Synthesis of Biocompatible Nanoparticulate Coordination Polymers for Diagnostic and Therapeutic Applications

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by

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Chapter 1: Introduction to Nanomaterials for Biomedical Applications.

1. Introduction

The combination of nanotechnology with medicinal chemistry has developed into a burgeoning research area.\textsuperscript{1-3} Nanomaterials (NMs) could be seamlessly interfaced with various facets in biology, biochemistry, medicinal chemistry and environmental chemistry that may not be available to the same material in the bulk scale.\textsuperscript{4,6} Nanotechnology involves in wide range of applications, for examples; NMs for energy storage,\textsuperscript{7} NMs for multimodal imaging,\textsuperscript{8,9} NMs for angiogenesis inhibitors for cancer treatments\textsuperscript{10} and NMs for environmental remedial applications.\textsuperscript{11} Surface of NMs plays a key role in deciding its characteristic features owing to nano-scale confinements. The effects of the surface are described by the fraction of atoms at surface of nanoparticles. The dispersion function is used to express surface influence on NMs in comparison to the volume. It is defined as the fraction of atoms at the surface and dispersion function \( F \) is calculated by the surface area divided by the volume.\textsuperscript{6} The smaller the particle’s size, the larger the fraction of atoms is at the surface. In other word, majority of atoms could participate in catalytic reactions and involve in ion-exchange reactions, or enhance the relaxation of protons in bulk water. Most visible examples of NMs in action are in diagnostic tools for CT\textsuperscript{12}, MRI,\textsuperscript{13} SPECT\textsuperscript{14,15}, PET\textsuperscript{16}, biosensors\textsuperscript{17}, therapeutics applications\textsuperscript{18,19} and targeted drug delivery systems.\textsuperscript{20} In general nanoparticulates in diagnostics offer a great opportunity to enhance efficacy of imaging agents with compared to the conventional approaches.

As majority of the elements that are used in imaging agents/probes have either no significant role in biological systems or they require trace amount to maintain biological processes, the premature releasing of those metal ions from imaging agents raise safety concerns. Interestingly, the usage of nanoparticulate based imaging probes has many advantages over the small molecular imaging agents. These advantages of nanoparticulate based imaging agents include; their excellent stability, cellular penetrability and easy with
which the surface can be functionalized for targeted delivery. Modern medical imaging techniques have gained an increasing interest in the early diagnosis of diseases in the last three decades. Modern imaging techniques include X-ray computed tomography (CT), magnetic resonance imaging (MRI), single photon emission computed tomography (SPECT) and positron emission tomography (PET). We have successfully developed several types of nanoparticulate diagnostics that have some potential usefulness in biomedicine.

For magnetic resonance imaging, metal chelates that contain paramagnetic metal centers are commonly employed as MR contrast agents. The majority of MRI contrast agents are based on Gd$^{3+}$-complexes owing to its highest magnetic moment ($S=7/2$) and favorable electronic relaxation of Gd(III). Free Gd(III) is a toxic heavy metal ion, with the size approximating calcium(II) and high charge density which lead to disruption of critical Ca(II)-dependent signaling pathways. By recent studies, the toxicity of the Gd(III)-based MRI contrast agents was also linked to the development of nephrogenic systemic fibrosis (NSF). As a result, we prepared a Gd(III) doped Prussian blue nanoparticles (PB NPs) as an alternative to smaller molecular complexes. Such Gd(III) doped PB NPs show excellent stability and high spin-lattice relaxivity ($r_1$). Further, Prussian blue platform can be doped with Ga(III) due to its similarities with Fe(III) including ionic radius and charge, and thence some of octahedral sites in face centered cubic (fcc) are occupied by gallium. Such solid solution of $^{68/67}$Ga$^{3+}$ and Gd$^{3+}$ doped PB in the form of nanoparticles provide both PET/SPECT and MRI properties to a single nanoparticulate platform. The multimodal imaging of MRI and PET/SPECT has centralized the strengths of individual MRI and PET/SPECT, providing anatomical and structural description of MRI along with molecular level diagnostic capabilities of PET and SPECT. Such multifunctional diagnostic agents offer several advantages in their synergistic operation. As a result, multifunctional nanoparticulate diagnostics have unique advantages in biomedical applications than their unimodal counterparts. Majority of the bimodal imaging agents reported so far comprise of MRI imaging and optical imaging. However, MRI-PET/SPECT imaging modality is the preferred modality for anatomic and functional imaging over MRI-optical imaging.
have been a few of combined MRI-PET/SPECT imaging agents and yet their clinical relevance is very uncertain because of the similar challenges as in unimodal operation. The co-administration of separate PET/SPECT and MRI contrast agents is not favored to examine tissues by hybrid MRI-PET/SPECT imaging modality because of their different pharmacokinetic properties. The PET/SPECT imaging agents and MRI contrasting agents have their own bio-distribution and tissue specificity along with different biological residence half-life times that deprive of intended synergistic effect in imaging application. Consequently, such paradoxical pharmacokinetic properties of each, viz MRI contrasting agent and PET imaging agent dispossess precise co-registration anatomical and functional information in a single scanning session. Hence, more smart approach would be the use of hybrid imaging agents to meet same pharmacokinetic properties that could guarantee the highly synergistic effect in bimodal imaging. Thus solid solution of KGd0.1Gd0.1Fe0.8[Fe(CN)6] in the form of nanoparticles assure both PET/SPECT and MRI properties to a single platform.

In vivo toxicity of the Gd(III)-based MRI contrast agents has set out our journey to search for non-gadolinium based T1 weighted CAs with high efficacy and low toxicity. Such a transition of standard model gained the attention toward the Mn(II) and Fe(III) that are most suitable candidates with many desirable properties; both have five unpaired electrons with long electronic relaxation time and naturally found in living systems. After Gd(III), high-spin Fe(III) and Mn(II) have the next highest unpaired electrons and hence the second highest total spin (S=5/2). Among these two transition metal ions, Fe3+ is considered to be more toxic to cells as the free ferric or ferrous ions can catalyze the production of reactive oxygen species via the Fenton reactions. All the Fe3+ based MR CAs are superparamagnetic iron oxide (SPIOs). SPIOs based T2-weighted CAs are far less desirable than that produced by the small molecule T1 agents. Therefore T1 weighted CAs have major clinical relevance accurately allocating pathogenic sites. To the best of our knowledge Mn(II) compounds are promising candidates for clinical use as MRI contrast agents because of their relatively high electronic spin, long electronic relaxation rate and relatively fast water exchange rate.
Furthermore, bioinorganic chemistry of Mn(II) ions in our body has been well researched and understood. However it is increasingly challenging to design and synthesize highly stable Mn$^{2+}$ complexes that could maintain the integrity when administered to living system. As Mn(II) is an essential metal ion, living systems has evolved a highly efficient mechanism to transmetallate Mn(II) from such chelates. For example; Mn(II) complex of dipyridoxyl diphosphate, (MnDPDP- TESLASCAN®) is already used in clinical practices as a liver imaging agent and complete its job by releasing Mn(II) ions. The toxicity is associated with exposure dose of Mn(II). Mn(II) concentration in mammalian tissues is minuscule, thus over exposure exhibits neurotoxicity with initial symptoms of Parkinson like syndrome following other sign of fatigue, poor memory, hallucinations and hepatic encephalopathy. As a consequence of the low stability and low efficacy of such Mn$^{2+}$ chelates, its domination in MR imaging field is far behind that of the Gd-chelates. We prepared a biocompatible colloidal solution of K$_2$Mn$_3$[Fe(CN)$_6$]$_2$ as T$_1$ weighted MR CAs. The Mn(II) has been locked in the lattice positions giving superb stability and citrate coating makes them highly stable and water dispersible.

Not only MR imaging requires contrasting agents, but computed tomography (CT) also needs contrast agents. They are usually called as “dyes” and enhance the image contrast in specific areas. Various CT contrast agents have been developed and most of them are iodinated organic compounds. However, they possess several disadvantages such as, rapid excretion from the body leading to short imaging time, low contrast efficacy, lack of targeted delivery and potential renal toxicity. Therefore, ideal CT contrast agents should have greater X-ray absorption coefficient or attenuation power. In general, heavy elements strongly attenuate transmittance of X-ray than that of iodinated compounds and led to higher X-ray attenuation coefficients. Therefore, inorganic compounds with heavy metals are better suited for X-ray computed tomography rather than the current iodinated CT contrast agents. We have set out to search for bismuth compounds that are hydrolytically stable and can readily be prepared as ultra-small nanoparticulate CT contrast agents that are biocompatible and renal.
clearable. As an alternative to the iodinated organics, we prepared ultra-small monodispersed bismuth-based nanoparticulate CT contrast agents.

Additionally, NMs could be employed as therapeutic agents or as drug carriers that effectively transport the high payload of drug molecules to targeted sites.43,44 Our size control synthesis of various nanoparticles has resulted in novel nanoparticulate diagnostics and therapeutics with good therapeutic index to be used in treatment for various pathological conditions. For example, pathological conditions that derived from dysfunction of copper homeostasis can be attributed to its facile redox chemistry. Copper is an essential trace element that serves as a catalytic and structural cofactor for many proteins and enzymes in all living organisms. However, when it is unbound to proteins or enzymes as the free ion, copper can exhibit cytotoxicity and trigger production of reactive oxygen species via Fenton-like reactions. Deficiencies in maintaining copper homeostasis are linked to several pathological conditions including Menkes disease, Wilson’s diseases (WD), familial amyotrophic lateral sclerosis, to name but a few.45,46 Despite the recent significant progress made in understanding the intracellular trafficking of copper, there are still a limited number of clinical drugs available in the form of chelation therapy to treat the diseases and disorders associated with copper overload.

The current drug treatment of WD is based on the removal of the toxic free copper ions using Cu^{2+}-chelators.45 As the current treatments for WD, D-penicillamine, triethylenetetramine, zinc acetate and tetrathiomolybdate are used as drugs with systemic toxicity but no organ-specificity.47 The above-mentioned drugs exhibit severe side effects including bone marrow and immune suppression, deterioration of various neurological functions, irreversible neurologic damage, negative copper balance in the body and drug-induced systemic lupus erythematosus.48 Also, none of these small molecular drugs can penetrate into cells to remove excess free copper ions.49 We have developed cell-membrane permeable chelating agents that have potential to become organ-specific with use of suitable targeting molecules as the next-generation copper detoxifying drugs for treating WD. We have prepared Au@D-PEN core-shell nanocomposite material to remove excess free copper.
ions from the cells. To the best of our knowledge, this is the first example to show that D-PEN can be tailor-made as a new-generation intracellular copper detoxifying drug.

In an attempt to make another copper detoxifying agent, we developed non-sulfur based nanoparticulate copper detoxifying agent to selectively sequestrate the copper ions. We have reported the synthesis, characterization and intracellular copper detoxification by the nanoparticles formed from a zinc analogue of Prussian blue K$_2$Zn$_6$[Fe(CN)$_6$]$_2$ (ZnPB NPs). These ZnPB NPs are highly water-dispersible, biocompatible and capable of penetrating cell membrane to selectively remove intracellular copper. The strategy we used here to design more selective to Cu(II) and effective nanoparticulate agent to treat the Cu(II) accumulation was derived from the empirical rule in coordination chemistry known as the Irving-Williams series. We noticed that the relative stability exhibited by homologous divalent 3d metal complexes would follow the trend Cr$^{3+}$<Mn$^{2+}$<Fe$^{2+}$<Co$^{2+}$<Ni$^{2+}$<Cu$^{2+}$>Zn$^{2+}$, regardless of the nature of ligand. Hence ZnPB NPs undergo ion-exchange exclusively with the Cu$^{2+}$ ion in the presence of the other endogenous divalent metal ions. Thus ZnPB NPs exhibits a high selectivity towards copper and traverse the cell membrane to act as an intracellular copper detoxifying agent.

Although many Cu(II) chelators have been developed to remove excess Cu(II), it is surprising to notice that a simple and reliable method or probe for detecting and monitoring in vivo copper levels has not been developed so far. Clinical diagnosis of copper overload is conducted exclusively by in vitro tests. Typically serum copper is paradoxically low regardless of the elevated level of copper in vital organs of WD patients. Again, simple blood test of copper may result in low or normal copper levels in such patients and masks the fact that they are suffering copper overload in the tissues. The gold standard diagnosis for WD is a liver biopsy. Such all in vitro tests are invasive and give confusing results. Magnetic resonance imaging (MRI) has ideal characteristics to develop such a noninvasive copper sensing probe. MR contrast agents that could change the relaxivity values as a response of analyte binding or recognition of guest molecules are called as smart MR contrast agent. The Gd-containing sensor molecules have developed to sense in vitro copper ions.
However, all Gd-chelates are known to be extracellular MRI contrast agents and such copper MR probes are not expected to penetrate cells.\textsuperscript{53,54} The use of Gd\textsuperscript{3+}-chelates as clinical MR contrast agents has been linked to nephrogenic systemic fibrosis (NSF). Therefore, further development of these Gd-chelates as \textit{in vivo} copper sensors for clinical applications does not seem to have a bright future.\textsuperscript{26,27} To the best of our knowledge, there has not been a single account of such nanoparticulate probe that has high selectivity for Cu(II). We noticed that our ZnPB NPs have an ability to sense Cu(II) as it undergoes ion exchange reaction. Free copper in the form of Cu(II) (S=1/2) shows reasonably high relaxivity value and copper deposit in vital organ could collectively enhance the visibility of affected sites. The free copper ions after being locked up in lattice positions in ZnPB NPs are not accessible for many H\textsubscript{2}O molecules as free copper ion does. Such irreversible conversion of ZnPB NPs into CuPB NPs could manifest its effect on T\textsubscript{1} weighted MRI images.

In cancer therapy, the number of different methods currently being developed to mitigate unregulated cell division is already beyond counting. However nanoparticulate drug delivery system to target the cancer cells is still a new approach to treating the cancerous cells.\textsuperscript{55} Even in the absence of suitable surface functionalization, nanoparticles could passively accumulate at tumor sites that have leaky, immature vasculature with wider fenestrations than normal mature blood vessels.\textsuperscript{56} This is known as the enhanced permeability and retention (EPR). The EPR effect combined with prolonged circulation times could increase concentrations of drug in tumors by 10 –100 fold with compared to the use of free drugs and thus reduces adverse side effect by lowering dosage of active pharmaceutical ingredient.\textsuperscript{57} Such nanoparticulate mediated drug delivery systems amply reflect its progression toward intended applications of this field. Typically, cancer therapy requires multiple lines of therapy that include radiation therapy and chemotherapy. Solid tumors of sizes less than 0.8-1mm\textsuperscript{3} can obtain oxygen and essential nutrients by simple diffusion. But solid tumor of size approximately 1-2mm\textsuperscript{3} creates new blood vessels giving rise to the tumor hypervascularity as response to the deprivation of oxygen (hypoxia), and pro-angiogenic factors.\textsuperscript{58,59} The cascade of such events can lead metastasis and transforms the primary tumor
into life threatening status of metastasis. In general, a commonly used mechanism for inhibiting the formation of tumor hypervascularity is the blockade of VEGF pathway. The angiogenesis inhibitor (Bevacizumab /Avastin) exploits biomolecular interactions of VEGF with Avastin (anti-VEGF antibodies) that has a higher affinity for VEGF than for VEGFR-2 receptor itself. Unfortunately, VEGF pathway inhibitors can be inactivated by the continuous genetic modification of cancer cells and increased risk of acquired resistance to therapy. Copper is considered as an essential cofactor for many pro-angiogenic growth factors that are required in blood vessel development, proliferation and migration of endothelial cells. Specifically, the activity of several angiogenic factors depends on copper availability, and by blocking the availability of copper, synthesis of pro-angiogenic factors can be down regulated. Thus our $K_2Zn_3[Fe(CN)_6]_2$ nanoparticles could be effective in two ways, decreasing the copper level in tumor to inhibit angiogenesis and restoring the zinc level in prostate tumors. Zinc is long known for its antagonist role to copper. Therefore ZnPB NPs has indirect role in inhibiting the angiogenesis.

1.1. Magnetic resonance imaging (MRI)

Magnetic resonance imaging has become an indispensable tool of noninvasive imaging in modern diagnosis procedures. MRI offers an opportunity to examine the anatomical details with high-resolution. It uses proton relaxation processes to generate the signal in the presence of external magnetic field. MR imaging uses the variation of the distribution of hydrogen nuclei’s spin density, the longitudinal ($T_1$) and transverse ($T_2$) relaxation times of these spins. The protons that have intrinsic spin (I) of half–integer value gives rise to angular momentum, $\mathbf{P}$ while angular motion of proton gives rise to magnetic dipole moment, $\mu$. Thus protons behave like a tiny bar magnet interacting with an external magnetic field giving rise to precession around $B_o$ owing to the angular momentum. 

$$\omega_L = \left(\frac{\gamma}{2\pi}\right) B_o \quad \text{Eq 1}$$

Precession of the protons occurs at a characteristic angular speed that is proportional to the strength of the applied magnetic field and precession angular frequency is called
Larmor frequency \( (\omega_L) \) (Eq1). The external magnetic field generates two energy states \( \alpha \) and \( \beta \), which correspond to the spin quantum number of +1/2 and -1/2 respectively. The orientation of the \( \alpha \) state is parallel to the field direction, whereas the orientation of the \( \beta \) state is antiparallel with external magnetic field. The equilibrium populations \( N_\alpha \) and \( N_\beta \) of the lower \( \alpha \) and the upper \( \beta \) energy states are given by the Boltzmann distribution.

\[
\frac{N_\beta}{N_\alpha} = \exp \left( - \frac{\Delta E}{k_B T} \right) \tag{Eq 2}
\]

The lower energy level is preferred; however thermal motion could provide the energy difference required for such transitions. There is only a small excess of spins in the lower energy state at thermal equilibrium. Such a small spin excess in low energy state causes to give very faint signal in MR imaging. Hence, MRI is a very insensitive technique. To increase the sensitivity, magnetic field strength \( B_0 \) could be increased or temperature could be decreased which is impractical with patients in MRI scanner. Thus, repetitive scanning of the same object increases the signal to noise ratio despite the small spin excess in \( \alpha \) states given by the Boltzmann distribution. So MR imaging allows seeing the interior of human body.

**Scheme 1.** (a) In the absence of an external magnetic field, spins rotate about their axes in random direction, (b) In the presence of a magnetic field, spins align mainly parallel to the magnetic field, producing, \( M_z \).

In the presence of external magnetic field, all protons in bulk water produce bulk magnetization called \( M_0 \) as shown in **Scheme 1**. This represents the summation of all aligned
spins in $B_0$ and population density is given by the Boltzmann distribution (Eq 2). The bulk magnetization has a longitudinal component only at equilibrium and all the individual magnetic moments precess around $B_0$ resulting zero transverse magnetization due to the no phase coherence of individual protons. In reality, the net effect of all the nuclei of interest in the sample will be observed, rather than the individual magnetic moments. Thus the application of the RF pulse that matches with Lamor frequency causes $M_0$ to tilt away from $B_0$, and to follow a spiral path and led to change in the population distributions. After the upper and lower levels have equal population, nuclei that precess in phase produce only a transverse magnetization in xy plane (Scheme 2c). The gradient coil magnet that is a key component in MRI scanner generates the coordinate dependent magnetic field and that allows protons to possess different precessions frequencies from slice to slice. Such a variation of precession frequency of proton could later decode for spatial information of imaging by computer analysis.

**Scheme 2.** (a) In the presence of a magnetic field with non-zero longitudinal magnetization ($M_z$); (b) A RF pulse with Lamar frequency, spin reversal into high energy state; (c) Generation of transverse magnetization, $M_{xy}$ shown in red arrow.

After the RF pulse is turned off, spin system relaxes; releasing the energy absorbed from the RF pulses and settles into a spin parallel-low energy state and producing longitudinal magnetization $M_z$ in the $z$-direction. During this relaxation, the receiver coil records the free inductive decay (FID) of transverse magnetization in xy plane and uses this information in
image construction. Relaxation of protons occurs via two independent processes that reduce transverse magnetization. Two independent relaxation processes use spin-lattice interaction and spin-spin interaction to return to the low energy state. The spin- lattice relaxation ($T_1$ relaxation) measures the rate of regrowth of longitudinal magnetization (Eq 3) and spin-spin relaxation measures the rate of decaying transverse magnetization (Eq 4).

$$M_z(t) = M_{z,eq}(1 - e^{-\frac{t}{T_1}}) \ldots \ldots \text{Eq 3}$$

$$M_{xy}(t) = M_{xy}(0)e^{-\frac{t}{T_2}} \ldots \ldots \text{Eq 4}$$

$T_1$ relaxation depends on the identity of biomolecules to which the hydrogen is embedded and the type of tissue. For instance, $T_1$ of body fluids is longer than that of solid tissues. $T_1$ relaxation occurs most efficiently when the molecular tumbling rate is near the Larmor frequency of proton precession. The body tissue that has large content of free water wobbles faster than the Larmor frequency which results in a long $T_1$. The transverse relaxation ($T_2$) losses phase coherence of transverse magnetization, $M_{xy}$. In phase transverse magnetization, $M_{xy}$ are fans out and leads to cancelation of coherence of resultant magnetization vectors. The transverse relaxation is entropy driven with no energy loss in the process of $T_2$ relaxation.

MR resonance imaging uses transverse magnetization component for image generations and employs the variation of the distribution of hydrogen nuclei’s spin density, the longitudinal ($T_1$) and transverse ($T_2$) relaxation times of these spins to manifest the contrast of image. There are two types of magnetic resonance imaging; $T_1$- weighted imaging ($T_1$W) and $T_2$-weighted imaging ($T_2$W). $T_1$W imaging accentuates the effect of $T_1$ relaxation while the $T_2$W imaging accentuates the effect of $T_2$ relaxation. The main parameters that decide either $T_1$W imaging or $T_2$W imaging are repetition time (TR) and echo time (TE). Repetition time (TR) is the interval between two successive excitations of the same slice and
echo time (TE) is the interval between application of the excitation pulse and collection of the MR signal. In other word, repetition time is how quickly we apply radiofrequency pulses and echo time is how quickly we listen to the returning signals. A short TR and TE pulse sequence accentuates the T₁ effect on signal intensity in MR imaging and long TR and TE pulse sequence accentuates T₂ effect on signal intensity of MR imaging.⁶²

1.1.1 Contrast agents for MRI

The natural contrast manifested by tissues alone is not enough to aid diagnosis of pathological sites. Therefore exogenous agents are required to enhance the natural contrast between normal and pathological regions. For MR imaging, metal chelates that contain paramagnetic metal centers are commonly employed as MR contrast agents (CAs).⁶³ The MRI CAs are divided into two classes based on the type of proton relaxation that is being prominently affected on MR imaging (i.e. T₁-weighted and T₂-weighted agents). T₁-weighted imaging accentuates the variation of T₁ relaxation of proton while T₂-weighted imaging accentuates the variation of T₂ relaxation protons with the help of T₁-weighted CAs and T₂-weighted CAs, respectively. Currently, various gadolinium-based coordination complexes⁶⁴ and magnetic nanoparticles including iron oxide⁶³, gadolinium oxide⁶⁵, and manganese oxide⁶⁶,⁶⁷ have been employed as MRI contrast agents.

Unlike radiographic contrast media, MRI contrast agents affect the relaxation properties of surrounding hydrogen atoms and are indirectly involved in imaging. The interactions of paramagnetic species’ electrons and protons of water/biomolecules in tissues have two parts; inner-sphere relaxation and outer sphere relaxation. Inner sphere relaxation occurs via the interaction of bound water onto CAs whereas outer-sphere relaxation occurs by an action at distance. Both inner and outer sphere relaxations contribute to the overall effect of MR contrast medium. The efficacy of contrast agent is expressed in term of relaxivity that measures the relaxation rate enhancement per unit concentration of paramagnetic species. The relaxivity is further a function of several factors. The observed longitudinal relaxation rate of water protons is the
sum of three contributions as shown in equation 5 which includes diamagnetic contribution, paramagnetic contribution from inner sphere water exchange with paramagnetic metal complex and outer sphere paramagnetic contribution of water molecules that diffuse in.\textsuperscript{64}

\[ R^{obs}_{1p} = R^{0}_{1} + R^{IS}_{1p} + R^{OS}_{1p} \ldots \ldots \text{Eq 5} \]

The inner-sphere relaxivity of metal chelates could be easily tuned than the outer sphere relaxivity. Solomon-Bloembergen-Morgan (SBM) equation (Eq 6) describes the inner sphere relaxation in terms of structural parameters of CAs for small molecular Gd(III) complexes.\textsuperscript{64} Inner sphere relaxation largely contributes to contrast enhancement of \text{T}_1-weighted MR imaging.

\[ R^{IS}_{1p} = \frac{1}{55.6} \frac{q[C]}{(T_{1M} + \tau_m)} \ldots \ldots \text{Eq 6} \]

In equation 6 where q is the number of water molecules directly coordinated to the Gd(III) center, [C] is molar concentration of CA in mM, \( T_{1M} \) is longitudinal relaxation time of bound water, \( \tau_m \) is mean residence time of bound water molecules. The identity of paramagnetic metal center can be seen in \( T_{1M} \) term. As can be seen from SBM equation, there are several possibilities for improving the relaxivity. By increasing the bound water molecules to metal center (e.g.-Gd(III)), relaxivity can be enhanced. Such a modification in molecular structure greatly reduces the thermodynamic stability and poses a safety concern of metal ion toxification. However the factor that could easily be modified is rotational motion of CAs by increasing the molecular weight. It affects on \( T_{1M} \); longitudinal relaxation time of bound water.
1.2 Multimodal imaging modalities (MRI-PET/SPECT)

Magnetic resonance imaging (MRI) provides anatomical details with high-resolution images as a major advantage, conversely MR imaging still lacks in delineating the metabolic changes of tissues which is presumed to occur earlier than changes of anatomical details. The PET/SPECT imaging modalities provide the information on molecular processes using radiolabeled imaging agents; on the other hand PET and SPECT give limited anatomical details and spatial resolution as a major disadvantage, regardless of their high-sensitivity \textit{in vivo} tracking of biomarkers. However, MRI and PET/SPECT imaging techniques have unique strength and intrinsic limitations over the other. The major drawbacks of each imaging modality can be offset by bimodal imaging technique.

![Scheme 3](image)

**Scheme 3.** Schematic diagram of a PET acquisition process

Both positron emission tomography (PET) and single photon emission computed tomography (SPECT) imaging use radiopharmaceuticals to image directly the area interest of, on the contrary to strong magnetic field and radio frequency pulses are used in MR imaging. The PET imaging agents generate positrons that are annihilated on their way with electrons and emit high energy $\gamma$ photons.\textsuperscript{68} Our body primarily contains a lot of organic and inorganic compounds having different energy interacting profiles and is transparent to the radiation with short wave lengths, while opaque at intermediate wave lengths. PET and SPECT modalities
use high energy $\gamma$ radiations which have short wavelengths to visualize internal structures. On the one hand, long wavelengths that are used in MR imaging are also transparent to body tissues. As depicted in Scheme 3, two high energy $\gamma$ photons (511 KeV) that are travelling at opposite direction are registered by detector rings. After the processing of concurrent emission of $\gamma$ photons, the final image is produced in computer. On other hand, SPECT uses the $\gamma$ photons directly emitted by decaying of radiotracer. The success of the radiographic imaging heartily lies in its biologically relevance in extracting the molecular level information with its high sensitivity ($10^{-10}$ - $10^{-12}$ mol/l).\(^{69}\)

**Scheme 4.** Designs of combined PET/ MRI systems (The RF-coil has omitted for clarity)

Although hybrid MRI/PET or SPECT multimodal imaging modality has shown a great promise in many ways, development of such bimodal imaging unit had faced many technological challenges in operating a PET and SPECT scanner within an MR instrument.\(^{29}\) The mutual interference between MRI (strong magnetic field) and PET hindered the progress of such hybrid imaging modality.\(^{70}\) However, the development of magnetic-field-insensitive, high-performance PET and SPECT detectors within MRI scanners permits the construction of fully working PET/SPECT-MRI scanners (Scheme 4).\(^{29,71}\)

### 1.2.1 Bimodal imaging agents for MRI-PET/SPECT

Metal oxide nanoparticles have been employed for multimodal imaging by many research groups. Nanoparticles are the ideal scaffold that allows the easy integration of several different imaging modalities into a single platform. To mention a few, Cheon et al.
have synthesized bimodal MnFe$_2$O$_4$ nanoparticles ($^{124}$I-SA-MnMEIO) which were stabilized by serum albumin. The tyrosine moieties in serum albumin are coupled with $^{124}$I ($t_{1/2}$ 4.2 days, $\beta^+$ emission). Magnetic core provides T$_2$ MR imagining activity and $^{124}$I on side arms gives action of PET. Similarly, CLIO-Tat nanoparticles (superparamagnetic iron oxide nanoparticles) coated with crosslink aminated dextrin conjugated to $^{111}$In(DTPA) complex (DTPA-diethylenetriamine pentaacetic acid, and $^{111}$In with $t_{1/2}$-62 hours for $\gamma$ ray emission) were shown to have potentials to be a bimodal imaging agent with SPECT-T$_2$ MR imaging capabilities by Weissleder’s and et al. Tian et al. have developed another MRI/PET dual contrast agents. In their approach, magnetic nanoparticle cores (Fe$_3$O$_4$) coated with 3,4-dihydroxy-D,L-phenylalanine were conjugated with $^{64}$Cu(DOTA) (DOTA-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid, $^{64}$Cu with $t_{1/2}$-12.7 hours for $\beta^+$ emission) to give T$_2$-weighted imaging activity and PET imaging activity into single platform. Misri et al. have recently reported another bimodal SPECT-MRI probe by conjugating $^{111}$In- labeled antimesothelin antibody with superparamagnetic iron oxide ($^{111}$In mAbMB). Such bimodal imaging agent of $^{111}$In mAbMB with SPECT-MRI activity was to probe accurately mesothelin-expressing cancers.

Among current radiopharmaceuticals, gallium-based imaging agents have several desirable properties that include easy access, in situ production, short half-life time and serval energy windows. However, the advent of $^{99m}$Tc in imaging field reduced the interest on gallium-based radiopharmaceuticals. Such a rapid transition in interest could be attributed to challenges of Ga coordination chemistry. However single molecular gallium complexes are still used as radiopharmaceuticals whereas coordination chemistry of gallium shows many challenges of its utilization. Single molecular Ga(III) complexes show strong affinity toward hydroxide ions, phosphate ions and demetallate from its complexes. The Ga (III) complexes are relatively unstable than the d-block metal complexes due to the lack of crystal field stabilization energy. The lack of ideal carrier to deliver the radionuclide of gallium has slowed the progress of $^{67/68}$Ga based PET and SPECT modalities. Both Ga and Gd ions doped
into Prussian blue render PET/SPECT and MRI imaging activity into single platform (i.e. Prussian blue nanoparticles).

1.3. X-ray computed tomography (CT)

X-ray computed tomography is another widely used imaging modality that could visualize internal structures such as bone and lungs with high resolutions. It has many advantages over the other imaging techniques, such as wide availability, cost effectiveness, high efficiency and deep tissue penetration. The X-ray computed tomography uses monochromatic X-rays as external energy sources and circular detectors that could record the intensity of X-rays transmitted across the body. X-ray radiations interact with matters that made up our body. The incident X-ray photons can either be absorbed or scattered by tissues. The reduction in beam intensity due to absorption and scattering defines the degree of X-ray attenuation and it obeys the Beer-Lambert law (Eq 7).

\[ I = I_0 e^{-\mu x} \quad \text{Eq 7} \]

where \( I \) is the transmitted X-ray intensity, \( I_0 \) is the incident intensity, \( \mu \) is the mass attenuation coefficient and \( x \) is the thickness of the matter (absorber).

