VISUALIZING ENZYMATIC ACTIVITY USING MAGNETIC RESONANCE
MOLECULAR IMAGING

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VISUALIZING ENZYMATIC ACTIVITY USING MAGNETIC RESONANCE MOLECULAR IMAGING

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ABSTRACT

Overexpression of ERK (MAP Kinase) and Pin1 is related to many types of cancer. Two pro-contrast agents, Gd-DOTA-APRTPGGRCKKK and Gd-DOTA-PRTPGGRCKK, which were hypothesized to be enzymatically activated to show greater contrast by overexpressed ERK and Pin1 were synthesized using solid phase peptide synthesis. ERK phosphorylation using $^{33}$P labeled ATP showed radioisotope incorporation in Gd-DOTA-APRTPGGRCKK only; Gd-DOTA-PRTPGGRCKK (missing alanine residue) did not undergo enzymatic phosphorylation. Contrast measurements of the Gd-DOTA-APRT(phospho)PGGRCKK showed 28% increase in relaxivity ($r_1$) over Gd-DOTA-APRTPGGRCKKK. Gd-DOTA-APRT(phospho)PGGRCKK underwent isomerization with the peptidylprolyl isomerase Pin1. It was initially hypothesized that this conformational change caused by the isomerization would further enhance the contrast by creating a more sterically open Gd coordination environment which would accommodate an inner-sphere water molecule. It was also possible for the isomerized structure to attenuate contrast by blocking the 9th Gd coordination site and preventing inner-sphere water coordination. Isomerization of the Gd-DOTA-APRT(phospho)PGGRCKKK with Pin1 was monitored using CD-spectroscopy and contrast measurements were done simultaneously which showed no change in contrast. The pro-contrast agents Gd-DOTA-APRTPGGRCKK and Gd-
DOTA-PRTPGGRCKK were tested for activity against other MAPK’s, such as p38 and JNK1, and showed negative results.

A novel contrast agent, Gd-DOTA-APRTPGGRCKK, which can directly and specifically monitor ERK activity, has been synthesized. Based on molecular modeling using Charmm27 MM3 we believe that the folding of the peptide upon phosphorylation causes a proline residue to recede away from the Gd coordination sphere, allowing free access to bulk water molecules. Based on literature comparisons we believe that the majority of the contrast enhancement is due to increased outer sphere relaxation rates resulting from phosphate incorporation.

This thesis describes the synthesis and relaxivity measurements of a new contrast agent for the non-invasive evaluation of signal transduction pathways that are up-regulated in cancer cells. This is the first example of a medical imaging agent, in any imaging modality, whose contrast generation or enhancement is responsive to kinase activity. Furthermore, this agent is specific to ERK, showing no activity towards other protein kinases, and can report on ERK signaling cascade within cancer cells with high specificity.
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PART I VISUALIZING ENZYMATIC ACTIVITY USING MAGNETIC RESONANCE MOLECULAR IMAGING
CHAPTER 1 INTRODUCTION

1.1 PROTEIN KINASES

Protein kinases play an essential role in nearly every aspect of cell biology. They mediate most of the signal transduction pathways by modifying the substrate activity. They orchestrate cellular processes like metabolism, transcription, cell cycle progression, cytoskeletal rearrangement, cell movement, apoptosis and differentiation. They also play a critical role in intercellular communication during development, in physical responses and in homeostasis, and in functioning of the nervous and immune system. The extracellular signal is transmitted from the plasma membrane to the nucleus of the cell through the cytosol by G protein and a cascade of mitogen-activated protein kinase (MAPK). The core module consists of MAPK kinase kinase (MAPKKK) that phosphorylates and activates a MAPK kinase (MAPKK), which in turn activates a MAPK. The Ras-Raf-MEK-ERK pathway is one of the five distinct pathways and is important for intracellular signaling. Cell proliferation, differentiation and survival are the key functions of this pathway and inappropriate activation is a common occurrence in human cancer. In this pathway Ras, Raf, MEK and extracellular signal-regulated kinase (ERK) correspond to the G-protein or guanine nucleotide-binding protein, MAPKKK, MAPKK and MAPK respectively. Kinases catalyze the reaction of transferring the phosphoryl group (PO$_3^{2-}$) from adenosine-5’-triphosphate (ATP) to the serine, threonine or tyrosine residues on its substrate and producing adenosine-5’-diphosphate as the byproduct as shown in scheme 1.1. Phosphorylation is a way to transmit signal from an upstream kinase to a downstream kinase. Phosphatases
hydrolyze the phosphorylated amino acid residue to its unphosphorylated form thereby acting like a deregulator.

Scheme 1.1 Phosphorylation and de-phosphorylation of amino acid residues catalyzed by kinases and phosphatases respectively.
Ras is a member of the large family of GTPase, proteins that bind and hydrolyze guanidine triphosphate (GTP) to guanidine diphosphate (GDP). Ras is anchored to the cytoplasmic face of the plasma membrane which brings it closer to nucleotide exchange factor son of sevenless (SOS) stimulating the protein to release the bound GDP in its inactive state and bind to GTP from cytosol. Ras cycles between the inactive-GDP bound state and the active GTP-bound states which is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). Ras is activated by receptor tyrosine kinases (RTKs) and other stimuli that are activated by growth factors. GTP binding to Ras causes conformational change to the protein turning it active. Raf comprises of a family of serine/threonine kinases which has multiple phosphorylation sites. It is indirectly activated by phosphorylation by the activated GTP-bound Ras and other multiply converging pathways. The activation of Raf initiates a cascade of protein phosphorylation starting with MEKs which phosphorylate ERK1/2 thereby activating them. The number of phosphorylations of Raf create a differential activation which is tailored for mitogen dependent activation. Phosphorylation of some sites on Ras causes deactivation of the protein causing release it to Ras-GTP. The released Ras-GTP can then be accessed by Ras GAP thereby deregulating Ras signaling. Along with other proteins regulating the Raf through protein-protein interactions, kinase suppressor of Ras (KSR) is a scaffolding protein for Ras-Raf-MEK-ERK pathway which facilitates phosphorylation of MEK1/2 by Raf and ERK1/2 by MEK1/2 by binding to Raf, MEK1/2 and ERK1/2. KSR modulates the intensity and duration of Raf/MEK/ERK pathway thereby which contributes to the specificity in MEK/ERK signaling. Along with activating the kinases, KSR is also responsible for bringing proteins together for
faster signaling by preventing de-phosphorylation by phosphatases in the system, keeping proteins apart to block signaling and control compartmentalization of proteins within the cell. KSR is regulated by phosphorylation for binding to the MAPKs. Phosphorylation of ERK1/2 causes it to move from the cytoplasm to the nucleus where it phosphorylates transcription factors causing it to turn on gene expression of a specific set of target genes.

1.2 EXTRACELLULAR SIGNAL REGULATED KINASE (ERK)

The activation of the MEKs and ERKs occur by phosphorylation at two conserved residues on each kinase. The reason for three kinases to be involved in the signal transduction is amplification of response at higher level of stimuli and lowering the response at lower level of stimuli which is known as “ultrasensitivity” and “subsensitivity” respectively. The subsensitivity helps in ignoring lower stimuli and avoiding a response. The enzyme is able to filter noise by using this strategy for signal transduction which is ideal for carrying responses for cell fate where there is no intermediate state. This makes the signal transmitted to the nucleus is almost like a binary signal, with an on and off corresponding to mitosis and apoptosis respectively.

ERK1/2 are hydrophilic serine/threonine kinases that phosphorylate and regulate numerous proteins including various transcription factors. ERK1 and ERK2 are 44- and 42-kDa respectively, with 84% of the sequence being identical. Human ERK1 and ERK2 activation require dual phosphorylation of tyrosine residue prior to the threonine residue by MEKs for complete activation. ERK2 has 60,000 fold increased rate of phosphorylation upon complete activation. ERK1 and ERK2 are both activated when transporting stimuli. When ERK1 is
removed, ERK2 is more activated and vice versa. Unlike Raf and MEKs which have a narrow set of substrates ERK1/2 have more 175 documented cytoplasmic and nuclear substrates. Phosphorylation catalyzed by ERKs occurs at a serine or threonine prior to a proline. The phosphorylation site is labeled as 0 and proline immediately after it is labeled as +1. The residue immediately before the phosphorylation site is labeled -1 where a basic amino acid residue like arginine or lysine is preferred. Another proline residue is preferred at the -2 position. Proline at the +1 position is a common feature for all of the MAPKs and hence they are regarded as proline-directed kinases. This sequence of amino acids is common between eukaryotic proteomes and even distantly related kinases like cyclin dependent kinases and hence it is not the cause for substrate specificity.

Substrates of ERK include upstream signaling proteins like SOS, GEFs, KSR which suggests that ERK plays a key role in feedback regulation as well. Also various transcription factors are also phosphorylated by ERK showing a transition phase between signaling and gene regulation. In spite of having large number of substrates ERK maintains its substrate specificity by using docking sites. Some proteins with the required (S/T)P residues are not phosphorylated and some proteins containing multiple (S/T)P residues get phosphorylated at a specific (S/T)P residue/s illustrating the importance of docking sites. The modular nature of docking sites on ERK mediates substrate binding and induces phosphorylation of a particular S/T residue if there are multiple phosphorylation sites present in the substrate. The two docking sites known for ERK are FXFP and D-domain docking site, which may or may not work synergistically to phosphorylate a particular S/T residue on the substrate. Another factor affecting phosphorylation of a substrate
is its accessibility to the active site and the position of nearby proline residues on the substrate.\textsuperscript{9} The docking site and active site are generally within 20 amino acid residues apart.\textsuperscript{8}

1.3 ROLE OF \textit{Ras/Raf/MEK/ERK} IN CANCER

In 2005, the American Cancer Society reported the number of deaths due to cancer has surpassed heart diseases and is the primary cause of death for patients under the age of 85.\textsuperscript{10} More than a third of all Americans will be diagnosed with cancer at some point in their life. The disease might not have been diagnosed now due to lack of symptoms leading to diagnosis but it is growing within. The detection of the cancer in early stages leads to complete cure by drugs or surgical methods. As the disease progresses towards stage III and IV, the chances of survival decrease exponentially in all types of cancer. Early detection is the key to successfully curing cancer. Biopsy is an invasive technique in which a sample of a lump of tissue which is suspected to be tumorous is surgically removed and chemically tested for cancerous behavior. The development of non-surgical or non-invasive techniques for detecting the precise location and spread of the tumor using Computed Tomography (CT), ultrasound, endoscopy, Positron Emission Tomography (PET), Magnetic Resonance Imaging (MRI) has made tremendous development in the last 15 years. These non-invasive imaging techniques have been used while doing biopsies in what is known at image guided biopsies and also during intraoperative procedures. Biopsies relying on histological determination are rarely reproducible and often fail to differentiate between benign and malignant forms of tumor. Imaging strategies help in
determining heterogeneity within tissue, define parameters to provide such distinction and also result in reproducible results making this much preferred than biopsies.11

Ras/Raf/MEK/ERK plays an important role in cell proliferation and prevention of apoptosis. Anomalous activation of this pathway is observed in malignant forms of tumor. Many members of the signaling cascade and their downstream transcription factors are proto-oncogenes. EGFR overexpression is a key feature in all forms of tumor leading to enhanced binding of mitogens to EGFR which leads to enhanced signaling. Overexpression levels and mutation characteristics vary for EGFR, Ras, Raf and MEK in different types of cancer and even within the same tumor due to different phenotypes as shown in the figure 1.1.

