and can be expressed by the following generalized linear expression given by,

\[ \frac{1}{T_{i, \text{obs}}} = r_i \times [\text{Mn}] + \frac{1}{T_{i,d}} \quad (i=1,2) \]

where \( \frac{1}{T_{i,d}} \) is the diamagnetic contribution to the relaxation rate taken as that of pure water in all the measurements and [Mn] is the total concentration of Mn(II) ions of dispersed C60M@PVPV NPs in water. The resulting values of relaxivities, determined from the slope, are \( r_1 = 4.12 \, \text{mM}^{-1} \, \text{s}^{-1} \) and \( r_2 = 5.71 \, \text{mM}^{-1} \, \text{s}^{-1} \). As suggested by the relaxivity data, C60M NPs shows positively enhanced \( T_1 \) relaxation.
Table 5.4. List of relaxivity data for selected Gd/Mn-chelates and NP based MRI CAs.

<table>
<thead>
<tr>
<th>MRI CA</th>
<th>Magnetic Field (T)</th>
<th>Particle size (nm)</th>
<th>$r_1$ (mM$^{-1}$s$^{-1}$)</th>
<th>$r_2$ (mM$^{-1}$s$^{-1}$)</th>
<th>$r_2/r_1$</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnevist</td>
<td>1.5</td>
<td>-</td>
<td>3.3</td>
<td>3.9</td>
<td>1.2</td>
<td>Rohrer et al.$^{153}$</td>
</tr>
<tr>
<td>MultiHance</td>
<td>1.5</td>
<td>-</td>
<td>4.0</td>
<td>4.3</td>
<td>1.1</td>
<td>-do-</td>
</tr>
<tr>
<td>Omniscan</td>
<td>1.5</td>
<td>-</td>
<td>3.3</td>
<td>3.6</td>
<td>1.1</td>
<td>-do-</td>
</tr>
<tr>
<td>Prohance</td>
<td>1.5</td>
<td>-</td>
<td>2.9</td>
<td>3.2</td>
<td>1.1</td>
<td>-do-</td>
</tr>
<tr>
<td>Gadovist</td>
<td>1.5</td>
<td>-</td>
<td>3.3</td>
<td>3.9</td>
<td>1.2</td>
<td>-do-</td>
</tr>
<tr>
<td>Mn (DPDP) (Teslascan®)</td>
<td>1.4</td>
<td>-</td>
<td>2.8</td>
<td>3.7</td>
<td>1.3</td>
<td>Pan et al.$^{154}$</td>
</tr>
<tr>
<td>HMnO@mSiO$_2$</td>
<td>1.5</td>
<td>15</td>
<td>1.72</td>
<td>11.30</td>
<td>6.6</td>
<td>Kim et al.$^{155}$</td>
</tr>
<tr>
<td>MnO nanplates</td>
<td>1.5</td>
<td>20</td>
<td>2.13</td>
<td>4.31</td>
<td>2.0</td>
<td>Park et al.$^{156}$</td>
</tr>
<tr>
<td>Hollow MnO spherical NPs</td>
<td>1.5</td>
<td>20</td>
<td>0.35</td>
<td>8.68</td>
<td>24.8</td>
<td>Kwangjin et al.$^{157}$</td>
</tr>
<tr>
<td>Hollow MnO NPs</td>
<td>1.5</td>
<td>20</td>
<td>1.15</td>
<td>6.74</td>
<td>5.9</td>
<td>-do-</td>
</tr>
<tr>
<td>K$<em>2$Mn$<em>3$[Fe$</em>{10}$(CN)$</em>{6}$]$_2$ 2.6H$_2$O NPs</td>
<td>1.4</td>
<td>21</td>
<td>4.54</td>
<td>18.33</td>
<td>4.0</td>
<td>Kandanapitiye$^{150}$</td>
</tr>
<tr>
<td>K$<em>2$Cu$</em>{1.80}$Mn$<em>{1.20}$[Fe(CN)$</em>{6}$]$_2$ xH$_2$O (C60M@PVP NPs)</td>
<td>1.4</td>
<td>19</td>
<td>4.12</td>
<td>5.71</td>
<td>1.4</td>
<td>This work</td>
</tr>
</tbody>
</table>