Specifically, X-ray attenuation is a result of three types of interactions between incident X-ray photons and the matter. These interactions include coherent scattering (\( \omega \)), the photoelectron effect (\( \tau \)) and compton scattering (\( \delta \)). The mass attenuation coefficient can be expressed as the sum of the three contributions. Coherent scattering produces scattered radiation and it contributes to noise on X-ray computed tomography. Photoelectron effect produces no random scattering and leads to a high quality image. The contribution of photoelectrons to \( \mu \) is high with low-energy X-ray radiation and with absorbers of high \( Z \). Compton scattering occurs when high energy photons collide with outer shell electron. The high energy incoming photons get deflected to new directions with lower energy. Compton scattering is responsible for scattering all radiation and thus it increases noise and decreases contrast.\(^{41}\)
1.3.1 Contrast agents for computed tomography

CT contrast agents are usually employed to enhance the image contrast in specific areas. The conventional 2-D X-ray projections are used to image hard tissue such as bone and teeth while the endogenous soft tissue types remain indistinguishable. The computed tomography provides multiple 2-D images slice by slice which could be used to reconstruct 3-D images. Exogenous contrast agents, usually called as “dyes” are used to distinguish soft tissue types. Such enhancement of contrast in soft tissues could reduce the average X-ray dose that a patient typically exposed in a single scan. Various CT contrast agents have been developed and most of them are iodinated organic compounds.

Superb CT contrast agents should have greater X-ray absorption coefficient or attenuation power. Heavy elements strongly attenuate transmittance of X-rays and as a result, they render high X-ray attenuation coefficients. Therefore, inorganic compounds with heavy metals are better suited as X-ray CT contrast agents rather than the current iodinated organic CT contrast agents. The accuracy with which the linear attenuation coefficient \( \mu \) is determined governs the ability to discriminate different materials with different X-ray attenuation coefficients. X-ray contrast is often reported by CT number and measured by Hounsfield unit. CT number has been defined as follows.

\[
\text{HU} = \frac{1000 \times (\mu_x - \mu_{\text{water}})}{\mu_{\text{water}}} \quad \ldots \ldots \ldots \ldots \quad \text{Eq 8}
\]

Typically CT numbers range from -1000 for air, 0 for water and \( \text{ca} \) 1500 for hydroxyapatite. Largely linear attenuation coefficients consist of the contributions from Compton scatter and photoelectric effect. Thus linear X-ray attenuation coefficient depends on photon energy of the X-ray beam (E), the electron density of the material (\( \rho_e \)), and the effective atomic number of the material (Z) and has shown in Eq 9. The variations of X-ray attenuation characteristics in biological tissues render the contrast in CT imaging.\textsuperscript{41,75}
\[ \mu = \rho_e \left( a + \frac{bZ^{3.8}}{E^{3.2}} \right) \ldots \ldots \ldots \ldots \text{Eq 9} \]

Iodine element (Z = 53) that has moderate atomic number has been a choice for most of commercial CT contrast agents. A few of the iodinated CT contrast agents are shown in Scheme 5.\textsuperscript{41} They can be categorized into ionic and non-ionic agents. The iodinated ionic contrast agents have a higher tendency to interact with biomolecules in cells such as peptides, cell membranes. Such ionic compounds have high osmolality, potentially leading to renal toxicity and other physiological problems such as vasodilation, bradycardia, and pulmonary hypertension.\textsuperscript{76}

**Scheme 5.** Chemical structures of representative of iodinated X-ray contrast agents.

On the other hand, X-ray CT contrast agents based on the non-ionic form of iodinated organics still possess several disadvantages such as, rapid excretion from the body leading to short imaging time, low contrast efficacy, lack of targeted delivery and potential renal toxicity. Superb CT contrast agents require a large X-ray absorption coefficient or attenuation
power. The Table 1 has listed X-ray mass attenuation coefficient for some of the heavy elements. In general, heavy elements strongly attenuate transmittance of X-ray than that of iodinated compounds and render higher X-ray attenuation coefficients. Therefore, inorganic compounds with heavy metals are better suited for this application than the current iodinated CT contrast agents. Bismuth (Z=83) is the heaviest stable nonradioactive element with a strong X-ray attenuation power. In general, bismuth compounds are considered to be nontoxic compared to those of its heavy-metal neighbors such as Hg, Tl and Pb. Nanoparticulate solutions of non-toxic compounds of heavy elements such as Au, TaO₉, Bi₂S₃ (Table 1) have the ability to show good CT contrast. They can be easily conjugated with various biomolecules to facilitate selective uptake of contrast agents. Despite the surface functionalization of nanoparticulate contrast agents, nanoparticles of larger sizes (greater than 400 nm) are preferentially taken up by macrophage cells due to the process of opsonization. Consequently, high concentrations of nanoparticulate contrast agents are accumulated in liver, spleen, and lymphatic tissues, which possess high level of phagocytic cells.

Table 1. X-ray mass attenuation coefficients of heavy elements used in CT imaging.

<table>
<thead>
<tr>
<th>Element</th>
<th>Atomic number</th>
<th>K-edge energy, KeV</th>
<th>( \mu \text{ at } 100 \text{ keV} \text{ [cm}^2\text{g}^{-1}] )</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>53</td>
<td>33.2</td>
<td>1.94</td>
</tr>
<tr>
<td>Ba</td>
<td>56</td>
<td>37.4</td>
<td>2.20</td>
</tr>
<tr>
<td>Gd</td>
<td>64</td>
<td>50.2</td>
<td>3.11</td>
</tr>
<tr>
<td>Dy</td>
<td>66</td>
<td>53.8</td>
<td>3.36</td>
</tr>
<tr>
<td>Yb</td>
<td>70</td>
<td>61.3</td>
<td>3.88</td>
</tr>
<tr>
<td>Lu</td>
<td>71</td>
<td>63.3</td>
<td>4.03</td>
</tr>
<tr>
<td>Ta</td>
<td>73</td>
<td>67.4</td>
<td>4.30</td>
</tr>
<tr>
<td>Pt</td>
<td>78</td>
<td>78.4</td>
<td>4.99</td>
</tr>
<tr>
<td>Au</td>
<td>79</td>
<td>80.7</td>
<td>5.16</td>
</tr>
<tr>
<td>Bi</td>
<td>83</td>
<td>90.5</td>
<td>5.74</td>
</tr>
</tbody>
</table>
1.4 The therapeutics for intracellular copper detoxifications.

Cellular copper plays a key role as an essential cofactor in numerous enzymes. Its essentiality stems from its ability to switch between Cu(I) and Cu(II) oxidation states. The copper-requiring enzymes or proteins span a wide range from cytochrome c oxidase to ceruloplasmin to Cu/Zn superoxide dismutase, to name just a few. The biological functions of such enzymes include catalytic reactions for cellular respiration, oxidative phosphorylation, antioxidant defense, free radical scavenging and neutralization. Although copper is an essential nutrient, copper in the form of free ions is a potent cytotoxin. When copper accumulated inside the cell more than required for cellular needs, free copper ions can catalyze the production of reactive oxygen species (ROS) including hydroxyl radicals. Hydroxyl radicals are known to cause deleterious cellular damages such as lipid peroxidation in membranes, oxidation of proteins and cleavage of DNA and RNA molecules, leading to oxidative stress in the body. Thus the deficiencies in maintaining copper homeostasis are linked to human diseases or disorders including Menkes disease, Wilson’s diseases, familial amyotrophic lateral sclerosis, to name but a few. An imbalance of copper metabolism is also implicated in Alzheimer’s disease, Parkinson’s disease and prion diseases. Despite the recent significant progress made in understanding the intracellular trafficking of copper, there are still a limited number of clinical drugs available in the form of chelation therapy to treat the diseases and disorders associated with copper overload. In the case of Wilson’s disease (WD), accumulation of excess copper in the liver and other vital organs damage the organs due to the production of excessive ROS, which renders the patient severe disability with a variety of hepatic, neurological, ophthalmic and psychiatric symptoms. If untreated, the disease can rapidly progress to liver cirrhosis, and then needs for liver transplantation or death. WD is a recessive genetic disorder characterized by a mutation in the ATP7B gene. Such mutation in turn causes abnormal copper metabolism resulting in a blockage in the secretion of copper from the body. According to the hard soft acid base theory...
Cu(I) and Cu(II) are considered to be a soft Lewis acid and intermediate Lewis acid respectively. Hence Cu(I) forms strong bonds with the ligands having soft donor atoms such as sulfur and nitrogen while the intermediate Cu(II) preferentially binds to nitrogen. As a result, clinical drugs that are clinically used in treatment for copper related disorders contain sulfur and nitrogen atoms. The chelation therapy was introduced as potential treatment for various metal homeostasis related diseases and disorders.

The symptoms of Wilson’s disease can be alleviated by chelation therapy using a chelating agent such as dimercaptopropanol (BAL),\textsuperscript{87,88} penicillamine (i.e. (2S)-2-amino-3-methyl-3-sulfanyl-butanoic acid)\textsuperscript{89} or tetrathiomolybdate (i.e. MoS\textsubscript{4}\textsuperscript{2−})\textsuperscript{90,91} to reduce copper absorption or remove the excess copper from the body. The chelation therapy was originally used as an antidote to heavy metal intoxication despite its modern uses in genetic disorder associated with copper metabolism. Although small-molecular chelating agents have been widely used to sequestrate the excess metal ions/toxic metal ions from bodies, chelation therapy can yield unexpected serious side effects. Despite the major challenges accompanied with chelation therapy, it has been practiced as the standard way of treating the metal homeostasis related diseases and disorders. On the other hand, chelation therapy is very often a lengthy procedure with unpredictable treatment outcomes. In spite of the need of good chelators with better pharmacological profile to treat the metal ion deposition in vital organs, only a few chelators have been developed or explored thus far, and they possess common disadvantages of chelation therapy. Furthermore, such small-molecule-based drugs seldom possess sufficient selectivity toward a specific metal ion from the body. Further, the efficacy of small molecular drugs has hampered by their inability to cross the cell membrane in order to function as intracellular detoxifying agents.\textsuperscript{49} On the other hand, some chelators may mobilize the metal ions from the primarily deposited organs and reroute them into circulation, thus increasing the risk of secondary exposure of such metal ions to the brain.\textsuperscript{92} Therefore, the development of novel copper chelating agents with less toxicity and excellent selectivity remains a challenge in this field of research.
1.4.1 Noninvasive copper detection using molecular probes.

The noninvasive methods for *in situ* cellular copper ion detection using molecular probes are still at their preliminary stages. Detection of cellular copper has greater practical significance due to the physiological and pathological role played by copper. A small number of fluorescent probes (Scheme 6-8) have been developed for detecting copper ions. These fluorescent probes can recognize copper ions and display an increase in fluorescence. Generally, small molecule-based fluorescence probes are not membrane-permeable and are sometimes toxic to live cells. In addition to these shortcomings, the optical imaging using fluorescence probes is limited by low penetration of radiation used.

**Scheme 6.** Fluorescent probes for Cu$^{2+}$ containing Spirolactam derivatives of rhodamine dyes.$^{93}$

**Scheme 7.** Nitrobenzofuran-functionalized Ni@SiO$_2$ core/shell magnetic nanoparticles.$^{94}$
Thus noninvasive MRI-based copper detection probes (Scheme 9) have been developed by Chang et. al in 2009 as a solution to fluorescence-based copper sensing probes whose function are limited by the poor penetration of radiation used. After, the analyte copper binds to the receptor part in the probe, Gd\(^{3+}\) containing metal chelate is now open for water exchange, thereby increasing the number of inner-sphere sites (q) available for water coordination at the paramagnetic center. As a consequence, the longitudinal relaxivity (r\(_1\)) of protons on exchangeable solvent molecules in the complex is similarly increased. They have designed and synthesized a number of Gd-containing sensor molecules that can selectively bind copper ions in vitro. Upon binding the paramagnetic Cu\(^{2+}\) ion by the 3S-N macrocyclic
ring, the longitudinal relaxivity of the Gd-chelate MR contrast agent \( (r_1) \) increases from 3.76 to 5.29 \( \text{mM}^{-1} \text{s}^{-1} \). Such an event can be readily detected by NMR spectroscopy. Although the enhancement of relaxivity is significant, such small molecular copper sensor has not shown the ability to transverse the cells membrane to target intracellular copper ions. Also it should be noted that all Gd-chelates are known to be extracellular MRI contrast agents.

1.4.2 Intracellular copper removal as a novel approach for angiogenesis inhibition

Angiogenesis is the formation of new blood vessels from preexisting ones. It is a vital event in growth and development as well as wound healing. Also it plays a crucial role in the transition of benign stage tumors into malignant state. The angiogenesis involves the migration, growth and differentiation of endothelial cells which line the inside wall of blood vessels. The angiogenesis is tightly controlled by the balance of both pro-angiogenic factors and anti-angiogenic factors.\(^9^7\) Such tight regulation leads to the formation of blood vessels only when they are really needed. There are several factors involved in angiogenesis and they are released as a chemical response to stimuli of angiogenesis. These chemical signals could stimulate the endothelial cells and the most studied factor is VEGF (vascular endothelial growth factor). A good blood supply is required for tumors to grow beyond a few millimeter in size. Tumor angiogenesis induces the secretion of VEGF molecules by cancer cells and recruits normal cells for the transition into malignant stage. Therefore, tumor angiogenesis is an excellent point to target tumor invasions. Further, the hypoxia condition in cancer causes production of hypoxia inducible factor 1a (HIF-1a) which increases the VEGF expression level. Copper is an essential trace element and is known to induce endothelial cell motility as well as stimulates the proliferation and \textit{in vitro} migration of endothelial cells.\(^9^8\) It has observed that serum copper concentration increases as cancer becomes more invasive. The role of copper in angiogenesis is linked to several pro-angiogenic factors that include basic fibroblast growth factors (bFGF), vascular endothelial growth factors (VEGF), tumor necrosis
factor-α (TNF-α) and interleukin (IL-1) as an essential cofactor. The current anti-angiogenic drugs include Avastin (Bevacizumab), sorafenib (Nexavar®) and sunitinib (Sutent®). Such anti-angiogenic inhibitors could block signaling pathways in angiogenesis. After one pathway is blocked, another new signaling pathway can be triggered and which in turn starts the angiogenesis again when the current drugs are used. Thus such anti-angiogenic drugs have limited success while our intracellular copper depletion strategy has shown the promise as a novel anti-angiogenic drug.
Chapter 2: Materials and Methods

2.1 Materials

All chemicals, unless specified otherwise, were purchased from Sigma Aldrich and used without further purification. All materials used in this dissertation are listed below.

Potassium ferrocyanide

Gallium nitrate

Bismuth nitrate

Ferric chloride

Gadolinium chloride

Polyvinyl pyrrolidone (MW=40 000, 8000)

Citric acid

Manganese chloride

Zinc sulphate

MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)

Tetrachloroauric acid

Sodium citrate

D-Penicillamine

2.2 Experimental design and techniques

Characterizations of synthesized nanoparticles were performed using a number of techniques, including powder X-ray diffraction spectroscopy (XRD), energy dispersive X-ray spectroscopy (EDS), high-resolution transmission electron microscopy (HR-TEM), STEM-
EDS based elemental mapping, Fourier-transformed infra-red spectroscopy (FT-IR), and thermogravimetric analysis (TGA). T₁ and T₂ measurements of nanoparticles were measured at different concentrations using a Minispec 60 MHz NMR analyzer or a 9.4 T MRI scanner.

2.3 Experimental methodology

Nanoparticles were prepared by the technique called coprecipitation method. Stabilization of nanoparticles was achieved by adding suitable coating agents. The syntheses of nanocrystallite by coprecipitation method include the concomitant events of nucleation, growth, coarsening, and agglomeration processes. Under the condition of supersaturating, large number of small nuclei seeds are formed and undergone the growth of particles. Such small particles formed during the nucleation process are subjected to Ostwald ripening and aggregation of nanoparticles in a later stage, which result in a dramatic change in the size, morphology and properties of the products. However coating agents or polymers added encapsulate the nanoparticles and stabilize them from further agglomeration.

2.4 Instrumentation

2.4.1 Powder X-ray diffraction spectroscopy (PXRD)

Powder X-ray diffraction spectroscopy (PXRD) is one of the most powerful techniques for the characterization of nanoparticles, phase identification and it provides a lot of information about the structural properties such as unit cell dimensions of the crystalline materials. The data were collected at room temperature on a Siemens D5000 powder X-ray diffractometer using monochromatic copper Kα radiation. All the XRD patterns were recorded using 0.1 increments and 5° per minutes in the range of 2θ from 10° to 50° using a voltage of 40 kV and current setting of 40 mA. Samples were finely ground to a powder and mounted on a microscope glass slide prior to analysis. The X-rays are produced by accelerating an electron beam under potential gradient. The high energy electrons then get decelerated on Cu target and produce X-rays. Ni- β monochromator is used to filter the X-rays and collimator makes the X-rays parallel. The parallel X-rays get diffracted following the
Bragg’s law, once they hit the sample. The intensities of the diffracted X-rays are monitored by a detector and provide diffraction patterns which could be compared with database of known crystal structures. For structure determination of the bulk sample, XRD patterns were recorded using a Bruker D8 Advance X-ray powder diffractometer (Cu Kα, Ni β-filter and LynxEye PSD detector) equipped with LynxEye position detector and an incident beam Ge 111 monochromator. Powder patterns were measured from 10 to 110° 20 with step size of 0.01446° and exposition time 800 sec per step.

2.4.2 Transmission electron microscopy (TEM) and energy dispersive X-ray spectroscopy (EDX)

TEM images provide information on size of the nanoparticles and their morphology. The nanoparticles were first suspended in water by sonication. Next, 5 µL droplet of the suspension was placed onto a carbon-coated copper TEM grid (400-mesh) and specimens were then allowed to air-dry and at 200KV using a FEI Tecnai F20 field emission transmission electron microscope (TEM) equipped with an integrated scanning TEM (STEM) unit. The energy dispersive X-ray spectroscopy (EDX) results were obtained with an EDAX spectrometer in STEM mode. The spatial resolution is <1 nm through the acquisition of high resolution high-angle angular dark field (HAADF) images, in which the contrast is sensitive to atomic number (Z). The elemental mapping of nanoparticles was obtained by using drift corrected EDX spectra in STEM mode for elements of interest at area selected. These maps were generated by combining the scanning electron microscopy (SEM) micrographs of the specimen and the collected EDX spectra.

2.4.3 Thermogravimetric analysis (TGA)

The thermal gravimetric analysis was performed using a TA instrument 2950 high-resolution thermogravimetric analyzer (New Castle, DE, USA) in nitrogen or air from room temperature to 800 °C with a heating rate of 5 °C/min or 10 °C/min. Thermal gravimetric
analysis displays the features of thermal decompositions, zeolitic/coordinated water content and average surface polymer coating of nanoparticles on the basis of weight.

**2.4.4 Fourier transform infrared spectroscopy (FTIR)**

Fourier transform infrared spectroscopic (FTIR) analysis is one of the most powerful techniques for identification of the functional groups in compounds. IR radiation is passed through the sample and transmitted light reaching the detector is converted to a spectrum. The wavelength absorbed by the sample is characteristic of its chemical bonds. Therefore the spectrum of an unknown can be identified by comparison to a library of known compounds. In these experiments described herein, Bruker Vector 33 Fourier Transform Infrared Spectrophotometer was used for product characterization.

**2.4.5 Confocal microscopy**

Confocal florescence microscopic studies were performed with an OLYMPUS FV1000 IX8 to confirm the internalization of nanoparticle by cells. Cells were seeded in a 6-well plate with $5 \times 10^4$ cells/well and incubated for 24 hours. Fluorescence dye conjugated nanoparticles were then introduced to each well with the serum free fresh medium and incubated for ca 4 hours. After ca 4 hours incubation time with dye-conjugated nanoparticles, cells were washed three times with HSSB/PBS and fresh culture medium was added before the images were acquired.

**2.4.6 T$_1$ and T$_2$ measurements**

Nanoparticles solutions of various concentrations were used for T$_1$ and T$_2$ measurements using a 1.5 T NMR analyzer ( Mq 60 Brucker) to measure relaxivity studies. For T$_1$ measurements, an inversion recovery gradient echo sequence with a TE = 4 ms was used. The inversion time was varied between 30–2000 ms. T$_2$ measurements were performed using a spin–echo sequence of TR of 10000 ms, and TE of 10.6–340 ms. The T$_1$ weighted MR imaging was performed using a T$_1$- weighted spin–echo sequence at room temperature. The T$_1$-weighted MR images were acquired using the 9.4 T scanner with a matrix size of
2.4.7 Atomic absorption measurements

All atomic absorption measurements were made using a Buck Scientific Model 210 VGP atomic absorption spectrophotometer. The primary line of the elements Mn (279.5 nm), K (766.5 nm), Fe (248.3 nm), Cu (324.8 nm), Au (242.8 nm), Ca (422.7 nm), Mg (285.2 nm), Bi (222.8 nm), Ga (287.4 nm) and Zn (213.9 nm) were used to generated calibration curves and analyzed metal ion concentrations using hollow cathode lamps that operated at 10 mA. An air-acetylene flame was used for all measurements. Also the concentrations of metal ions in an aqueous solution were measured by inductively coupled plasma atomic emission spectroscopy (ICP-OES, Perkin Elmer Optima 3300-DV ICP).

2.4.8 Cell viability of nanoparticles

Cytotoxicity studies were performed using an MTT viability assay. HeLa cells were seeded in a 96-well plate at a density of $2 \times 10^4$ cells per well with the DMEM low-glucose medium and incubated for 24 hours at 37 °C in an atmosphere of 5% CO$_2$ and 95% air allowing cells to attach to the surface. Cells in each well were then treated with 100 μL of fresh medium containing varying concentrations of the nanoparticles and then incubated for 24 hours. Control wells contained the same medium without nanoparticles. After 24 hours incubation period, the cells were incubated with fresh DMEM media containing MTT reagent 10 μL, 1% (w/v) for 4 hours. Purple colored insoluble formazan dye was formed by the activity of mitochondrial reductase. After the MTT solution was removed, the precipitated violet crystals were dissolved in 100 μL of detergent. The absorbance was measured at 560 and 630 nm using a microplate reader. The assay results were presented as percentage of viable cells.
2.4.9 Determination of acid dissociation constants and binding constant of D-penicillamine toward the Cu$^{2+}$ and characteristics of complexation equilibria.\textsuperscript{101}

The experimental method used to measure thermodynamic stability constants consist of series of potentiometric titrations of D-penicillamine ligand in the absence of metal ions and the D-penicillamine ligand, in the presence of the M$^{2+}$. The ionic strength of the solutions were maintained at constant by using 0.05 M KClO$_4$ and employing relatively low concentration of ligand and the metal ions being investigated. Proton ligand stability constants and metal ligand stability constants were calculated directly from the formation curves of the systems for proton-ligand and M$^{2+}$ ion-ligand generated by the pH measurements made during the titrations. The titration curve was produced from the pH versus volume measurements obtained to the accuracy of 0.001 units in pH and 0.005 mL of alkali volume.

2.5 Experimental calculations

2.5.1 Relaxivity ($r_1$ and $r_2$ values)

$T_1$ and $T_2$ relaxation times measured from using a 1.5 T NMR analyzer (Mq 60 Brucker) are sum of the diamagnetic and paramagnetic terms.

\[ (1/T_{1i})_{obs} = (1/T_{1i})_d + (1/T_{1i})_p \quad i = 1, 2 \]

\[ r_i = (1/T_{1i})_p/[C] \]

\[ (1/T_{2i})_{obs} = (1/T_{2i})_d + r_i[C] \]

Where $T_1$ = longitudinal relaxation time, $T_2$ = transverse relaxation time, $r_1$ = longitudinal relaxivity, $r_2$ = transverse relaxivity, and C = concentration of paramagnetic species in term of mM. The slope of the graph of $(1/T_{1i})_{obs}$ vs concentration of paramagnetic species in term of mM gives the relaxivity value $r_i$. 

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2.5.2 Stability constant of metal complexes using Irving Rossetti method

The following sets of solutions were prepared for pH titrations. The set 1, set 2 and set 3 were prepared, set 1- HClO₄ acid (0.1173 moldm⁻³, 2.000 mL), KClO₄ (4.00 mL, 0.50 moldm⁻³) and deionized water 34.00; set 2-2.000mL of HClO₄ (0.1173 moldm⁻³), 25.00 D-PEN (5.0157 mM), 9.00 mL deionized water and 4.00 mL KClO₄ (0.05M); set 3- 2.000 mL HClO₄ (0.1173 moldm⁻³), 25.00 D-PEN (5.0157 mM), 8.50 mL deionized water and 4.00 mL KClO₄ (0.05M) and 0.50mL of M²⁺ solution whose concentration was predetermined by AAS. Total volume of each set was 40.00 mL. Each of the set was titrated with standardized KOH solution at 25 °C±0.5°C. The formation functions (n_H̅̅̅̅̅, n_L̅̅̅̅̅) and pL were calculated using following equations. By plotting the graph of n_H̅̅̅̅̅ vs pH, acid ionization constants (pKa₁,pKa₂) can be extracted and pKa₁,pKa₂ and pKa₃ correspond to the pH values at which n_H̅̅̅̅̅ is equal to 2.5, 1.5 and 0.5 respectively. By plotting n_L̅̅̅̅̅ vs pL, formation constants (logK₁, logK₂) can be extracted and logK₁ and logK₂ values correspond to the pL values at which n_L̅̅̅̅̅ is equal to 0.5 and 1.5 respectively.

\[
\overline{n}_H = \left( yT_0^0 + \frac{(v_1-v_2)(N+E^0)}{(V_0+v_1)} \right) / T_0^0
\]

\[
\overline{n}_L = \frac{(v_3-v_2)(N+E^0 + T_0^0(y-n_H̅))}{(V_0+v_2)n_H̅ T_M}
\]

\[
pL = \log_{10}\left\{ \sum_{j=0}^{j=I} \beta_j[H^+]^j / (T_L - \overline{n}_HT_M) \right\}
\]

y = Number of ionizable proton present in the ligand
T_L = Concentration of ligand
E^0= Mineral acid, concentration of HClO₄
V₀ = Total volume of the titration medium
N = Concentration of alkali
(v₁ - v₂) = Displacement of pH curve of set 1 and set 2 along the x axis at a given pH
\( (v_3 - v_2) \) = Displacement of pH curve (in the presence of metal ion) of set 3 and set 2 along the x axis at a given pH.

\( \beta_j^H \) = Overall proton-ligand association constant
Chapter 3: Synthesis of Novel Coordination Polymers KGa[Fe(CN)₆], KGₐ₋ₓ[Fe(CN)₆] and KGₐₓGdₓFe₄₋ₓ(CN)₆ Solid Solutions for Diagnostic Applications.


The gallium analogue of the soluble Prussian blue with the formula KGa[Fe(CN)₆]·nH₂O was prepared and structurally characterized. A simple aqueous synthetic procedure for preparing nanoparticles of this novel coordination polymer was investigated. The stability, in vitro ion exchange with ferrous ions, cytotoxicity, and cellular uptake of such nanoparticles coated with poly(vinylpyrrolidone) was explored for potential applications of delivering Ga³⁺ ions into cells or removing iron from cells.

3.1 Gallium Analogue of Soluble Prussian Blue KGa[Fe(CN)₆]·nH₂O

Metal hexacyanometallates AₓMᵧ[M'(_CN)₆]z (A = alkali metal ions, M = main-group-, transition-, or lanthanide metal ions, and M' = Cr, Mn, Fe, or Co), constitute an important class of inorganic coordination polymers that have recently attracted increasing research attention.¹⁰² PB can be prepared in two different forms, i.e., the soluble PB (SPB) with the idealized formula KFe(III)[Fe(II)(CN)₆] and the insoluble PB (IPB) with the idealized formula Fe₄(III)[Fe(II)(CN)₆]₃. Prussian blue was considered to be the first synthetic coordination compound and it was accidentally invented by the artists’ color maker Diesbach in Berlin, Germany in 1704.¹⁰³ In recent years, transition- and lanthanide-metal analogues of PB have been
intensively investigated for their magnetic and photomagnetic properties. However, PB analogues of main-group metals have only previously been studied to a lesser extent. Recently, there have been growing interests in the synthesis and investigations of novel nanoparticles derived from PB and its analogues. Particularly scarce is the exploration of the coordination chemistry of the main-group-metal hexacyanometallates that have potential biomedical relevance. In living systems, gallium(III) may be viewed as a redox inactive mimic of iron(III), as manifested by their similar biological transport and binding properties because of the identical charge and similar ionic radii of the two ions. Recently, Singh et al. showed that certain gallium(III) compounds exhibit antimicrobial activities against the multidrug-resistant strains of Pseudomonas aeruginosa isolated from patients with cystic fibrosis by disrupting the bacterial uptake and metabolism of iron. On the other hand, both $\text{Ga-67}$ (γ emitter with $t_{1/2} = 78.3$ hours) and $\text{Ga-68}$ (positron emitter with $t_{1/2} = 68$ minutes) are delivered as the citrate complex in diagnostic nuclear medicine for γ-radiation or positron-emission imaging, respectively. However, because of rapid decomplexation and hydrolysis, the mechanism of the uptake and biodistribution of these two radioisotopes in vivo is complicated and less desirable. In general, the lack of stability and the complex hydrolysis of gallium compounds at physiological pH have hampered the further development of radioactive and nonradioactive gallium pharmaceuticals.

In light of the resurgence of interest in the medicinal and radiopharmaceutical chemistry of gallium, here we describe the synthesis and characterization of the gallium analogue of SPB. Thus far, the gallium analogue of SPB has not been reported in the literature, while the preparation of thin films consisting of the gallium analogue of ISB has been described before.

### 3.1.1. Preparation of Gallium Analogue of Soluble Prussian Blue KGa[Fe(CN)$_6$]·nH$_2$O

Nanoparticles of KGa[Fe$^{II}$(CN)$_6$]n.H$_2$O (KGaPB NPs) were synthesized by a simple aqueous reaction. To better control the size distribution of KGaPB NPs and to impart biocompatibility to KGaPB NPs, an aqueous solution of Ga(NO$_3$)$_3$ (1 mM, 50 mL) containing...
200 mg of PVP 8000 was added to an aqueous mixture of K₄[Fe(CN)₆] (1 mM, 50 mL) under vigorous stirring for ca. 4 hours. It afforded a pale yellow colored colloidal solution. However, the continuous stirring of the resultant solution at room temperature for more than 24 hours afforded a slight color change into off-green. For purification of nanoparticulate KGa[Fe(CN)₆], an approximately equal volume of acetone was added to the above aqueous solution, but, the reaction product still did not separate from the liquid as a precipitate. Only after centrifugation at 13000 rpm for ca. 10 min, a pale-yellow pellet was formed at the bottom of the Eppendorf tube. The isolated precipitate could be redispersed back into water to form an almost colorless solution. This allowed for purification of the product through a water–acetone resuspension followed by centrifugation. The large scale purification of KGa[Fe(CN)₆] nanoparticles was carried out using regenerated cellulose tubular membrane (MWCO is 12000-14000) against distilled water for 2 days. The solid product was collected by lyophilization of the above solution. Bulk KGa[Fe(CN)₆] was prepared using the same procedure without adding PVP. Briefly molar ratio of 1:1 at concentrations of aqueous solutions Ga(NO₃)₃ (100 mM, 20 mL) and K₄[Fe(CN)₆] (100 mM, 20 mL) were mixed at room temperature under vigorous stirring. The reaction product was dialyzed using regenerated cellulose tubular membrane (MWCO=12000-14000) against distilled water for 48 hours, followed by lyophilization. The solid material obtained from this process has shown a good crystallinity for powder XRD analysis.

The KGa[Fe(CN)₆] nanoparticles were characterized by transmission electron microscopy (TEM), Energy dispersive spectroscopy (EDX), powder X-ray diffraction (XRD), Fourier-transformed infra-red spectroscopy (FT-IR), and thermal gravimetric analysis (TGA). The Bulk KGa[Fe(CN)₆] sample was used characterized by transmission electron microscopy (TEM), Energy dispersive spectroscopy (EDX), powder X-ray diffraction (XRD), Fourier-transformed infra-red spectroscopy (FT-IR), and thermal gravimetric analysis (TGA). Also the bulk KGa[Fe(CN)₆] sample obtained was used to solve the crystal structure.
Figure 3.1. TEM image of as prepared KGaPB nanoparticles.

Transmission electronic microscopy (TEM) images of the PVP-coated nanoparticles in Figure 3.1 revealed quasispherical nanoparticles with a narrow distribution of size at ca 15 ± 2 nm which was obtained from counting and averaging the size of 160 particles in Figure 3.2.