![Ras/Raf/MEK/ERK signaling pathway showing kinase mutations in various types of cancer](image)

Figure 1.1 Ras/Raf/MEK/ERK signaling pathway showing kinase mutations in various types of cancer.( Roberts, P. J.; Der, C. J. Oncogene 2007, 26, 3291) 12
Physiological conditions like hypoxia in the tumor cells trigger the release of hypoxia inducible factor (HIF) which is a transcription factor and drives the production of genes which upregulate EGFR protein levels and helps the tumor cell survive the hypoxic conditions by using aerobic glycolysis as the primary source for ATP. This upregulation of EGFR protein due to tumor microenvironment rather than genetic mutation gives us clues as to why some of the current anti-cancer drugs based on EGFR inhibition have not been successful. The overexpressed EGFR now activates the intercellular receptor tyrosine kinase function of the EGFR and results in autophosphorylation of the receptor at an enhanced rate. The Ras oncogenes and activating mutations lead to activation of Ras protein which is observed in 30% of all human cancers. Oncogenic Raf has been shown to activate oncogenic Ras. Depending on the Raf isoform, Raf overexpression can lead to cell proliferation or apoptosis. Similarly the oncogenic Ras has been shown to activate downstream MEK which instead activates ERK. MEK and ERK are not found to be mutated in tumor cells till now but the enhanced activated upstream kinases causes enhancement in the activity of these enzymes.\(^\text{13}\) As has been discussed before the signaling protein activations occur by phosphorylation. The difference between normal and tumor cells, is the heightened signaling in case of tumor cells, which leads to enhanced cell division. The use of a downstream kinase like ERK is more favorable since the signal is the highest at this step of the signaling cascade. Also since there is no mutation in ERK the substrate in tumor cells remains the same as in the normal cells which makes targeting it as an imaging biomarker viable.\(^\text{14}\)
1.4 ROLE OF PIN1 IN CANCER

The peptidyl-prolyl \textit{cis/trans} isomerase (PPlase) Pin1 is a member of the parvulins family and catalyzes the \textit{cis/trans} isomerization of imidic peptide bonds in a phosphorylated-S/T-P moieties of polypeptides. The phosphorylated -S/T-P- has 1300 fold greater selectivity than the unphosphorylated -S/T-P. Other members of the PPlase family do not isomerize the same substrate as Pin1. Pin1 is a small protein with two structurally and functionally distinct domains. A WW domain that binds to the phosphorylated -S/T-P-moiet and a catalytic PPlase domain that isomerizes the phosphorylated -S/T-P-moiet.\textsuperscript{15}

Figure 1.2 Pin1 catalyzed \textit{cis/trans} isomerization(Velazquez, H. A.; Hamelberg, D. Biochemistry (Mosc). \textbf{2011}, \textit{50}, 9605.).\textsuperscript{16}

Pin1 plays an important role in switching the conformation of the phosphorylated enzymes thereby modulating the protein activity, phosphorylation status, protein-protein interaction,
subcellular localization and stability. Phosphorylated MAP kinases like ERK, p38 and c-JNK are substrates for Pin1. The substrate for Pin1 in these enzymes lies in the conserved phosphorylation loop of the kinases. It has been shown that binding of JNK1 with Pin1 and the isomerization of JNK1 by Pin1 causes conformational changes in the protein which activates it and also increases the binding affinity of the enzyme to its downstream substrates. Also the Pin1 bound JNK1 is less resistant to de-phosphorylation which sheds light on the fact that the duration of activation of JNK can be modulated by Pin1. Since there is a one to one binding event for complete activation of MAP kinase the overexpression of Pin1 in tumor cells along with MAP kinase is not surprising. Especially in the case of breast cancer the high levels of Pin1 can be used a potential biomarker.\textsuperscript{17}

1.5 Magnetic Resonance Molecular Imaging

To visualize the anatomy of the body clearly in an MRI certain contrast agents are introduced in the body either orally or intravenously. These contrast agents increase the relaxation rates of the water proton’s nuclear relaxation rates thereby increasing the brightness of water in its vicinity. Several kinds of contrast agents are available and based on their bio-distribution and application they can be classified as intravenous fluid agents, intravascular contrast agents, organ specific agents, cell labeling agents, smart or responsive contrast agents and pH sensitive agents.\textsuperscript{18}

Gadolinium based MRI contrast agents have come a long way as a diagnostic tool for imaging of human tissue. Their use started in 1988 when Gd (III) diethylenetriaminepentaacetate
[Gd(DTPA)(H20)]2− was approved for injecting in human bodies. Since then there have been a variety of contrast agents which have been approved under different brand names with Gd(III) as the metal ion and variety of ligands. Contrast agents with metal centers like Mn(II) and iron have also been approved but the Gd(III) remains a favorite for reasons discussed later.19

In 1948 Bloch20 observed that the use of paramagnetic metal ions could enhance the relaxivity of water protons. In 1961 it was found that binding the paramagnetic metal ion to DNA or a macromolecule enhances the water proton relaxation efficiency. Lauterbur20 in 1973 did studies on tissue samples in which water protons in proximity to paramagnetic metal ions (Mn(II)) could be differentiated from the ones without them based on their relaxation rates. The relaxation rates were also correlated to the concentration of Mn(II). With the approval of [Gd(DTPA)(H20)]2− in vivo 3D visualization of tissue was possible. This was a totally non-invasive technique in which the Gd-complex was injected to the patient’s blood and MRI scan showed increased contrast in the regions of the body accessible by blood. Gd(III) was preferred as the paramagnetic metal ion because it is the only metal ion which had seven unpaired electrons being in the middle of the lanthanide series. Gd(III) has a coordination number of nine and the contrast agents are designed in such a way that the ligand chelates the metal ion’s eight coordination sites and leaves the ninth coordination site open for bulk water molecule to coordinate. The high coordination number leads to very stable Gd-chelate complexes, which is important from a toxicity point of view.20

The addition of a paramagnetic metal ion to water changes the longitudinal and transverse relaxation rates, 1/T1 and 1/T2, respectively, for water protons. The observed relaxation rate is a
summation of the contribution from diamagnetic solvent and the paramagnetic metal ion contributions, \((1/T)_d\) and \((1/T)_p\), respectively.

Longitudinal Relaxation Rate = \((1/T_1)_{\text{observed}} = (1/T_1)_d + (1/T_1)_p\) (1.1a)

Transverse Relaxation Rate = \((1/T_2)_{\text{observed}} = (1/T_2)_d + (1/T_2)_p\) (1.1b)

The paramagnetic relaxation rate is linearly dependent on the concentration of the paramagnetic metal ion ([M]), proportionality constant is relaxivity, \(R(\text{mM}^{-1}\text{s}^{-1})\) and hence can be expressed as

\[(1/T_1)_{\text{observed}} = (1/T_1)_d + R_1[M]\] (1.2a)

\[(1/T_2)_{\text{observed}} = (1/T_2)_d + R_2[M]\] (1.2b)

The positively charged paramagnetic metal ion binds water in its inner coordination sphere through dative bonding and outer coordination sphere via hydrogen bonding as shown below in figure 1.3A and 1.3B.

![Diagram](image)

Figure 1.3 Different modes in which water can interact with the metal ion.
The large fluctuating magnetic field around the paramagnetic species provides an additional relaxation pathway for water molecules that are associated with it, and is the basis for differentiating them from bulk water. The translation diffusion of water close to the metal ion complex also contributes to the outer sphere relaxation mechanism as shown in Fig 1.3c. The total contributions from the inner sphere relaxation to the longitudinal relaxation can be expressed in a form shown in equation 3.

\[
\frac{1}{T_1} = P_M q / (T_{1M} + \tau_M)
\]

(3)

In equation 3, q is the no. of water molecules in the inner coordination sphere, \(P_M\) is the mole fraction of metal ion, \(T_{1M}\) is the relaxation time of the inner sphere water protons, \(\tau_M\) is the residence lifetime of the inner sphere water. At the magnetic field strengths at which typical MR imaging is performed, the \(T_{1M}\) is almost equal to the rotational correlation time of the complex which means the slower the contrast agent rotates or reorients itself, the greater is the relaxation rate. Again based on this equation, the relaxation rate \((1/T_1)\) is directly proportional to the number of bound water molecules (q). The increase in relaxation rates results in positive contrast (brighter image) where the Gd(III) is located. Hence, maximizing the number of water molecules bound directly to the contrast agent is a way to increase its contrasting efficiency. But this comes at the price of in vivo stability of the complex which is of a very high priority due to the high toxicity of free Gd(III) ions. Any free Gd(III) will have undesirable consequences to the health of the patient. Therefore, most Gd(III) based contrast agents have only one coordinated water and
the remaining eight coordination sites are occupied by polydentate chelate arms. Model studies have shown that there are about twelve to fifteen water molecules in the outer coordination sphere which are about 4 Å away from the metal center which makes the outer sphere relaxation effect equally important for the increase in longitudinal relaxation rates. The functionalities on the complexing ligand used for the contrast agent could also be affected by the orientation of the water molecules in the surrounding. The orientation of the water molecules in turn would affect the relaxivity of water protons. The actual mechanism of outer sphere relaxation has not been completely understood, but studies on the particular contrast agent of interest could help us in maximizing the relaxivity.\textsuperscript{19,20}

To find an effective complexing agent for Gd(III) comparative studies on relaxivity of DTPA and DOTA (structures shown below) complexes of Gd(III) have been performed which showed that DOTA and DTPA had relaxivities which were comparable to typical $q = 1$ agents. Hence, we may use DTPA or DOTA macrocyclic ligands as the framework for our contrast agent.\textsuperscript{20}

![DOTA](image1.png)  ![DTPA](image2.png)