Furthermore, these values imply several favorable characteristics that qualifies the C60M NPs as MRI CA. For an effective “positive” MRI CA, a higher value of $r_1$ and an optimal ratio $r_2/r_1$ of 1.0 is desired.$^{155}$ Table 5.4 lists the relaxivity values of selected commercial Gd and Mn-chelates and literature reported manganese-based nanoparticulate MRI CAs for comparison purposes. C60M@PVP PBANPs exhibits better $r_1$ value (4.14
mM$^{-1}$s$^{-1}$) compared to several commercial Gd-chelates and MnDPDP (Teslascan®). The high $r_1$ value may be attributed to the high surface area of the NPs due to the presence of inherent vacant sites in the PBA lattice which allow access to water molecules and interact with coordinatively unsaturated surface Mn (II) centers. Furthermore, the $r_1$ value is comparable to that of K$_2$Mn$_3$[Fe(CN)$_6$]$_2$·2.6H$_2$O NPs reported by our previous colleague in our lab.$^{150}$ It is interesting to note that $r_1$ value is nearly same despite only 40 % of Mn (II) centers present in C60M@PVP PBANPs compared to the pure K$_2$Mn$_3$[Fe(CN)$_6$]$_2$ 2.6H$_2$O NPs. Moreover, the $r_2$ value of 5.71 mM$^{-1}$s$^{-1}$ is significantly lower when compared to the pure MnPB analog for which the $r_2$ value was reported as 18.33 mM$^{-1}$s$^{-1}$. Such a reduced $r_2$ value translated to lower $r_2/r_1$ value of 1.34 and is closer to the optimal ratio of 1 shown by commercial Gd-contrast agents. C60M@PVP PBAs contains only 40 % Mn (II) ions in the framework that are homogeneously distributed and well separated in the framework compared to the pure K$_2$Mn$_3$[Fe(CN)$_6$]$_2$ 2.6H$_2$O NPs. Therefore, the decrease in the $r_2$ value may be attributed to the decreased spin-spin interactions.

The anti-proliferative ability coupled with the high $r_1$ relaxivity makes C60M@PVP NPs suitable for multifunctional image-guided drug delivery applications. Although, the cytotoxicity (IC$_{50}$) and magnetic relaxation properties ($r_1$, $r_2$ and $r_2/r_1$) can be tuned with the change in the relative amounts of Cu (II) and Mn (II) in the CxM PBA framework, no further studies were performed to optimize these parameters.

### 5.4 Conclusion

In conclusion, a series of homogeneous solid solutions of copper-manganese Prussian blue analog nanoparticulates were synthesized. The theranostic ability of the NP...
platform arises from the presence of surface copper (II) ions capable of elevating oxidative stress in cancer cells and manganese (II) paramagnetic centers capable of increasing the relaxation rate of coordinated water protons. A representative sample C60M@PVP NPs from this series exhibited $r_1$ relaxivity which is comparable to that of several commercial Gd-chelates. The major highlight of this work is the preparation of an effective theranostic agent by a single step salt metathesis reaction.
6. Conclusion

This dissertation work focused on the design and synthesis of several copper-based nanomaterials for anti-cancer and diagnostic applications.

Chapter 2 described the room temperature synthesis of ultrasmall polymer coated copper tetrathiomolybdate (Cu₂MoS₄) nanoparticles (5.5 ± 1.8 nm) in a mixed solvent system of formamide and water. These NPs were found to inhibit the proliferation of cancer cells more than that of the normal kidney cells. Furthermore, based on the stability studies under different conditions, these NPs are found to be suitable for preparing intrinsically radiolabeled $^{64}$Cu-CTM NPs for PET application.

In Chapter 3, aqueous synthesis of polymer coated Libethenite (Cu₂(OH)PO₄) nanosheets (CHP NSs) was reported. No reports that describe the colloidal Libethenite synthesis have appeared in the literature. These NSs (97 ± 27 nm) were found to be highly toxic to HT29 and PC3 cancer cells with an IC$_{50}$ value of 47 ± 3 and 50 ± 2 μM respectively. Furthermore, the intracellular ROS elevation by CHP NSs was demonstrated by the DCFDA assay which confirms the hypothesis. It is interesting to explore the favorable NIR absorption property of CHP to determine its potential as a candidate for photothermal therapy.

The reported biological applications of nanoparticles of Prussian blue and its analogs rely on their biocompatible nature. Although, a copper-based Prussian blue analog NPs (30-75 nm) was reported in literature to be biocompatible and used for drug
delivery applications, we have demonstrated in Chapter 4 that nanoparticulates of copper Prussian blue analogs (copper ferro and ferricyanide analogs) with sizes 15 nm and below are detrimental to cancer cells. Moreover, the ultrasmall size of the copper ferrocyanide NPs together with their stability towards copper and free cyanide release under various conditions show promise towards preparation of intrinsically labeled $^{64}$Cu PBA NPs for PET applications.

Using DCFDA assays, we determined that in all cases the nanoparticles elevated the intracellular ROS level in cancer cells.

The major highlight of Chapter 5 is the facile one-step synthesis of nanoparticulate copper-manganese Prussian blue solid solution with potential theranostic application. With Mn(II) content only 40% of that of the pure phase, this NP exhibited $r_1$ relaxivity (4.12 mM$^{-1}$ s$^{-1}$) and $r_2/r_1$ (1.38) on a par with several commercial Gd-chelates.

To the best of our knowledge, these NP systems were not reported previously for the intended bio-applications.
7. Appendix

A) PXRD patterns of CxM PBA, K₂Cu₃[Fe(CN)₆]₂ (CuPBA) and K₂Mn₃[Fe(CN)₆]₂ (MnPBA) bulk powders
8. References


20. http://www.abraxane.com/hcp/?b=1&gclid=CNyVvpq-4NECFc64wAod_vcHCQ.


134. Ho, Y.-P.; Leong, K. W., Quantum Dot-Based Theranostics. Nanoscale 2010, 2 (1), 60-68.