Figure 3.2. TEM image, STEM images and particles size distributions of as prepared KGaPB nanoparticles.
Additionally in Figure 3.3, Energy-dispersive X-ray spectroscopic analysis (EDS) on a nanoparticle shows distinctive signals of elements; K, Ga, and Fe. Metal ion analysis was carried out for purified bulk materials by atomic absorption spectroscopy (AAS). A sample of 50 mg of KGaPB bulk material was first decomposed at 620 °C for ca. 6 hours to obtain an amorphous oxide powder that was then dissolved in 5 mL of concentrated HNO₃. The elemental analysis of the purified bulk materials by the AAS gave a Ga–Fe molar ratio of 0.85:1.00 or an empirical formula KGa₀.₉₂Feₐ₀.₀₈[Fe(CN)₆]. These observations strongly suggest that our synthesis has yielded the target compound reminiscent of the SBP system.

Figure 3.4. The FT-IR spectra of PVP, KGaPB bulk compound and KGaPB NPs.
Fourier transform infrared spectroscopy (FT-IR) data shown in Figure 3.4 provides information on the surface of the KGaPB nanoparticles and core of the nanoparticles. The Fourier transform infrared (FT-IR) spectra of the bulk sample exhibited a strong and broad C≡N stretching vibration centered at 2111 cm$^{-1}$, attributable to the Fe$^{II}$–C≡N–Ga$^{III}$ bonding mode structures. Next to this peak, a shoulder appeared at 2075 cm$^{-1}$. The latter is the characteristic stretching vibration attributed to the Fe$^{II}$–C≡N–Fe$^{III}$ bonding mode found in pure PB samples. This observation indicates that the formation of a trace amount of PB due to the slow dissociation of [Fe(CN)$_6$]$_4^{4-}$ under acidic conditions during the synthesis and oxidation of the dissociated Fe$^{2+}$ to Fe$^{3+}$ by air. Also the KGaPB NPs contain inorganic cores of Fe$^{II}$–C≡N–Ga$^{III}$ bonding mode in structures and giving rise to characteristic stretching vibration at 2119 cm$^{-1}$. Further PVP coated KGaPB NPs exhibited peaks at 1662 cm$^{-1}$ as well as 1600 cm$^{-1}$ that are characteristic stretching vibrations of the amide group confirming the presence robust PVP around the nanoparticles.

The quantitative yield of good crystalline sample of bulk KGaPB was obtained by following lyophilization under reduced pressure and at low temperature. Such synthesis process and product isolation were known to increase the crystallinity at low temperature and reduced pressure. The advantage of such method is that the heating of the reaction is avoided to prevent the decomposition of the reaction product into Prussian blue. The structure determination of KGaPB bulk sample was performed by using a Bruker D8 Advance X-ray diffractometer.
Figure 3.5. Observed XRD data for the bulk KGa[Fe(CN)₆]·nH₂O given in black with calculated pattern given in red. The difference between observed and calculated is given in grey. The tick marks denote the peak location for the known structure.

Figure 3.6. The unit cell structure of KGa[Fe(CN)₆]. Color code: dark yellow=Fe(II), turquoise=Ga(III), pink=K, gray=C and blue=N.

The final refinement in space group Fm$\overline{3}$m yielded $a = 10.1274(3)$ Å and $V = 1038.7(1)$ Å³. The structure can be best viewed as the face-centered-cubic lattice defined by one type of ion, i.e., the Fe²⁺ or Ga³⁺ ions. The Ga³⁺ or Fe²⁺ ions occupy the octahedral holes. The infinite 3D framework coordination polymer of KGa[Fe(CN)₆].nH₂O consists of Fe²⁺ octahedra and Ga³⁺ octahedra joined in a 3D framework by CN⁻ groups. The Fe²⁺ ion is
coordinated by six C atoms of the CN\(^-\) groups while the Ga\(^{3+}\) ion is bonded by six N atoms, affording ideal octahedral geometry for both metal centers. The bond distances are Fe-C = 2.0065(7) Å, Ga-N = 1.9133(7) Å and C-N = 1.1445(4) Å. The tetrahedral sites in the framework are filled up by the K\(^+\) ions.

Table 2. Crystallographic data for KGa[Fe(CN)\(_6\)] \(\cdot\) nH\(_2\)O

<table>
<thead>
<tr>
<th>Chemical formula, KGa[Fe(CN)(_6)]</th>
<th>(F.w = 320.77 \text{ g mol}^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a = b = c = 10.1274 (3) \text{ Å})</td>
<td>space group Fm-3m (225)</td>
</tr>
<tr>
<td>(V = 1038.7 (1) \text{ Å}^3)</td>
<td>(\lambda = 1.54178 \text{ Å})</td>
</tr>
<tr>
<td>(V = 1038.7 (1))</td>
<td>(R_{wp} = 5.697, R_{exp} = 5.354)</td>
</tr>
</tbody>
</table>

Table 3. Atomic positions for KGa[Fe(CN)\(_6\)] \(\cdot\) nH\(_2\)O.

<table>
<thead>
<tr>
<th>Atom</th>
<th>Wyckoff Position</th>
<th>x</th>
<th>y</th>
<th>z</th>
<th>Occupancy</th>
<th>Beq</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe</td>
<td>4a</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1.03(3)</td>
</tr>
<tr>
<td>Ga</td>
<td>4b</td>
<td>0</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>1.03(3)</td>
</tr>
<tr>
<td>K</td>
<td>8c</td>
<td>0</td>
<td>0.25</td>
<td>0.25</td>
<td>0.5</td>
<td>1.03(3)</td>
</tr>
<tr>
<td>C</td>
<td>24e</td>
<td>0</td>
<td>0.2052</td>
<td>0</td>
<td>1</td>
<td>1.03(3)</td>
</tr>
<tr>
<td>N</td>
<td>24e</td>
<td>0</td>
<td>0.3140</td>
<td>0</td>
<td>1</td>
<td>1.03(3)</td>
</tr>
</tbody>
</table>
The thermal gravimetric analysis (TGA) was performed on both bulk and nanoparticle using a TA instruments 2950 high-resolution thermogravimetric analyzer (New Castle, DE, USA) in nitrogen from room temperature to 600 °C with the heating rate of 10 °C/min. Thermal gravimetric analysis on the bulk sample showed a clean one-step 17.3% weight loss of water before heating to 150 °C, indicating the presence of ~3.7 zeolitic water molecules per formula that is similar to the number of zeolitic water molecules in SPB that could varies from 3.7 to 4.0 from one batch to another.

Also the thermal gravimetric analysis of KGaPB NPs sample showed a weight loss of 57.4% which could be attributed to average polymer coating of polyvinylpyrrolidone. The stability of PVP-coated KGaPBNPs dispersed in aqueous solution was monitored over a period of 4 weeks by dynamic light scattering. There was not a change in the particle size (ca 27 nm) during this time period indicating the stability of nanoparticulate solution.
Figure 3.8. TGA curve of the PVP-coated KGa[Fe(CN)$_6$]·nH$_2$O nanoparticles

Figure 3.9. Dynamic Light Scattering of the PVP-coated KGa[Fe(CN)$_6$]·nH$_2$O nanoparticles

The leaching studies of Ga$^{3+}$ ions due to dissociation and hydrolysis from PVP-coated nanoparticles to the solution were conducted. For leaching of Ga$^{3+}$ and CN$^-$ ions from the nanoparticles, KGaPB NPs of 200 mg were sealed in a membrane dialysis bag (MWCO=3,500) and it was gently stirred in 25 mL distilled water solutions at neutral pH for
24 hours. The resulted solutions were then analyzed for free CN\textsuperscript{-} ions released from the nanoparticles by a fluorometric method using the cyanide test kit developed by LaMotte Co. (Chestertown, Maryland; Code 7387-01). The analysis of leached Ga\textsuperscript{3+} ions was carried out by AAS. It was found that the gallium concentrations were below the detection level of 20 ppm by AAS. The release of free cyanide ions from nanoparticles into the solution was studied by the Konig reaction\textsuperscript{117} and the highest cyanide concentration detected after 24 hours of incubation with KGaPBNPs was below \(~0.15 \pm 0.05\) ppm at neutral pH. As an indirect measurement of stability constant, solubility products K\textsubscript{sp} values of bulk KGa[Fe(CN)\textsubscript{6}] were determined by the static method. In this method, KGaPB bulk phase of ca 200 mg was allowed to equilibrate with 1000 mL deionized water. The solutions were stirred at 25 °C for 48 hours. After 48 hours, the leachate solution 1000 mL was reduced to small volume and then transferred into crucible and was heated to dryness. The crucibles were kept at 630 °C for 6 hours. Next nitric acid was added and gently heated to dissolve all completely. After gentle heating the final volume was measured again and metal concentration was determined by AAS. The solubility product of KGa[Fe(CN)\textsubscript{6}] was found to be \((2.7\pm0.5)\times10^{-13}\) mol\textsuperscript{3}dm\textsuperscript{-9}.

\[
2H^{+}(aq) + KGa[Fe(CN)\textsubscript{6}](s) + Fe^{2+}(aq) \xrightarrow{O_2(g)} KFe[Fe(CN)\textsubscript{6}](s) + Ga^{3+}(aq) + H_2O(l)
\]

The \textit{in vitro} ion-exchange studies were conducted to show the ability of Fe\textsuperscript{2+} exchange with KGaPB NPs (see the above equation). The KGaPB NPs (10 mM, 10 mL) were sealed in a dialysis bag (MWCO=3,500) which was brought in contact with a freshly prepared FeCl\textsubscript{2} solution (50 mL) with the initial concentration at \(~100\) ppm level. The iron concentrations of the solution outside the dialysis bag were periodically analyzed by AAS to check the progression of ion exchange kinetics.
Rate law

\[
- \frac{d[Fe^{2+}]}{dt} = k[KGa\{Fe(CN)\}_6][Fe^{2+}]
\]

At the beginning of the experiment KGa[Fe(CN)_6] is in excess.

\[
- \frac{d[Fe^{2+}]}{dt} = k'[Fe^{2+}]
\]

\[k' = k[KGa\{Fe(CN)\}_6]} (Pseudo first order rate constant)\]

\[[KGa\{Fe(CN)\}_6] >> [Fe^{2+}]\]

\[\int \frac{d[Fe^{2+}]}{[Fe^{2+}]} = -k' \int dt\]

\[This will yeild\]

\[ln[Fe^{2+}] = ln[Fe^{2+}]_0 - k't\]

The raw data can be fitted to two separate rate laws, i.e. the pseudo first order and the second order reactions with the first order rate constant \[k_1 = 2.0 \times 10^{-4} \text{ s}^{-1}\] or \[1.2 \times 10^{-2} \text{ min}^{-1}\] and the second order rate constant of \[k_2 = 1.0 \times 10^{-4} \text{ mM}^{-1}\text{s}^{-1}\] or \[0.60 \text{ M}^{-1}\text{min}^{-1}\].

**Figure 3.10.** Kinetic curve of Ga^{3+}/Fe^{2+} ion-exchange in aqueous solution.
Figure 3.11. Curve fitting results to the pseudo first-order reaction (right), and second-order reaction for the ion-exchange reaction (left).

The in vitro ion exchange studies of KGaPB NPs at neutral pH with ferrous ions showed that the ion exchange reaction followed a pseudo-first-order reaction up to a reaction time of \( \sim 50 \) min with a rate constant of \( k_1 = 1.2 \times 10^{-2} \, \text{min}^{-1} \) or a half-life of \( t_{1/2} = 58 \) min (Figure 3.11). There is an abrupt change of the reaction rate after this point to a slower and second-order reaction with a rate constant of \( k_2 = 0.60 \, \text{M}^{-1} \, \text{min}^{-1} \) (Figure 3.11 left panel) presumably because of the need for \( \text{Fe}^{2+} \) ions to penetrate the KGaPB NPs to react with the inner \( \text{Ga}^{3+} \) ions after the surface \( \text{Ga}^{3+} \) ions are consumed. The exchanged \( \text{Fe}^{2+} \) ions are concomitantly oxidized by O atoms from air to become \( \text{Fe}^{3+} \) ions during ion exchange.

The effect of PVP-coated KGa[Fe(CN)₆] \( \cdot \) nH₂O nanoparticles on the viability of cells was evaluated using a Trypan Blue exclusion viability assay. HeLa cells were seeded in a 96-well plate at a density of \( 1 \times 10^4 \) cells per well with the DMEM (Dulbecco’s Modified Eagle’s Medium) low glucose medium containing 10% FBS (fetal bovine serum) plus penicillin-streptomycin and incubated for 5 hours at 37 °C in an atmosphere of 5% CO₂ and 95% air to allow cells to attach to the surface. Cells in each well were then incubated with 100 mL of fresh medium containing various concentrations of the KGaPB NPs for 24 hours. Control wells contained the same medium without nanoparticles. The cells were then trypsinized and re-suspended in 100 μL full medium, then added to 100 μL of 0.4% Trypan. Viable and non-
viable cells were counted using a hemocytometer and each concentration was tested in replicates of three. The assay results were presented as percent viable cells.

![Cell Viability Curve](image)

**Figure 3.12.** The cell viability curve of PVP-coated KGaPB NPs.

The cytotoxicity results of KGaPB NPs (Figure 3.12) show that Hela cell viability measured by cell counting was *ca.* 95 ± 4% after 24 hours of incubation with a concentration of 1.1 mM. This suggests that cell killing by the PVP-coated KGaPBNPs does not occur for these types of cells under these experimental conditions.

The KGaPB nanoparticles for cellular uptake studies were prepared by modified synthesis protocol. An aqueous Ga³⁺ (1 mM, 50 ml) solution containing PVP (average MW=8000, ~ 500 mg) and ~ 2.0 ml of 0.5 mM of ethylenediamine was slowly added to an aqueous K₄[Fe(CN)₆] (1 mM, 50 ml) solution under vigorous stirring. The resulted solution was further stirred at room temperature for *ca.* 5 hours. These nanoparticles have pendent terminal –NH₂ groups. NPs were concentrated to ~ 2.5 mM and N-(3-dimethylaminopropyl)-N-(ethylcarbodiimide hydrochloride (EDC, 0.0018 g) and 6-carboxyfluorescene dye (0.0030 g) were reacted with each other first. The final fluorescence dye concentration was adjusted to ~ 0.8 mM. The fluorescence dye (~ 1 ml) was added to the nanoparticles (10 ml) and stirred overnight to allow the coupling reaction to occur. The reaction mixture was then dialyzed for
2 days to remove unreacted dye molecules using the regenerated cellulose tubular membrane (MWCO is 12000) against distilled water.

**Scheme 10.** Synthesis of PVP-coated KGaPB NPs.

**Scheme 11.** EDC coupling reaction for activation of carboxyl group.
Scheme 12. Surface functionalization of nanoparticles with carboxyfluorescein dye.

KGaPB NPs were first conjugated with fluorescence dye molecules by a water-soluble carbodiimide coupling reaction (Schemes 10-12) and the fluorescence dye conjugation was confirmed by fluorescence spectroscopic measurements (Figure 3.13).
Figure 3.1. Fluorescence spectrum of carboxyfluorescein dye and dye labeled KGaPB NPs.

Figure 3.1. (a) bright-field images, (b) confocal-fluorescence images of dye-labeled KGaPBNP-treated HeLa cells, (c) bright-field for control cells and (d) confocal-fluorescence of control cells.

An 8-well plate was seeded with cells and incubated for 24 hours to allow the cells to attach to the surface. The cells were then exposed to fluorescence dye conjugated KGaPB NPs for 4 hours and washed thoroughly several times with PBS buffer before they were
viewed under a confocal microscope. The cellular uptake of PVP-coated KGaPB NPs in HeLa cells was visualized using the confocal fluorescence microscopy technique. The fluorescent images of the live HeLa cells treated with dye-labeled nanoparticles showed strong fluorescent signals in the perinuclear region of the cell, indicating an untargeted distribution of nanoparticles in the cytoplasm without specific binding to any of the small organelles in the region (Figure 3.14). This observation seems to suggest that the cellular uptake of these nanoparticles is \textit{via} endocytosis.

3.2 Synthesis and characterization of KGa$_{0.1}$Fe$_{0.9}$[Fe(CN)$_6$] for diagnostic applications in PET/SPECT scans.

The significance of radionuclides in imaging and therapeutic modalities has gained an increasing attention over last six decades. The brief history of the uses in medicine extended up to late 1940 when Iodine-131 was first used radionuclide tracer to treat the thyroid cancer. Over the last 50 years of developments in nuclear reactors and accelerators, various radionuclides were artificially created. All the man-made radionuclides are not equally qualified for medicinal applications and most of the current diagnostic agents are coordination complexes of radionuclides. Such radiopharmaceuticals are required to meet several desirable characteristics such as short half-life times of radioactivity decay, easy accessibility -easy production of radionuclides and excellent pharmacological properties.\textsuperscript{113,114,119} On the other hand, radiopharmaceutical agents need to have a half-life long enough to do the imaging of pathological tissues and the preparation of imaging agent. All radionuclide tracers are essentially delivered to the target sites with a suitable protection to avoid the potential toxicity of metal ions. The use of $^{68}$Ga and $^{67}$Ga isotopes meets several desirable properties including easy access, \textit{in situ} production, short half-life time and several energy windows for multispectral scanning.\textsuperscript{74} Moreover, the differences between Ga(III) and other biological relevant ions leads to minimized potential toxicity which permits the use of it for therapeutic applications.\textsuperscript{120} However the advent of $^{99m}$Tc into imaging field reduced the interest on
gallium based radiopharmaceuticals. Such a rapid transition in interest could be partly attributed to challenges of Ga coordination chemistry and the pronounced diversity of stable $^{99m}$Tc imaging agents with varying pharmacological properties. However, single molecular gallium complexes are still used as radiopharmaceuticals, though they have many drawbacks. The lack of ideal carrier to deliver the radionuclide of gallium has slowed the progress of $^{67/68}$Ga-based PET and SPECT modalities and yet they remain as one of the frequently used radionuclides in nuclear medicine. These two isotopes are employed for positron emission tomography (PET) and gamma scintigraphy. $^{68}$Ga is positron-emitting nuclides with a half-life of 68 min and energy of ($\beta^+ \cdot 1.9$MeV) while $^{67}$Ga ($t_{1/2}$ 78 hours) is used for $\gamma$ scintigraphy and is produced by cyclotron. The production of $^{68}$Ga by a generator of parent nuclide $^{68}$Ge makes $^{68}$Ga radionuclides readily available and such an in situ generator of $^{68}$Ga provides a constant supply of $^{68}$Ga for the radiopharmaceuticals of PET.

Typically $^{18}$F and $^{11}$C radionuclide tracers, which are used in diagnosis of colorectal tumor and prostate cancers, have covalently bonded in fludeoxyglucose (18F-FDG) and choline C-11 respectively. Similarly, the utilization of $^{68/67}$Ga(III) in radiomedicinal applications requires robust complexation with suitable ligand. The complexation of the predominant oxidation state, Ga$^{3+}$ is dominated by ligands containing oxygen and nitrogen donor atoms and frequent clinical practices in PET/SPECT diagnostic imaging by $^{68/67}$Ga have inspired to explore various possible ligands containing nitrogen, oxygen donor atoms. The commonly used ligands include citric acid, NOTA- $2,2',2''-(1,4,7$-triazanonane-1,4,7-triyl)triacetate, DOTA- 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid, and NOPO-1,4,7-triazacyclononane-1,4,7-tris methylene phosphinic acid just to name a few. They are either multidentate ligand or multidentate macrocylic ligands. Among the gallium based radiopharmaceuticals, $^{67}$Ga-citrate was the first tumor imaging agent. The Ga(III)-citric acid complex gets transmetalated easily with transferrin (blood plasma protein) and form Ga(III)-transferrin with stability constant of logK-19.8. The stability constants of some Ga(III) complexes are as follows; logK$_{(Ga\text{-citrate})}^{+}$ 10.0, logK$_{(NOTA)^+}$ 30.1, logK$_{(DOTA)^+}$21.3, and logK$_{(NOPO)^+}$25.0. All the ligands discussed above are
polyaminicarboxylic ligands with N and O centers which could hold Ga(III) so tightly, except the citric acid. The NOPO ligand contains additional phosphinic acid moiety on N atoms in triazacyclononane macrocycle (TACN). Using such macrocyclic chelates, the pharmacological properties of Ga(III) complexes can be tailored by modifying the ligands. Principally, the complexation of Ga(III) to such a macrocyclic or multidendate ligand shows slower rate than the open chain ligands, despite its highest stability. Therefore chelation of Ga(III) into macrocyclic ligand requires elevated temperature and longer reaction time to overcome the activation energy barrier, imposed by the steric hindrance of highly strained macrocyclic ligand. Thus specially the $^{68}$Ga complexation has to be done in a limited time frame by reason of its short half-time ($t_{1/2}$ = 68 min). Such difficulties in incorporation of Ga(III) into macrocyclic ligands exemplifies challenges in coordination chemistry.

Also Ga(III) is oxo-phile hard Lewis acid which undergoes hydrolysis above pH 3 and forms insoluble Ga(OH)$_3$. These undesirable chemical characteristics make its aqueous coordination chemistry difficult and challenging. The biomedical use of Ga$^{3+}$ requires stable and safe Ga carrier which could overcome the above mentioned challenges. Moreover Ga(III) is subjected to in vivo exchange with plasma protein, transferrin and formed Ga(III)-transferrin complex. The demetalation and formation of oxo-complexes essentially occur due to lack of crystal stabilization energy and the rapid precipitation of Ga(OH)$_3$.\textsuperscript{111,113,114}

The challenges in coordination chemistry of such small molecular gallium complexes could be addressed by preparing solid state gallium doped Prussian blue coordination polymers. Interestingly, Ga(III) and Fe(III) are similar in size and charge, which results in the similar charge density for both Fe(III) and Ga(III) i.e. 69 pm and 62 pm. On the other hand, Ga(III) is a redox-inactive twin of the Fe(III) ion with regard to the similar properties due to its outermost electron configuration. The Ga(III) has filled 3d$^{10}$ configuration and Fe(III) has half-filled 3d$^5$ configuration which can easily take another electron, as a result, to be reduced to Fe(II). Despite the similarities in sizes and charge between Fe(III) and Ga(III), octahedral geometry adopted by Ga (III) in complexes displays a relatively less stability than Fe(III)-octahedral complexes due to the lack of crystal field stabilization of Ga(III) octahedral.
complexes. However, a small amount Ga(III) ions could easily be inserted into some of the octahedral lattice position and locked up in their lattice positions in Prussian blue and preventing it from unwanted leaching.

The challenges including the stability, susceptibility to hydrolysis, ligand exchange or transmetallation in the biological medium inspired us to assess the suitability of our nanoparticulate platform to develop novel radiopharmaceuticals which contain \(^{67/68}\text{Ga}\). For the preparation of \(^{68}\text{Ga}\)-radiopharmaceuticals, fast formation kinetics are required owing to the short half-life of \(^{68}\text{Ga}\). Our accelerated synthesis involving the aqueous solution and efficient-easy purification of PB NPs would be highly desirable. In addition, easy preparation and fast purification allow physicians to gain considerable time for imaging even with relatively low concentration of radiopharmaceuticals. To the best of our knowledge, we here demonstrate for the first time the use of Ga(III) doped colloidal solutions of Prussian blue (PB) as a novel radioactive Ga(III) delivering agent.

### 3.2.1 Preparation of PVP-citrate coated KGa\(_{0.1}\)Fe\(_{0.9}\)[Fe(CN)\(_6\)] nanoparticles

In a typical synthesis of 10% Ga(III) doped Prussian blue nanoparticles (10-GaPB NPs), PVP (MW-40000, ~ 400 mg) and citric acid (~ 200 mg) were first added to the solution of 40 mL of 0.9 mM aqueous Fe(NO\(_3\))\(_3\) and 0.1 mM Ga(NO\(_3\))\(_3\) under stirring. To this solution was added a 40 mL 1.0 mM aqueous K\(_4\)[Fe(CN)\(_6\)] solution under stirring at room temperature for 2 hours. A clear bright blue dispersion was formed immediately. The pH of this dispersion was measured to be 2.20. For TEM grid preparation, an equal volume of acetone was then added to the dispersion. Centrifugation at 10,000 rpm for 10 min resulted in the formation of a small pellet of nanoparticles. The latter was re-dispersed in deionized water by sonication and separated again by the addition of equal volume of acetone and centrifugation. The purification process was repeated one more time. After stirring for 2 hours, the solution was then transferred into dialysis bag and dialyzes against the deionized water for 12 hours at room temperature with continuous stirring. Bulk KGa\(_{0.1}\)Fe\(_{0.9}\)[Fe(CN)\(_6\)] materials were prepared in the absence of a coating agent. Specifically, an aqueous solution of 40 mL containing both 0.2 mM Ga(NO\(_3\))\(_3\) and 1.8 mM Fe(NO\(_3\))\(_3\) was added to an aqueous
solution of 40 mL K₄Fe(CN)₆ (2.0 mM) under vigorous stirring at room temperature. This reaction afforded a dark blue precipitate in an hour and stirred for another 3 hours. The product obtained was dialyzed for 24 hours and for every ca 3 hours outside solution was replaced with deionized water. The purified product was then collected by lyophilization.

**Figure 3.15.** TEM images of PVP-citrate coated 10-GaPB and selected area electron diffraction map.

The Figure 3.15 shows the TEM image of nanoparticles. The TEM studies revealed that these nanoparticles have cubic shape with the size distribution in the range of 21±5.5 nm. Furthermore, 10-GaPB NPs prepared by this method exhibited polycrystalline features, as confirmed by selected area electron diffraction (SAED) pattern of randomly selected individual nanoparticles, as shown in left panel inset of Figure 3.15. Citric acid-PVP40K couple acts as pre-chelating agent and form transiently stable metal complex with trivalent cations. Such transiently stable complexes of Ga(III) and Fe(III) slowly release trivalent cations to the incoming [Fe(CN)₆]⁴⁻. The formation of pre chelated complexes reduces the rate of nucleation and prevents the concomitant growth of nanoparticle. Strong chelating agent, [Fe(CN)₆]⁴⁻ can displace off loosely bound pre-chelating agent and form network structure. The controlled release of metal ions is preferable to form nanoparticulate network structure. Additionally pre-chelation of citric acid prevent formations of insoluble Ga(OH)₃ which would otherwise unavoidable above pH 3 in the preparation of nanoparticles. The chemistry
of synthesis we developed here allows us to overcome undesirable chemical characteristics associated with Ga(III) chemistry which make its aqueous coordination chemistry difficult. The particles are kinetically stabilized by the coating agents which prevent the further growth of the particles.

**Figure 3.16.** EDX spectrum on a PVP-citrate coated 10-GaPB NPs.

Energy-dispersive X-ray spectroscopic analysis (EDS) on a nanoparticle illustrates the composition of nanoparticles. As can be seen in Figure 3.16, nanoparticles contain gallium, potassium and iron.

**Figure 3.17.** EDS-STEM elemental mapping of 10-GaPB NPS of selected nanoparticle acquired by different energy window.

As shown in Figure 3.17 elemental mapping of PVP-citrate coated 10-Ga PB NPs acquired by drift corrected STEM-EDX mode showed distinct signals for Ga, Fe and K.
Elemental mapping of selected particles established the presence of doped Ga in PB structure as a single phase without forming mechanical mixture of all products.

**Figure 3.18.** X-ray diffraction pattern of the PVP-coated 10-GaPB NPs

Furthermore, the powder XRD pattern of 10-GaPB NPs exhibited all the characteristic peaks of bulk phase (fcc structure) confirming that the inorganic core of the PVP-coated 10-GaPB NPs is a single-phase. All peaks have inevitably broadened due to the effect of Debye-Scherrer broadening. On the basis of the assumed Gaussian-type peak broadening, the average particle size can be estimated to be ca 8 nm using the Scherrer formula.

To solve the crystal structure, a series of bulk KGa$_{x}$Fe$_{1-x}$[Fe(CN)$_6$] samples (where x=0.05 - 0.20) were individually prepared by the hydrothermal reactions including KGa$_{0.05}$Fe$_{0.95}$[Fe(CN)$_6$], KGa$_{0.1}$Fe$_{0.9}$[Fe(CN)$_6$], KGa$_{0.15}$Fe$_{0.85}$[Fe(CN)$_6$] and KGa$_{0.2}$Fe$_{0.8}$[Fe(CN)$_6$]. For the preparation of KGa$_{0.05}$Fe$_{0.95}$[Fe(CN)$_6$], mass of 0.2747 g, K$_4$[Fe(CN)$_6$].3H$_2$O, 0.0083g Ga(NO$_3$)$_3$ and 0.2364g Fe(NO$_3$)$_3$.9H$_2$O were added into teflon autoclave, and dissolved in 4.0 mL distill water with a few drops of concentratred HCl acid. For the preparation of KGa$_{0.1}$Fe$_{0.9}$[Fe(CN)$_6$], mass of 0.2741 g, K$_4$[Fe(CN)$_6$].3H$_2$O, 0.0166 g Ga(NO$_3$)$_3$ and 0.2359g Fe(NO$_3$)$_3$.9H$_2$O were added into teflon autoclave and dissoved in 4.0 mL distill water with a few drops of concentratred HCl acid. For the preparation of
KGa$_{0.15}$Fe$_{0.85}$[Fe(CN)$_6$]· mass of 0.2735 g, K$_4$[Fe(CN)$_6$].3H$_2$O, 0.0248 g Ga(NO$_3$)$_3$ and 0.2354g Fe(NO$_3$)$_3$.9H$_2$O were added into teflon autoclave, and dissolved in 4.0 mL distill water with a few drops of concentratred HCl acid. For the preparation of KGa$_{0.2}$Fe$_{0.8}$[Fe(CN)$_6$]· mass of 0.2729 g, K$_4$[Fe(CN)$_6$].3H$_2$O, 0.0330 g Ga(NO$_3$)$_3$, and 0.2349g Fe(NO$_3$)$_3$.9H$_2$O were added into teflon autoclave and dissolved in 4.0 mL distill water with a few drops of concentratred HCl acid. All the closed containers were incubated at 90 °C over 24 hours. The resulted blue-color crystallite products were washed with deionized water, and acetone following the centrifugation. The products were dried in air at room temperature for 24 hours.

**Figure 3.19.** a) KGa$_{0.05}$Fe$_{0.95}$[Fe(CN)$_6$]·nH$_2$O; b) KGa$_{0.10}$Fe$_{0.90}$[Fe(CN)$_6$]·nH$_2$O; c) KGa$_{0.15}$Fe$_{0.85}$[Fe(CN)$_6$]·nH$_2$O; d) KGa$_{0.20}$Fe$_{0.80}$[Fe(CN)$_6$]·nH$_2$O; Observed XRD data for the bulk KGa$_x$Fe$_{1-x}$[Fe(CN)$_6$]·nH$_2$O given in black with calculated pattern given in red. The difference between observed and calculated is given in grey. The tick marks denotes the peak location for the known structure.
The final rietveld refinement of KGa$_{0.05}$Fe$_{0.95}$[Fe(CN)$_6$] yielded crystal structure parameters including a=10.1958 (1) Å, V=1059.90 (4) Å$^3$, space group $Fm\bar{3}m$, (No. 225), $\rho_{\text{calc}}=2.023$ g/cm$^3$ and converged at $R_{wp}=3.602 \%$ and $R_p=2.856\%$. For the KGa$_{0.1}$Fe$_{0.9}$[Fe(CN)$_6$] PB, rietveld refinement yielded a= 10.1925(7) Å, $V=1058.87(2)$ Å$^3$, space group $Fm\bar{3}m$, (No. 225), $\rho_{\text{calc}}=1.832$ g/cm$^3$ and converged at $R_{wp}=3.539\%$ and $R_p=2.815\%$. The final rietveld refinement of KGa$_{0.15}$Fe$_{0.85}$[Fe(CN)$_6$] yielded a= 10.1847(1) Å, $V=1056.44(3)$ Å$^3$, space group $Fm\bar{3}m$, (No. 225), $\rho_{\text{calc}}=1.841$ g/cm$^3$ and converged at $R_{wp}=3.358\%$ and $R_p=2.677\%$. The final rietveld refinement of KGa$_{0.2}$Fe$_{0.8}$[Fe(CN)$_6$] yielded a= 10.1856 (1) Å, $V=1056.72 (5)$ Å$^3$, space group $Fm\bar{3}m$, (No. 225), $\rho_{\text{calc}}=1.845$ g/cm$^3$ and converged at $R_{wp}=3.406\%$ and $R_p=3.359\%$.