Figure 1.4 Commonly used macrocyclic chelators DOTA and DTPA.
1.6 SMART OR ACTIVATABLE CONTRAST AGENTS

The first generation of contrast agents, such as [Gd-DOTA(H$_2$O)]$^{1-}$ and [Gd-DTPA(H$_2$O)]$^{2-}$ were nonspecific and would distribute throughout the blood plasma in a more or less generic pattern. Our project is based on designing contrast agents which will be responsive to specific disease biomarkers. MRI contrast agents whose activity is triggered by interaction with specific biomarkers are referred to as “activatable MR agents”. The “off” state of these pro-contrast agents that we envision have a coordinatively saturated Gd(III) ion, which leads to a diminished relaxivity, by exclusion of water from the inner coordination sphere of Gd(III), relative to the “on” or activated state. The change in the ligand environment is brought about by the activity of the biomarker that we intend to visualize. As discussed earlier the increase in relaxivity can be caused by either increasing the number of water molecules in the Gd(III) inner coordination sphere or by reducing the tumbling motion of the Gd(III) complex. A small number of contrast agents have been reported that are responsive to pH changes, presence of metal ions like Ca(II), Zn(II), Fe(III), presence of O$_2$, proteins like Human Serine Albumin(HSA) and β-galactosidase.$^{21}$

The pH sensitive contrast agents were made in such a manner that change in pH would change the number of water molecules in the inner coordination sphere. The pH sensitive contrast agents are supposed to distinguish between normal cells (pH=7.4) and tumor cells (pH=6.8 – 6.9). The way these work is that at lower pH the Gd(III) inner coordination sphere is occupied by two water molecules but at pH more than 6 the concentration of the HCO$_3^-$ ion increases and this now binds to the Gd(III) more effectively than water as shown in figure 1.5.
Note that the number of water molecules \((q)\) coordinated to the Gd(III) ion changes from 2 to 0 which results in a large change in relaxivity \((\sim 280\%)\).\textsuperscript{22}

\[\text{Intermolecular Anion Binding}\]

\[
\begin{array}{c}
\text{GdDO5ala} \quad R = \text{CH(CH}_3\text{)CO}_2^- \\
\end{array}
\]

Figure 1.5 Intermolecular anion binding (Lowe, M. P. Curr. Pharm. Biotechnol. 2004, 5, 519.)\textsuperscript{21}

A more sophisticated way of achieving pH responsive Gd contrast agents is that have intramolecular ligands that bind Gd(III) ion at higher pH but become labile when they are protonated at lower pH as shown in figure 1.6. This creates an opportunity to change ligands in such a way to fine tune contrast activation to specific pH ranges. Some other ligating groups like \(p\)-nitrophenols have also been reported to show relaxivity changes with pH change.\textsuperscript{23}
Metal ion activated contrast agents have been used to monitor changes in the concentration of metal ions like Ca(II), Zn(II) and Fe(III). For the detection of Ca(II) the ligands are designed in such a manner that in the presence of Ca(II) the ligands dissociate from Gd(III) and bind with Ca(II) causing structural changes which allow water molecules to coordinate with Gd(III) leading to increased relaxivity as shown in figure 1.7(left). In case of detection of Zn(II) the binding of the contrast agent with the Zn(II) prevents water molecules from coordinating to the ninth coordination site of Gd(III) which results in a decrease in the relaxation rate as shown in figure 1.7(right). This results in a Zn(II)-triggered negative contrast as opposed to, the Ca(II) case where Ca(II) binding results in contrast enhancement.
One of the most interesting examples of enzyme activatable MRI contrast agents was the seminal work performed by Tom Meade and coworkers in which the pro contrast agent was activated in vivo by the enzyme β-galactosidase. The activation is caused by the enzymatic cleavage of the galactopyranose as shown by the figure 1.8.
Figure 1.8 Activation of contrast agent by β-galactosidase.\textsuperscript{27}

As the bond highlighted in red is cleaved by catalytically by β-galactosidase, water molecules from the bulk can displace the galactopyranose ring from the inner coordination sphere of Gd(III) thereby increasing the relaxivity of bulk water. Hence enzyme activity can be directly correlated to the increase in contrast. It is to be noted that in the pro contrast agent the exact coordination mode of the galactopyranose ring is not very well defined. In general, bonding of lanthanide ions with ligands is largely coulombic in nature, rather than covalent, due to the limited radial extension of the 4f valence orbitals. The presence of the galactopyranose above the 9\textsuperscript{th} coordination site is enough to prevent the coordination of water molecules making it inactive.\textsuperscript{27}
1.7 **PROPOSED ACTIVATABLE CONTRAST AGENT TO MONITOR ENZYMATIC ACTIVITY**

Our objective is to design an activatable contrast agent which is responsive to the enzyme MAP kinase which overexpressed in the tumor cells with respect to a normal cell. It plays an important role in the signal transduction pathway for mitosis. Visualization of the excess MAP Kinase activity is strong indication of the rapid cell division in tumorigogenesis. There is a greater degree of restricted rotation about peptidyl proline bonds relative to other amino acid residues. Alteration in the stereochemistry at this bond is a common activation mechanism for proteins. Substrates of MAP kinase are activated by MAP kinase phosphorylation and Pin 1 isomerization sequence.

![Scheme 1.2 Proposed Activation of Contrast Agent by MAP kinase and Pin1](image)

**Pro MRI Contrast Agent**  
**Activated MRI Contrast Agent**
The structure of the simplest example of our desired pro-agent is shown in scheme 1.2. This pro MRI contrast agent does not have any water in its inner coordination sphere limiting the relaxivity of bulk water around it to residual outer sphere relaxation. As shown in the scheme 1.2, one of the nitrogens of the tetraazacyclododecane ring is conjugated to a dipeptide (serine-proline) through a linker. MAP kinase recognizes this specific amino acid sequence and catalyzes the phosphorylation of the serine hydroxyl group. Pin 1 is responsible for isomerization of the cis orientation between the phosphorylated serine and proline to the trans isomer. We hypothesize that this conformational change moves the peptide fragment away from Gd(III) and creates a vacant site for water coordination. This process triggers the inner sphere relaxivity to come into play increasing the overall relaxivity of the bulk water protons. Hence, cancer cells expressing MAP kinase and Pin 1 will show increased relaxivity, i.e., greater contrast relative to normal cells. One of the most critical parts of this project is to obtain the pro contrast agent in the conformation shown above. The lifetime of the conformation should be long enough so that they do not isomerize on their own. Also, the strength of the ligand in the ninth coordination site should not be too high which might make Pin 1 isomerization thermodynamically unfavorable. All these thermodynamic parameters must be optimum to achieve the perfect contrast agent. Such a process of optimization has to be done using trial and error method. A large number of prototype contrast agents with minor variations will be required for this to get the perfect contrast agent which not only a substrate of ERK and Pin1 but also performs in such a way which leads to increase in relaxivity of the contrast agent.
2.1 INTRODUCTION

Solid phase peptide synthesis (SPPS) is a synthetic technique in which amino acids are covalently attached to an insoluble solid polymer and subsequent amino acids are attached in a stepwise manner till the required peptide sequence is achieved. The stepwise synthesis is carried out from the C-terminal to the N-terminal of the target peptide. This method was conceived by Merrifield in 1963 when he made the first tetrapeptide illustrating its superiority over the classical solution phase peptide coupling techniques. In SPPS, the reagents in the solution phase react with the functional group on the solid polymer. The technique uses orthogonal protecting group chemistry so that required functional groups can be selectively deprotected.

The advantage of this technique lies in the fact that the growing peptide chain is bound to the solid polymer which helps easy separation of products from bye products and excess reagent by washing with copious amounts of appropriate solvents. The presence of only one functional group available for reaction helps improve reaction yields by using excess of reagent and by repeating reactions with same reagents to ensure complete product formation. Hence, multiple stepwise reactions can be performed without the requirement for purification in between reactions. The increasing insolubility of the growing peptide is not an issue in SPPS. Final products are separated from solid phase using appropriate cleavage cocktails depending on the functionalities present in the molecule. The cleavage step does not degrade the peptide sequence.
This technique has been extensively used for synthesis of peptides, deoxyribonucleic acid sequences and other molecules which are in series.

The solid support is designed so that it can sustain the conditions of functionalization and synthesis during SPPS. Peptides when bound to the solid polymer have limited intermolecular interactions and hence have a lower tendency to form aggregates. The restricted movement of the bound peptide could sometimes be a disadvantage. Poly(styrene-co-divinylbenzene) (PS-DVB) is the most commonly used solid polymer support. The amount of crosslinking in the polymer governs whether the solid support was stable and swollen enough for mass transfer through the micro-channels of the beads. The solid polymer support is swollen using polar organic molecules. A swollen polymer allows for proper solvation of the growing peptide which lowers the network free energy. PS-DVB with 1% crosslinking has ideal swelling and stability for SPPS.

The strength of the covalent bond between the growing peptide chain and the efficient with which the cleavage takes place governs the purity of the peptide. The design and development of a large number of covalent linkers has extended the scope and application of SPPS. The linkers are designed in such a way so that one end is labile during cleavage conditions and the other end can be coupled to the functionalized solid polymer.

2.2 Peptide Coupling Reagents

Peptides are synthesized by the coupling of the carboxylic group of the one amino acid to the amino group of another amino acid. Coupling reactions are done in presence of additives called coupling reagents as shown in scheme 2.1.
Scheme 2.1 General Peptide Coupling

Three major routes for racemization that are known are elimination of alpha-proton, reversible beta-elimination and through azlactone formation as shown in scheme 2.2. Among the three routes the racemization by azlactone formation is the most important mechanism which is dependent on the acidity of the alpha-proton to be picked up by a base resulting in a resonance stabilized aromatic structure. Electron donating groups like benzyloxyxarbonyl, tert-butoxycarbonyl and other alkoxy carbonyl groups destabilize reduce the acidity of the alpha-proton and also destabilize the azlactone anion preventing racemization.