Figure 3.20. Unit cell structure of KGa$_x$Fe$_{1-x}$[Fe(CN)$_6$].

The final rietveld refinements yielded lattice parameters for all four samples and metal analysis of these samples was carried out using inductive coupled plasma optical emission spectroscopy (ICP-OES) with a PerkinElmer Optima 3200 system. A small sample of this solid (~ 50 mg) was transferred to a crucible and was heated in an oven at 620 °C for 6 hours to decompose the compound into oxides. After cooling it to room temperature, 5.0 ml of concentrated HCl/HNO$_3$ 2:3 were added to the residue. The resulting mixture was diluted, so
that, Ga\(^{3+}\) and Fe\(^{2+}\) concentrations fall into the linear dynamic range of metal analysis. Metal ions were analyzed against respective standards to determine the composition of each compound. As can be seen in Table 4, the actual chemical formula have a little discrepancy with the nominal formula suggesting that Ga(III) incorporation is an approximately half of the theoretically estimates. This observation could be attributed to competition for octahedral sites in fcc structure. The presence of Ga(III) in O\(_h\) sites does not provide any extra crystal field stabilization energy, on the other hand, Fe(III) in O\(_h\) sites in fcc does. The trend in the lattice parameters of these solid solutions is consistent with the trend anticipated by ionic radii of Ga(III) and Fe(III) ions for the different level of gallium doping. i.e. Gallium doping could slightly shrink cell constant of fcc (Ga(III) 62 pm vs 69 pm Fe(III)).

**Table 4.** Elemental analysis of gallium doped PB and lattice parameters.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Empirical formula</th>
<th>Chemical formula suggested by ICP-OES elemental analysis</th>
<th>a (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Ga PB</td>
<td>KGa[Fe(CN)](_6)</td>
<td>KGa(<em>{0.92})Fe(</em>{0.08})[Fe(CN)](_6)</td>
<td>10.1274</td>
</tr>
<tr>
<td>5% Ga doped PB</td>
<td>KGa(<em>{0.05})Fe(</em>{0.95})[Fe(CN)](_6)</td>
<td>KGa(<em>{0.02})Fe(</em>{0.98})[Fe(CN)](_6)</td>
<td>10.1958</td>
</tr>
<tr>
<td>10% Ga doped PB</td>
<td>KGa(<em>{0.10})Fe(</em>{0.90})[Fe(CN)](_6)</td>
<td>KGa(<em>{0.05})Fe(</em>{0.95})[Fe(CN)](_6)</td>
<td>10.1925</td>
</tr>
<tr>
<td>15% Ga doped PB</td>
<td>KGa(<em>{0.15})Fe(</em>{0.95})[Fe(CN)](_6)</td>
<td>KGa(<em>{0.07})Fe(</em>{0.93})[Fe(CN)](_6)</td>
<td>10.1847</td>
</tr>
<tr>
<td>20% Ga doped PB</td>
<td>KGa(<em>{0.20})Fe(</em>{0.95})[Fe(CN)](_6)</td>
<td>KGa(<em>{0.10})Fe(</em>{0.90})[Fe(CN)](_6)</td>
<td>10.1856</td>
</tr>
</tbody>
</table>

Such a trend in lattice parameters can be described by the Vegard's Law that is an approximate empirical rule which holds a linear relationship with crystal lattice parameter vs
constituent elements in solid solutions at constant temperature.\textsuperscript{128-130} As can be seen in Figure 3.21, a good linear correlation exists for the Ga doped PB solid solutions to follow the Vegard's Law.

![Figure 3.21. Vegard plot for lattice parameter of Ga doped Prussian blue.](image)

Fourier transformation infrared (FT-IR) spectra of bulk KGa\textsubscript{0.1}Fe\textsubscript{0.9}[Fe(CN)\textsubscript{6}] displayed a splitted C≡N stretching vibration at 2165 cm\textsuperscript{-1} and 2061 cm\textsuperscript{-1} which can be attributed to Fe(II)-C≡N-Ga(II)/Fe(III) stretching vibration mode. The presence of broad band around 3200 cm\textsuperscript{-1} is originated from zeolitic water trapped inside the cavities. On the other hand, 10-GaPB NPs exhibited a weak C≡N stretching vibration centered at 2080 cm\textsuperscript{-1}, attributable to the Fe(II)-C≡N-Fe(III) bonding mode in the structure. On contrary to the bulk sample, no shoulder peak appeared at around 2165 cm\textsuperscript{-1} due to poor resolution of weak signal in FT-IR spectrum. The appearance of the C=O stretching vibration of PVP amide at 1652 cm\textsuperscript{-1} suggested the amide unit in PVP has coordinated to the inorganic core. The coordinated PVP has C=O stretching vibration that has shifted to lower energy due to the effect of bond weakening in C=O as a consequence of lone pair donation from oxygen atom.
Figure 3.22. FT-IR spectra of citric acid (green), PVP40K (blue), PVP-citrate coated 10- Ga PB NPs (brown) and 10- Ga PB bulk (red).

Figure 3.23. The TGA curve of KGa$_{0.1}$Fe$_{0.9}$[Fe(CN)$_6$] sample.

We found that the use of both PVP40K and citric acid as coating agents is essential for long term stability in aqueous solution. The formation of pre-chelated complexes with
citric acid reduces the hydrolysis of Ga(III) in aqueous solution and the rate of nucleation in synthesis. The TGA curve confirmed the presence of inorganic core and average citrate-PVP coating of ca 42.6wt %, which cannot washed off from dialysis against deionized water.

For the radioactive Ga-68 (\(^{68}\)Ga) labeling of PB nanoparticles and Ga-68 dialysis study, a 2 mM solution of K\(_4\)[Fe(CN)\(_6\)] (8.0 mL) and 2 mM solution of Fe(NO\(_3\))\(_3\) (8.0 mL) containing PVP 20 mg and 20 mg citric acids were used for labelling and leaching studies. The gallium generator was rinsed and then eluted with 1 mL of 0.1 M HCl; an activity of 1.5-1.9 mCi of Ga-68 was obtained. An aliquot of 100 µL (Ga-68, 1.5-1.9 mCi) was added to a solution of Fe(NO\(_3\))\(_3\) (2 mM, 2.0mL) containing PVP\(_{8000}\) and citric acid and then stirred for 1 minute. To 2.00 mL solution of 2 mM Fe(NO\(_3\))\(_3\) containing \(^{68}\)Ga(III) 1.5-1.9 mCi, PVP\(_{8000}\) and citric acid was added 2.0 mL of a 2 mM K\(_4\)[Fe(CN)\(_6\)] solution and stirred 1 min. The resulted solution immediately yields a blue color colloidal solution. Colloidal solution of \(^{68}\)Ga doped PB NPs was immediately dialyzed against the deionized water (100 mL). Initially, three leachates (A, B and C cups of 100 mL deionized water) were used to rinse off any unbound radioisotopes. After first three cups were taken, the fourth cup, D (100.0 mL deionized water) was used to examine the stability of the compound. Aliquots of 5.00 mL were pulled from the leachates A, B, and C and counted on the HPGe detector. For cup D, 0.5 mL aliquots were pulled at the specified time intervals: 5, 10, 15, 20, 30, 45, and 60 minutes and counted using the HPGe detector. The sample aliquots were counted using gamma spectroscopy using the 511 keV annihilation gamma associated with Ga-68. A sample of original \(^{68}\)Ga solution was also counted to determine the original activity inside the dialysis bag. The activity of leachate solution was converted to a percentage of the original activity incorporated into KGa\(_{0.1}\)Fe\(_{0.9}\)[Fe(CN)\(_6\)] nanoparticles.
Figure 3.24. Radioactive leaching studies (left) and dialysis of 10$^{68}$GaPB NPs (right).

Our primary investigations turned out that Ga(III) could easily and tightly be incorporated into extremely stable PB NPs without undergoing any leaching and undesirable hydrolysis reaction. More importantly our $^{68}$Ga-doped nanoparticulate agent permits fast-easy preparation and purification of radiopharmaceutical in comparison to small molecular macrocyclic complexes. The radioactive labeling results confirmed that $^{68}$Ga isotope has successfully incorporated into PB structures and the activity of leachate solution was so low that the radioactive counting was equal to background level of counting, even after the spontaneous decay of $^{68}$Ga was accounted for. The development of novel cells permeable nanoparticulate radiopharmaceutical would definitely have major clinical relevance with selective targeting moiety attached onto the surface.

As an indirect measurement of KGa$_{0.1}$Fe$_{0.9}$[Fe(CN)$_6$] stability, solubility product constant was determined by conductivitymetric method.$^{131}$ A bulk samples (KGa$_{0.1}$Fe$_{0.9}$[Fe(CN)$_6$]) of ca 100 mg were placed in a dialysis bag and resuspended deionized water. The dialysis bags containing bulk samples were allowed to equilibrate with deionized water. Before the conductivity measurements were taken, the conductivity probe was calibrated by calibration standard of electrolyte solution whose electrical conductivity is known to be $1413\mu$Scm$^{-1}$. Electrical conductivity (κ) of the leachate solution of
KGa$_{0.1}$Fe$_{0.9}$[Fe(CN)$_6$] was measured to be 2.90±0.1 $\mu$S cm$^{-1}$ after 48 hours at 20 °C. The electric conductivity of deionized water was deducted to get the net conductivity of leachate solution. The limiting molar conductivity values were calculated using the tabulated $\Lambda^\circ_m$ values of individual ions in aqueous solution. The equilibrium concentration of each ion was calculated to be $2.81 \times 10^{-10}$ moldm$^{-3}$ by equation: $[KGa_{0.1}Fe_{0.9}[Fe(CN)_6]]_{eq} \equiv \frac{K}{\Lambda^\circ_m}$. The solubility product of KGa$_{0.1}$Fe$_{0.9}$[Fe(CN)$_6$] was found to be $2.2 \times 10^{-29}$±0.3 mol$^3$dm$^{-9}$ and thus showing that leachate contained nM level of released metal ions. The 10-GaPB NPs samples were also used for the metal ion leaching studies. A dialysis bag containing 0.5 mM solution of nanoparticles was kept in deionized water 1.0 L for 24 hours and the leachate solution was pre-concentrated before the determination of metal ion concentration by AAS. The leachate solution contains ca 49.7±1.3 nM concentration of Fe and no Ga released is detected and it is below minimum detection limit of AAS.

![Figure 3.25. Plots of 1/T$_1$ (i = 1, 2) vs the Fe$^{3+}$ concentration at magnetic field strengths of 1.4 T for 10-GaPB NPs.](image)

We performed a series of proton T$_1$ and T$_2$ relaxation measurements in order to determine their longitudinal and transverse relaxivity values, $r_1$ and $r_2$ at 1.4 T. The 1.4 T relaxometry results were obtained using a Bruker Minispec 60 MHz NMR analyzer.
Experimentally, the change of relaxation rate of water with the increasing concentration of contrast agent was measured, and the numeric value of relaxivity, $r_1$ or $r_2$, was then extracted from the slope of plot $1/T_1$ (or $1/T_2$) vs. concentration of Fe ions in nanoparticles. The $r_1$ and $r_2$ values were found to be 0.43 mM$^{-1}$s$^{-1}$ and 0.77 mM$^{-1}$s$^{-1}$ respectively. The good $T_1$ weighted contrast agent should have a ratio of $r_2/r_1$ that is closer to unity while $r_1$ relaxivity become large. For example Gd(DTPA) has a $r_1$ of 3.4 mM$^{-1}$s$^{-1}$ and $r_2$ of 3.7 mM$^{-1}$s$^{-1}$ ($r_2/r_1 = 1.1$). In the present work, we have achieved a decent $r_2/r_1$ ratio of 1.8 whereas the spin lattice relaxivity is relatively small. We suggested that the incorporation of Gd(III) ions with Ga(III) could impart high $r_1$ relaxivity while the $r_2/r_1$ ratio remain unchanged.

**Figure 3.26.** Confocal microscopic images of HeLa cell line (a) fluorescence image of cells incubated with dye conjugated nanoparticles for 4 hours, (b) bright field image of cells incubated with dye conjugated nanoparticles for 4 hours, (c) fluorescence image of untreated cells and (d) bright field image of untreated cells.

The cellular internalization of 10-GaPB NPs was shown by the fluorescent dye-labeled nanoparticles. Specifically for the preparation of $K_{Ga_{0.1}Fe_{0.9}[Fe(CN)_6]}$, an aqueous Ga$^{3+}$ (0.1 mM), Fe$^{3+}$ (0.9 mM) (total M$^{3+}$ concentration-1 mM, 40 mL) solution containing PVP and citrate (average MW=40,000, ~ 400 mg, citric acid-200 mg) and 1.0 mL of 1 mM of ethylenediamine were slowly mixed and reacted with $K_4[Fe(CN)_6]$ (1.0 mM, 40 mL) under
vigorously stirring. The anchored primary ethylene diamine functionalities allow the conjugation of carboxyfluorescein dye on 10-GaPB NPs (ca 4 mM). The fluorescent labeled 10-GaPB NPs were prepared following the Scheme 12. Hela cells were seeded in a 8-well chamber at a density of approximately 1.0 × 10^5 cells per well in complete medium and then incubated for 24 hours at 37 °C. The cells were then incubated with dye-labelled nanoparticles at 37 °C for 4 hours. The nanoparticle internalization into the cells was examined by confocal microscope using a 488 nm excitation wavelength. The internalized dye-labeled nanoparticles show green fluorescence inside the cytoplasm as shown in the confocal fluorescence microscopic images (Figure 3.26). This confirms that the fluorescent dye labeled nanoparticles are efficiently taken up by the HeLa cells.

![Viability of HeLa cells after incubation with 10-GaPB NPs for 24 hours (MTT viability assay).](image)

**Figure 3.27.** Viability of HeLa cells after incubation with 10-GaPB NPs for 24 hours (MTT viability assay).

The effect of PVP-citrate-coated 10-GaPB NPs on cell viability was assessed using a MTT viability assay. HeLa cells were seeded in a 96-well plate at a density of 1 × 10^4 cells per well with the DMEM (Dulbecco’s Modified Eagle’s Medium) low glucose medium containing 10% FBS (fetal bovine serum) plus penicillin-streptomycin and incubated for 5 hours at 37 °C in an atmosphere of 5% CO₂ and 95% air allowing cells to attach to the surface. Cells in each well were then treated with 100 μL of fresh medium containing varying
concentrations of the NPs and then incubated for 24 hours. Control wells contained the same medium without NPs. The cells were incubated in media containing 0.1 mg/mL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (i.e., the MTT dye) for 3 hours. The precipitated violet crystals were dissolved in 100 μL of detergent. The absorbance was measured at 570 and 630 nm using a microplate reader. The assay results were presented as percent viable cells. After 24 hours of incubation with NPs at concentration of 1.16 mM, the cell viability was found to be ca. 94 ± 4%, suggesting that the 10-GaPB NPs are not toxic to HeLa cells.

3.3 Synthesis and characterization of KGa$_{0.1}$Gd$_{0.1}$Fe$_{0.8}$[Fe(CN)$_6$] for bimodal imaging applications in MRI-PET/SPECT scans.

The modern imaging modalities (eg: CT, MRI, PET) have a wide spectrum of research applications including biomedical research, clinical diagnosis and studies of molecular level interactions in living systems. MRI and PET/SPECT are very powerful noninvasive diagnostic techniques and offer noninvasive examination of internal organs, body cavities, molecular processes. Magnetic resonance imaging (MRI) provides anatomical details with high-resolution images as a major advantage, conversely MR imaging lacks the ability to delineate the anomalous metabolic changes. The PET/SPECT imaging modalities provide information on molecular processes using radiolabeled imaging agents; on the other hand PET and SPECT gives limited anatomical details and spatial resolution as a major disadvantage, regardless of their high-sensitivity in tracking in vivo biomarkers. However, the major drawbacks of each imaging modality can be offset by bimodal imaging techniques.

Thus, the transition of paradigm into multimodal imaging has encouraged by unmet clinical needs. Such hybrid imaging techniques allow physicians to precisely co-register anatomical and functional information in a single scanning session without removing the patient from the bed, which would offer increased diagnostic accuracy. The relevance of
hybridized PET-MRI multimodal imaging in diagnosis was recognized even before the advent of PET-CT predecessor in hybrid imaging modalities.\textsuperscript{134} The PET-MRI unification is well qualified in many ways; amazingly synergistic to one another, usage of nonionizing radiation MR imaging, excellent for soft-tissues and temporal resolution, just to name a few.\textsuperscript{70,135} The multimodal imaging MRI-PET/SPECT reduces discomfort associated with multiple sessions and scan times required for separate imaging procedures.\textsuperscript{14} Generally, all imaging modalities including MRI, PET/SPECT and CT require the administration of exogenous agents which typically consist of small molecular compounds. The small molecular complexes and small molecule-based imaging agents possess major drawbacks; they are unable to penetrate cells, and still have susceptibility to undergo the displacement of active metal centers out of MRI contrast agents and PET/SPECT imaging agents, which leads to adverse side effects later. On the other hand, multimodal contrast agents are cost effective and they enhance the safety by limiting the number of contrast agents administrated to patients.\textsuperscript{136} The majority of bimodal imaging agents reported so far are going along with MRI imaging and optical imaging. MRI contrasting agents and optical imaging agents with near IR fluorescence (NIRF) dye complement each other to improve overall imaging quality. However, NIR has limited depth of penetration for tissues and is mainly absorbed by the hemoglobin and myoglobin present in a given volume. Therefore, MRI-PET/SPECT imaging modality is the preferred modality for anatomic and functional imaging over MRI-optical imaging. It should be noted that MR imaging requires high concentration of contrasting agents while the positron emission and single photon emission tomography needs lower concentrations of imaging agents owing to the discrepancy of sensitivity factor in each modality. Thus the ability to tune the activity of MRI-PET/SPECT bimodal imaging agents and their therapeutic dosage could enhance the diagnostic relevance of the bimodal imaging agents.

Nanoparticulate approaches could be used to prepare the multimodal imaging agents and they are less problematic. Nanoparticulate materials are the ideal scaffold that allows the easy integration of several different imaging modalities onto a single platform. Even though
the bimodal imaging agents with superb thermodynamic stability and excellent pharmacokinetic properties are imperative; the reliable, simple, robust and reproducible synthetic route is still not available for the preparation of bimodal imaging agents. To the best of our knowledge, we have described for the first time a novel nanoparticulate solid-state compound that contains both Gd(III) ($f^7$, $S=7/2$) and Ga(III) as dopants in the network structure, whereas all nanoparticulate bimodal imaging agents reported so far contain macrocyclic ligand bearing radionuclide tracer as side arm anchored onto magnetic nanoparticle cores. The dopant Gd(III) and radioactive Ga(III) render the $T_1$-weighted MRI imaging active and PET-SPECT imaging active, respectively. Our Prussian blue nanoparticles are the smartest option as a scaffold material for the development of multimodal agent due to their biocompatibility, high water dispersability and easy with which its surface can be modified to get better pharmacokinetic properties.

### 3.3.1 PVP-citrate coated KGa$_{0.1}$Gd$_{0.1}$Fe$_{0.8}$[Fe(CN)$_6$] nanoparticles

In this study, we have synthesized KGa$_{0.1}$Gd$_{0.1}$Fe$_{0.8}$[Fe(CN)$_6$] nanoparticles (10-GaGdPB NPs) stabilized by PVP and citric acid and characterized them by X-ray powder diffraction (XRD), transmission electron microscopy (TEM), EDX, STEM-EDX elemental mapping, elemental analysis (ICP-OES), UV-vis spectroscopy and FT-IR spectroscopy.

\[
[M_{\text{citric,PVP40K}}][\text{NO}_3]_3 + K_4[\text{Fe(CN)}_6] \rightarrow KM[\text{Fe(CN)}_6] + K\text{NO}_3
\]

$M \equiv$ Fe(III)- 0.8 mM, Gd(III)-0.1mM and Ga(III)-0.1mM

**Scheme 13.** Preparation of KGa$_{0.1}$Gd$_{0.1}$Fe$_{0.8}$[Fe(CN)$_6$] nanoparticles.

In a typical synthesis of KGa$_{0.1}$Gd$_{0.1}$Fe$_{0.8}$[Fe(CN)$_6$] nanoparticles, polyvinylpyrrolidone (PVP - MW- 40000, ~ 500 mg) and citric acid (~ 400 mg) were first added to the solution of 40 mL of 0.8 mM aqueous Fe(NO$_3$)$_3$ , 0.1 mM Ga(NO$_3$)$_3$ and 0.1 mM Gd(NO$_3$)$_3$ under stirring.
To this solution was slowly added a 40 mL 1.0 mM aqueous K₄[Fe(CN)₆] solution under stirring at room temperature for 3 hours (Scheme 13). Upon slow mixing, a clear pale green solution was initially formed and later tuned into clear a bright blue dispersion. After stirring for 3 hours, the solution was transferred into a dialysis bag and dialyzed against deionized water for 12 hours at room temperature with continuous stirring. The direct combination of such solution with K₄Fe(CN)₆ in aqueous solution results in a dark blue precipitate. The product was dialyzed in water to remove unreacted ions. The nanoparticle solution was lyophilized to afford a bright blue solid.

![Figure 3.28](#)

**Figure 3.28.** TEM image (left), SAED insets with histogram of particle size (right)

As shown in Figure 3.28, transmission electronic microscopy (TEM) analysis revealed that the nanoparticles have an average diameter of ca. 36.0±5.7nm, although the size distribution appeared to be relatively wide, ranging from 30 to 41.7 nm (Figure 3.28, left panel). Moreover, NPs prepared by this method exhibited polycrystalline features, as confirmed by selected area electron diffraction (SAED) pattern of randomly selected individual nanoparticles of 10-GaGd PB NPs, as shown in Figure 3.28 left panel inset.
Figure 3.29. EDX spectrum of PVP-citrate coated 10- GaGd PB NPs

Energy dispersive spectroscopy studies (Figure 3.29) and elemental mapping acquired by drift corrected STEM-EDX mode for PVP-citrate coated 10-GaGd PB NPs (Figure 3.30-31) showed distinct signals for Ga, Gd, Fe and K. Elemental mapping of selected particles and intensity line profiles (Figure 3.30) established the presence of Ga and Gd ions in the same phase without forming a mechanical mixture of all products. The synthetic method shown in Scheme 13 has successfully yielded a Ga and Gd doped solid solution as a single phase.
Figure 3.30. Intensity line profile of PVP-citrate coated KGa$_{0.1}$Gd$_{0.1}$Fe$_{0.8}$[Fe(CN)$_6$] nanoparticles (orange bar).

Figure 3.31. EDS-STEM elemental mapping acquired by different energy windows for 10-GaGd PB NPs of selected nanoparticle
The elemental analysis of the sample digested with nitric acid suggested that the elemental ratio of Ga/Gd/Fe to be 0.07:0.08:1.85 over the expected ratio 0.1:0.1:1.8, giving the average experimental formula of KG\textsubscript{0.07}Gd\textsubscript{0.08}Fe\textsubscript{0.85}[Fe(CN)\textsubscript{6}].

![FT-IR spectra of bulk 10-GaGd PB](image)

**Figure 3.32. FT-IR spectra of bulk 10-GaGd PB**

Fourier transformation infrared (FT-IR) spectra of bulk KG\textsubscript{0.1}Gd\textsubscript{0.1}Fe\textsubscript{0.8}[Fe(CN)\textsubscript{6}] displayed a splitted C≡N stretching vibration at 2161 cm\(^{-1}\) and 2071 cm\(^{-1}\) which can be attributed to Fe(II)-C≡N-M(III) stretching vibration mode where M(III) represent Ga(III), Gd(III) or Fe(III). The appearance of two distinct peaks in IR indicates the formation of solid solution containing Fe(III), Ga(III) and Gd(III) in single phase. However, Fe(II)-C≡N-Gd(III) could not clearly be distinguish from the two distinct CN\(^{-}\) stretching vibration peaks as stretching vibration of cyanide in Fe(II)-C≡N-Gd(III) and Fe(II)-C≡N-Fe(III) unit is similar in wave number (i.e. 2078 cm\(^{-1}\)). Similarly, the presence of a broad band around 3200 cm\(^{-1}\) is originated from zeolitic water trapped inside the cavities.
Figure 3.3. FT-IR spectra of citric acid, PVP40K and PVP-citrate coated 10- GaGd PB NPs.

The FT-IR spectrum of NPs (Figure 3.33) shows the characteristic feature of C≡N stretching vibration at 2070 cm⁻¹ accompanying the stretching vibration of carboxyl group and O-H group of water at 3300 cm⁻¹ along with C=O stretching vibration of PVP amide unit at 1652 cm⁻¹. The appearance of the latter peak suggested that a part of the amide unit in PVP has coordinated to the inorganic core. The coordinated PVP has C=O stretching vibration shifted to lower energy indicating that there is an interaction between carbonyl group of PVP with KGa₀.₁Gd₀.₁Fe₀.₈[Fe(CN)₆] NPs. Such coordination in turn protects the nanoparticles from further growth.

For phase identification, a high crystalline sample of KGa₀.₁Gd₀.₁Fe₀.₈[Fe(CN)₆] was prepared by the hydrothermal synthesis. A mass of 0.6631 g K₄[Fe(CN)₆].3H₂O, 0.0402 g Ga(NO₃)₃, 0.0709g Gd(NO₃)₃ and 0.3395g Fe(NO₃)₃.9H₂O were added into a teflon autoclave and with 8.0 mL distill water followed by adding a few drops of concentratred HCl acid. The closed container was heated at 90 °C over 24 hours. The resulted blue-color crystalline sample was washed with deionized water, and dialyzed against deionized water for 24 hours. The product was lyophilized for 24 hours to obtain the bulk sample.
Figure 3.34. Observed XRD data for the bulk KGa$_{0.1}$Gd$_{0.1}$Fe$_{0.8}$[Fe(CN)$_6$]·nH$_2$O given in black with calculated pattern given in red. The difference between observed and calculated is given in blue, the tick marks denotes the peak location for the known structure.

The final refinement in space group Fm$\overline{3}$m gave $a = 10.1944(40)$ Å, $V = 1059.46(120)$ Å$^3$ and $1.97065$ g/cm$^3$ and converged at $R_{wp} = 4.531\%$ and $R_p = 4.518\%$. The selected interatomic distances are summarized in Table 5.

Table 5. Selected interatomic distances.

<table>
<thead>
<tr>
<th>Atom 1</th>
<th>Atom 2</th>
<th>Distance, Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>M$^{3+}$</td>
<td>N1 x 6</td>
<td>2.2540</td>
</tr>
<tr>
<td></td>
<td>O1</td>
<td>3.4200</td>
</tr>
<tr>
<td>Fe1</td>
<td>C1 x 6</td>
<td>1.7432</td>
</tr>
<tr>
<td></td>
<td>N1 x 6</td>
<td>2.8432</td>
</tr>
<tr>
<td></td>
<td>O1</td>
<td>4.1893</td>
</tr>
<tr>
<td>N1</td>
<td>O1</td>
<td>2.8079</td>
</tr>
</tbody>
</table>

The structure can be best described as the face-centered-cubic lattice defined by one type of ion, i.e., the M$^{3+}$ or Fe$^{2+}$ ions occupying the octahedral sites where M$^{*}$ represent
Ga(III), Gd(III) or Fe(III). The infinite 3D network consists of Fe$^{2+}$-octahedral and M$^{3+}$-octahedral connected by CN$^-$ bridges in the coordination polymer as shown in Figure 3.35. The cavities in the framework structure are filled up by the K$^+$ ion and water of crystallization, showing site-occupancy disorder in the same cavity. The interatomic distance of oxygen to the nearby N and M(III) (i.e. Ga, Gd or Fe) are in the range of 2.80 to 3.42 Å and they correspond to the adsorbed water molecules. Such an unusual structural feature renders some of the M$^{3+}$ (Gd$^{3+}$, Fe$^{3+}$ ions) centers accessible to water coordination. Thus the inner sphere relaxation mechanism is active in 10-GdGa PB NPs, offering a unique platform for developing nanoparticle-based T$_1$ agents with high relaxivity.

![Figure 3.35. Unit cell structure of KGa$_{0.1}$Gd$_{0.1}$Fe$_{0.8}$[Fe(CN)$_6$].](image)

Thermal gravimetric analysis (TGA) on bulk sample (Figure 3.36) shows a two-steep mass loss from room temperature to 200 °C which could be assigned to adsorbed water loss. Such a stepwise loss of water could be attributed to the loosely bound water molecules and tightly bound water which can be identified as crystallization water. The first water loss was found in the range from room temperature to about 100 °C corresponding to the loss of 3.7 H$_2$O water molecules, and the second step of weight loss was found in the range from 100 °C and 200 °C, corresponding to the loss of 1.6 H$_2$O molecules (Figure 3.36). This observation is
consistent with the existence of zeolytic water in the crystal structure revealed by powder X-ray structure determination. Furthermore for bulk phase, a prominent mass loss of ca 30% has occurred when the temperature is brought up to 300 °C, suggesting that robust cyanide ligand could withstand such elevated temperature to secure the position of metal center inside the coordination polymer.

Figure 3.36. The TGA curve of 10-GaGdPB bulk (black) and 10-GaGdPB NPs (red).

The TGA curve of such NPs (Figure 3.36) confirmed the presence of inorganic core and average coating citrate-PVP of ca 51 wt %, which cannot be washed off by prolonged dialysis against deionized water.

The bulk 10-GaGd PB solid is insoluble in water and hydrolytically stable at neutral pH. We have used an electro-analytical technique to calculate equilibrium concentration of metal ions.\textsuperscript{131} At 22 °C, the solubility product (K\textsubscript{sp}) of the dissociation reaction KM[Fe(CN)\textsubscript{6}]\textsubscript{s} = K\textsuperscript{+} (aq) + M\textsuperscript{3+}(aq) + [Fe(CN)\textsubscript{6}]\textsuperscript{4-}(aq) was found to be 2.6×10\textsuperscript{-29} ± 0.2 mol\textsuperscript{3}dm\textsuperscript{-9}, thus giving saturated concentrations of each ion in the range of ca 2×10\textsuperscript{-10} M. We also monitored the leaching of the Fe(III) and Ga(III) ions from the PVP-citrate coated 10-GaGdPB NPs to aqueous solution by soaking ∼50 mg of NPs sealed in a dialysis bag.
(MWCO-3500) and periodically checking the solution concentrations of Fe(III), Ga(III) ions outside the dialysis bag using atomic adsorption spectrometry (AAS) for 1 week. In all the analyzed solution samples, the iron, and gallium concentration fell below the detection limit of the analytical technique indicating that 10-GaGdPB NPs has sufficient thermodynamic stability and kinetic inertness to maintain its structural integrity throughout the period of PET and MRI scanning application.

To investigate the potential use of our NPs on MR imaging, we performed a series of proton T<sub>1</sub> and T<sub>2</sub> relaxation measurements in order to determine their longitudinal and transverse relaxation values, r<sub>1</sub> and r<sub>2</sub> at 1.4T. The relaxation rates were obtained on a Bruker Minispec 60 MHz for varying concentration of nanoparticles. Both the longitudinal (r<sub>1</sub>) and transverse relaxivity values (r<sub>2</sub>) were extracted from the slope of the plot of 1/T<sub>i</sub> and 1/T<sub>2</sub> (Figure 3.37) versus Gd(III) concentration using the following equation.

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

Where 1/T<sub>i,d</sub> (i=1,2) is relaxation of proton in the absence of paramagnetic species and [Gd] is the concentration of Gd(III) ions in NPs.