Peptide coupling is performed in the presence of additives which allow the reaction to occur under mild conditions while maintaining the stereochemical integrity of the amino acids involved, minimizing side reactions. These additives are useful in SPPS and generally their activity is similar to that in solution phase.
Scheme 2.2 Routes for Racemization of a Peptide

2.2A CARBODIIMIDE REAGENTS

Carbodiimide reagents have been widely used in peptide synthesis to activate the carboxylic acid group of an amino acid. Some of the commonly used reagents are shown in figure 2.1. Dicyclohexylcarbodiimide (DCC) was the first reported by Sheehan in 1955. 29
The general mechanism of activation is shown in scheme 2.3. Generally the initial step is the addition of the carboxylic group of the amino acid to the carbodiimide to form the O-acylisourea, a very powerful acylating agent. The O-acylisourea would react with amine of another amino acid to form the peptide bond and produce the corresponding carbodiimide urea as byproduct. DCC was extensively used as a coupling reagent for solution phase peptide coupling. Insolubility of the formed byproduct (urea) in organic solvents made it popular for solution phase peptide
synthesis but was inconvenient for SPPS. The solubilities of the corresponding urea from the CIC, DIC and DCC were 30, 5.2 and 1.5 g/L in CH₂Cl₂. For SPPS, use of DIC was preferred due to increased solubility of the byproduct, N, N′-diisopropylurea. Also DMF was a potent solvent for keeping N, N′-diisopropylurea solubilized during peptide coupling reaction. The high reactivity of carbodiimides resulted in fast reaction times but the side reactions and caused racemization of the amino acid.
Scheme 2.3 Activation of Amino Acid by Carbodiimide with possible side product formation
2.2B **ONIUM REAGENTS**

To suppress the racemization and side reactions caused by carbodiimides nucleophilic additives are added to the system, which react with the *O*-acylisourea to form a new acylating species as shown in figure. These new acylated species are designed so that they can discriminate which amine to react with without undergoing racemization. HOAt/DIC mixtures were better at retaining amino acid stereochemistry during coupling reaction than HOBt/DIC systems. Further attempts to improve preservation of configuration by introducing more electronegative substituents to the HOBt core did not lead to any improvement.\(^{31}\)

![Scheme 2.4 Activation of Amino Acid by Onium Reagents](image)
### 2.2c Phosphonium Reagents

A slightly different approach for the activation process was to deprotonate the carboxylic acid of an amino acid with a base which then reacted with an activating group. Phosphonium based activating reagents were known to activate by substitution of chloride as shown in scheme 2.5.

![Scheme 2.5 Activation of Amino Acid by Phosphonium Reagents](image)

<table>
<thead>
<tr>
<th>X</th>
<th>R₂</th>
<th>Activating agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl</td>
<td>NMe₂</td>
<td>CloP</td>
</tr>
<tr>
<td><img src="image" alt="Ring 1" /></td>
<td>NMe₂</td>
<td>BOP</td>
</tr>
<tr>
<td><img src="image" alt="Ring 2" /></td>
<td>NMe₂</td>
<td>AOP</td>
</tr>
<tr>
<td><img src="image" alt="Ring 3" /></td>
<td></td>
<td>PyBOP</td>
</tr>
<tr>
<td><img src="image" alt="Ring 4" /></td>
<td></td>
<td>PyAOP</td>
</tr>
</tbody>
</table>

Scheme 2.5 Activation of Amino Acid by Phosphonium Reagents
The chloride leaving group was replaced by anions of HOBt and HOAt to get BOP and AOP respectively. The toxic byproduct of BOP was HMPA and hence the for safety reasons the functionality of phosphonium was modified which led to PyBOP and PyAOP. PyBOP has been shown to be useful for coupling hindered amino acids. It can be used in excess and more can be added during the coupling. Purity of PyBOP is important since presence of pyrrolidine can cause unwanted derivatization of the amino acid carboxylic group.\textsuperscript{22}

Scheme 2.6 Different Activation Routes of Amino Acid by Phosphonium Reagents

Two different activation routes for phosphonium based systems have been known. N-guanidinium salt of HOBt and HOAt gave HBTU and HATU respectively both having better
reactivity than their precursors. It has to be noted that changing the non-nucleophilic counterion for these salts did not change the reactivity of the coupling agent. Uronium reagents are generally more stable than phosphonium reagents whereas in the presence of a base the phosphonium reagents were more stable. Uronium reagents derived from HOAt were more efficient than the ones derived from HOBt because of the stabilization of the activated ester intermediate by hydrogen bonding with the additional nitrogen atom of HOAt. Inspite of better efficiency the higher cost of HOAt and its derivated than that of HOBt and its derivatives kept its use somewhat restricted to situations where product yield was of utmost importance.

![Figure 2.2 Structures of HBTU and HATU](image)

**X = CH for HBTU**  
**X = N for HATU**

### 2.3 N-Terminal Protecting Groups

The N-terminal of the amino acid is protected with a temporary protecting group which is generally a urethane derivative and can be removed under mild conditions without racemization. *tert*-Butyl carbamate (BOC) and 9-fluorenymethyl carbamate (Fmoc) are the two commonly used protecting groups as shown in figure 2.3.
Boc protecting group is removed using 25-50% trifluoroacetic acid (TFA) in dichloromethane. The final peptide cleavage from the solid phase is done using hydrofluoric acid. The side group protecting groups during Boc based peptide synthesis is chosen so that they are stable to TFA treatment during Boc deprotection. Boc protection is easy to introduce, results in good coupling yields and TFA deprotection is facile. Acidic conditions required for the removal of Boc is a disadvantage as it might induce removal of some of the permanent side chain protecting group like t-butyl. For a long peptide chain repeated treatment of TFA might lead to degradation of sensitive peptide sequences. Also some peptides cannot maintain their integrity during the final HF cleavage. Also HF is considered expensive and requires expensive laboratory apparatus.

![Boc and Fmoc Protecting Groups](image)

**Figure 2.3** Structure of Boc and Fmoc Protecting Groups

Fmoc protecting group can be used with an orthogonal protection scheme since it can be removed using a mild base like piperidine which is a much milder condition compared to using TFA to deprotect Boc. Fmoc protected amino acids were chosen for our peptide synthetic schemes.
2.4 RESULTS AND DISCUSSION

2.4a SYNTHESIS OF DOTA-SP-NH$_2$

![Reaction setup for Solid Phase Peptide Synthesis](image)

Figure 2.4 Reaction setup for Solid Phase Peptide Synthesis

Rink Resin HS 100-200 mesh, 1% DVB, 1.2mmol/g (0.5g, 0.6mmol) was suspended in ~ 3ml DMF and allowed to soak overnight in a fluorinated fritted funnel under nitrogen purge. The next day the Fmoc deprotection was removed using 20% piperidine in DMF (3 x 2.5ml) for 30min, washed with (3 x 5 ml) DMF for 10min. The complete removal of Fmoc protection was checked using was tested with standard free amine tests. Attachment of the amino acid started from the last amino acid in the desired peptide sequence. The Fmoc protected amino acid (1.8mmol), HOBt (243.2mg, 1.8mmol) and DIC (227.2mg, 1.8mmol) was dissolved in minimum volume of DMF in a 10ml round bottomed flask under nitrogen and stirred for 30 min. After 30 min any
precipitate observed was removed by filtration. The filtrate was then added to the free amine on the solid phase and purged under nitrogen for 3 hours. After 3 h the reaction mixture was washed with dimethylformamide and few resin beads were tested with standard free amine test. If test results showed colored beads, meaning incomplete coupling, coupling was repeated with same stoichiometry. This cycle of deprotection and coupling was repeated for corresponding amino acids. After the required amino acid sequence was achieved the peptide sequence was washed with dry NMP and then suspended in NMP. The free amine of the peptide was mixed with Bromoacetyl Bromide (0.521 ml, 6mmol) and DIEA (0.261 ml, 1.5 mmol) stirred for 3 hours. The same standard tests for free amine were done to check if Bromoacetyl Bromide was completely attached and if the reaction was to be repeated. The reaction mixture was washed with NMP (3 x 3 ml) and DCM (3 x 3 ml). The resins were suspended in DCM and cyclen (6.718 g, 39 mmol) was added in two parts and purged under nitrogen for 2.5 hours. The filtrate from the reaction mixture was stored to recover excess cyclen from the reaction mixture. The resin was washed with DMSO (5 x 5 ml) and NMP (5 x 5 ml). The resin was suspended in 5ml NMP and tert-butyl bromoacetate (1.776 ml, 12 mmol), DIEA (2.088 ml, 12 mmol) were added and purged under nitrogen for 19 hours. The resins were washed with NMP (3 x 3 ml), DMF (3 x 3 ml) and DCM (3 x 3 ml). Resins were dried under vacuum. The DOTA-peptide conjugate was removed from solid phase using cleavage cocktail made (3 x 10 ml)) consisting of 95% trifluoroacetic acid, 2.5% triethylsilane and 2.5% water for 45 min each. The filtrate from cleavage mixture was rotary evaporated to get the crude DOTA-peptide conjugate. This protocol is illustrated in scheme 2.7.
Scheme 2.7 Solid Phase Peptide Synthesis using Cyclen as the macrocycle.

30% Piperidine in DMF (v/v) was used for deprotection. Amino acid, HOBt and DIC were used in 3-fold molar excess, tert-Butyl Bromoacetate was used in 10-fold molar excess, DIEA
was used in 2.5 molar excess and Cyclen was used in 65-fold molar excess with respect to the solid phase.

**Recycling Cyclen**

The washings from the reaction of cyclen with the solid phase were combined and rotary evaporated. The solid mixture (0.3g) of cyclen and its cyclen.HBr salt was dissolved in NaOH solution (80ml, 3M) and was extracted with chloroform (3 x 25 ml). The combined chloroform layer was dried over anhydrous MgSO₄ and rotary evaporated to get a white residue. The white residue matched reported NMR for cyclen and was used without further purification.

### 2.4b Characterization of DOTA-SP-NH₂

Yellow oil was isolated after removal of TFA under rotary evaporation keeping the temperature of the water bath below 30°C. The crude was extracted with 10ml diethyl ether and kept at -20°C for 30 min. The ether layer became cloudy which was centrifuged at 4°C to get a title compound as a white residue (220mg, 62%). The LCMS spectrum is shown in figure 2.5.

![Figure 2.5 LCMS spectra](image)

Figure 2.5 LCMS spectra of crude showing DOTA-SP-NH₂ (peak1) and DOTA-(SP-NH₂)₂ (peak2). Integrated ESI spectra of peak 1 shown in figure 2.6. Structure of DOTA-(SP-NH₂)₂ shown in figure 2.7.
The top spectrum shows the overall spectra where the two peaks are observed. The integral of the peak 1 gives major ions of 588.3 m/z and 610.3 m/z which corresponds to the [M+H]$^{+1}$ and [M+Na]$^{+1}$. ions from the title compound as shown in figure 2.6.

Figure 2.6 Integration of LCMS peak 1 in figure 2.5 to get the ESI spectra showing 610 m/z corresponding to [M+Na]$^{+1}$ ion for DOTA-SP-NH$_2$

The major ion in the peak corresponded to 771.4 m/z which correspond to the disubstituted macrocycle as shown in figure 2.7.
Figure 2.7 Compound corresponding to peak 2 in LCMS of figure 2.5. Relative position of the peptide on the macrocycle was not determined.

The LCMS in figure 2.5 shows that the disubstituted macrocycle is of considerable amounts if not equal. The formation of the disubstituted macrocycle was unexpected due to the nature of the synthetic route taken. Since SPPS favours linear chain growth it was unforeseen that there could be a disubstitution possible. During peptide synthesis the side chain of the peptide were protected using permanent protecting groups but the last coupling of the macrocycle to the peptide strand could happen on more than one amine on the macrocycle since they were not protected. The cyclen could react with one strand on the solid phase and then react with another strand which is within reach on the solid support. The large amounts of disubstituted macrocycle formed showed that the peptide strands on the solid phase might be close enough for this unwanted reaction to occur. To improve the synthesis future modifications could be to change the solid phase support so that the distance between the peptide stands are large enough to avoid attachment of different
strands to the same macrocycle. Other protocol could be to use a macrocycle which was functionalized in such a way that only one stand of peptide could be attached to the macrocycle.

2.4c SYNTHESIS OF Gd-DOTA-SP-NH₂ AND Gd-DOTA-(SP-NH₂)₂

The mixture of ligands DOTA-SP-NH₂ and DOTA-(SP-NH₂)₂ were coupled to GdCl₃ hydrate as shown 2.8 without prior purification to observe the solubility characteristics of the complex. The ligand mixture was dissolved in basic water due to poor solubility in the acidic water. After addition of GdCl₃, the pH was maintained at 6.8 using 0.1N sodium hydroxide. On the pH had stabilized the reaction mixture was stirred for 72 hours since the complexation process in slow. The mixture yielded a precipitate which was isolated by centrifuging at a rate of 8000rpm at 4°C. The precipitate was further washed with chilled water to remove unreacted Gd(III) ions. The precipitate was solubilized in a mixture of acetonitrile, isopropanol and water with minute amounts of formic acid due to insolubility in water. Formation of Gd-DOTA-SP-NH₂ was confirmed using LCMS as shown in figure 2.8.

![Scheme 2.8 Complexation of DOTA-SP-NH₂ with GdCl₃ hydrate maintained at a pH of 6.5.](image-url)
Figure 2.8 LCMS of crude Gd-DOTA-SP-NH₂ (top) showing the a single peak. Integral of the single peak of the LCMS corresponding showing [M+H]⁺ ions of Gd-DOTA-SP-NH₂ in the ESI spectra (bottom).

The insolubility of Gd-DOTA-SP-NH₂ in water is makes it impossible to be useful for cell membrane permeation. Also, enzymatic activity on Gd-DOTA-SP-NH₂ is highly improbable if it is not soluble in aqueous medium which is where the MAP kinases are localized. These limitations make the use of Gd-DOTA-SP-NH₂ as a contrast agent for visualizing enzymatic activity unviable. Gd-DOTA is highly soluble in water due to the overall negative charge on the complex. Although upon formation of the amide bond with one of the acetates on DOTA with
the peptide the source of negative charge is lost resulting in a neutral Gd-DOTA-SP-NH₂ complex. The polar functional groups of the DOTA-SP-NH₂ are expected to have ionic interactions with Gd(III) making as ionic shell where the Gd(III) ion resides. The exterior non-polar regime of the molecule is exposed to the surrounding water which renders it insoluble. It is perceived that Gd-DOTA-SP-NH₂ complex would have a very low contrast due to no water molecules in the inner coordination sphere. This would be considered an ideal OFF state for the contrast agent which if could have been phosphorylated by MAP kinase and isomerized by Pin1, introducing be water molecule to the inner coordination sphere possibly turning it an ON contrast agent. To improve the solubility properties of the contrast agent, lysines were attached to the peptide sequence as described in chapter which led to the subsequent molecules.

2.4d Alternative Route to Conjugate Peptide with Macrocycle

In pursuit of attaching only one peptide in the solid phase to one macrocycle, coupling of DOTA was attempted to AAAA bound to the solid phase as shown in scheme 2.6. The product obtained after cleavage from solid phase was analyzed using mass spectrometer which did not show any ion which correlated to any of the possible products. This route was hence discarded without further investigation.
2.5 CONCLUSION

Cyclen was not an ideal synthon for the synthesis of the DOTA-peptide conjugates. There were unexpected side products resulting from over substitution of the macrocyclic ligand. Hence alternative macrocyclic molecules have to be used which have only one functionalizable side chain to avoid any over substitution of the macrocycle. Also, the solubility characteristic of the Gd-DOTA-SP-NH2 was very poor suggesting that hydrophilic amino acids should be attached to the peptide chain to obtain a more soluble contrast agent. Also changing the macrocyclic synthon from cyclen to DOTA resulted in no product formation.
CHAPTER 3 MACROCYCLE SYNTHESIS

3.1 INTRODUCTION

Cyclen is one of the common scaffolds used for building macrocyclic chelators for lanthanides like Gd$^{3+}$. As described in section 2.4a, the direct functionalization of the cyclen on the solid phase using SPPS as shown in scheme 2.5 resulted in considerable bifunctionalization of cyclen. To solve this problem, cyclen was modified to achieve a macrocyclic ligand which has only one reactive side arm. The modified cyclen can be typically shown in the scheme 3.1 which has three $t$-butyl esters as permanent protecting group and one free carboxylic acid moiety.

![Scheme 3.1 DOTA with one free carboxylic acid](image)

This modification allows the only one free carboxylic acid as the reactive site. Using SPPS the carboxylic end can be coupled to the deprotected amine of an amino acid moiety. The reaction times are expected to be greater than an amino acid coupling due to the steric reasons. The $t$-butyl esters are compatible with the Fmoc based SPPS where they are deprotected in the
last step of SPPS using the TFA cleavage cocktail. This is quite different from the synthetic scheme used previously where the linker, cyclen and the three α-esters are added in three subsequent steps. Whereas, using DOTA –tris-(t-bu) ester the SPPS is cut down by two steps which is beneficial in improving reaction yields as shown in the scheme 3.2.

Scheme 3.2 Target reaction schematic using DOTA-tris (t-Bu) ester
3.2 RESULTS AND DISCUSSION

3.2A SYNTHESIS OF 1,4,7-TRIS(3-TERTY-BUTOXYCARBONYLMETHYL)-1,4,7,10-
TETRAAZACYCLODODECANE, HYDROBROMIDE SALT.

Scheme 3.3 Synthetic strategies for 1,4,7-Tris (tert-butoxycarbonylmethyl)-1,4,7,10-
tetraazacyclododecane, hydrobromide salt

Direct trisubstitution of cyclen was achieved by Parket et al., without the need for protecting
the fourth nitrogen. Under the mild alkylation conditions of using sodium bicarbonate as base,
acetonitrile as solvent, temperature of 20°C and stirring it for 48 h resulted in only the
trisubstituted product. The alkylation of the 4th nitrogen on cyclen did not occur until the
temperature was increased or a stronger base like cesium carbonate was used. Another method
published in 2008 used the insolubility of the trisubstituted salt of the product in
dimethylacetamide which prevents alkylation of the fourth nitrogen. The setup in this reaction
was quite unique as it required a mechanical stirrer since the reaction mixture is heterogeneous
and performing the reaction using an ordinary stir bar did not result in good reaction yields. Due to the heterogeneous nature of the reaction mixture, maintaining the right temperature by proper stirring was crucial. The water bath needed to be cooled towards the last third of the addition of the tert-butyl bromoacetate because of the exothermic nature of the step. Low temperature requirements were similar to the previous procedure. Reaction temperatures were maintained between 25°C-26°C. Extended period of exposure to temperatures above 35°C has been reported to result in tetraalkylation. Both methods were used for synthesizing the trisubstituted cyclen but the former methods was preferred due to easier setup conditions and comparable yields. LCMS of title compound is shown in figure 3.1.

![LCMS graph](image)

Figure 3.1 LCMS of 1,4,7-Tris (tert-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane, hydrobromide salt
3.2b Synthesis of tris-t-butyl DOTA using Ethyl Bromoacetate

A stronger base and reflux conditions were used for alkylation of the fourth nitrogen as shown in scheme 3.4. The reaction took 2 days to complete but quantitative conversion was observed in LCMS as shown in figure 3.2.

Scheme 3.4 Synthesis of tris (t-Bu) mono ethyl DOTA ester using 4 equivalents of K$_2$CO$_3$ and 1.5 equivalents of Ethyl Bromoacetate at room temperature.

Figure 3.2 LCMS of tris (t-Bu) mono ethyl DOTA ester

The alkaline hydrolysis of the ethyl ester was done at elevated temperatures as shown in the scheme 3.5. The reaction was monitored over time using LCMS.
Scheme 3.5 Hydrolysis of tris (t-Bu) mono ethyl DOTA ester in 3:1 ratio of Dioxane and 0.1 M NaOH at 50 °C.

Allowing the reaction to run longer resulted in some product formation as observed by LCMS shown in the figure 3.3. Also, yields from the hydrolysis were low and surprisingly not very reproducible. The low yields and poor reproducibility of the hydrolysis reaction led us to change the synthetic strategy.

Figure 3.3 LCMS of the partially isolated tris (t-Bu) mono ethyl DOTA from the hydrolysis of tris (t-Bu) mono ethyl DOTA ester showing other impurities.
3.2c Synthesis of tris-t-butyl DOTA in one step using bromo acetic acid

A modified route which allowed direct preparation of the monoacid was attempted conditions as reported in literature\textsuperscript{37,38} was attempted. The protocol was first attempted with equimolar amounts of trisubstituted cyclen and bromoacetic acid. LCMS showed poor yields. Using four times the moles of bromoacetic acid resulted in yield to increase. Eventually an eight time molar excess of bromoacetic acid was used. Converting the trisubstituted cyclen salt to its free base form did not improve yields or product distribution. The LCMS from the crude mixture from a reaction using eight molar excess of bromoacetic acid is shown in figure 3.4.

![Diagram](image)

Scheme 3.6 One step synthesis of tris (t-Bu) DOTA using 8 equivalents of K$_2$CO$_3$ and 3.5 equivalents of bromoacetic acid.

As shown in figure 3.4 the shaded peak corresponding to tris (t-Bu) DOTA along with peaks of compounds shown in the figure 3.5 which coeluted with tris (t-Bu) DOTA. The product was partially formed with considerable amounts of starting material still remaining, represented by
the unshaded peak. The poor yields of the reaction and multiple side products lead in modification of the synthetic scheme. It also shows the importance of using an ester protected bromoacetate in eliminating the formation of side products.

Figure 3.4 LCMS of the reaction mixture shown in scheme 3.6. Shaded peak refers to product peak along with side products and unshaded peak at 7.5 min refers to starting material.