![Graph of 1/T<sub>i</sub> versus Gd<sup>3+</sup> concentration](image)

**Figure 3.37.** The graph of 1/T<sub>i</sub> (i=1,2) versus Gd<sup>3+</sup> concentration at the magnetic field strength of 1.4 T.
It has been previously reported that KFe$^{III}[\text{Fe}^{II}(\text{CN})_6]$ has a large magnetic susceptibility with magnetic coupling of adjacent magnetic centers (without hysteresis loop) at room temperature which favors the spin-spin relaxation (T$_2$ relaxation) over the more desired spin lattice relaxation mechanism. Both T$_1$ and T$_2$ relaxation mechanisms are operative in magnetic resonance imaging experiment. The effect of two relaxations in MR imaging are complementary to one another. High r$_1$ relaxivity renders a brightening effect and on the other hand high r$_2$ relaxivity renders a darkening effect. As a result, a lower value of r$_2$/r$_1$ ratio is more desirable to develop T$_1$-weighted contrasting agents. Most of nanoparticulate MRI contrasting agents have a high value of r$_2$/r$_1$ ratio. Our primary investigation of PB NPs has shown that the colloidal solution of KFe[Fe(CN)$_6$] NPs possesses T$_1$-weighted imaging activity. Despite the promising results of PB NPs as a T$_1$-weighted contrasting agent, the reported lower value of r$_1$ and larger r$_2$/r$_1$ ratio hampered its biomedical application in the process of developing better T$_1$-weighted contrasting agent. The novel 10-GaGd PBNP has assured its role as T$_1$-weighted contrasting agent along with the PET/SPECT imaging activity by radioactive counterpart of dopant gallium.

The commercial contrast agents Magnevist (i.e. Gd-DTPA) and several other nanoparticulate MRI contrasting agents have presented below, and all of the data have been reported per Gd(III) concentration (in mM) at 1.4T. Specifically, numeric values of relaxivity (i.e. r$_1$, r$_2$), r$_2$/r$_1$ ratio and thermodynamic stabilities are summarized for 10- GaGdPB NPs, PB NPs, 100-GdPB NPs, 10-GaPB NPs and Gd(DTPA) in Table 6.
Table 6. Comparison of relaxivity data of several selected nanoparticulate Gd(III) based MRI contrast agents

<table>
<thead>
<tr>
<th>Nanoparticulate based MRI CAs</th>
<th>Size (nm)</th>
<th>( r_1 ) (mM(^{-1})s(^{-1}))</th>
<th>( r_2 ) (mM(^{-1})s(^{-1}))</th>
<th>Stability constant ( r_2/r_1 )</th>
<th>ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-GaGd PB NPs</td>
<td>36</td>
<td>51.73</td>
<td>73.37</td>
<td>( ca \ 10^{28} )</td>
<td>1.4</td>
</tr>
<tr>
<td>KGa(<em>{0.1})Fe(</em>{0.9})[Fe(CN)(_6)](_n) NPs</td>
<td>21</td>
<td>0.43</td>
<td>0.77</td>
<td>( ca \ 10^{28} )</td>
<td>1.8</td>
</tr>
<tr>
<td>KFe[Fe(CN)(_n)] NPs</td>
<td>22</td>
<td>0.20</td>
<td>1.22</td>
<td>( ^{*}ca \ 10^{40} )</td>
<td>6.1</td>
</tr>
<tr>
<td>KGd(H(_2)O)(_2)[Fe(CN)(_n)].H(_2)O NPs</td>
<td>27</td>
<td>35.2</td>
<td>38.4</td>
<td>-</td>
<td>1.1</td>
</tr>
<tr>
<td>Gd(DTPA)</td>
<td>-</td>
<td>3.4</td>
<td>3.7</td>
<td>( ca \ 10^{22} )</td>
<td>1.1</td>
</tr>
<tr>
<td>GdPO(_4) (at 0.47 T)</td>
<td>23</td>
<td>13.9</td>
<td>15.0</td>
<td>( ca \ 10^{14} )</td>
<td>1.1</td>
</tr>
</tbody>
</table>

All clinically used \( T_1 \)-weighted MRI contrast agents are dominated by molecular Gd-complexes, and they possess \( r_1 \) relaxivities of 3-5 mM\(^{-1}\)s\(^{-1}\), whereas the nanoparticulate agents have shown higher longitudinal relaxivities (\( r_1 \)) than small molecular complexes as multiple metal centers in them can induce water proton relaxation. \(^{64,141}\)

As shown in Figure 3.37, the relaxivity values of our NPs are \( r_1 = 51.73 \) mM\(^{-1}\)s\(^{-1}\) and \( r_2 = 73.37 \) mM\(^{-1}\)s\(^{-1}\). For comparison, the relaxivity values of Gd-DTPA are \( r_1 = 3.4 \) mM\(^{-1}\)s\(^{-1}\) and \( r_2 = 3.7 \) mM\(^{-1}\)s\(^{-1}\) at 1.4 T. It is intuitive to conjecture that Gd(III) ions in the close-packed cubic structure and high content of adsorbed water per formula unit may have enhanced the inner sphere relaxation mechanism as evidenced by the significantly higher \( r_1 \) relaxivity (51.73 mM\(^{-1}\)s\(^{-1}\)) and reduced \( r_2/r_1 \) ratio (1.4) for 10-GaGd PB NPs as compared to the typical \( r_2/r_1 \) value of >10 found in many SPIO systems in which only the outer-sphere relaxation mechanism is operative. Also it is very interesting to notice that the solid-state compound 10-GaGd PBNPs has stability of six orders of magnitude higher than that of the commercially used Gd(DTPA). Such extraordinary stability of this compound has clearly linked to the structure of coordination polymer where all metal ions and cyanide ligands are completely locked in their corresponding lattice positions to prevent them from being released to
solution by self-dissociation and yet having the ability to work as a T$_1$-weighted imaging agent and PET imaging agent. Such an increased thermodynamic stability and kinetic inertness could reduce the potential health hazards and safety concerns.

To further confirm the efficacy of 10-GaGd PB NPs as T$_1$-weighted MR contrast agent, we acquired the T$_1$-weighted MRI phantom images of 10-GaGdPB NPs in aqueous solution with various concentrations on a 9.4 T scanner (Figure 3.38). The T$_1$-weighted images rapidly become brighter with increasing concentration of NPs as shown in Figure 3.38. These results suggest that our bimodal nanoparticulate formulation can act as an effective T$_1$-weighted contrast agent at a high magnetic field.

Figure 3.38. T$_1$-weighted MRI phantoms of aqueous solutions of 10-GaGd PB NPs with various concentrations nanoparticles in term of Fe$^{3+}$ using a 9.4 T scanner.

The ability of NPs to cross the cell membrane is a critical prerequisite for cellular MR imaging applications. We studied the cell membrane penetrability of our NPs for HeLa cells using the fluorescent confocal microscopic imaging technique. Our nanoparticles alone do not show any fluorescence. As a result, carboxyfluorescein dye (CbF) conjugated NPs were used to cellular uptake studies. For live cell imaging, HeLa cells were treated with carboxyfluorescein dye conjugated NPs, washed with PBS, and directly imaged under a laser scanning confocal microscope without fixation. The carboxyfluorescein molecule tagged on NPs surface acts as a molecular beacon in cellular uptake studies, since the CbF dye itself is
not traverse across the membrane because of its higher negative charge which leads to a feature of the membrane impermeability. The images of confocal microscopy displayed the presence of bright green fluorescein signal inside the cells that were incubated with CbF dye labelled nanoparticles for 4 hours. The untreated HeLa cells were used as the negative control. The confocal laser microscopic images shown in Figure 3.39 represent HeLa cells treated with CbF dye conjugated NPs and control cells. The uniform green fluorescence emission from the cytoplasm indicates an untargeted accumulation of NPs in cytoplasm. Such significantly high green fluorescence intensity in comparison to the untreated cells suggested that the internalization of NPs is most likely to occur via endocytosis.

**Figure 3.39.** Confocal microscopic fluorescence image of HeLa cells incubated with dye-conjugated NPs for 4 hours (panel a), the bright-field image of the cells from (panel b), the fluorescent image of untreated HeLa cells as the negative control (panel c), and the bright-field image of the cells from (panel d).
Although Prussian blue (KFe[Fe(CN)₆]) capsules have been used as the antidote for Tl and ¹³⁷Cs poisoning even before the Chernobyl nuclear reactor accident in 1986, the toxicity of 10-GaGd doped PB has remained completely unexplored. Additionally toxicity of bulk materials (i.e. Radiogardase®) cannot be used to deduce the toxicity of nanoparticles. Especially, nanoparticles may exhibit unexpected or increased cytotoxicity because of their unusually large surface area relative to the total mass, which could increase the chance of interaction with endogenous biomolecules and initiate severe toxicity. We examined *in vitro* cytotoxicity of such NPs using the MTT assay with various concentrations of 10-GaGd PB NPs for 24 hours incubation. As shown in Figure 3.40, more than 81% of the cells were viable after 24 hours of incubation with 0.9 mM of NPs, indicating that PVP-citrate coated 10-GaGd PB NPs exhibit low cytotoxicity. This can be attributed to its high structural integrity of coordination polymer with proper surface coating.

**Figure 3.40.** Cell viability of HeLa cells after incubation with 10-GaGdPB NPs for 24 hours.
Despite the nontoxic behavior of our NPs in cell viability assay, a major concern regarding uses of NPs in biomedical application may arise due to the potential toxicity of the released cyanide. We carried out a series of leaching experiments under different conditions to assess cyanide leaching. The NPs were sealed in dialysis membrane and allowed to equilibrate with external solutions. The CN⁻ concentrations of leachate solutions were studied by the Konig reaction. The highest cyanide concentration detected after 24 hours of incubation with 10-GaGd PB NPs was below ~ 0.25 ppm at pH-1.0 and in phosphate saline buffer solution (Figure 3.41). In comparison, the maximum allowable level of cyanide in drinking water is 0.2 ppm as set by the environmental protection agency. Cigarette smokers are usually exposed to a high concentration of cyanide (35-65 ppm) right after a cigarette is smoked.

Figure 3.41. Cyanide-releasing studies for different conditions
The suitability of 10- GaGd PB NPs as T₁-weighted MR contrast agent was assessed by acquiring T₁-weighted MR phantom images of aqueous solution containing various concentrations of 10- GaGd PB NPs. Aqueous solutions containing 10- GaGd PB NPs were imaged on a Bruker 9.4-T MRI scanner at 37 °C using a conventional gradient echo acquisition with an inversion recovery preparation. The T₁-weighted MR images were acquired using the 9.4 T scanner with a matrix size of 128×128, a field of view of 3.0cm²×3.0 cm², a slice thickness of 0.5 mm, TE of 9.4 ms, and TR of 13.9~1500 ms. The T₁-weighted MR images of 10- GaGd PB NPs solutions show an increasing brightness with increasing the concentration of NPs and this suggests its T₁-weighted imaging ability (Figure 3.42).

To determine whether the internalized PVP-citrate coated 10-GaGd PB NPs could enhance the brightness of T₁-weighted MR images in cell pellets. We incubated HeLa cells with various concentrations of PVP-citrate coated 10-GaGd PB NPs and acquired a T₁-weighted image for each sample at 37 °C using a spin-echo saturation sequence on a Bruker 9.4 T MRI scanner.

Figure 3.42. T₁-weighted MRI phantoms of aqueous solutions containing 10- GaGd PB NPs using a 9.4 T scanner (NP concentrations given in term of Fe).
**Figure 3.43.** $T_1$-weighted MRI phantoms of untreated HeLa cells (right), HeLa cells treated with 10% GaGd PB NP (middle and left).

As shown in Figure 3.43, the cells treated with NPs at a concentration equivalent of 63 $\mu$M Gd(III) ions prior to imaging exhibited some contrast in comparison to the untreated control cells. These results tend to indicate that PVP-citrate coated NPs have the potential to be used as an effective $T_1$-weighted contrast agent for cellular imaging in addition to its potential uses in nuclear medicine.
Chapter 4: Non-gadolinium based T\textsubscript{1}-weighted MRI Contrast agent: Biocompatible Nanoparticulate based T\textsubscript{1}-weighted MRI Contrasting agent using Mn Analogues of Prussian blue K\textsubscript{2}Mn\textsubscript{3}[Fe(CN)\textsubscript{6}]\textsubscript{2}.

As previously discussed on magnetic resonance imaging (MRI), the nanoparticulate approach has a potential to deliver high payloads of contrasting agents, i.e., iron oxide particles\textsuperscript{13,63}, gadolinium oxide\textsuperscript{65}, gadolinium phosphate\textsuperscript{139} and gadolinium-containing micelles, liposomes and emulsions\textsuperscript{142,143}. As a consequence of efficient delivery, the MR images could be acquired using relatively low concentrations of CAs. Additionally, nanoparticle-based MRI CAs contain multiple metal centers which could greatly enhance the relaxation rate of protons in tissues of interest. Thus, low sensitivity problem associated with MRI imaging can be compensated by nanoparticulate CAs that contain multiple paramagnetic metal centers\textsuperscript{144}.

As far as the solely effectiveness of relaxation is concerned, Gd-based T\textsubscript{1}-weighted MRI contrast agents have excellent enhancement of image contrast but they have risks of biological toxicity. Specifically, Gd(III) has similar ionic radius as Ca(II) and can disrupt the critical Ca(II) -triggered signaling pathways. Free Gd\textsuperscript{3+} metal ions are toxic, so they should be coordinated to a protective chelate to form a stable non-toxic complex\textsuperscript{25}. For example as MRI CAs, Gd-DTPA and Gd-HP-DO3A have been approved for clinical use\textsuperscript{24,28}. However, such complexes still undergo metal-ligand exchange reactions with the \textit{in vivo} abundant cations and the released Gd(III) has a high affinity for phosphate, citrate, and carbonate ions and will bind to proteins such as serum albumin. By recent studies, the toxicity of the Gd(III)-based MRI contrast agents was also linked to the development of nephrogenic systemic fibrosis (NSF) in renal impaired patients. NSF is clearly a growing concern for the continued use of Gd(III) contrast agents\textsuperscript{26,27}. Consequently, the search for T\textsubscript{1}-weighted CAs with high efficacy and low toxicity has gained attention toward the Mn(II) and Fe(III), the two most suitable
candidates with many desirable properties; both have five unpaired electrons with long
electronic relaxation time and naturally found in living systems.

All of the Fe(III)-based CAs are superparamagnetic iron oxides (SPIOs) and such
nanoparticulate SPIOs have strong super exchange magnetic coupling which lead to large
values of $r_2$ and be qualified as $T_2$-weighted CAs. So they are used as $T_2$-weighted
contrasting agents for both clinical diagnosis and cellular imaging. However, the negative
contrast manifested by $T_2$ CAs is far less desirable than positive contrast of $T_1$ CAs. For
example, negative contrast is often confused with a low-level MR signal arising from
bleeding, calcification and metal deposits or other artifacts from the background and led to
indistinguishable from other hypo-intense regions, such as hemorrhage and blood clots. Two iron oxide-based $T_2$-weighted CAs have already been marketed under the names of
Endorem® and Resovist®, whereas the Fe$^{3+}$ is considered to be more toxic to cells because
free ferric or ferrous ions can catalyze the production of reactive oxygen species via the
Fenton reactions. SPIOs based $T_2$-weighted CAs (vide supra disadvantages) are less desirable
than that produced by small molecule $T_1$-weighted CAs. Therefore $T_1$-weighted CAs have
major clinical relevance for accurate diagnosis of pathogenic sites.

On the other hand, paramagnetic chelates of Mn(II) could be employed as $T_1$-
weighted CAs. However, it is challenging to design and synthesize highly stable Mn$^{3+}$
complexes that could maintain the integrity when administered to living system. As Mn(II) is
an essential metal ion, living systems possess highly efficient mechanism to sequestrate the
Mn(II) from such chelates. However, Mn(II) complex of dipyridoxyl diphosphate,
(MnDPDP- TESLASCAN®) is already used in clinical practices as a liver imaging agent and
done the job by releasing Mn(II) ions. Essential Mn(II) ion is a conundrum of nature,
because of both essentiality and toxicity associated with diagnostic uses. The Mn(II)
concentration in mammalian tissues are minuscule, thus over exposure exhibits neurotoxicity
with initial symptoms of Parkinson-like syndrome followed by other signs of fatigue, poor
memory, hallucinations and hepatic encephalopathy. The drawbacks of Mn-chelates used in
MR imaging can be circumvented by employing nanoparticulate platform, for example, MnO, Mn$_3$O$_4$@SiO$_2$, MnO@mesporous SiO$_2$ have been reported as Mn(II)-base new MR CAs. Many approaches have been developed to synthesize manganese oxide (MnO or Mn$_3$O$_4$) nanoparticles. The biocompatible mesoporous silica coating enables increased water accessibility across the shell, and as a result provides enhanced T$_1$-weighted contrast. The relaxivity measurements of HMnO@mSiO$_2$ nanoparticles in water showed that $r_1$ and $r_2$ values are 1.72 mM$^{-1}$s$^{-1}$, $r_2= 11.30$ mM$^{-1}$s$^{-1}$ respectively and $r_2/r_1$ ratio is 6.56 at 1.4 T field strength. The good T$_1$ contrast agents have a low ratio of $r_2 / r_1$ (close to 1) in order to generate positive contrast on T$_1$-weighted images. Conversely, large transverse relaxivity abolishes the hyper-intense MR signal generated by brightening effect of T$_1$-weighted contrast agents. In general, MR imaging experiment makes use of transverse magnetization component to generate the MR signal when the detector coil is on for imaging. The T$_1$-weighted imaging accentuates the T$_1$ relaxation of protons by providing many RF pulses in short repetition time (TR) and quick listening of the signal generated by the echo of transverse magnetization. However, large values of $r_2$ diminish the coherence of transverse magnetization before the spin echo signals were recorded by the detector. As a result, it neutralizes the brightening effect caused by enhanced longitudinal relaxation. Such a challenge of independently tuning longitudinal relaxivity and transverse relaxivity tempted us to test the suitability of the oldest synthetic coordination polymer to prepare an efficient T$_1$-weighted CA.

4.1 Citrate-coated K$_2$Mn$_3$[Fe(CN)$_6$]$_2$ nanoparticles as a T$_1$ weighted contrast agent for MRI.

The coordination polymer we described here, Prussian blue is a manganese hexacyanoferrate and has a face-centered cubic structure (space group Fm$\overline{3}$m) in which two metal centers Mn(II) and Fe(II) are bridged by the CN$^-$ groups. Mn analogues of PB contains carbon-bound Fe(II) with low-spin (S = 0), while the Mn(II) is nitrogen-bound and high-spin (S = 5/2), which gives rise to a total of five unpaired electrons per Fe$^{2+}$–C≡N–Mn$^{2+}$ unit.
Hence, this compound has the ability to shorten the longitudinal and transversal relaxation times \((T_1 \text{ or } T_2)\) of protons from bulk water. Hence inner sphere relaxation mechanism is active in Mn PBNPs due to the presence of zeolitic water in crystal structure and offers a unique platform for developing nanoparticle-based \(T_1\) agents with enhanced relaxivity.

In this study, we demonstrate the synthesis and utility of nanoparticulate Mn analogue of Prussian blue \((K_2Mn_3[Fe^{II}(CN)_6]_2)\) as an effective \(T_1\) MRI contrast agent for cellular imaging. Our previous studies on \(KMn[Fe^{III}(CN)]_6\) showed that it could act as good \(T_2\) weighted CAs in that unusually large \(r_2\) relaxation rate \((ca. 170 \text{ mM}^{-1}\text{s}^{-1})\) of \(KMn[Fe^{III}(CN)]_6\) and huge \(r_2/r_1\) ratio \((ca. 37 \text{ mM}^{-1}\text{s}^{-1})\) generate negative contrast on \(T_2\)-weighted images. The strong magnetic coupling of adjacent paramagnetic metal centers (Mn(II) and Fe(III)) makes the nanoparticles superparamagnetic and leads to have large transverse relaxivity, \(r_2\). As means of lowering the large value of \(r_2\), we have prepared \(K_2Mn_3[Fe^{II}(CN)]_6\) nanoparticles in that paramagnetic centers (Mn(II)) are not in a close proximity (>10 Å) to communicate the spin via bridging cyanides, thus inhibiting the strong magnetic coupling.\(^{151}\) Moreover, MRI contrast agents with high \(r_1\) values offer good contrast for \(T_1\)-weighted images at lower concentrations of the CAs.\(^{36,152}\) These Mn PB nanoparticles have several distinct advantages (1) ease of synthesis and post-synthesis modification with small molecules, (2) the ability to carry large payloads of active magnetic centers, (3) decreased tumbling rates which lead to increased relaxivity values, (4) decreased transverse relaxivity and \(r_2/r_1\) ratio.\(^{148}\) We have also shown that such NPs have no toxicity and are readily taken up by cancer cells, suggesting their use in drug delivery, and attesting to their potential as a new class of diagnostic probes.

For the synthesis of citrate-coated \(K_2Mn_3[Fe(CN)]_6\)·\(n\)·\(H_2O\) nanoparticles (MnP NPs), an aqueous solution of MnCl\(_2\) (1 mM, 15 mL) containing 100 mg of trisodium citrate was added dropwise to an aqueous mixture of \(K_4[Fe(CN)]_6\) (1 mM, 10 mL) under vigorous stirring for \(ca. 2\) hours. The reaction product was dialyzed using a regenerated cellulose tubular membrane (MWCO is 12 000–14 000) against distilled water for 24 hours. The solid product was collected by lyophilization. After the mixture had been stirred for 2 hours, 10 mL aliquots of the solution were each added with an equal volume of acetone and centrifuged at
13000 rpm for ~15 min, resulting in the formation of a pellet of nanoparticles in each centrifuge tube. The nanoparticles were redispersed in ~10 mL of distilled water by sonication and separated again by the addition of an equal volume of acetone and centrifugation. The purification process was repeated twice. Bulk K$_2$Mn$_3$[Fe(CN)$_6$]$_2$ material was prepared in the absence of coating agent using the same procedure. Specifically, aqueous solutions of MnCl$_2$ (1 mM, 75 mL) and K$_4$[Fe(CN)$_6$] (1 mM 50 mL) were mixed at room temperature under vigorous stirring. The solution turned into white and cloudy after continuous stirring at room temperature for ca. 60 min. The reaction product was dialyzed using a regenerated cellulose tubular membrane (MWCO = 12 000–14 000) against distilled water for 12 hours, followed by lyophilization. The solid bulk material was collected.

**Figure 4.1.** Histograms of the size distribution for MnPB NPs corresponding to panel on the right and TEM images of as-prepared MnPB NPs

The transmission electronic microscopic (TEM) imaging revealed that the NPs have near cubic shape with an average diameter of 21.1±3.2 nm as determined by counting and averaging the size of 61 particles in this TEM picture frame (see Figure 4.1). The average particle size of ca 21 nm is amenable for the intended biological application due to high water dispersability and subcellular size. Furthermore, the energy dispersive X-ray spectroscopic
EDX measurements showed the characteristic signals of Mn, Fe and K from several individual NPs randomly selected from the TEM grid (Figure 4.2).

![EDX spectrum on a typical citrate-coated MnPB nanoparticles](image)

**Figure 4.2.** EDX spectrum on a typical citrate-coated MnPB nanoparticles

The quantitative elemental analysis of the MnPB NPs, carried out by the inductive couple plasma spectroscopy (ICP) gave the average molar ratio of K:Mn:Fe to be 1.15/1.58/1.00 indicating that the composition of the NPs matches $K_2Mn_3[Fe^{II}(CN)_6]_2$. 
Here, we have employed a simple titrimetric method for stoichiometric ratio determination of \( K_2\text{Mn}_3[\text{Fe}(\text{CN})_6]_2 \). In above conductivitymetric titration, \( \text{MnCl}_2 \) solution was titrated with \( K_4[\text{Fe}(\text{CN})_6] \) solution at 25 °C. The free \( \text{Mn}^{2+} \) and \( \text{Cl}^- \) ions in initial solution give rise to electrical conductivity of ca 240 \( \mu \text{S cm}^{-1} \). Upon addition of \( K_4[\text{Fe}(\text{CN})_6] \) solution into \( \text{MnCl}_2 \), white precipitate of MnPB was immediately formed. As a result of removing the Mn(II) from the solution, electric conductivity of solution decreases. The equivalence point at which the volume ratio of \( K_4[\text{Fe}(\text{CN})_6] \) and \( \text{MnCl}_2 \) is equal to 2:3 gave a lowest electrical conductivity as shown in Figure 4.3 with only \( K^+ \) and \( \text{Cl}^- \) ions present. Further, addition of extra \( K_4[\text{Fe}(\text{CN})_6] \) leads to increase the electrical conductivity. Interestingly, the conductivitmetric titration studies (Figure 4.3) independently confirmed stoichiometric ratio of 3:2 for \( \text{MnCl}_2 : K_4[\text{Fe}(\text{CN})_6] \) giving the empirical formula, \( K_2\text{Mn}_3[\text{Fe}^{II}(\text{CN})_6]_2 \). The empirical formula we have derived is consistent with previous studies of the same compound.
The X-ray powder diffraction pattern shown in Figure 4.4 showed the presence of diffraction peaks with indices 200, 220, 400 and 420 at the corresponding Bragg’s angles, which are consistent with the systematic absence conditions of $h + k = 2n + 1$, $h + l = 2n + 1$ and $k + l = 2n + 1$ in the cubic face-centered PB lattice (space group Fm$\bar{3}$m) with a calculated lattice constant $a = 10.105$ Å.

As shown in Figure 4.5, our Mn analogue of Prussian blue adopt a structure, in which octahedral $[\text{Fe(CN)}_6]^{4-}$ complexes are linked via octahedrally coordinated, nitrogen-bound...
Mn$^{2+}$ ions. According to the stoichiometric ratio of Mn:Fe in $K_2Mn_3[Fe(CN)_6]_2$ discussed above, some of the $[Fe(CN)_6]^{4+}$ units have been removed from simple cubic framework. Therefore the unit cell structure of MnPB has been deviated from $KFe[Fe(CN)_6]$ $fcc$ structure. One-third of $[Fe(CN)_6]^{4+}$ units are missing from the perfect $fcc$ structure and it leads some of the Mn(II) centers to be available for coordination of water.\textsuperscript{156} The coordinated water molecules on Mn(II) ions turn on inner-sphere $T_1$ relaxation and result in $r_1$ relaxivity of 4.54 mM$^{-1}$s$^{-1}$ (Figure 4.10).

![Graph showing number against diameter (nm)](image)

**Figure 4.6.** Dynamic light scattering of the citrate-coated MnPB NPs

The purified and isolated MnPB NPs can be freely dispersed in water and the hydrodynamic diameter of such MnPB NPs dispersed in water was determined by the dynamic light scattering (DLS) and hydrodynamic diameter to be 99.5 nm (Figure 4.6).
The Fourier transform infrared (FT-IR) spectra of the bulk sample exhibited a strong sharp C≡N stretching vibration centered at 2060 cm\(^{-1}\) and MnPB nanoparticle sample showed relatively less intense peak at same wavenumber, which can be easily attributed to the Fe\(^{II}\)−C≡N−Mn\(^{II}\) bonding mode in the structure (Figure 4.7). The peaks at ~1563 cm\(^{-1}\) and 1387 cm\(^{-1}\) can be assigned to asymmetric carboxyl stretching band and symmetric carboxyl stretching band respectively. IR spectrum of trisodium citrate has lot of vibration peaks, which correspond to different vibration modes. Once it coordinated to metal centers, lesser number of peaks has been observed. The Δ[\(v_a\)(COO) - \(v_s\)(COO)]\(=176\) cm\(^{-1}\) is the indication of bidentate metal carboxylate coordination.\(^{157}\) Without trisodium citrate coating, we have noticed that the preparation of MnPB NPs was challenging as it agglomerate and separate from solution if the aqueous dispersion of NPs was left to stand at room temperature for 30 minutes. According to the HSAB concept, Mn(II) has been categorized in to the family of hard Lewis acid. As a result, trisodium citrate could complex Mn(II) and control the growth of nanoparticles. However, the protonated form of citric acid could not slow down the
nucleation of particles and to stabilize MnPB NPs. We have amply witnessed citric acid protected Mn(II) immediately yields white sludge without leading to the formation of nanoparticles. Citrate anchored on NPs could render extra electrostatic stabilization besides the steric stabilization of MnPB NPs while giving highly water dispersible colloidal solution. Such solid-state coordination polymer has always greater stability than the small molecular chelate counterparts owing to the large lattice energy of association reaction, and it guides the formation of MnPB NPs from Mn(II)-citrate complex.

Solubility product of bulk $K_3Mn_3[Fe(CN)_6]_2$ was evaluated as an indirect measurement of its stability against the release of Mn$^{2+}$ ions in solution. The $K_{sp}$ value was determined by the static method. Bulk phase of ca 800 mg was allowed to equilibrate with 1000 mL deionized water. The solutions were stirred at 25 °C for 48 hours. After 48 hours, volume of the solution (1000 mL) was reduced to a small amount and then heated to dryness. To disintegrate the compound, the crucibles containing the residue were kept at 630 °C for 6 hours. Next nitric acid was added and gently heated to dissolve the residue completely. The final volume of solution was setup to 5.00 mL and metal concentration was determined by AAS. The solubility product of $K_3Mn_3[Fe(CN)_6]_2$ was found to be $(3.5\pm0.2)\times10^{-38}$ mol$^7$dm$^{-21}$. Although many paramagnetic chelates of Mn(II) agents have been practiced as clinical MRI CAs, the main downside is related to the poor stability of complexes. Thus, one of the main requirements of small molecular chelates is its in vivo stability. There is amounting evidence to suggest that small molecular manganese complexes are unstable in vivo, which may hamper their use as contrast agents due to the release of free Mn(II) ions into the blood, because of the transmetallation with Zn(II). As a consequence, the elevated level of Mn(II) in the body can cause neurological disorder (Manganism), although the adverse toxicity caused by Mn(II) is usually less severe than that caused by Gd(III). The reciprocal of solubility product ($1/K_{sp} \sim 2.8\times10^{37}$) manifests its thermodynamic stability against the dissociation of $K_3Mn_3[Fe(CN)_6]_2$. Such a large stability of solid-state coordination compound could be attributed to the robust 3-D structural network and large lattice energy.
Figure 4.8. The TGA curve of citrate-coated MnPB NPs

Figure 4.9. The TGA curve of bulk MnPB sample
The thermal gravimetric analysis was conducted from 30° to 600° C under nitrogen or air flow using heating rate of 5 °C/min. Thermogravimetric analysis (Figure 4.8) of K₂Mn₃[Fe(CN)₆]₂ nanoparticles shows that the average citrate-coating is to be ca 21 wt % and TGA result of bulk sample (Figure 4.9) gives two-step 6.48% weight loss of water before heating to 150 °C. This indicates the presence of ca 2.6 zeolitic and coordinated water molecules per formula in accordance with the crystal structure as shown in Figure 4.5. The presence of approximately three water molecules in the structural cavities plays an important role in contributing to the inner-sphere longitudinal relaxation of the water protons.

![Figure 4.10. Plots of 1/Tᵢ (i=1,2) vs the Mn concentration at magnetic field strengths of 1.4T for citrate coated -MnPBNPs.](image)

The efficiency of CAs is evaluated by their relaxivity values and relaxivity measures the relaxation rate of protons per unit concentration of CAs in mM. To evaluate the efficacy of our NPs as MRI contrast agents, we performed a series of proton T₁ and T₂ relaxation measurements in order to determine their longitudinal and transverse relaxivity values, r₁ and r₂, at 1.4 T magnetic field strengths. The 1.4 T relaxometry results were obtained on a Bruker Minispec 60 MHz relaxometer. Experimentally, the change in relaxation rate with increasing concentration of CA was measured, and the numeric value of the relaxivity, r₁ or r₂, was then
extracted from the plot of 1/T₁ (or 1/T₂) vs the concentration of Mn(II) ions in NPs using the following equation.

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

where 1/T₁,₂ (i = 1, 2) is the diamagnetic contribution to the relaxation rate and [Mn] is the concentration of Mn(II) ions in NPs. The relaxation rate of pure water is taken as the diamagnetic contribution in all of the experiments.