Figure 3.5 Side products formed from reaction scheme 3.6
3.2d **Synthesis of tris-t-butyl DOTA using Benzyl Bromoacetate**

![Chemical Structure]

Scheme 3.7 Synthesis of tris (t-Bu) mono benzyl DOTA ester using 5 equivalents of NaHCO₃ and 1.3 equivalents of benzyl bromoacetate at 100 °C.

Using benzyl bromoacetate with sodium bicarbonate as a base under reflux conditions resulted in the quantitative formation of the tris (t-Bu) mono benzyl DOTA ester in 24h as shown in scheme 3.7. The reaction was clean and no impurities were observed in the LCMS of the crude product shown in figure 3.6.

![LCMS Graph]

Figure 3.6 LCMS of crude tris (t-Bu) mono benzyl DOTA ester
tris (\textit{t}-Bu) mono benzyl DOTA ester was hydrogenated in a Parr low pressure reactor as shown in scheme 3.8 using hydrogen at a pressures of 80 psi using 10\% Pd/C as catalyst. Trace amounts of ethanol was added to the 10\% Pd/C to avoid flash fire. The conversion was continued till benzyl protection was removed. Generally, 24 h of hydrogenations was required for complete deprotection as shown by LCMS in figure 3.6. The ease of alkylation of trisubstituted cyclen using benzyl bromoacetate and its facile removal using hydrogenation made this is the preferred route for tris (\textit{t}-Bu) DOTA synthesis.

Scheme 3.8 Synthesis of tris (\textit{t}-Bu) DOTA by hydrogenation of tris (\textit{t}-Bu) mono benzyl DOTA ester using Parr low pressure reactor at 80 psi of H2 using 10\% Pd/C as catalyst at room temperature.
Figure 3.7 LCMS of crude tris (t-Bu) DOTA ester showing predominant product peak at 11 min.

3.2E SYNTHESIS OF TRIS- T-BUTYL MONO NHS DOTA ESTER

The free carboxylic acid of tris (t-Bu) DOTA ester was activated as an N-hydroxysuccinimide ester. N-hydroxysuccinimide was coupled to the carboxylic acid using HATU as the coupling reagent as shown in scheme 3.9.

Scheme 3.9 Synthesis of tris (t-Bu) mono NHS DOTA ester using 1.1 equivalents of N-hydroxysuccinimide, 1.1 equivalents HBTU and at room temperature.
Crude product was crystallized from ethyl acetate resulting in title compound in pure form as shown by LCMS in figure 3.8. The NHS-ester prepared from tris (t-Bu) DOTA ester was used without any further purification for coupling to a free amines on a peptide strand on the solid phase.

Figure 3.8 LCMS of crystallized tris (t-Bu) mono NHS DOTA ester

3.2F COUPLING OF TRIS (T-BU) MONO NHS DOTA ESTER TO PEPTIDE ON SOLID PHASE

This test experiment was run to understand the characteristics of the coupling reaction along with the potency of the cleavage mixture to deprotect all the permanent t-butyl protecting groups. The test coupling was done using a single alanine attached to the solid phase which was coupled to tris (t-Bu) mono NHS DOTA ester as shown in scheme 3.10.
Scheme 3.10 Optimization of cleavage and deprotection duration

LCMS of the cleaved product after 1 h showed only one LC peak although there were multiple ions in the MS of the peak integral as shown in figure 3.8. Apart from the [M+1]$^+$ and [M+Na]$^+$ ions, some other quite intense ions under the same peak area were characterized as [M+t-Bu+H]$^+$, [M+t-Bu+Na]$^+$, [M+(2*t-Bu)+H]$^+$, [M+(2*t-Bu)+Na]$^+$ as shown by the MS spectra. Allowing the reaction mixture to stir over a period of 20 h at room temperature showed complete product cleavage with [M+1]$^+$ as the predominant ion in the LCMS as shown in figure 3.9.
Figure 3.9 LCMS of the reaction mixture after 1 h showing ESI spectra from integral of peak at 1.8 min.
Figure 3.10 LCMS of the reaction mixture after 20 h showing ESI spectra from integral of peak at 5.5 min ran at a slower gradient compared to LCMS in figure 3.9.

3.3 CONCLUSION

The best route to synthesis of large scale amounts of tris (t-Bu) mono NHS DOTA ester was achieved. The functionalization of the trisubstituted cyclen with benzyl bromoacetate and then deprotecting the benzyl functionality by hydrogenation gave the best yields and reproducible results for synthesizing tris (t-Bu) DOTA ester. The further functionalization of the tris (t-Bu) DOTA ester with NHS ester resulted in the desired tris (t-Bu) mono NHS DOTA ester with only
one activated carboxylic acid. Also coupling of tris (t-Bu) mono NHS DOTA ester with a simple amino acid on the solid phase was successful. The time required for complete deprotection of all the t-Butyl groups in tris (t-Bu) mono NHS DOTA ester was optimized for SPPS cleavage conditions. The t-Butyl groups in the tris (t-Bu) mono NHS DOTA ester are the most sterically hindered protecting groups and need the most time to get complete deprotection. Hence, the deprotection times optimized for these t-Butyl groups are enough for the complete removal of any side chain permanent protecting group on the amino acid functionality.
CHAPTER 4 SYNTHESIS OF A LIBRARY OF COMPOUNDS

4.1 INTRODUCTION

The synthetic approach for building a library of compounds is fundamentally different from that of conventional organic synthesis. In general organic chemistry, the target is to synthesize one compound whereas in a library synthesis the goal is to synthesize a large number of different compounds simultaneously. The library of compounds synthesized contains compounds in all required configurations of the building blocks. The library synthesis results in the preparation of the desired compounds in pure form but as an inseparable mixture. This technique is extensively used in drug discovery and material science where a large number of compounds are screened for a particular function. The target compound is found using a high throughput screening. Three different approaches to preparing this library are mixture synthesis, parallel synthesis and combinatorial synthesis.

4.1A MIXTURE SYNTHESIS

This method requires the introduction of all different reagents to the whole pool of resins at every step as shown in figure 4.1. Even though this method results in a large number of compounds in a few steps there are some serious disadvantages. One is the analysis of the purity of the individual compound and their relative amount. Additional work is needed to determine which one is the biologically active compound. Generally a smaller library is synthesized to
determine the biologically active compound but sometimes it is hard to pin point the actual active molecule due to synergistic activity of a few molecules to get the desired activity.

![Diagram of mixture synthesis](image)

Figure 4.1 Strategy for mixture synthesis.

### 4.1b Combinatorial Synthesis

This technique evolved in the early nineties and its use has increased exponentially since then. The general schematic for this protocol is shown in figure 4.2. The splitting of resins into smaller batches and then individually reacting each with A1, A2 and A3 incorporates the first level of diversity. The number of batches is equal to the number of variants of the first reagent. This way individual batch of reins can be tested for completion of reaction. The smaller batches
are pooled together after washing and deprotection. It is then split into smaller batches to be reacted with B1, B2 and B3. After washing and deprotection the batches are again pooled together. At this point there are 9 different compounds in the pool. As above, splitting into smaller batches and reacting each batch with C1, C2 and C3 results in 27 different compounds. After this protocol one single compound is bound to the resin and hence the term one bead one compound (OBOC) was coined which is synonymous with split-pool synthesis. Advantages of this method are that it requires a very few number of reaction vessels and yet results in a large library. Disadvantage is the amount of synthesized compound synthesized in very small since basically one bead is used to characterize the compound. Hence, sensitive equipment is required for analyzing such low concentrations. The reactions should not be sensitive to steric or presence of functional groups.

4.2 APPLICATION IN ISOLATING THE IDEAL Gd-DOTA-Peptide Conjugate

The potential of these library synthesis schemes lie in identifying the ideal Gd-DOTA-peptide conjugate which is a substrate for ERK and Pin1 and also, showed the desired increase in contrast upon activity. Although the peptide substrates for MAP kinases are known, the substrate recognition might change if there is a Gd-DOTA moiety close to the phosphorylation site on the peptide. Gd-DOTA complex can be considered as a large non polar molecule since most of the polar groups are inwards in the macrocyclic chelate to interact with the Gd(III). There appropriate distance between the phosphorylation site on the peptide and Gd-DOTA will allow the phosphorylated moiety of the peptide to enter the enzyme phosphorylation pocket without
any steric interactions from Gd-DOTA. Also, if the enzyme phosphorylation pocket is too hydrophilic the Gd-DOTA might have unfavorable interactions prohibiting proper enzyme substrate interaction. For this technique to work we had to make sure that the available Varian 500 LCMS we use to analyze our compounds are detecting the molecules from a single bead and the intensity is higher than the detection limit of the machine. Hence, individual beads had to be analyzed for moles of compound obtained per bead to get an idea of detectability by the LCMS.
Figure 4.2 Strategy for combinatorial synthesis.
4.3 Results and Discussion

4.3A Estimation of a Bead Size

A high resolution microscope was used to measure the diameter of a sample of resin beads solvated overnight with DMF. The swollen beads were bigger which made it easier to analyze. The data set is tabulated in table 4.1.

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Table 4.1 Diameter (D) of 64 beads after swelling them overnight in DMF.
Average bead diameter was found to be 153 µm with a standard deviation of 24 µm which shows the diversity of the bead sizes. The varying bead sizes indicate that the amount of functionalization available on the bead surface also varies bead to bead. For beads showing sizes as low as 110 µm the cleaved sample from the bead is going to be considerably small which could possibly hamper the final analysis of the compound. Also snapshots of the resin beads showed imperfection on the bead surface and were not all spherical as assumed. Also some beads appeared completely dark under the microscope which probably meant that these were defective beads with no micro channeling or were not functionalized properly.

Figure 4.3 a) Normal resin beads showing size distribution b) Distorted bead c) Defective bead
### 4.3b Average Functionalization per Bead

<table>
<thead>
<tr>
<th>Resin Weight (mg)</th>
<th>Number of Resin Beads</th>
<th>Count 1</th>
<th>Count 2</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4</td>
<td></td>
<td>136</td>
<td>124</td>
<td>130</td>
</tr>
<tr>
<td>0.9</td>
<td></td>
<td>310</td>
<td>322</td>
<td>316</td>
</tr>
<tr>
<td>0.8</td>
<td></td>
<td>300</td>
<td>290</td>
<td>295</td>
</tr>
</tbody>
</table>

Average mass per bead = 2.9 µg

**Moles per bead with 1.2 mmol/g loading = 3.5 nmol**

Number of functional groups per bead = \(2.1 \times 10^{15}\)

**Minimum area per functional group on the bead = 3.5 \times 10^{-3} \text{ Å}^2**

Table 4.2 Calculations for moles per bead and minimum area per functional group on the beads.