All of the data are reported on a per Mn(II) ion basis and compared to commercial contrast agent, Teslascan, Gd-based MR contrast agents and several known nanoparticulate Mn(II) MR contrast agents (Table 7). As shown in Figure 4.10, the resulting values for these NPs are \( r_1 = 4.54 \text{ mM}^{-1}\text{s}^{-1} \) and \( r_2 = 18.33\text{mM}^{-1}\text{s}^{-1} \) at 1.4 T, respectively. However, the KMn[FeIII(CN)₆] nanoparticles prepared by Mn(II) ions and K₃[Fe(CN)₆] yielded a large value of \( r_2 \) relaxivity and thus a high \( r_2/r_1 \) ratio. Thus nanoparticles of KMn[FeIII(CN)₆] act as a good T₂ contrast agent. The observed large \( r_2 \) relaxivity is due to the magnetic coupling of low spin Fe(III) (S=1/2) in [Fe(CN)₆]³⁻ with Mn(II) (S=5/2). It causes fast transverse relaxation of protons in water and let KMn[FeIII(CN)₆] NPs be a good T₂ contrast agents. On the other hand, K₂Mn₃[FeII(CN)₆] nanoparticles exhibit small \( r_2 \) relaxivity and it gives a low \( r_2/r_1 \). Our K₂Mn₃[FeII(CN)₆] nanoparticles contain the [FeII(CN)₆]⁴⁻ anion with a total spin number zero. Therefore, Mn(II) centers in these NPs can be viewed as isolated paramagnetic centers rather than coupled with adjacent magnetic centers to give the desirable properties of T₁-weighted contrast agents.²¹
Table 7. Comparison of relaxivity data of several selected nanoparticulate based contrast agents.

<table>
<thead>
<tr>
<th>MRI contrast agent</th>
<th>Magnetic Field (T)</th>
<th>Diameter of core</th>
<th>$r_1$ (mM(^{-1}\mathrm{s}^{-1}))</th>
<th>$r_2$ (mM(^{-1}\mathrm{s}^{-1}))</th>
<th>$r_2/r_1$</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>K(_2)Mn(<em>3)[Fe(</em>{\text{III}})(CN)(_6)](_2).2.6H(_2)O NPs</td>
<td>1.4T</td>
<td>ca 21 nm</td>
<td>4.54</td>
<td>18.33</td>
<td>4.03</td>
<td>This work</td>
</tr>
<tr>
<td>Mn(DPDP) TESLASCAN®</td>
<td>1.4T</td>
<td>-</td>
<td>2.8</td>
<td>3.7</td>
<td>1.32</td>
<td>(^{146})</td>
</tr>
<tr>
<td>HMnO@mSiO(_2)</td>
<td>1.4 T</td>
<td>ca 15 nm</td>
<td>1.72</td>
<td>11.30</td>
<td>6.56</td>
<td>(^{37})</td>
</tr>
<tr>
<td>HMnO@mSiO(_2) (MnO@dSiO(_2))</td>
<td>11.7 T ((11.7 \text{ T}))</td>
<td>ca 15 nm ((\text{ca 15 nm}))</td>
<td>0.99 ((0.08))</td>
<td>11.02 ((2.27))</td>
<td>11.13 ((28.37))</td>
<td>(^{57})</td>
</tr>
<tr>
<td>Hollow MnO NPs</td>
<td>3T</td>
<td>ca 20 nm</td>
<td>1.42</td>
<td>7.74</td>
<td>5.45</td>
<td>(^{147})</td>
</tr>
<tr>
<td>MnO NPs</td>
<td>3T</td>
<td>ca 20 nm</td>
<td>0.21</td>
<td>1.49</td>
<td>7.1</td>
<td>(^{147})</td>
</tr>
<tr>
<td>Gd(DTPA)</td>
<td>1.4T</td>
<td>-</td>
<td>3.4</td>
<td>3.7</td>
<td>1.1</td>
<td>(^{132})</td>
</tr>
</tbody>
</table>

Table 7 summarizes the features of a few MRI contrast agents including Gd(III) complexes, high spin Mn(II) complexes and nanoparticles of manganese oxides. Our MnPB NPs have a larger $r_1$ value as well as a smaller $r_2/r_1$ ratio than those of the other CAs listed in Table 7. As previously mentioned, water accessibility to metal center is essential for the inner-sphere relaxation to be operative. Typically, nanoparticulate MRI contrasting agents have a high value of $r_2/r_1$ ratio because of unusually large $r_2$ value or small $r_1$ value. In MR imaging, the effect of two relaxations, $T_1$ and $T_2$ are complementary to one another in such a way that high $r_1$ relaxivity renders the brightening effect and high $r_2$ relaxivity renders darkening effect. As a result, the $r_2/r_1$ ratio that is close to one is more desirable to develop $T_1$-weighted contrasting agents. Our K\(_2\)Mn\(_3\)[Fe(CN)\(_6\)]\(_2\) has a good $r_1$ value and a reasonable $r_2/r_1$ ratio to be a good $T_1$ weighted contrast agent.
Figure 4.11. Mn leaching results of MnPB NPs under different conditions

For metal leaching studies, $K_2Mn_5[Fe(CN)_6]_2$ nanoparticle samples (~5 mL of 3mM) were placed in a dialysis bag (MWCO-3500) and soaked in solutions each of 50 mL containing 100 ppm Mg(II), 100 ppm Ca(II), 100 ppm Zn(II), 100 ppm HCO$_3^-$, and another solution at pH~1. The containers were tightly stopped and incubated at 37 °C for 48 hours. The leachates were analyzed for manganese by atomic absorption spectroscopy. We concluded that the MnPB NPs do not release Mn(II) ions to a toxic level (Figure 4.11). The highest Mn(II) concentration was found to be approximately 4 ppm or 0.07 mM, which is less than the minimal toxic level of 0.1 mM. The strong bonding between [Fe(CN)$_6$]$^{4-}$ and Mn(II) via cyanide ligand offers greater stability for our solid-state compound and particularly both Fe(II) and Mn(II) ions are completely secured in the lattice positions.
The release of free cyanide ions from nanoparticles into the solution was studied under different conditions (Figure 4.12). The cyanide leaching studies as shown in Figure 4.12 suggest that the leaching level of CN$^-$ ions is less than 0.3 ppm after 24 hours of incubation for test solutions. These levels of free cyanide ions are comparable to those found in drinking water or certain plants and fruit seeds. The maximum allowed contaminant level of CN$^-$ ions in safe drinking water is 0.2 ppm set by environmental protection agency (EPA). However, such small intake of cyanide by humans can be rapidly detoxified by the mitochondrial enzyme Rhodanese that converts cyanide into thiocyanate.$^{159}$
Figure 4.13. Cell viability curve of MnPB NPs

The cytotoxicity of the NPs in HeLa cells was evaluated using trypan blue exclusion test of cell viability assays with various concentrations of NPs for 24 hours incubation. As shown in Figure 4.13, more than 90% of the cells were still viable after 24 hours of incubation with 0.5 mM citrate coated MnPB NPs, revealing the biocompatibility of NPs. The low cytotoxicity of our NPs could be attributed to high structural integrity and proper surface coating.

To confirm the efficacy of MnPB NPs as T$_1$-weighted MR contrast agent, we acquired the T$_1$-weighted MRI phantom images of MnPB NPs in aqueous solutions. MR imaging of aqueous solutions containing NPs were imaged on a Bruker 9.4-T MRI scanner at 37 °C using a conventional gradient echo acquisition with an inversion recovery preparation. The T$_1$-weighted MR images were acquired using the 9.4 T scanner with a matrix size of 128×128, a field of view of 3.0cm$^2$×3.0 cm$^2$, a slice thickness of 0.5 mm, TE of 9.4 ms, and TR of 13.9~1500 ms. The MR images of aqueous solution containing various concentrations of MnPB NPs show that the brightness of T$_1$-weighted images rapidly increases with
increasing the concentration of NPs (Figure 4.14) and these results demonstrate that MnPB NPs have potentials to be used as a T<sub>1</sub>-weighted contrast agent. Also it is more like to be the next generation of Mn based T<sub>1</sub>-weighted contrast agents as an alternative to the extracellular Gd based T<sub>1</sub> weighted contrast agents.

![Figure 4.14](image)

**Figure 4.14.** T<sub>1</sub>-weighted MR phantom images of aqueous solution containing citrated-coated MnPB NPs using 9.4 T scanner.

Also we have prepared fluorescence dye labeled nanoparticles to study the cellular uptake ability of these nanoparticles. To conjugate the fluorescence dye molecules to the surfaces of NPs, we used a slightly modified synthesis procedure for preparing dye labeled citrate-coated MnPB NPs. Specifically, 100 mg of trisodium citrate and 1.0 mL of 1 mM ethylenediamine were added to an aqueous solution of MnCl<sub>2</sub> (1mM, 15 mL) under stirring, followed by the addition of a 10 mL K₄[Fe(CN)<sub>6</sub>] solution (1 mM). The resultant solution was stirred for ca. 3 hours before it was dialyzed and lyophilized. After pre-concentrating the NPs solution, for the dye coupling reaction, the 6-carboxyfluorescence dye (CbF, 0.012 g) was first reacted with N-[3-(dimethylamino)propyl]-N-ethylcarbodiimide hydrochloride (EDC,0.004 g) in a water/ethanol mixture (3.2 mM, 10 mL). The primary -NH₂ on K₂Mn₃[Fe(CN)₆]₂ (10 mL, ~3 mM) NPs was then reacted with ~1 mL of the EDC-coupled carboxyfluorescence dye (CbF) and stirred overnight. The reaction mixture was dialyzed against distilled water using a regenerated cellulose tubular membrane (MWCO of 12000) for 2 days to remove unreacted dye. Confocal scanning microscopy was used to visualize the cellular uptake of the fluorescence dye-labeled NPs in HeLa cells. The cells were first seeded in an 8-well chamber at a density of approximately 1.5 × 10<sup>5</sup> cells per well and incubated at 37 °C for 24 hours in a
complete medium without antibiotics. The cells were then incubated with the fluorescence dye-labeled NPs for 3 hours at the same temperature. After the cells were washed with the PBS buffer solution three times to remove free NPs, the cellular uptake of NPs was directly imaged on the living cells under the confocal microscope with the 488 nm excitation wavelength.

Figure 4.15. Confocal laser microscopic fluorescence images; HeLa cells incubated with dye-conjugated NPs for 3 hours (top left), the bright-field image of the cells (top right), the fluorescent image of untreated HeLa cells as the negative control (bottom left), and the bright-field image of the cells (bottom right).

We found that the CbF dye conjugated MnPB NPs have been internalized by HeLa cells as evidenced by the presence of green fluorescent signal in the cytoplasm of cells and the untreated HeLa cells were used as the negative control (Figure. 4.15). Such a significant increment of green fluorescence intensity in comparison to the untreated cells suggested that the internalization of NPs occur via endocytosis.
Chapter 5: Nanoparticulate based CT Contrast agents for X-ray Computed Tomography


X-ray computed tomography is capable of delineating the 3-D images of soft tissues with superb quality. The variation of X-ray attenuation from one tissue to another is used to generate the well spatial resolved superb quality images. As inherent contrast alone does not convey better delineation of soft tissue to make right decision, exogenous radiopaque agents are necessary for the superb visualization of different types of soft tissues. In the search for suitable media to enhance X-ray contrast for medical imaging of the human body, the development of metal based contrast agents predates the advent of the iodinated organics. Heavy metals with high atomic number are better suited for biomedical applications to enhance the image contrast due to their high mass attenuation coefficient. Thus several compounds with metallic elements with high atomic numbers, including silver, cesium, bismuth, thorium, and tungsten, to name but a few, were explored for potential contrast applications because of their high X-ray attenuation power.

The colloidal solution of ThO$_2$ formulation was once introduced into clinical use as an X-ray contrast agent (Thorotrast®). However, thorium has a naturally-occurring radioactive isotope $^{232}$Th that is an $\alpha$-emitter with an extremely long radioactive half-life. This problem, compounded with the unusually long biological half-life of the ThO$_2$ colloidal formulation ($t_{1/2}= ca\ 22\ yr$), would later cause liver cancer and leukemia in millions of patients who were injected with Thorotrast®. The unusually long biological half-life of this formulation is partially attributable to the relative large particle size of 10 nm. This can be
shortened if the particle size is reduced to < 8 nm so that renal clearance of the NPs can occur readily. On the other hand, Gold (Z-79) NP formulations have recently been investigated as potential CT contrasting agents due to its high mass attenuation coefficient and in vivo nontoxicity in order to fill the unmet clinical needs created by the banning of Thorotrast®. However, Au nanoparticle formulations have little or no chance to become the viable alternative as the clinical CT contrasting agent in replacement of Thorotrast® due to its scarcity and cost. Bismuth (Z- 83) is the nonradioactive, heaviest, nontoxic element available among the other closest neighbors (Hg, Tl, Pb and Po) of the periodic table. Recently, a polymer-coated Bi₂S₃ nanoparticle preparation was found to have high X-ray adsorption, long circulation time and excellent imaging efficacy. But, the in vivo hydrolytic stability of such foul-smelling formulations will remain as an unsolved problem. It should be noted that hydrolysis of Bi₂S₃ under the endogenous acid conditions may lead to the releases of hydrogen sulfide (H₂S), a gas that is known to be more toxic than hydrogen cyanide (HCN) to the neural and circulating system. We have set out to search for compounds that are hydrolytically stable, more efficient and more amenable in terms of biocompatibility. Moreover this new discovery can significantly reduce the average radiation dose in one CT scan.

Herein we describe an important technological breakthrough in the development of bismuth-based nanoparticulate and organ-specific CT contrast agents. Prior to this work, the field of heavy metal-based and organ-specific CT contrast agents was faced with two major problems: (1) finding a suitable bismuth compound that is chemically nontoxic and inert, synthetically feasible and cost-effective for this specific application; and (2) developing a scalable procedure to produce ultra-small nanoparticles of the selected bismuth compound to reduce the biological half-life of the agent inside the human body and to properly surface-engineer the NPs produced by our new method in order to impart the required water solubility (dispersability) and biocompatibility for the intended application. We have discovered a simple one-step aqueous solution route for preparing biocompatible and ultra-small bismuth oxyiodide BiOI nanoparticles and investigated their potential application as an efficient CT
contrast agent. Our ultra-small monodisperse BiOI NPs have excellent water dispersability, thermodynamic stability and kinetic inertness, high biocompatibility and superior attenuation power, suggesting their potential as an organ-specific CT contrast agent that may fill the gap left by the other nanoparticulate and iodine-based CT contrasting agents.

5.1 Ultra-small nanoparticles of BiOI as a potential contrast agent for computed tomography

Herein, we describe a simple and reproducible one-step aqueous solution route for preparing biocompatible and hydrolytically stable BiOI nanoparticles. Polyvinylpyrrolidone (PVP40K) was used as a surface coating agent to control the size and prevent the agglomeration of nanoparticles. PVP is a water-soluble and biocompatible polymer widely used in pharmaceuticals and cosmetics.\textsuperscript{172}

\[
2Bi^{3+} + 2H_2O(l) + I^{-}(aq) + PVP \xrightarrow{Decelerated hydrolysis} 2BiOI(s) + 4H^+(aq)
\]

To synthesize the PVP-coated BiOI nanoparticles, a solution of 1.0 mM Bi(NO\textsubscript{3})\textsubscript{3} (0.1940 g of bismuth nitrate pentahydrate in 400 mL at pH ~2.7) containing 2.0 g of PVP (average MW = 40000) was added slowly with a solution of 20 mM NaI (0.0600 g of sodium iodide in 20 mL) at room temperature to give a clear colorless to pale yellow solution. After stirring the mixture for 30 min, 10 mL aliquots of the solution were added with an equal volume of acetone and centrifuged at 10000 rpm for ~15 min, resulting a pellet of nanoparticles in each centrifuge tube. The nanoparticles were redispersed in ~10 mL of distilled water by sonication and separated again by the addition of an equal volume of acetone and centrifugation. The purification process was repeated twice. Bulk BiOI materials were prepared using solutions of Bi(NO\textsubscript{3})\textsubscript{3} and NaI in the absence of a coating agent. Specifically, an aqueous solution of 400 mL of Bi(NO\textsubscript{3})\textsubscript{3} (1.0 mM) was added to an aqueous solution of 20 mL of NaI (20 mM) while being vigorously stirred at room temperature. This
reaction resulted in a pale yellow precipitate in an hour. After the mixture had been stirred for
an additional 8 hours at room temperature, the product was filtered and washed with water
twice and with acetone twice. The product was then dried in air at room temperature for 24
hours.

\[ [\text{Bi}(\text{H}_2\text{O})_n]^{3+} (\text{aq}) + \text{H}_2\text{O}(l) \rightarrow [\text{Bi}(\text{H}_2\text{O})_{n-1}(\text{OH})]^{2+} (\text{aq}) + \text{H}^+ (\text{aq}) \]

**Scheme 14.** Hydrolysis reaction of Bi\(^{3+}\) in aqueous solution

Interestingly, a simple aqueous solution-based synthetic procedure we have
developed here yielded ultra-small monodisperse nanoparticles. The essence of our new
method is the decelerated hydrolysis of the hydrated Bi\(^{3+}\) ion under acidic conditions (i.e., pH
2.75) to produce the Bi\(^{O+}\) ion that in turn reacts with the I\(^-\) ion to form BiOI. In the presence
of a PVP in solution, such a hydrolysis reaction in an acidic solution is so slow that it
becomes the rate-limiting step for the nucleation of BiOI NPs, as well, thus leading to the
formation of ultra-small and monodispersed NPs.\(^{173}\)

**Figure 5.1.** Histogram of particle size (left), TEM image (top right) with HRTEM and SAED
insets, and Z-contrast STEM image (bottom right) of BiOI NPs.
Transmission electronic microscopy (TEM) studies revealed the ultra-small BiOI NPs with a narrow size distribution of $2.8 \pm 0.5$ nm as shown in Figure 5.1. The size distribution of BiOI NPs was determined by measuring and averaging the size of 105 NPs from the TEM images. The shape of these NPs is also nearly spherical. The microstructure of individual NPs was examined in detail by high-resolution TEM (HRTEM) and selected area electron diffraction (SAED) on randomly selected NPs. The typical HRTEM image along with the SAED patterns of an NP, given as insets in Figure 5.1, revealed the crystalline nature of the NPs with a $d$ spacing of 0.287 nm. This $d$ spacing value matches well with the layer repeating distance found in BiOI from the X-ray structure determination, i.e., 2.876 Å.

![Figure 5.1. HRTEM image and SAED pattern of BiOI NPs](image)

**Figure 5.1.** HRTEM image and SAED pattern of BiOI NPs

The energy dispersive X-ray spectroscopy (EDX) results revealed the presence of Bi, O and I (Figure 5.2) and confirmed the identity of the nanoparticles. However the hydrolysis of the hydrated Bi$^{3+}$ ions in acidic solutions often leads the formation of polynuclear...
Thus we prepared the bulk samples of BiOI via this hydrolysis reaction under the same conditions by omitting the use of the polymer coating agent for phase determination.

**Figure 5.3.** Rietveld refinement plot of BiOI; observed pattern (gray) and calculated patterns (red) the difference between observed and calculated shown at the bottom (blue) and the reflection positions shown as the vertical lines.

**Table 8.** Summary of structure determination for BiOI by X-ray powder diffraction

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<th>Value</th>
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<tr>
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<tr>
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</tbody>
</table>
The X-ray powder diffraction (XRD) patterns of the bulk samples revealed that the product obtained from the procedure of slow aqueous hydrolysis reaction was phase-pure and could be readily indexed in space group P4/nmm (No. 129), indicating that BiOI is isostructural with BiOCl. The final refinement yielded $a = 3.9940(1) \text{ Å}$, $c = 9.1549(1) \text{ Å}$, $V = 146.038(3) \text{ Å}^3$, and $\rho_{\text{calc}} = 8.002 \text{ g/cm}^3$ and converged at $R_{wp} = 3.6\%$ and $R_p = 2.7\%$. The structure of BiOI can be viewed as consisting of layers of BiOI perpendicular to the c direction as shown in Figure 5.4. Within each BiOI layer, there are five alternating I–Bi–O–Bi–I sublayers. The O layer is situated at the center of the double sandwich structure. Each O atom has tetrahedral geometry. The Bi layer is cushioned by an O layer from the bottom and capped by an I layer from the top. Each Bi atom is eight-coordinated and bound by four O atoms from a tetragonal base and four I atoms from a tetragonal cap to form a tetragonal antiprism. The fractional atomic coordinates and isotropic displacement parameters are listed in Table 9.

<table>
<thead>
<tr>
<th></th>
<th>$x$</th>
<th>$y$</th>
<th>$z$</th>
<th>$U_{iso}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bi1</td>
<td>0.25</td>
<td>0.25</td>
<td>0.13358 (3)</td>
<td>0.01169 (7)</td>
</tr>
<tr>
<td>O1</td>
<td>0.25</td>
<td>0.75</td>
<td>0.0</td>
<td>0.01169 (7)</td>
</tr>
<tr>
<td>I1</td>
<td>0.25</td>
<td>0.25</td>
<td>0.66586 (5)</td>
<td>0.01169 (7)</td>
</tr>
</tbody>
</table>
Figure 5.4. X-ray structure of BiOI viewed along the b axis showing the layers stacked in the direction perpendicular to the c axis (top), the view of a single layer (bottom left), and the coordination geometry of the Bi atom (bottom right).

Figure 5.5. X-ray powder diffraction patterns of the BiOI NPs.
Also the powder X-ray diffraction pattern of BiOI NPs shown in Figure 5.5 was consistent with characteristic Bragg’s reflection of crystalline BiOX. All the peaks observed were indexed to a pure tetragonal phase. The powder XRD pattern of BiOI nanoparticles shows the broaden peaks and some of the peaks have been merged. Specifically, the XRD peaks with the extension of hk0 of BiOI nanoparticles are little broadened in comparison to the other peaks. As a result, some of characteristic reflections have not been properly resolved.

![Figure 5.6. Particle size distribution of PVP-coated BiOI NPs in water dispersion.](image)

Our PVP-coated BiOI NPs are highly dispersible in water and stable against aggregation for more than six months. The dynamic light scattering (DLS) shows that the hydrodynamic diameter of such NPs dispersed in water to be ~16 nm. Thermal gravimetric analysis (TGA) was conducted on a PVP-coated BiOI and bulk BiOI sample using a TA instruments 2950 high-resolution thermogravimetric analyzer (Universal V3.9A) in air from room temperature to 600 °C with a heating rate of 10 °C/min (Figure 5.7). Thermal
gravimetric analysis (Figure 5.7) and FT-IR (Figure 5.8) confirm the presence of robust PVP coating and average surface loading of PVP to be 28 wt % respectively. It suggests that the robust polymer coating cannot be stripped off from the surface by prolonged dialysis against distilled water.

Figure 5.7. The TGA curve of PVP-coated BiOI NPs
Figure 5.8. The FT-IR spectrum of PVP-coated BiOI NPs.

The bulk BiOI solid is highly insoluble in water and hydrolytically stable at neutral pH. To quantitatively determine the concentrations of the dissociated [BiO\(^+\)] and [I\(^-\)] ions at equilibrium, the solubility product of the BiOI bulk phase was measured using the spectrophotometric method. A bulk BiOI sample was allowed to equilibrate with deionized water at 22 °C for 48 hours.

\[
\text{BiOI} (s) \rightarrow \text{BiO}^+ (aq) + I^- (aq)
\]

\[
K_{sp} = [\text{BiO}^+ (aq)][I^- (aq)]
\]

The equilibrium I\(^-\) can be oxidized by following reaction.

\[
2I^- (aq) + 2NO_2^- (aq) + 4H^+ (aq) \rightarrow I_2 (aq) + 2NO(g) + 2H_2O(l)
\]

The released I\(_2\) by oxidation reaction can be quantified by measuring the absorbance at 525 nm. Specifically, the I\(^-\) concentration at equilibrium were individually prepared and the 1.00 mL of that solution was added with 1.00 mL of KNO\(_2\) (0.1 M) and 1.00 mL of HNO\(_3\) (0.1 M) before the mixture was transferred into a cuvette. A solution containing the same amount of KNO\(_2\) and HNO\(_3\) was used as the reference blank. Absorbance at 525 nm was
measured for the produced iodine. Standard solution series (5.0, 10.0, 25.0, 50.0, 100.0, and 300.0 μM) of KI were prepared and mixed with KNO$_2$ and HNO$_3$ using the same procedure to obtain a calibration curve. The $K_{sp}$ value of BiOI was calculated to be $(3.34 \pm 0.9) \times 10^{-11}$ mol$^2$ dm$^{-6}$, based on the measured $\Gamma$ concentration by assuming the following equilibrium

$$\text{BiOI} (s) = \text{BiO}^+ (aq) + \Gamma (aq),$$

thus giving saturated concentrations of the BiO$^+$ and $\Gamma$ ions of $\sim 5.8 \times 10^{-6}$ M each by the spectrophotometric method.\textsuperscript{178} We also monitored the leaching of the BiO$^+$ ions from the PVP-coated BiOI NPs to aqueous solution by soaking $\sim 5$ mg of NPs sealed in a dialysis bag [MWCO- 3500] and periodically checking the solution concentrations of BiO$^+$ ions outside the membrane bag using atomic adsorption spectrometry for 1 week. In all the analyzed solution samples, the bismuth concentration fell below the detection limit of this analytical technique for bismuth of 1 ppm, indicating that BiOI NPs has sufficient thermodynamic stability and kinetic inertness to maintain its structural integrity for the intended application as a CT contrast agent.

To explore the possibility of developing the BiOI NPs as an intravenous CT contrast agent, we studied the \textit{in vitro} cytotoxicity of such NPs using the Trypan Blue exclusion method. The effect of PVP-coated BiOI NPs on cell viability was evaluated using a Trypan Blue exclusion viability assay. HeLa cells were seeded in a 96-well plate at a density of $1 \times 10^4$ cells/ well with the DMEM (Dulbecco’s modified Eagle’s medium) low glucose medium containing 10% FBS (fetal bovine serum) with penicillin/streptomycin and incubated for 5 hours at 37 °C in an atmosphere of 5% CO$_2$ and 95% air allowing cells to attach to the surface. HeLa cells in each well were then treated with 100 μL of fresh medium containing varying concentrations of the NPs and then incubated for 24 hours. Control wells contained the same medium without NPs. After the cells had been stained with Trypan Blue, viable and nonviable cells were counted using a hemocytometer.
Figure 5.9. Cell viability of HeLa cells after being incubated with PVP-coated BiOI NPs for 24 hours.

After 24 hours of incubation with the highest dispersible concentration, i.e., 4.15 mg/mL BiOI NPs, the cell viability was found to be ∼92 ± 0.8%, indicating that the PVP-coated BiOI NPs exhibit no significant cytotoxicity (Figure 5.9).

The ability of the NPs to cross cell membranes provides a possibility to develop such NPs as a cellular CT contrasting agent. Currently, CT is not considered as a cellular imaging modality, partly because of the lack of suitable contrast agents that are either cell-permeable or surface-modified with targeting agents that can selectively bind to certain receptors of the cell exterior. Therefore confocal laser scanning microscopy was used to investigate the cellular uptake studies. BiOI NPs were conjugated with the carboxyfluorescein dye. The typical synthesis procedure used to prepare PVP-coated BiOI NPs was slightly modified to covalently attach the fluorescence dye molecules onto surfaces of NPs, specifically, an aqueous Bi^{3+} (1 mM, 50 mL) solution containing PVP (average MW = 40000, ∼500 mg) and 1.0 mL of 1 mM ethylenediamine was slowly added to the aqueous solution of KI (1 mM, 50 mL) while being vigorously stirred. In the dye coupling reaction, the 6-carboxyfluorescenc
dye (CbF, 0.012 g) was first reacted with N-[3- (dimethylamino)propyl]-N-ethylcarbodiimide
hydrochloride (EDC, 0.004 g) in a water/ethanol mixture (3.2 mM, 10 mL). The primary -NH₂ on BiOI (10 mL, ~5 mM) NPs was then reacted with ~1 mL of the EDC-coupled carboxyfluorescence dye (CbF) and stirred overnight. The reaction mixture was dialyzed against distilled water using a regenerated cellulose tubular membrane (MWCO of 12000) for 2 days to remove unreacted dye. Hela cells were first seeded in an eight-well chamber at a density of approximately 1.5 × 10⁵ cells/well and incubated at 37 °C for 24 hours in DMEM medium. The culture medium was then replaced with a medium containing dye-labeled NPs (0.5 mM). The cells were then incubated with fluorescence dye-labeled NPs for 4 hours. After the cells had been washed three times with the PBS buffer solution to remove free NPs, fresh medium was added to the cells and the living cells without fixing were directly imaged under the confocal microscope with the 488 nm excitation wavelength.

Figure 5.10. Confocal microscopic fluorescence image (top left) and bright field image (top right) of HeLa cells incubated with dye conjugated BiOI NPs. Also shown are the fluorescence image (bottom left) and the bright field image (bottom right) of untreated HeLa cells as a negative control.
As shown in confocal microscopic images (Figure 5.10), HeLa cells treated with the dye-labeled NPs, as compared to the control cells, exhibited strong and uniform green fluorescent signals, showing an even and untargeted distribution of NPs in the cytoplasm. This finding is consistent with a cellular uptake mechanism of the NPs via endocytosis.

X-ray CT numbers of BiOI NPs for various concentrations were obtained by Gamma Medica Xspect instrument and the CT phantom imaging studies were conducted with the following parameters: 512 slices/360° rotation; 75 kVp, 110 μA; field of view, 39.47; resolution, 150 μm. The X-ray attenuation power of PVP-coated BiOI NPs was measured and reported as density values in Hounsfield units (HU). The Hounsfield is defined as

$$ HU = \frac{\mu_x - \mu_{\text{water}}}{\mu_{\text{water}}} \times 1000 $$

where $\mu_x$ and $\mu_{\text{water}}$ are the linear attenuation coefficients of the sample and water, respectively. By definition, the density value of water is assigned as zero. When the HU values of unknown materials are measured, the CT scanner is needed to be calibrated by measuring the density value of distilled water as the external reference. In this study, six different concentrations of NPs dispersed in aqueous media and a sample of distilled water were used in the measurement performed with a Gamma Medica Xspect microCT scanner. As shown in Figure 5.11, the CT values expressed in HU exhibit a linear relationship with the Bi(III) concentration of NPs. The slope of this linear curve indicates the X-ray attenuation efficiency of the contrast agent in Hounsfield units per millimolar.

In this regard, the X-ray attenuation efficiency of the current CT agent has a value of $\sim 20$ HU/mM. In comparison, the PEGylated gold NPs gave a value of 5.3 HU/ mM (i.e., 1.27 M PEG-coated Au NPs have a CT value of 6690 HU), while this value for PVP-coated Bi$_2$S$_3$ was reported to be 9.3 HU/mM in one article and 6.7 HU/mM in another. Similarly, the X-ray attenuation efficiency was measured and reported for a polymer-coated TaO$_x$ NP system to be 6.0 HU/ mM.
Figure 5.11. CT values and phantom images of the PVP-coated BiOI NPs as a function of Bi$^{3+}$ concentration in aqueous solution for (1) 5.07, (2) 12.7, (3) 25.4, (4) 30.4, (5) 38.1, and (6) 50.7 mM.

It is clear that our PVP-coated BiOI NPs appear to have the highest value on a molarity basis because of the combined X-ray attenuation effect of bismuth and iodine, as all the other reported inorganic NPs show a value of <20 HU/mM. It should be noted that the molar mass of BiOI, 351.88 g/mol is also the highest of those of the inorganic substances mentioned above, suggesting that when a given molar concentration of each contrast agent is to be injected into the body, the amount of mass required for BiOI NPs would turn out be the largest as well. However, even when the CT value is converted to the per milligram mass basis, the X-ray attenuation efficiency of BiOI NPs can still rival those of all of the inorganic nanoparticle based CT contrast agents mentioned above.
Chapter 6: Nanoparticulate for Therapeutic and Diagnostic Applications

The recent advances in nanomedicine have increased the interest in nanoparticulates for diagnostic and therapeutic applications. The nanoparticulate therapeutics have potential to enhance the efficacy of treatments for pathological conditions including neurological disorders, cancer, tumors, and Alzheimer’s disease. Nanoparticle-based therapeutics consist of sub micrometer-sized particles connected to therapeutic entities, such as small-molecular drugs, peptides, proteins or nucleic acids. In addition, molecular components such as lipids and polymers are assembled to form hybrid nanoparticles, nano-spheres, polymeric micelles, and vesicular systems like liposomes with the therapeutic entities. 43 For an example, micelle-based nanoparticles loaded with doxorubicin and paclitaxel were systematically studied for targeted delivery of drugs because of their ability to localize the activity at the sites of action, thereby lowering its concentration at other organs in the body. Particularly, such a selective targeting of an anticancer drug on an organ or a tissue minimizes the exposure of healthy tissues and it could significantly reduce the adverse side effects. 55 The accumulation of therapeutic agents at cancerous sites can be attributed to leaky fenestration of epithelial cells of blood vessels and it is known as enhanced permeable retention (EPR effect). The entities in sub micrometer sizes can enter into tumors at high concentration due to the EPR effect. For instance, superparamagnetic iron oxide nanoparticles (SPIO) have been investigated to treat the cancer with exquisite selectivity. 181 In treatment, SPIO NPs will be directed to cancer cells using a magnetic field and heated by being exposed to an alternative magnetic field. The heat produced, increases the tumor temperature up to 40-42 °C which result in the destruction of the tumor and the technique is called as hypothermia.