From the above analysis it was shown that that the Rink bead beads from Advanced Chemtech had 3.5 nmol of compound per bead. The limit of detection on the Varian 500 LCMS is in the nanomolar concentration so the compounds obtained from cleavage of a single bead is definitely enough for LCMS detection. Obviously the sample dilution is important in this case to maintain nanomolar concentrations. Also typically it is seen in the LCMS that the detection limit lower for higher molecular compounds and drops down immensely if the compounds have metal
incorporation. The area per functional bead was calculated based on the assumption that there are no micro-channels within the bead which is not the case. The calculated surface area is actually much higher due to the presence of micro-channels in the bead. So the calculated value for functional groups per unit area on the bead is the minimum proximity of two functional groups on the bead surface. The value suggests that the functional groups can be close enough if the functionalization of the resin is not properly homogenized. Also as seen from the microscope images of the bead in figure 4.3(c) there could be beads which have improper or no channeling which will lead to majority of functional groups localized on a particular region of the bead leading to very close proximity of functionalization.

4.3c Single Bead Analysis

One single resin bead functionalized with Fmoc-SPK was taken in a 5ml round bottomed flask and cleaved with the 1.5 ml of cleavage cocktail (95% TFA, 2.5% Water and 2.5% TIS) and stirred overnight. The round bottomed flask was rotary evaporated and re-suspended in 10 µL of DMSO. A duplicate sample was run with another single resin bead. These samples were taken in inserts designed for micro-liter pickup in the LCMS and minimum dilution was used so that the sample would be of the highest concentration possible yet the tip of the autosampler needle would be below the sample in the sample vial. Sample concentrations attained were approximately 70 µL. Fmoc-SPK-NH₂ was the compound bound to the resin which was used for this analysis as shown in figure 4.4. LCMS data from the single bead cleavage did not show required peaks for Fmoc-SPK-NH₂ as shown in figure 4.5(1).
Figure 4.4 Structure of Fmoc-SPK-NH₂ with corresponding m/z distribution.

Figure 4.5 LCMS analysis of Fmoc-SPK-NH₂ cleaved from the resin using (1) Single Bead (Top) and (2) Multiple Beads (Bottom).

To check the consistency of the compound obtained by LCMS of the single bead a large scale cleavage was done. The cleavage was performed with the same cleavage cocktail and stirred
overnight. The sample was diluted to the required LCMS sample concentrations. As shown in figure 4.5(2). The LCMS clearly showed the required compound as \([\text{M+H}]^+\) and \([\text{M+Na}]^+\) ions. The failure of the single bead to show the desired peak suggests that detecting the functionalization on a single bead is not possible with our current LCMS. Possibly a mass spectrometer of greater sensitivity will be required for conducting such experiments. This failure to detect the mass of the compound cleaved from a single bead implies that we cannot analyze a library of peptides synthesized by either combinatorial synthesis or mixture synthesis using Varian 500 LCMS.

4.4 CONCLUSION

![Figure 4.6 Hypothetic Coupling Scheme of pre-complexed Gd-DOTA with a peptide on the solid phase.](image)

Figure 4.6 Hypothetic Coupling Scheme of pre-complexed Gd-DOTA with a peptide on the solid phase.

It should also be noted that the final goal is to test these Gd-DOTA-peptide conjugates as substrates for MAP kinases and Pin1 and finally test them for proton relaxivity changes in the MRI. Even if a synthetic scheme as shown in figure 4.6 is tried where a precomplexed Gd-DOTA is conjugated to a peptide strand immobilized on the solid phase to avoid the synthetic
challenges of conjugating the Gd to the DOTA-peptide on the solid phase there are other problems that should be recognized. There is no certainty that the substrate recognition of the Gd-DOTA-peptide conjugate bound to the solid phase will correlate to the substrate recognition of Gd-DOTA-peptide conjugate in solution phase since the bead itself might keep the substrate to enter the active site of the enzyme or might cause steric hindrance so that the enzymatic reaction too slow. Also, Pin1 activity on the Gd-DOTA-peptide conjugate has to fold the peptide, which might be hampered due to the presence of the bead. Similar argument applies for relaxivity measurements between solid phase bound contrast agent and unbound contrast agent. In fact it is known that a bound contrast agent has greater relaxivity than an unbound contrast agent due to increase in rotational correlation time of the bound contrast agent. These drawbacks show that even if the synthetic and analytical challenges of the OBOC technique are overcome, there are other challenges in the assays and relaxivity measurements which need to be overcome.
CHAPTER 5 SYNTHESIS OF LIGANDS FOR CONTRAST AGENT

5.1 INTRODUCTION

5.1a PARALLEL SYNTHESIS

In parallel synthesis each reaction is run at different conditions much like organic synthesis. The concept used here is based on one compound one vessel. The general schematic is shown in figure 5.1. The reaction resins are split into separate building blocks the number of which depends on the total number of combinations possible. The compounds are thus always in spatially separated compartments. One third of the building blocks react with either with A1, A2 or A3, washed and deprotected. One third of the building blocks react with either B1, B2 or B3, washed and deprotected. One third of the building blocks react with either C1, C2 or C3, washed and deprotected. The advantage of this technique lies in the fact that the exact structure of the compound on the bead is always known. Relatively large quantity of the sample is available which allows semi-prep or even prep scale purification. Also biological evaluations are easier and more accurate since there is no dilemma of whether the assay technique which worked in the solid phase may or may not work in the solution phase. Drawbacks of this method, as can be envisioned, is that only a small sized library of compounds can be synthesized.
Figure 5.1 Strategy for parallel synthesis
5.1b Synthetic Peptide Substrate for ERK

Myelin basic protein (MBP) is a substrate for a number of kinases like MAP kinase family, cAMP-dependent protein kinases, Calmodulin-dependent protein kinases, protein kinase C and phosphorylase kinase. ERK phosphorylates MBP exclusively on the Thr-97 of the highly conserved partial sequence –Pro-Arg-Thr<sup>97</sup>-Pro-Pro-Pro.<sup>43</sup> Other ERK specific substrates like EGFR at Thr-669, human c-myc protein at Ser-62 and rat c-jun protein at Ser-246 gave a common phosphorylation sequence of Pro-Leu-Ser/Thr(P)-Pro. It was shown that the substrate requirements for ERK1 and ERK2 were very similar.<sup>44</sup> Using this basic sequence point mutation at the different amino acid residues provided a better understanding of the substrate requirements of ERK in specific. It was shown that replacement of the Leu residue with a basic amino acid like Arg or Lys causes phosphorylation rates to remain as fast or increase. The relative positioning of proline near the phosphorylation site is of utter importance for substrate recognition. Also, the N-terminal Pro had to be separated by one amino acid from the phosphorylated Thr. The substrate specificity outside the recognized phosphorylation sequence is known to be unconserved.<sup>45</sup> Further studies using synthetic peptide Ala-Pro-Arg-Thr-Pro-Gly-Gly-Arg-Cys attached to a poly-L-lysine and Lys-Lys showed increased phosphorylation rates with MAP kinase. It was also observed that the peptide lost activity towards cAMP-dependent protein kinase, protein kinase C and multifunctional calmodulin-dependent protein kinases making it a specific substrate for MAP kinase.<sup>46</sup>....
5.2 Desired DOTA-Peptide Conjugates

Parallel synthesis was used to obtain the desired DOTA-peptide sequence in a semi-quantitative scale. This was desired since the as there could be some variations incorporated into the peptide strand. The PRTP moiety is the minimum requirement for ERK to recognize the peptide as a substrate. The variations in the peptide chain are designed such that the PRTP moiety is placed at an incremental distance to the bulky, unnatural DOTA macrocycle. Also the presence of proline at a -1 position to the phosphorylated threonine is preferred but not required so for one of the peptide strands only the TP moiety was retained. The loss of PR and direct attachment of threonine to Gd-DOTA moiety brings the phosphorylation site very close to the Gd and could have some interesting activation mechanism. The desired DOTA-peptide conjugates synthesized were Gd-DOTA-TPGGRCKK-NH₂, Gd-DOTA-PRTPGGRCKK-NH₂ and Gd-DOTA-APRTPGGRCKK-NH₂ as shown in figure 5.2.

Figure 5.2 Desired DOTA-peptide conjugates
5.3 RESULTS AND DISCUSSION

The parallel synthesis was carried as shown in the figure 5.1. The common peptide strand, TPGGRCKK bound to the Rink resin was synthesized using peptide coupling strategies showed in scheme 2.5. One third of the resin was removed to be coupled with tris(t-Bu) mono NHS DOTA ester and then cleaved from the solid phase and deprotected with optimized conditions as shown in scheme 3.10. Two thirds of the beads were coupled to R and P, split in two parts. One part was coupled to tris(t-Bu) mono NHS DOTA ester and cleaved off the resin. The other part was coupled to A and tris(t-Bu) mono NHS DOTA ester after which it was cleaved off the resin.

5.3a PURIFICATION AND CHARACTERIZATION OF DOTA-TPGGRCKK-NH₂

A crude LCMS showed three peaks with the first peak being the desired compound as shown in top figure 5.3. The presence of lysine’s allowed the Gd-DOTA-TPGGRCKK-NH₂ to be extracted in the aqueous phase while other impurities to be retained in the ethyl acetate wash. A semiprep C18 column was used to purify the crude. LCMS of one of the HPLC fraction collected showed pure title compound with the ionization of the ion as [M+2H]⁺² as shown in figure.5.3.
5.3b Purification and Characterization of DOTA-PRTPGGRCKK-NH₂

The crude product was dissolved in water and impurities were extracted using ethyl acetate. LCMS of crude showed two peaks with the first peak being the desired one as shown in figure
5.4. A semiprep C18 column was used to purify the crude. LCMS of one of the HPLC fraction collected showed pure title compound as shown in figure 5.4.