Nanoparticle-based therapeutics and diagnostics have several interesting features over the small molecular therapeutics entities. Nanoparticles can be tuned to have long or short circulation times by careful control of size and surface properties and directed to target organs with a large payload of therapeutic agents. Moreover, nanoparticles can easily accommodate multiple targeting ligands that can allow multivalent bindings to cell-surface receptors. More
importantly, nanoparticle-based therapeutics could have the potential to circumvent the multidrug resistance in which the drugs are effluxed by cell surface protein pumps. On contrary to selective transport of drugs via transmembrane proteins, the cellular uptake of nanoparticles occurs via endocytosis avoiding acquired multidrug resistance.\textsuperscript{43} However, the toxicity of nanoparticles associated with the accumulation in vital organs is a major concern. Such long term accumulation could be avoided by suitably tailoring the particle size and surface modification of nanoparticles with biocompatible polymers such as polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP).


Copper is an essential trace element that serves as a catalytic and structural cofactor for many proteins and enzymes in all living organisms.\textsuperscript{46,182} However, when it is unbound to proteins or enzymes, the free copper ions can exhibit deleterious effects due to its ability to trigger Fenton-like reactions that produce hydroxyl radicals to cause oxidative damage of proteins, lipids, and nucleic acids.\textsuperscript{82,183}

\begin{center}
\begin{tikzpicture}
\node at (0,0) {GSH/ Ascorbic acid};
\node at (1,1) {Cu(I)};
\node at (2,1) {Cu(II)};
\node at (2,-1) {H$_2$O$_2$ \rightarrow \dot{\text{OH}} + \dot{\text{OH}}};
\draw[->] (1,1) -- (2,1);
\draw[->] (1,1) -- (2,-1);
\end{tikzpicture}
\end{center}

\textbf{Scheme 15.} Copper catalyzed Fenton reaction.

Under normal conditions, copper is sequestered and tightly regulated at every stage of its uptake, transport, delivery and excretion in cells to achieve copper homeostasis in order to prevent oxidative stress.\textsuperscript{184} Deficiencies in maintaining copper homeostasis are linked to a number of human diseases or disorders including Menkes disease, Wilson’s diseases, familial amyotrophic lateral sclerosis, to name but a few.\textsuperscript{45,185} An imbalance of copper metabolism is
also implicated in Alzheimer’s disease, Parkinson’s disease and prion diseases. Despite the recent significant progress made in understanding the intracellular trafficking of copper, there is still a limited number of clinical drugs available in the form of chelation therapy to treat the diseases and disorders associated with copper overload. Such situation is particularly manifesting for Wilson’s disease (WD) that is also known as hepatolenticular degeneration. WD is a recessive genetic disorder characterized by excess copper accumulation in the liver and other vital organs.

In 1951, the British anti-Lewisite molecule (BAL) was introduced as the first clinical drug for WD. This chelating agent had been initially developed in World War II (WWII) as an antidote to the chemical warfare agent Lewisite and was later adopted for use in detoxifying heavy metal poisoning by arsenic, gold, antimony, lead or mercury (Scheme 1). Because of some serious side effects including nephrotoxicity and hypertension of BAL, D-Penicillamine (D-PEN; i.e. (2S)-2-amino-3-methyl-3-sulfanyl-3-butanoic acid; Scheme 1), a metabolite of penicillin was introduced in 1956 as a better clinical drug for WD. In 1982, triethylenetetramine (tiensine; Scheme 1), a less effective copper chelating agent than D-PEN, was introduced as another clinical drug for WD, mainly for the patients who showed intolerance to D-PEN. Currently, the clinical use of triethylenetetramine is limited in the USA because such application has not been approved for the European market. In 1997, the US Food and Drug Administration (FDA) approved the use of zinc acetate as a clinical drug for WD. Unlike other three clinical drugs for WD, this compound is not a chelating agent, but zinc ions from the drug can stimulate the production of metallothionein in gut cells, which in turn binds with copper ions to inhibit their absorption and transport to the liver. It has been shown that zinc acetate is only effective as a maintenance therapy for WD. Recently, tetrathiomolybdate (TTM; Scheme 1) was introduced as an investigational drug for WD. Research has shown that TTM forms a non-bioabsorbable form of ternary complexes with copper and food proteins in the gastrointestinal tract to block the intestinal absorption of copper from the diet, thus creating a negative copper balance in the body.

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Among all these drugs for WD, D-PEN has the highest efficacy, and hence is currently the most widely used drug for WD across the world. However, the side effects of D-PEN are numerous, and several of these are severe. They include bone marrow and immune suppression, skin rash, mouth ulcers, nausea, and deterioration of various neurological functions. The latter side effect is believed to be caused by the ability of D-PEN to mobilize copper ions that are stored in the body tissues and reroute them into circulation, thus increasing the concentrations of copper in the brain. It has been estimated that about half of the WD patients treated with D-PEN would show neurologic deterioration, and a quarter of such patients would suffer irreversible neurologic damage for use of D-PEN. All of these side effects are attributable to the fact that this drug is delivered systemically with no organ-specificity, hence causing a variety of side-effects due to the systemic toxicity of the drug. Furthermore, all the above-mentioned small molecule based drugs are unable to penetrate cells to target intracellular free copper ions for detoxification.

Scheme 16. Molecular structures of the clinical and investigational drugs based on copper chelation for WD.

We and some others have recently begun to develop cell membrane permeable chelating agents that have potential to become organ-specific with the use of suitable
targeting molecules as the next-generation of copper detoxifying drugs for treating WD and other copper-overload related diseases and disorders such as, fibrosis, inflammation and autoimmune diseases (e.g. rheumatoid arthritis). Such copper detoxifying drugs have also shown the potential to inhibit the angiogenesis in metastatic cancer; acting as antiangiogenic agent.\textsuperscript{194} We have developed a novel delivering system based on gold nanoparticles (Au NPs) for D-PEN that can remove excess free copper ions from the cell. Au NPs were chosen as the carrier for cellular delivery of D-PEN due to their unique properties including surface thiophilicity for covalent conjugation of the chelating agent, the high surface-area to volume ratio for carrying a high payload, and multivalent surface architecture for potential incorporation of multiple therapeutic agents and targeting molecules on the same surfaces.\textsuperscript{195,196} We have prepared the D-PEN-conjugated Au NPs of the average size of 16 ± 2 nm with superb water dispersability, and examined the kinetics and selectivity of copper binding of such NPs in aqueous solution. We also studied the cellular uptake, cytotoxicity and intracellular copper removal of these NPs to demonstrate their potential as a novel cell-penetrable copper detoxifying agent. To the best of our knowledge, this is the first attempt to show that D-PEN can be tailor-made as a new-generation biocompatible intracellular copper detoxifying drug.

The citrate-coated Au NPs were synthesized using the modified Turkevich method. In the typical synthesis, an aqueous solution of HAuCl\textsubscript{4} (0.25 mM, 100 mL) was first heated to boiling point under vigorous stirring, and a small amount of sodium citrate in distilled water (1\%, 5 mL) was added to the HAuCl\textsubscript{4} solution. This solution turned from pale yellow to wine red within a minute signifying the formation of AuNPs. The reaction mixture was boiled with continuous stirring for 30 min before it was cooled to room temperature. The as-synthesized citrated coated Au NP solution was placed in a dialysis bag made of regenerated cellulose tubular membrane (MWCO = 12000-14000) and dialyzed in distilled water for two days to remove the excess of sodium citrate. The Au@D-PEN NPs were prepared by a displacement reaction between the surface-anchored citrate and free D-penicillamine molecules. Specifically, the solution of purified citrate-coated Au NPs (0.25 mM, 100 mL) was allowed
to react with D-penicillamine (0.075 g) for 24 hours. During this time, small aliquots of NPs
dispersion were periodically taken out from the dialysis bag, diluted with equal amount of
acetone and centrifuged to separate the NPs. The resulting solution was dialyzed again in
distilled water for two days to remove unbound D-penicillamine. The solid product was
obtained by lyophilization.

**Figure 6.1.** TEM images of as-prepared Au NPs (upper left) and Au@D-PEN NPs (lower
left) and the histograms of the particle size distribution for NPs corresponding to each panel
on the right.

The transmission electron microscopic (TEM) studies as shown in Figure 6.1
revealed that both the citrate-coated Au NPs and the Au@D-PEN NPs are approximately
spherical in shape. However, a careful comparison of their size and particle size distribution
shows that there is a statistically significant change in the average size and particle size
distribution after the ligand displacement reaction. The average size of Au NPs, obtained by
measuring and averaging the size of 86 NPs, is 20 ± 4 nm, while the particle size distribution
is near Gaussian. The average size of the Au@D-PEN NPs, obtained by measuring and averaging 91 NPs, is 16 ± 2 nm, while the particle size distribution is asymmetric with a negatively skewed tail. Such changes are caused by a process known as the “digestive ripening”, first observed by Klabunde and co-workers in the Au NP-alkanethiol system.\textsuperscript{197,198}

![EDX spectrum of a typical Au@D-PEN nanoparticle.](image)

**Figure 6.2.** EDX spectrum of a typical Au@D-PEN nanoparticle.

The energy-dispersive X-ray spectroscopy (EDX) analysis of individual Au@D-PEN NPs showed the presence of S, C and N in addition to Au, confirming that D-PEN molecules exist as a shell on the Au core rather than being present as a separate phase in the material (Figure 6.2). Interestingly, we also observed a slight blue shift in the localized surface plasmon resonance (LSPR) of the Au@D-PEN NPs in comparison to the citrate-coated AuNPs, which is consistent with the decrease in the average particle size from Au NPs to Au@D-PEN NPs as shown in Figure 6.3.
Fourier transform infrared (FTIR) spectroscopy and near-UV CD spectrum of Au@D-PEN NPs were used to analyze the reaction product in order to ensure that the ligand displacement had been completed. As shown in Figure 6.4, the FTIR spectra indicate that the final product is D-PEN coated NPs and it is citrate free. By careful examinations of FTIR data, we noticed that the peaks around ~ 2586.4 cm\(^{-1}\), attributable to the stretching vibrations of the thiol group (\(\nu_{\text{S-H}}\)) found in the free D-PEN ligand, have completely disappeared in the Au@D-PEN NPs, indicating that D-PEN molecules are covalently bound to the Au surfaces via the S atom. Additionally, substantial shifts in the vibrational modes and the reduction in the number of peaks for both amine and carboxylate groups were found in the Au@D-PEN NPs as compared to those in the free D-PEN ligand, suggesting that both amine and carboxylate groups also interact with the Au surface (Figure 6.4). Similar changes of IR spectroscopic features were previously observed in the D-PEN molecules adsorbed on the bulk Au surface.\(^{196,199}\) The observed covalent bonding of D-PEN molecules to the Au surface is fully consistent with the well-established thiophilic nature of Au NPs’ surface, and also confirms the existence of the robust coating layers of D-PEN molecules on the Au NPs. It suggests that the prolonged dialysis against distilled water did not cause any loss of D-PEN molecules from the Au@D-PEN NPs.
Figure 6.4. Fourier transform infrared (FT-IR) spectra of Au@D-PEN NPs (left) and free D-PEN molecules (right).

Figure 6.5. Near-UV CD spectrum of Au@D-PEN NPs.

D-penicillamine is an optical active compound and could impart chirality to the Au NPs. As shown in Figure 6.5, the characteristic peak at ca 220 nm in near-UV CD spectrum of Au@PEN NPs confirmed the presence of optical active D-penicillamine on Au NPs surface. Thermogravimetric analysis (TGA) of Au@D-PEN NPs was conducted on a TA
instrument 2950 high-resolution thermogravimetric analyzer from 30° to 800° in air. The TGA analysis as shown in Figure 6.6 showed a total weight loss of 60%, which corresponds to the decomposition of D-PEN molecules attached to the Au NPs, when the sample was heated to 600 °C in air.

![Figure 6.6. Thermogravimetric analysis of Au@D-PEN NPs.](image)

We have studied the copper removal kinetics of Au@D-PEN NPs in aqueous solution and selectivity of copper binding on Au@D-PEN NPs based on the distribution of the metal ions between the solid and liquid phases. It was evaluated by monitoring the removal of Cu^{2+} ions from aqueous solution by Au@D-PEN NPs over a time period of 8 hours. Specifically, 10 mL of NPs (3 mM) solution was sealed in a dialysis bag (MWCO = 3,500), which was brought in contact with a CuCl₂ solution (25 mL) having the initial concentration at ~50 ppm level. The Cu^{2+} concentrations of the solution outside the dialysis bag were periodically analyzed by AAS. In parallel, selectivity studies were performed by soaking another dialysis bag containing 10 mL of NPs (3 mM) in a solution containing the magnesium (II), calcium (II), iron (II), manganese (II), zinc and copper (II) ions. The concentration of each metal ion in the solution was ~50 mg/L. After 24 hours, an aliquot of the solution was taken out and diluted with 2% HNO₃ acid and analyzed for each metal ion by AAS.
Figure 6.7. Copper removal kinetics of Au@D-PEN NPs from the aqueous solution and the inset shows the curve-fitting plot of a pseudo first order law.

The results indicate that the copper binding reaction follows a pseudo first order law up to the time point of 54 min with a rate constant $k_1 = 7.0 \times 10^{-5} \text{ s}^{-1}$ or the half-life of $t_{1/2} = 164 \text{ min}$ as shown in Figure 6.7 and Figure 6.8. These binding measurements suggest that our NPs are kinetically suitable for the removal of intracellular copper ions.

Figure 6.8. The kinetic data curve-fitting for time between 0 to 3200 seconds
It should be noted that the intracellular copper predominantly exists as Cu(I) because Cu(II) is reduced by various metallo reductases before transported into the cell by the copper transport protein (Ctr1).\(^{200}\) Since Cu(II) is invariably reduced to Cu(I) upon binding to D-PEN, we could use Cu(II) in place of Cu(I) in these and all of our subsequent studies involving copper in aqueous solution.

Figure 6.9. Selectivity studies of biologically essential divalent metal ions by Au@D-PEN NPs in aqueous solution.

To evaluate the selectivity of Au@D-PEN NPs towards Cu\(^{2+}\) ions in the presence of the other biologically essential divalent metal ions including Mg\(^{2+}\), Ca\(^{2+}\), Fe\(^{2+}\), Mn\(^{2+}\) and Zn\(^{2+}\), we investigated the competition of Au@D-PEN NPs for binding different ions in aqueous solution. The competitive binding studies were performed by soaking the dialysis bag with 10 mL Au@D-PEN NPs (3 mM) in a solution containing Cu\(^{2+}\), Mg\(^{2+}\), Ca\(^{2+}\), Fe\(^{2+}\), Zn\(^{2+}\) and Mn\(^{2+}\) ions each at the 50 ppm level. After 24 hours of incubation time, an aliquot of solution was taken out, diluted with 2% HNO\(_3\) acid and analyzed by AAS to determine the concentration for each metal ion. Figure 6.9 clearly shows that Au@D-PEN NPs are most selective toward the Cu\(^{2+}\) ion in the presence of all the other divalent metal ions tested. These results suggest that a proper therapeutic window should exist for selectively removing the Cu\(^{2+}\) ion while
maintaining the homeostasis of all the other biologically essential divalent metal ions when Au@D-PEN NPs are used as a drug for cellular copper detoxification.

For cellular copper detoxification by Au@D-PEN NPs, first the elevated copper level in HeLa cells was induced by incubating the cells with DMEM medium supplemented with 250μM of copper (II) solution for 12 hours. After such incubation, the cells remained healthy and thriving. The cells were washed three times with PBS and then incubated with the culture medium containing the NPs (50 μM) for 4 hours at 37 °C. After 4 hours of incubation, the cells were washed three times with PBS to remove the non-internalized NPs and further incubated with fresh culture medium for another 4, 6 and 8 hours. The cells grown in separate flasks were then trypsinized, centrifuged, re-suspended in PBS, and counted using a hemocytometer for 4, 6 and 8 hours respectively. The cells were then collected by centrifugation and lysed with 0.50 mL of concentrated nitric acid. The cell lysates were diluted to 5.00 mL with deionized water and filtered through a 200 μm filter. The intracellular copper concentrations were determined for the cell lysates as a function of time by AAS. The results were normalized by taking the copper concentration divided by the number of cells to obtain the intracellular copper content per cell.

Figure 6.10. Kinetics of intracellular copper removal from HeLa cells.
As can be seen from the results given in Figure 6.10, the cells treated with Au@D-PEN NPs showed a substantial decrease in the cellular copper level. Specifically, the intracellular concentration of copper dropped from the highly elevated level of 1040 ± 15 fg/cell to 516 ± 10 fg/per cell after the first 4 hours of incubation, and then to 285 ± 29 fg/cell after 6 hours of incubation in the NP-treated cells, indicating that intracellular copper was restored to a level close to the normal endogenous copper concentration of 263 ± 13 fg/cell in the control cells. In contrast, the cellular copper content of the control cells with the elevated copper level that were not treated with Au@D-PEN NPs showed a slight decrease to 870 ± 40 fg/cell after 8 hours of incubation with medium only, probably due to automatic effusion of copper ions from the cells. On the other hand, if D-PEN molecules alone were used to incubate the cells with the elevated copper level, the copper concentration would only decrease from 1040 ± 15 fg/cell to 753 ± 19 after 4 hours of incubation, and would remain at this level even after 8 hours of incubation. That latter observation is consistent with the notion that as a free ligand, D-PEN is not cell-permeable because it exists in the zwitterion form with an extremely high hydrophilicity. It should be noted that the cells remained alive and viable during the entire duration of the copper detoxifying studies (i.e. 8 hours). It is tempting to conjecture that our Au@D-PEN NPs show potential to be developed as an organ-specific drug if coupled with a suitable targeting agent. In contrast, the current systemic delivery of D-PEN with poor organ-specificity might be the cause of many undesirable side effects.

To assess the cytotoxicity, we performed cell viability assays in HeLa cells using the MTT method. HeLa cells were seeded in a 96-well plate at a density of 2 × 10^4 cells per well with the DMEM low-glucose medium and incubated for 24 hours at 37 °C in an atmosphere of 5% CO₂ and 95% air allowing cells to attach to the surface. Cells in each well were then treated with 100 μL of fresh medium containing varying concentrations of the nanoparticles and then incubated for 24 hours. Control wells contained the same medium without nanoparticles. After 24 hours of incubation, the cells were incubated with fresh DMEM media containing MTT reagent 10 μL, 1% (w/v) for 4 hours. After the MTT solution was removed, the precipitated violet crystals were dissolved in 100 μL of detergent. The absorbance was
measured at 560 and 630 nm using a microplate reader. The assay results were presented as the percentage of viable cells. As shown in Figure 6.11, the viability results clearly indicate that the NPs are nontoxic to cells. For example, more than 80% of the cells were viable even after incubation with the NPs at the concentration of 88 μM for 24 hours, and more than 89% of the cells were viable after incubation with the NPs at 52 μM for 24 hours (Figure 6.11).

![Viability Graph](image)

**Figure 6.11.** Effect of Au@D-PEN NPs on viability of HeLa cells after 24 hours of incubation.

We studied cellular uptake of Au@D-PEN NPs in HeLa cells using the fluorescent confocal microscopic imaging technique. As Au@D-PEN NPs themselves are non-fluorescent, 5-carboxyfluorescein dye (CbF) was covalently anchored onto the Au@D-PEN NPs surfaces.
Figure 6.12. Confocal microscopic images of HeLa cells: (upper left) bright-field image of cells incubated with dye-conjugated NPs for 4 hours; (upper middle) fluorescence image of cells incubated with Hoechst dye (nucleus stain); (upper right) fluorescence image of cells incubated with dye-conjugated NPs for 4 hours; (lower left) bright field image of the untreated cells as the control; (lower middle) fluorescence image of untreated cells incubated with Hoechst dye; and (lower right) fluorescence image of untreated cells

To prepare dye-conjugated Au@D-PEN NPs, 500 μL of 0.25 mM ethylenediamine solution was added to a 10.0 mL of Au@D-PEN NP solution (~ 250 μM) under vigorous stirring. The resulting mixture was continuously stirred for 12 hours. The product was purified by dialysis to remove unreacted ethylene diamine molecules. Next, 10 mL of nanoparticle solution obtained from previous step was reacted with carboxyfluorescene dye (3 mg) in the presence of the coupling agent 1-ethyl-3-(3- dimethylaminopropyl)-carbo diimide (EDC; 1.8 mg) for 24 hours. To remove the unconjugated dye molecules, the resulting product was dialyzed against distilled water for two days and analyzed by fluorescence spectroscopy to confirm that the dye molecules are covalently bound to Au NPs. An 8-well plate was seeded with HeLa cells at a density of approximately 2×10^4 cells per well and incubated for 24 hours allowing the cells attachment to the surface. The cells were then
exposed to fluorescence dye-conjugated Au@D-PEN NPs for 4 hours of incubation and washed thoroughly several times with PBS buffer. Hoechst nuclear staining dye was then introduced to stain the nuclei before the living cells were imaged under a confocal microscope. The living cells were imaged under a laser scanning confocal microscope without fixation. Figure 6.12 shows the representative confocal fluorescent images of HeLa cells treated with the dye-conjugated Au@D-PEN NPs and the control cells. As can be seen in the confocal images, strong and uniform green fluorescence signals attributable to the surface-conjugated CbF dye are present in the perinuclear region but not in the nucleus of the cell, indicating an untargeted cytoplasmic distribution of NPs. Furthermore, no specific binding was found to any small organelle in the region, which is consistent with cellular uptake via endocytosis. These results suggest that Au@D-PEN NPs possess the ability to penetrate the cell membrane, which provides an important prerequisite for developing them as a potential cellular copper detoxifying agent.

6.2 Selective Ion-Exchange Governed by the Irving-Williams Series in $\text{K}_2\text{Zn}_3[\text{Fe(CN)}_6]_2$ Nanoparticles: Toward a Designer Prodrug for Wilson’s Disease

In the case of Wilson’s disease (WD), accumulation of excess copper in the liver and other vital organs disrupt their function due to the excessive production of ROS, which renders the patient severe disability with a variety of hepatic, neurological, ophthalmic and psychiatric symptoms. If untreated, these conditions can rapidly progress to liver cirrhosis, and then a need for liver transplantation or death.\textsuperscript{46} WD is a recessive genetic disorder

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characterized by a mutation in the ATP7B gene. Such mutation in turn causes abnormal copper metabolism resulting in a blockage in the secretion of copper from the body. Before chelation therapy using D-penicillamine (D-PEN) was introduced in 1956, WD had been always progressive and fatal. D-PEN or (2S)-2-amino-3-methyl-3-sulfanyl-butanoic acid is an α-amino acid cysteine-like chelating agent originally discovered as a metabolite of penicillin. It contains O, N and S donors, and exhibits a preferential binding, but not sufficient selectivity toward either Cu(I) or Cu(II) ion. Furthermore, D-PEN is an oral drug with systemic toxicity but no organ-specificity. Therefore, the use of D-PEN causes severe side effects as previously discussed in 6.1. Among the all numerous side effects, the most detrimental and deplorable one is the irreversible neurologic damage caused by the mobilization of copper ions stored in the body tissues and rerouting them into blood circulation by D-PEN. Consequently, two new oral drugs, i.e. triethylenetetramine (Trientine®) and zinc acetate (Galzin®), were later introduced to clinical treatment of WD. As compared to D-PEN, Trientine® is a less effective copper chelator, but proven to be beneficial to the patients who show intolerance to D-PEN. Although zinc in Galzin® is not a chelating agent, it acts as an antagonist to copper by stimulating the production of metallothionein in cells. Metallothionein is a low molecular weight cysteine-rich protein capable of binding to various metal ions including copper ions to inhibit their absorption and transport to the liver. However, Galzin® has been shown to be only effective as a maintenance drug for WD. An investigational drug, tetrathiomolybdate (TTM) has recently emerged as a potential alternative oral drug for WD. TTM forms insoluble ternary complexes with copper and food proteins in the gastrointestinal tract, which reduces the bioavailability of copper for intestinal absorption of this metal from diet, and thus creates a negative copper balance in the body. Despite of its many adverse side effects, D-PEN remains as a treatment for WD because of its proven efficacy. It seems clear that there is an unmet clinical need for a novel WD drug with improved organ-specificity and reduced systemic toxicity. Our approach of tackling these problems focuses on the development of cell-permeable copper-depleting
nanoparticles that can be surface-engineered to be potentially organ-specific when targeting agents are used to form new-generation drugs for WD.\textsuperscript{19,192}

Here we describe the synthesis, characterization and intracellular copper detoxification of the NPs formed from a zinc analogue of Prussian blue ( K\textsubscript{2}Zn\textsubscript{3}[Fe(CN)\textsubscript{6}]\textsubscript{2}·ZnPB).\textsuperscript{201} Prussian blue and its many analogues have recently gained a huge attention due to their potential applications in MRI contrast enhancement, bio-molecular sensing and cancer treatment. We have found that ZnPB NPs are highly water-dispersible, biocompatible and capable of penetrating cells and selectively remove the intracellular copper. As a novel strategy for ensuring exclusive selectivity of these NPs toward the copper, we have explored a pre-determined ion-exchange reaction rather than relying on the normal metal-chelation process. Specifically, we noticed that the relative stability exhibited by homologous divalent 3d metal complexes would follow the trend Cr\textsuperscript{2+}<Mn\textsuperscript{2+}<Fe\textsuperscript{2+}<Co\textsuperscript{2+}<Ni\textsuperscript{2+}<Cu\textsuperscript{2+}>Zn\textsuperscript{2+}, regardless of the nature of ligand. The properties governing the stability of these homologous complexes are atomic radii and ligand field stabilization energy (LFSE). Across the series, decreasing atomic radius results in shorter, stronger metal-ligand bonds. This trend approximately coincides with larger LFSE across the series to give rise to more stable complexes.\textsuperscript{50} This empirical law, known as the Irving-Williams series suggests that the ZnPB NPs should undergo ion-exchange exclusively with Cu\textsuperscript{2+} ions in the presence of the other endogenous divalent metal ions including Mn\textsuperscript{2+}, Fe\textsuperscript{2+}, Mg\textsuperscript{2+} and Ca\textsuperscript{2+} ions. To the best of our knowledge, this is a novel discovery that the zinc analogue of Prussian blue NPs can be employed to design the intracellular copper detoxifying prodrug.
We prepared polyvinylpyrrolidone (PVP)-coated ZnPb NPs by slowly adding 75 mL of 1.0 mM ZnSO$_4$ solution containing 0.80 g of PVP (average MW = 40,000) into a 50 mL of 1.0 mM K$_4$[Fe(CN)$_6$] solution at room temperature to afford a white to pale yellow solution. After stirring for 30 minutes, the solution was transferred into a dialysis bag made of regenerated cellulose tubular membrane (MWCO = 3,500) and dialyzed against distilled water for two days. The solid product was collected by lyophilization. As the control, we also prepared the bulk materials of this compound by mixing the two above solutions in the absence of the surface-coating polymer, PVP. This reaction resulted in a white precipitate in about an hour of stirring at room temperature. The transmission electron microscopy (TEM) imaging revealed that the NPs have nearly spherical shape with an average diameter of 44 ± 8 nm as determined by counting and averaging the size of 51 particles in this TEM picture frame (Figure 6.13).
The energy dispersive X-ray spectroscopic (EDX) measurements showed the characteristic signals of Zn, Fe and K from several individual NPs randomly selected from the TEM grid as shown in Figure 6.14, while the quantitative elemental analysis of the ZnPB NPs carried out by the atomic absorption (AAS) method gave the molar ratio of K:Zn:Fe to be 0.059/0.074/0.055, indicating that the composition of the NPs matches the expected formula $K_2Zn_3[Fe^{II}(CN)_6]_2$. The C, H and N elemental analysis showed C% = 17.67%, N% = 21.41% and H% = 0.98%, suggesting an empirical formula of $K_2Zn_3[Fe^{II}(CN)_6]_2 \cdot nH_2O$.

ZnPB NPs and bulk prepared in the same manner as described above were purified and isolated for powder X-ray diffraction analysis. The X-ray powder diffraction (XRD) studies in Figure 6.15 showed that the powder patterns of the bulk materials and nanoparticles match with those calculated from the previously reported X-ray crystallographic data for $K_2Zn_3[Fe^{II}(CN)_6]_2$, confirming the phase identity and purity of both materials prepared in this work. The previous X-ray structure determination showed that this compound crystallizes in the rhombohedral space group ($R\overline{3}c$) with a three dimensional framework structure consisting of octahedral $[Fe(CN)_6]^{4-}$ units linked to tetrahedral $Zn^{2+}$ ions to create large
hexagonal bipyramidal cages encapsulating K$^+$ ions and water molecules, as shown in Figure 6.16.

**Figure 6.15.** Powder XRD patterns for the bulk K$_2$Zn$_3$[Fe(CN)$_6$]$_2$ (left), and the PVP-coated K$_2$Zn$_3$[Fe(CN)$_6$]$_2$ nanoparticles (right).

**Figure 6.16.** Structure of a zinc-ferrocyanide frameworks of compounds of the type K$_2$Zn$_3$[Fe(CN)$_6$]$_2$.xH$_2$O, yellow tetrahedral ZnN$_4$ and green FeC$_6$ octahedral.
As can be seen Figure 6.16, large cavities defined by the Zn₃[Fe(CN)₆]₂⁻ framework are significantly more accessible than those present in Prussian blue and consequently, this compound has desired properties for intended biomedical applications.

Thermal gravimetric analysis (TGA) was conducted on a PVP coated ZnPB bulk sample using a TA instruments 2950 high-resolution thermogravimetric analyzer in air from room temperature to 800 °C with a heating rate of 10 °C/min (Figure 6.17). The results from thermal gravimetric analysis (TGA) on the bulk sample showed a 13.26% weight loss of water before reaching to 150 °C, indicating the presence of ca 5.9 zeolitic water molecules per formula (Figure 6.17). On contrary to varying content of zeolitic water (3.7 - 4.0) found in SPB, our ZnPB could accommodate ca 5.9 zeolitic water molecules. Herein the presence of high content of zeolitic water in ZnPB is due to the large hexagonal bipyramidal cavities in the polymeric structure. Further, thermal gravimetric analysis of ZnPB NPs showed the average surface loading of PVP to be 40 wt % (see Figure 6.17).

**Figure 6.17.** The TGA curves of the bulk K₂Zn₃[Fe(CN)₆]₂ sample (right), and the PVP-coated K₂Zn₃[Fe(CN)₆]₂ NPs (left).
The Fourier transform infrared (FTIR) spectra of the PVP-coated ZnPB NPs exhibit a characteristic C≡N stretching vibration at 2094 cm\(^{-1}\), the peak which is also present in the bulk sample, in addition to the other characteristic stretching and bending vibrations of PVP (Figure 6.18).

![IR spectra](image)

**Figure 6.18.** The IR spectra of the bulk K\(_2\)Zn\(_3\)[Fe(CN)\(_6\)]\(_2\) sample and the PVP-coated K\(_2\)Zn\(_3\)[Fe(CN)\(_6\)]\(_2\) NPs in comparison to that of a PVP sample.

The kinetics of the ion-exchange between K\(_2\)Zn\(_3\)[Fe(CN)\(_6\)]\(_2\) NPs and Cu(II) ions were investigated to evaluate the suitability of nanoparticles for copper detoxification. The kinetic studies of copper removal were done by using a solution containing 100 ppm of CuCl\(_2\) (50 mL). A water dispersion of ZnPB NPs (6.7 mM and 10 mL) was sealed in a dialysis bag (MWCO-3,500) which was brought in contact with the above copper solution. The copper and zinc concentrations of the solution outside the dialysis bag were periodically analyzed by AAS. The kinetics of copper removal were followed by measuring the concentration changes of both Cu\(^{2+}\) and Zn\(^{2+}\) ions in outside aqueous solution, in which the dialysis bag containing the PVP-coated ZnPB NPs was immersed. The simultaneous decrease in copper concentration coincided with the increase of zinc concentration in solution as determined quantitatively by AAS, signifying an ion exchange reaction between the ZnPB NPs and Cu\(^{2+}\) ions to give NPs
with a composition: $K_2Cu_{x}Zn_{3-x}[Fe^{II}(CN)_{6}]_2$ determined by AAS. The $x$ value ranges from 0.07 to 0.11 depending on the extent of ion-exchange (Figure 6.19 and Figure 6.20).

**Figure 6.19.** Kinetics of ion-exchange between ZnPB NPs and the Cu(II) ion in aqueous solution.

**Figure 6.20.** The TEM image (left) and histogram (right) of the Cu(II) ion-exchanged ZnPB NPs ($K_2Cu_{x}Zn_{3-x}[Fe^{II}(CN)_{6}]_2$).
The kinetics data of this ion-exchange reaction can be fitted into two separate rate laws, i.e. a pseudo first-order reaction up to the time point of ~7 hours with a rate constant of $k_1 = 5.7 \times 10^{-5} \text{ s}^{-1}$ and a half-life of $t_{1/2} = 202 \text{ min}$, and a second-order reaction with a rate constant of $k_2 = 4.02 \times 10^{-2} \text{ M}^{-1} \text{s}^{-1}$ (Figure 6.21).

**Figure 6.21.** (a) The kinetics data fitting curve to a pseudo first-order reaction for the ion-exchange (b) the kinetic data fitting curve to a second-order reaction for the ion-exchange between $K_2Zn_3[Fe(CN)_{6}]_2$ NPs and $Cu^{2+}$ ions in aqueous solution.

The dependence of the reaction rate on concentration of NPs in the second part may be attributable to the fact that now Cu$^{2+}$ ions needed to penetrate into the inner layers of the ZnPb NPs to react with Zn$^{2+}$ ions after the surface Zn$^{2+}$ ions had been replaced. Indeed, when we examined the NPs isolated at this point and studied by TEM, EDX and metal elemental analysis, we found that the NPs now consisted of a solid solution $K_2Cu_xZn_{3-x}[Fe^{II}(CN)_{6}]_2$ as shown in Figure 6.20.

It should be noted that $+1$ is the predominant oxidation state for intracellular copper ions. Thus we performed another the ion exchange studies between the Cu$^+$ ion and ZnPb NPs in aqueous solution. Initially, a 0.3 mM Cu$^+$ solution was prepared by dissolving CuCl in a small amount of concentrated HCl. A proper amount of $K_2Zn_3[Fe(CN)_{6}]_2$ NPs was added to
this solution to reach the dispersion level of \( \sim 0.6 \text{ mM} \). The solution was quickly sonicated and transferred to a quartz cuvette. The ion-exchange was monitored using a UV-vis spectrophotometer over a period of 4 hours. This reaction showed the identical spectroscopic feature as the ion-exchange reaction occurred between \( K_2Zn_3[Fe(CN)_6]_2 \) NPs and the Cu(II) ion in the presence of air, and found that such ion-exchange would still take place with the concomitant oxidation of the Cu\(^+\) into Cu\(^{2+}\) ion in the product (Figure 6.22). Such observations are consistent with the notion that the N-donor in the CN\(^-\) ligand is not soft enough to prefer the +1 over +2 oxidation state for this metal once it is incorporated into the solid structure.

![UV-vis spectroscopic scanning results for ion-exchange between ZnPB NPs and the Cu(I) ion (left panel) and kinetics of Cu(I) removal by ZnPB NPs (right panel).](image)

**Figure 6.22.** UV-vis spectroscopic scanning results for ion-exchange between ZnPB NPs and the Cu(I) ion (left panel) and kinetics of Cu(I) removal by ZnPB NPs (right panel).

To quantitatively evaluate the selectivity of the Cu(II) removal by ZnPB NPs in the presence of other biologically relevant divalent metal ions including Mg\(^{2+}\), Cu\(^{2+}\), Fe\(^{3+}\) and Mn\(^{2+}\), we studied the ion-exchange competition among all these ions. Selectivity studies were performed by soaking the dialysis bag containing 5 mL of nanoparticles (10 mM) in a solution of Cu(II), Mn(II), Fe(II), Mg(II) and calcium(II). The concentration of each metal in
the competitive solution was ~100 mg/L. After 24 hours, an aliquot of the solution was taken out and diluted with 2% HNO₃ acid and analyzed for each metal ion using AAS.

![Figure 6.23](image)

**Figure 6.23.** Selectivity of metal ion removal by ZnPB NPs from aqueous solution.

The results were normalized against the percent removal of the Cu²⁺ ion from the solution. In a separate batch reaction, the ZnPB NPs were brought in contact with Zn²⁺ ions at the same concentration for 24 hours, and showed no ion exchange. As predicted by the Irving Williams series, the ZnPB NPs showed a high selectivity toward Cu²⁺ ions over all the divalent ions used in the selectivity study with the Ca²⁺ ion being an exception (Figure 6.23). We found that the Ca²⁺ ions in the solution were undergoing ion exchange with the K⁺ ions in the crystal lattice.
Figure 6.24. Changes of the Ca$^{2+}$ and K$^{+}$ concentrations of the aqueous solutions indicating the ion-exchange reaction between the K$^{+}$ ion in the NPs and the Ca$^{2+}$ ion from the aqueous solution.

To confirm the ion-exchange between the K$^{+}$ ion in NPs and the Ca$^{2+}$ ion in aqueous solution, we set up a separate experiment. A water dispersion of as-synthesized ZnPB NPs (5 mM and 2 mL) was sealed in a dialysis bag (MWCO-3,500) which was immersed in an aqueous solution containing about 100 ppm of CaCl$_2$. After stirring for 4 hours, the concentrations of both calcium and potassium of the outside solution were analyzed by AAS. The results in Figure 6.24 showed that there is an ion-exchange reaction between the K$^{+}$ ion in the NPs and the Ca$^{2+}$ ion from the aqueous solution with a molar ratio of 2:1 for K to Ca. In a separate experiment, a similar amount of ZnPB NPs was soaked overnight in an aqueous solution containing ~0.1 M Ca(II) ion. After centrifugation and washing with distilled water for 3 times, the Ca(II) exchanged NPs were sealed in a dialysis bag and submerged in another aqueous solution containing about 100 ppm of CaCl$_2$ for 18 hours. The elemental analysis of the solution for both calcium and potassium showed that there is essentially no change in their
concentrations due to the ion-exchange. Therefore we concluded that such unwanted ion-exchange can be suppressed by pre-treating our ZnPB NPs with CaCl$_2$ solution (Figure 6.25).

![Figure 6.25. Ca(II) removal kinetics of ZnPB NPs pretreated with Ca(II).](image)

As an indirect measurement of stability, the solubility products $K_{sp}$ for above compounds were determined by the static method. Approximately 800 mg of each compound was sealed in dialysis bag and allowed to equilibrate with 1000 mL of deionized water. The solutions were stirred at 25 °C for 48 hours and then the volume of each solution (1000 mL) was reduced to small amount and then transferred into porcelain crucible. The crucibles containing the solutions were further heated to dryness and kept at 630 °C for 6 hours. The residue was dissolved in nitric acid and finally metal concentration was determined by AAS for each of solution.

$$K_{sp} = [K^+]^2[M^{2+}]^3[Fe(CN)_6]^{4-} \quad (M=Zn \text{ or Cu})$$

The solubility product constants of $K_{Zn}Zn_3[Fe(CN)_6]_2$ and $K_{Cu}Cu_3[Fe(CN)_6]_2$, as measured by a static method, are $1.1 \pm 0.2 \times 10^{-38}$ mol$^7$/dm$^{21}$ and $3.8 \pm 0.1 \times 10^{-46}$ mol$^7$/dm$^{21}$.
respectively, indicating that such ion-exchange reaction is driven by the difference in the thermodynamic stability of the two analogous compounds.

To determine the stability constant of Cu(II) with D-penicillamine, we performed series of potentiometric titrations and pH and volume measurements were taken to the accuracy of 0.001 units of pH and 0.005 mL of alkali volume respectively. Data of potentiometric titrations (pH vs volume of standardized KOH) was analyzed by Irving Rossetti methods (See chapter 2.5.1).

For potentiometric titrations, following sets of solution were prepared and the compositions of solution sets are as follows, set 1 - HClO₄ acid (0.1173 moldm⁻³, 2.000 mL), KClO₄ (4.00 mL, 0.50 moldm⁻³) and deionized water 34.00 mL: set 2 - 2.000mL of HClO₄ (0.1173 moldm⁻³), 25.00 D-PEN (5.0157 mM), 9.00 mL deionized water and 4.00 mL KClO₄ (0.05 moldm⁻³): set 3 - 2.000 mL HClO₄ (0.1173 moldm⁻³), 25.00 D-PEN (5.0157 mM), 8.50 mL deionized water and 4.00 mL KClO₄ (0.05 moldm⁻³) and 0.50mL of M²⁺ solution whose concentration was predetermined by AAS respectively. Each solution was kept at room temperature to reach thermal equilibrium (25 °C ± 0.5°C). Each set was titrated with freshly prepared and standardized KOH solution (Figure 6.26). The ionic strength of the each solution was maintained at constant by using 0.05 M KClO₄ and employing relatively low concentration of ligand and the metal ions being investigated. Proton ligand stability constants and metal ligand stability constants were calculated directly from the formation curves of proton- ligand and M²⁺ ion-ligand association (Figure 6.27), derived from the titration data shown in Figure 6.26.
Figure 6.26. Titration curves for each set prepared, (set1- green curve, set 2- magenta curve and set 3-brown curve (containing Cu$^{2+}$ 1.919×10$^{-4}$ moldm$^{-3}$).

Figure 6.27. (a) Formation curve for proton-ligand association of D-PEN, (b) Formation curve for metal ion–ligand association (Cu$^{2+}$ and D-PEN).

Acid dissociation constants of D-PEN were calculated to be pK$_{a1}$-2.10, pK$_{a2}$-8.14, pK$_{a3}$-10.82 by using Figure 6.27a, when $n_H$ is equal to 2.5, 1.5 and 0.5.
As can be seen in Figure 6.28a, predominant form of the D-PEN exists as zwitterionic form in physiological pH range of 6.0 to 7.40 including human plasma pH and intestinal pH. Further, overall stability constant $\beta_2$ of Cu(D-Penicillamine)$_2$ was found to be $\log\beta_2$ 18.77. Such large stability constant of Cu(D-Penicillamine)$_2$ is highly desirable to sequester the copper and it has been the reason for D-penicillamine to remain in clinical use. Interestingly, these thermodynamic stability constants suggests that our novel ZnPBNPs have more affinity towards Cu(II) than that of D-penicillamine.

Figure 6.29. Competition for copper by ZnPBNPs over D-PEN.

Therefore, we performed separate competitive studies with Cu(II) and Cu-(D-PEN) complex. Herein, ZnPBNPs were separately treated with an aqueous solution of Cu(II) 100-
ppm and with another aqueous solution containing D-PEN complexes of copper (pH = 5.6) at same concentration. The results demonstrated that the ZnPB NPs can effectively compete against D-PEN for copper ions in aqueous solution (Figure 6.29). CuPB solid-state compound has greater thermodynamic stability than Cu(D-penicillamine)$_2$. As a result, ZnPB NPs could compete even for copper in Cu(D-penicillamine)$_2$ to form CuPB NPs and the Figure 6.29 confirms the anticipated behavior of ZnPB NPs.

**Figure 6.30.** Fluorescence spectrum of the dye-labeled ZnPB NPs.

To prepare dye-labeled ZnPB nanoparticles, an aqueous Zn$^{2+}$ solution (1 mM, 75 mL) containing PVP (average MW=40000, ~ 800 mg) and ~2.0 mL of 0.5mM of ethylenediamine was slowly added to an aqueous K$_4$[Fe(CN)$_6$] (1 mM, 50 mL) solution under vigorous stirring. The resulting solution was further stirred at room temperature for ca. 2 hours. These NPs have terminal $\text{–NH}_2$ groups. After dialyzed for 24 hours, the NPs were concentrated to ~2.5 mM. Then EDC (i.e. N-(3-dimethylaminopropyl)-N- ethylcarbodiimide hydrochloride, 0.0018g) and 6-carboxyfluorescene dye (0.0030 g) were allowed to react in order to form the activated 6-carboxyfluorescene dye. The fluorescence dye (~ 1mL) was added to the NPs (10 mL) and stirred for overnight allowing the coupling reaction to occur. The reaction mixture was then dialyzed for 2 days to remove unbound dye molecules using the regenerated
cellulose tubular membrane (MWCO is 12000) against distilled water. The covalent binding of fluorescence dye molecules to the surface of the NPs was confirmed by fluorescence spectroscopic measurements (Figure 6.30).

The fluorescence dye-labeled NPs were then incubated with HeLa cells to visualize the cellular uptake of these NPs by confocal microscopy. HeLa cells were seeded in an 8 well chamber at a density of approximately $1.0 \times 10^5$ cells per well for 24 hours at $37^\circ$C. The cells were then incubated with dye-labeled NPs for 4 hours at $37^\circ$C and washed thoroughly with PBS buffer before they were viewed under a confocal microscope. Then the cells were directly imaged without fixation using a confocal microscope with 488 nm excitation wavelength. We observed that PVP-coated ZnPB NPs have been taken up by HeLa cells. The cellular fluorescent signals showed a uniform and non-compartmentalized cytoplasmic distribution of the dye-labelled ZnPB NPs in the peripheral region of the nucleus as shown in Figure 6.31, suggesting that the cellular uptake of such NPs is most likely through endocytosis.

**Figure 6.31.** Confocal microscopic images of Hela cells: (upper left) fluorescence image of cells incubated with dye-conjugated NPs for 4 hours; (upper right) bright field image of cells incubated with dye-conjugated NPs for 4 hours; (lower left) fluorescence image of the untreated cells; (lower right) bright field image of the untreated cells.
The elevated copper level in HeLa cells was induced by incubating the cells with a 250 μM CuCl$_2$ solution for 12 hours. The cells were washed three times with PBS and then incubated with the culture medium containing the NPs (550 μM) for 4 hours at 37 °C. After 4 hours incubation period, the cells were washed with PBS three times to remove the non-internalized NPs and further incubated with fresh culture medium for another 2, 4 and 8 hours. The cells grown in separate flasks were then trypsinized, centrifuged, re-suspended in PBS, and counted using a hemocytometer for 2, 4 and 8 hours, respectively. The cells were then collected by centrifugation and lysed with concentrated nitric acid. The lysates were diluted to 5.00 mL with deionized water and filtered through a 200 μm filter. The copper contents in the lysates were analyzed by AAS.

![Figure 6.32](image.png)

**Figure 6.32.** Kinetics of intracellular copper removal from HeLa cells.

As shown in Figure 6.32, there was a steady decrease in the copper concentration in the copper-saturated HeLa cells as revealed by the ICP analysis of the cell lysates. In essence, after incubation with NPs, the copper concentration in the cells decreased from the saturated level of 1093 ± 54 fg/cell, to 907 ± 45 fg/cell in 2 hours, to 533 ± 45 fg/cell in 4 hours and to 369 ± 18 fg/cell in 8 hours. The latter is comparable to the copper concentration of 262 ± 18 fg/cell in the control cells. Therefore we could confirm that the internalized ZnPB NPs can remove Cu$^{2+}$ ions that were previously introduced into HeLa cells.
We studied the cytotoxicity of PVP-coated ZnPb NPs using an MTT viability assay. HeLa cells were incubated for 24 hours with different amounts of NPs at 37 °C. Cells in each well were then treated with 100 μL of fresh medium containing varying concentrations of the nanoparticles and then incubated for 24 hours. Control wells contained the same medium without nanoparticles. After 24 hours, DMEM medium containing nanoparticles was removed and then, the cells were incubated with fresh DMEM media containing MTT reagent 10 μl, 1% (w/v) for 4 hours. After the MTT solution was removed, the precipitated violet crystals were dissolved in 100 μL of detergent. The absorbance was measured at 560 and 630 nm using a microplate reader. Each concentration was tested in replicates of three. The assay results are presented as percent viable cells. We found that more than 93% cells were viable after incubating with NPs at 0.67 mM, and more than 83% cells were viable after incubating with NPs at 1.2 mM, indicating that such NPs exhibit no significant cytotoxicity in the concentration range for the intended application as shown in Figure 6.33.

6.2.1 Antiangiogenic activity and copper sensing properties of $\text{K}_2\text{Zn}_3[\text{Fe(CN)}_6]_2$ nanoparticles.

Copper is identified as an essential cofactor for several pro-angiogenic factors that include basic fibroblast growth factors (bFGF), vascular endothelial growth factors (VEGF), tumor necrosis factor-α (TNF-α) and interleukin (IL-1). For cell migration assay, human
umbilical vein endothelial cells (HuVEC) were used as a model of angiogenesis. Migration of HuVEC cells were studied in Boyden chambers in 24-wells plate. The 80% confluent HuVEC cells in 25cm² flask were starved 24 hours in basal EBM-2 medium prior to harvesting and seeding them into Boyden chamber. Basal EBM-2 (300 μL) containing cells of 1.5×10⁷/ mL was added to top chamber along with the antiangiogenic inhibitors. In the bottom chamber of each well was added Basal medium 500 μL with chemoattracter, VEGF. The cells were allowed to migrate at 37 °C in an atmosphere of 5% CO₂ for 24 hours. The cells which migrated through the membrane in Boyden chamber were stained with 0.5% crystal violet solution for 10 min after aspirating the cells that failed to migrate. The excess dye was washed off carefully with deionized water. After blotting the Boyden chamber, they were transferred into wells containing 400 μL of extraction solution and incubated for 10 min. After the crystal violet stain was transferred into new 24-well plate, each well was read at 540 nm. For migration assay, we have used testing variables as shown below. Standard set up: well 1- basal medium; well 2- basal medium + VEGF 50 ng/mL; well 3- basal medium + VEGF 50 ng/mL + ZnPB NPs 6.2 nM; well 4- basal medium+ VEGF 50 ng/mL + Cu 100 nM; well 5- basal medium + VEGF 50 ng/mL + Cu 100 nM + ZnPB NPs 6.2 nM.
As can be seen in the Figure 6.34, HuVEC cells have ability to migrate through membrane as a response to chemoattracter, VEGF. In comparison to HuVEC cells in basal medium, HuVEC cells treated with VEGF has high ability of migration. In order to determine whether $K_2Zn_3[Fe(CN)_6]_2$ NPs could inhibit the migration of cells in angiogenesis, HuVEC cells were exposed to ZnPB NPs of 6.2 nM. As shown in Figure 6.34, ZnPB NPs inhibited the migration of VEGF-induced HuVEC cells. On the other hand, migration ability of VEGF-induced HuVEC cells that were already inhibited by ZnPB NPs (6.2 nM), could be restored by replenishing copper again (Figure 6.34). This observation clearly suggests that our ZnPB NPs can inhibit the angiogenesis by blocking the availability of copper.

Besides the copper detoxification ability of $K_2Zn_3[Fe(CN)_6]_2$, the concomitant copper sensing activity of ZnPB NPs was investigated owing to its selective copper ion exchange kinetics. To evaluate its suitability for copper sensing, homogeneous aqueous dispersions of NPs were used for the relaxivity measurements at 25 °C using a 60 MHz Bruker MiniSpec.
relaxometer. Also the T₁-weighted MR images of Eppendorf tubes containing aqueous solution were acquired at 9.4T scanner.

Figure 6.35. T₁ relaxation rate of (a) Zn PB NPs, (b) CuPB NPs (c) free Cu(II)

Magnetic resonance imaging (MRI) is widely used as it is the most appropriate modality for noninvasive clinical practice to identify and quantify the brain mineral deposition. The development of bimodal theranostic agent could link MR imaging with treatment follow-up for metal ions detoxification. As a result of copper accumulation, the abnormalities in MR imaging has been observed among the WD patients due to the occurrence of high T₁ intensity of MR signal in the globus pallidus, putamen, and esencephalon. The elevated level of copper ions is presumed to induce the oxidative stress and successively led to cell destruction and pathological changes. In generally, it is not
currently possible to evaluate copper accumulation in vital organs in patient without having tissue biopsy.

**Figure 6.3.** $T_1$-weighted phantom images (left) and $R_1$ relaxivity values (right)

The free Cu(II) ions increase the longitudinal relaxation rate of water protons as Cu(II) is a paramagnetic ion ($S = 1/2$). Thus accumulation of free Cu(II) causes to the hyperintensity of $T_1$ MR signal. The $r_1$ relaxivity values of free Cu(II) ions, ZnPB NPs and Cu PB NPs were found to be 0.56 mM$^{-1}$s$^{-1}$, 0.036 mM$^{-1}$s$^{-1}$ and 0.12 mM$^{-1}$s$^{-1}$ respectively (Figure 6.35). Small $r_1$ value of ZnPB NPs was anticipated in that Zn(II) and $[\text{Fe(CN)}_6]^{4-}$ form the coordination polymer and contain low spin Fe(II) in ZnPB. On the other hand, free copper ions have large $r_1$ relaxivity and increase the brightness of $T_1$-weighted images. The conversion of ZnPB NPs to CuPB NPs was shown to be fast and irreversible. As a consequence of ion exchange, longitudinal relaxivity $r_1$ significantly dropped from 0.56 mM$^{-1}$s$^{-1}$ to 0.12 mM$^{-1}$s$^{-1}$ which could manifest it in $T_1$ weighted images as darkening effect relative to the free copper ion which is not treated with Zn NPs (Figure 6.36 left panel).
Figure 6.37. Aqueous solutions’ ROS assay for Cu(II) induced oxidative stress, Exp 1-\(\text{H}_2\text{O}_2+\text{Cu(II)}\), Exp 2-\(\text{H}_2\text{O}_2+\text{Cu(II)}+\text{Zn PB NPs}\), Exp 3-\(\text{Zn PB NPs}\), Exp 4-\(\text{H}_2\text{O}_2+\text{Zn PB NPs}\)

Also we have studied the effect of our ZnPB NPs on copper assisted reactive oxygen species (ROS) production. The redox cycle of free copper ions catalyze the production of ROS by \(\text{H}_2\text{O}_2\) via Fenton chemistry. We conjectured that ZnPB NPs particles have the ability to inhibit the production of ROS via sequestrating the free copper ions. Fluorogenic probe for ROS, 2', 7'-Dichlorodihydrofluorescin diacetate (DCFH-DA), was used to identify the copper assisted oxidative stress. In brief, DCFH-DA is non fluorescence and oxidized form of dye, DCF is fluorescent. Upon exposure to the ROS, non-fluorescent dye can be easily converted into highly fluorescent dye. The fluorescence intensity is directly proportional to the ROS levels. The effect of antioxidant/free radical compounds on DCF-DA can be measured by the intensity of fluorescence. Reactive oxygen species could be artificially generated by \(\text{H}_2\text{O}_2\) in
the presence of catalytic amount of Fe(II) ions or Cu(II) ions. We have studied the effect of K₂Zn₃[Fe(CN)₆]₂ NPs on Cu(II) induced oxidative stress in a 96-well cell culture plate.

For this ROS assay, we have set up testing variables as follows, setup-1, 50 µL of H₂O₂ (100 µM), 50 µL of Cu(II) (10µM), 100 µL HSBB and 25 µL DCFH-DA 1X. For setup-2, 50 µL of H₂O₂ (100 µM), 50 µL of Cu(II) (10µM), 50 µL HSBB, 50 µL of Zn PB NPs (12 µM) and 25 µL DCFH-DA 1X. For setup-3, 50 µL of Zn NPs (12 µM), 150 µL of HBSS and 25 µL of DCFH-DA 1 X were mixed. For setup-4, 50 µL of H₂O₂ (100 µM), 100 µL HSBB, 50 µL of Zn PB NPs (12 µM) and 25 µL DCFH-DA 1X were added. All the setups were incubated at 25 °C for 30 minutes. The trace amount of Cu(II) catalyze the production of ROS in the presence of H₂O₂. The fluorescence intensity was measured at 530 nm (emission) with excitation at 480 nm. The fluorescence intensity of DCF is directly proportional to the ROS activity.

As shown in Figure 6.37, K₂Zn₃[Fe(CN)₆]₂ nanoparticles have ability to reduce the copper assisted ROS production by removing free copper ions in solution. In this experiment, H₂O₂ was employed to mimic in situ generation of transiently stable byproduct, H₂O₂ by copper dependent enzyme. Monoamine oxidase metabolism produces H₂O₂ by metabolizing biological amines such as dopamine in brain areas. Dysfunction of highly elaborative mechanisms for ROS regulation yields highly reactive hydroxyl radicals by mild H₂O₂. The DCF fluorescence intensity of H₂O₂ + Cu(II) combination in setup-1, is significantly higher compared to the combination of H₂O₂ + Cu(II) + Zn PB NPs in setup-2 and H₂O₂ + Zn PB NPs in setup-3. The H₂O₂ + Cu(II) + Zn PB NPs combination showed a very low fluorescence intensity and it can be attributed to removal of free copper ions. On other hand, the combination of H₂O₂ + Zn PB NPs does show slightly higher fluorescence signals than that of H₂O₂ + Cu(II) + Zn PB NPs in setup-2 which indicates the background level of ROS generated by H₂O₂. Our Zn PB NPs do not show measurable increase in fluorescence signals indicating that no ROS production associated with Zn PB NPs.
Chapter 7: Conclusion and Outlook

The design and the preparation of several novel nanomaterials for biomedical applications have been described in this dissertation. The utility of Prussian blue nanoparticles as the prototype to design the analogues of Prussian blue has been demonstrated in this dissertation. These analogues were shown to be highly biocompatible and highly water dispersible. Main group analogues of Prussian blue and the dopant controlled synthesis of Ga(III) and Gd(III) doped PB were synthesized and structurally characterized. Then nanoparticles of solid solutions of KGa[Fe(CN)$_6$], KGa$_{0.1}$Fe$_{0.9}$[Fe(CN)$_6$] and KGa$_{0.1}$Gd$_{0.1}$Fe$_{0.8}$[Fe(CN)$_6$] were evaluated for potential biomedical applications. Solid solutions of such nanoparticles exhibit novel properties including ion exchange properties, the ability to deliver $^{68/67}$Ga for PET imaging with an increased relaxation rate of water when Gd(III) is incorporated while they deliver Ga(III) to give bimodal imaging agents.

Specifically, KGa[Fe(CN)$_6$]·nH$_2$O nanoparticles can sequester Fe$^{2+}$ ions and release Ga$^{3+}$ ions via a rapid ion exchange reaction, suggesting possible antimicrobial activities of these nanoparticles. Particularly, the development of novel gallium radiopharmaceuticals and bimodal imaging agents of MRI-PET/SPECT using this nanoplatform is due to its ability to form highly water dispersible, but yet hydrolytically stable nanoparticles at physiological pH. We have also developed a proper method to stabilize the nanoparticles of KGa[Fe(CN)$_6$], KGa$_{0.1}$Fe$_{0.9}$[Fe(CN)$_6$] and KGa$_{0.1}$Gd$_{0.1}$Fe$_{0.8}$[Fe(CN)$_6$] and assessed their biocompatibility using HeLa cells. Subsequently, we have shown that such nanoparticles can penetrate the cell membrane and hence are suitable for cellular and molecular imaging applications. Further the easy with which surface can be functionalized with small molecular markers allows to develop organ-specific bimodal modal contrasting agents.

In chapter 4, synthesis and characterization of manganese analogue of PB nanoparticles for MR imaging was reported. We have developed a simple one-step method for preparing extremely stable and biocompatible NPs of K$_2$Mn$_3$[Fe(CN)$_6$]$_2$ as an alternative
to the Gd based T\textsubscript{1}-weighted contrast agents. Such a remarkable stability of K\textsubscript{2}Mn\textsubscript{3}[Fe(CN)\textsubscript{6}]\textsubscript{2} lowers the potential toxicity associated with leaching of manganese and cyanide. We have found that K\textsubscript{2}Mn\textsubscript{3}[Fe(CN)\textsubscript{6}]\textsubscript{2} NPs are readily taken up by cells and show no cytotoxicity. Furthermore, we have demonstrated that such NPs exhibit high T\textsubscript{1}-weighted relaxivity, suggesting the potential of non-gadolinium based new generation of T\textsubscript{1}-weighted MR contrast agents. The handy surface functionalization with target specific biomolecules and its cellular penetrability could make it possible to probe biological receptors or markers within the cell in biomedical research and clinical applications.

In chapter 5, we have discussed the use of bismuth and iodine in a single compound that gives rise to a favorable structural platform with an X-ray attenuation power that is among the best of those of all the known inorganic nanoparticulate systems thus far reported in the literature for CT contrast applications. Conceptually, BiOI can be viewed as a compound consisting of the BiO\textsuperscript{+} and I\textsuperscript{−} ions. This notion has led to the development of a controlled hydrolytic procedure in this work for the preparation of biocompatible ultra-small NPs of BiOI. Such ultra-small nanoparticulate formulation assures the fast renal clearance. On the other hand, BiOI is a covalent solid-state compound with a layered structure and a very low solubility product constant. The latter is a desirable feature for ensuring low osmolality if these NPs are to be developed as an intravenously injectable CT contrast agent. Our preliminary studies of the cytotoxicity and cellular internalization suggest that such NPs may have potential for CT cellular imaging and image-guided drug delivery applications.

The last chapter of this dissertation focuses on the synthesis of therapeutic nanoparticles. The first part of the chapter 6 describes the synthesis and characterization of Au@PEN nanoparticles and non-sulfur based compound K\textsubscript{2}Zn\textsubscript{3}[Fe(CN)\textsubscript{6}]\textsubscript{2} as copper depleting agent for the treatment of copper accumulation in vital organs such as liver. Their utility as copper depleting agent has been clearly demonstrated \textit{in vitro}. The non-cytotoxicity and the ability for such nanoparticles to be internalized into cells provide encouraging results for the novel nanocomposite material to be used as a copper detoxifying agent. Currently, the
treatment of choice for copper related pathogenic disease relies on the use of D-PEN and tetrathiomolybdate (TTM) which is a slow-acting oral chelating agent with numerous adverse side effects. From our results, we concluded that the our nanoparticulate therapeutic agents will be a new generation of drugs for targeted sites owing to their remarkable characteristics such as cell permeability, remarkable stability, lack of toxicity, and the potential for surface modification of target specific biomolecules for drug delivery. This novel approach provide a novel possibility of developing a safer and more effective treatment to reverse the progression of Wilson’s disease and other copper related pathogenic conditions at the late onset of symptoms. Future research should be focused on testing the safety and efficacy.

In the last part of Chapter 6, utilization of non-sulfur based copper depleting agent, K$_2$Zn$_3[\text{Fe(CN)}_6]_2$ is discussed for its role in angiogenesis inhibition and copper sensing ability with the concomitant detoxification. This nanoparticulate approach is a more reliable and novel way to treat copper overload in vital organs and to monitor \textit{in vivo} depositions as a way of following up the therapy. Our studies have shown that K$_2$Zn$_3[\text{Fe(CN)}_6]_2$ NPs are readily internalized by cells \textit{via} endocytosis and are gradually ejected by cells \textit{via} exocytosis. Moreover, all the compounds in the class of PB analogues we have examined so far have showed a remarkable stability. Our preliminary studies have clearly showed that K$_2$Zn$_3[\text{Fe(CN)}_6]_2$ nanoparticles can sequester copper ions and exhibit a turn-off response in MR images after ion-exchange with copper. The contrast generated by such ion-exchange reaction was a negative contrast in T$_1$-weighted images. This effort opens up new avenues to develop dual functional MR probes for copper detection and detoxification. Further, our K$_2$Zn$_3[\text{Fe(CN)}_6]_2$ NPs exhibit anti-angiogenic activity and such effect on HuVEC cells could easily be reversed by replenishing the copper supply. This observation suggested that our nanoparticles show their activity on angiogenesis inhibition by depleting copper which acts as a cofactor in a number of angiogenesis promoters.
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