Figure 5.4 LCMS of crude (top) and pure (bottom) DOTA-PRTPGGRCKK-NH₂
5.3c SYNTHESIS AND CHARACTERIZATION OF DOTA-APRTPGGRCKK-NH₂

The crude product was dissolved in water and impurities were extracted using ethyl acetate. The LCMS of the crude showed multiple peaks. Although some of these peaks were due to the variety of possible salts formed which were getting resolved in the LC. The crude was purified using semi-prep HPLC and checked again on LCMS for purity as shown in figure 5.5. A semiprep C18 column was used to purify the crude. LCMS of one of the HPLC fraction collected showed pure title compound with the ionization of the ion as [M+2H]²⁺, [M+3H]³⁺ and [M+3H+HCO₂H]⁴⁺ as shown in figure 5.5.
5.4 CONCLUSION

The ligands required for complexation to Gd(III) were successfully synthesized. The crude had minor impurities, considering the large number of consecutive synthetic steps involved in the SPPS. Also, as the peptide chain gets bulkier the coupling to the macrocycle gets more steric hindrance. Also the ligands obtained had high water solubility which was desired for achieving a
soluble contrast agent after Gd(III) coupling. The high solubility of the ligand also helped in purification by simple extraction techniques.
CHAPTER 6 SYNTHESIS OF THE DESIRED CONTRAST AGENTS

6.1 INTRODUCTION

Gd(III) ion is the preferred lanthanide for the preparation of contrast agent complexes. Firstly, the stability of complexes is highest for Gd(III) amongst all the other lanthanides. Secondly, it has a half filled 4f shell corresponding to an \( ^8S_{7/2} \) electronic ground state which gives it the high magnetic moment. The large number of unpaired electrons and low electronic relaxation rates make it ideal as a contrast agent. Across the lanthanide series, as the size of the lanthanide ions goes down, the coordination number for the smaller lanthanide ions drops from nine to eight due to steric reasons. The ligand field effects are small in case of lanthanides since the 4f orbitals do not effectively interact with the ligands of the complex. Gd(III) is the seventh element in the lanthanide series where the complex stabilization energy is maximum while still preferring a high coordination number of nine, although eight coordinate compounds are also known.\(^{47,48}\) The coordination modes of a nine coordinate Gd(III) is shown in figure 6.1. According to valence shell electron pair theory (VSEPR) tricapped trigonal prism is the most stable geometry for a coordination number of nine, while the capped square antiprism is slightly lower in energy.

![Figure 6.1 Coordination geometry of \([\text{Gd(H}_2\text{O)}_8]\)\(^{3+}\) (left) and \([\text{Gd(H}_2\text{O)}_9]\)\(^{3+}\) (right).](image)

Tricapped Trigonal Prism  
Capped Square Antiprism
The formation of macrocyclic chelates is thermodynamically favored and is particularly important for high coordination Gd(III) ions. Three ligands with two, three and four acetate groups attached to the nitrogens atom of cyclen have been of prime importance for the development of Gd(III) chelates. The equilibrium constant of the complexes are shown in table 6.1.

<table>
<thead>
<tr>
<th>Complex</th>
<th>log K at 25°C</th>
<th>q value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gd-EDTA</td>
<td>16.8&lt;sup&gt;26&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td>Gd-DO2A</td>
<td>19.4&lt;sup&gt;27&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td>Gd-DO3A</td>
<td>22.0&lt;sup&gt;27&lt;/sup&gt;</td>
<td>2</td>
</tr>
<tr>
<td>Gd-DOTA</td>
<td>24.0&lt;sup&gt;28&lt;/sup&gt;</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 6.1 Various Gd(Ligand) complexes showing correlation between the number of water molecules coordinated to the Gd(III) (q) and the thermodynamic stability of the complex (log K)

Gd-DO2A has much higher thermodynamic stability than Gd-EDTA due to the cyclic nature of the cyclen moiety giving it a more stable geometry. As the number of acetate groups on the cyclen is increased the stability of the complex is increased due to chelation effect. There is a two and a half log unit increase in the equilibrium constant going from Gd-DO2A to Gd-DO3A. There is a two log unit increase in equilibrium constant going from Gd-DO3A to Gd-DOTA which has a maximum number of acetate groups on the cyclen moiety. The high thermodynamic stability is one of the reasons for Gd-DOTA to be one of the first MRI contrast agents used for medical purposed. Crystal structure of Gd-DOTA shows a square antiprism arrangement where
the eight coordination sites are occupied by the four nitrogens of cyclen and the four carboxylate oxygens. The four five-membered chelates formed confer a high stability to this complex as shown in the figure 6.2.

![Diagram of Na[Gd(DOTA)(H2O)]·H2O](image)

Figure 6.2 The nine-coordinate molecular structure of Na[Gd(DOTA)(H2O)]·H2O as viewed along the crystallographic xy plane.54 (Viola-Villegas, N.; Doyle, R. P. Coord. Chem. Rev. 2009, 253, 1906)

As shown from the crystal structure of Gd-DOTA the ninth coordination site is occupied by a water molecule labeled O5 in the figure 6.2. The macrocyclic chelate structure in the solid phase is expected to be maintained in the solution phase. From relaxivity values the calculated number of water molecules in the inner coordination sphere of Gd(DOTA) is also one. The contrast achieved using a contrast agent is directly proportional to the number of water molecules in the inner coordination sphere (q value). Increasing the number of water molecules is at the expense of the complex stability as observed in the table 6.1.
6.2 SYNTHESIS, COMPLEXATION AND PURIFICATION TECHNIQUES OF Gd(III) TO DOTA-PEPTIDE CONJUGATE

The complexation of Gd\(^{3+}\) to the DOTA-peptide conjugates were done according to known literature methods. A wide variety of pH ranges exist for this complexation. Generally the pH is adjusted using a base and kept between 5.5 and 7.5 by quenches the liberated acid with an inorganic base\(^{55}\) as shown in the figure 6.3. Higher pH had the problem of formation of insoluble Gd(OH)\(_3\) \((K_{sp} = 1.8 \times 10^{-23})\)^\(^{56}\), whereas a pH too low results in protonation of the carboxylic oxygens leading to inefficient coordination to the Gd(III).

![Scheme 6.1 General scheme for chelation of Gd(III) with DOTA-peptide conjugates.](image)

Reaction times were long and generally stirring was continued for a week. The Gd\(^{3+}\) salt was used in excess and the complete removal of free Gd\(^{3+}\) from the sample was important because of two factors. Firstly, the free Gd\(^{3+}\) has a much higher proton relaxivity than the complexed-Gd which would lead to erroneous measurement of relaxivity for the actual Gd-complex. Secondly, free Gd\(^{3+}\) is highly toxic in living organisms. It is known to interfere with Ca\(^{2+}\) ions and has a LD\(_{50}\) of 0.4 mmol/kg in rats.\(^{20}\) Removal of free Gd\(^{3+}\) was carried out in more than one
subsequent step. Ultrafiltration if one the techniques where the product is washed using a membrane with the right porosity such that all the smaller components of the reaction mixture can pass through the membrane leaving the required molecular weight compounds\textsuperscript{55}. Cation exchange resins like Chelex 20, Sephadex or Amberlite in the K\textsuperscript{+}/Na\textsuperscript{+} form are commonly used to remove the excess Gd\textsuperscript{3+} where the Gd\textsuperscript{3+} binds to the resin releasing K\textsuperscript{+}/Na\textsuperscript{+} from the cation exchange resin.\textsuperscript{57} Ethylenediaminetetraacetic acid (EDTA) is added in some cases after the complexation is thought to be complete. EDTA is an excellent chelator and forms a Gd-EDTA complex thereby facilitating in removal if there is possibility for free Gd\textsuperscript{3+} to bind to other moieties on the required molecule. HPLC is also used to remove free Gd\textsuperscript{3+} ions and also purifying the compound from chelated and unchelated molecules. While using a reverse phase column this is done by initially using 100% aqueous phase as the mobile phase under isocratic conditions for a few column volumes to ensure that the free Gd3+, which does not stick to a reverse phase column, get flushed away leaving the pure complex to be eluted from the column. Finally the presence of free Gd3+ ions in the purified compounds can be tested with a colorimetric test known as the Xylenol orange test. The Xylenol Orange which is an organic dye is colorless in absence for free Gd\textsuperscript{3+}. In the presence of free Gd\textsuperscript{3+}F it shows a yellow color in an acidic solution and a violet color in a basic solution. The Gd-(Xylenol Orange) complex formed has a low thermodynamic stability (log K = 5.8) which makes free Gd3+ as its only substrate since it cannot abstract the Gd3+ from highly stable Gd-DOTA or Gd-DOTA-derivative complexes making it specific for Gd\textsuperscript{3+} ions. This is very sensitive test for free Gd\textsuperscript{3+} and not only does eye detection help in determination of presence of free Gd\textsuperscript{3+} of but also spectroscopic
techniques can be used for accurate determination of the amount of free Gd3+ ions in solution.\textsuperscript{58-59}

\textbf{6.3a Characterization of Gd-DOTA-TPGGRCKK}

The crude was purified was first treated with Chelex 20. Fractions collected from reverse phase semi-prep HPLC were directly infused in a QTOF-MS. Surprisingly; none of the peaks in the MS corresponded to the required peaks for Gd-DOTA-TPGGRCKK. No free ligand was observed. Some of the ions did have the profile of a Gd(III)-complex but did not correspond to any known ionization patterns. The title compound was hence not isolated.

\textbf{6.3b Characterization of Gd-DOTA-PRTPGGRCKK}

The crude was purified was first treated with Chelex 20. Fractions collected from reverse phase semi-prep HPLC were directly infused in a QTOF-MS. The fraction with the required $[\text{M+HCOOH+3H}]^{3+}$ as shown in figure 6.3 was collected. Xylenol orange test was negative showing absence of free Gd(III) ions.
Figure 6.3 QTOF of the HPLC fraction containing Gd-DOTA-PRTPGGRCKK

6.3c Characterization of Gd-DOTA-APRTPGGRCKK

The crude was purified was first treated with Chelex 20. Fractions collected from reverse phase semi-prep HPLC were directly infused in a QTOF-MS. The fraction with the required \([\text{M+3H}]^{3+}\) as shown in figure 6.4 was collected. Xylenol orange test was negative showing absence of free Gd(III) ions.
6.4 CONCLUSION

The contrast agents Gd-DOTA-PRTPGGRCKK and Gd-DOTA-APRTPGGRCKK were successfully synthesized. The contrast agent Gd-DOTA-TPGGRCKK was not detected in the QTOF but other ions with Gd-complex profiles were observed.
CHAPTER 7 RELAXIVITY MEASUREMENTS AND IN-VITRO ENZYME ASSAYS

7.1 INTRODUCTION

Relaxivity of the contrast agents was measured using inversion recovery experiment using a Bruker 7T MRI. Upon magnetization of the protons, the time required for it to decay to 63% of its initial magnetization is given by the longitudinal relaxation time ($T_1$). Relaxivity ($r_1$) is given by the slope of a $1/T_1$ vs [Gd] plot as described in section 1.5. Gd-DOTA was chosen as the standard contrast agent to compare relaxivity ($r_1$) of the synthesized contrast agents since the Gd(III) surrounding is very similar in both systems with the exception of the number of water molecules in the inner/outer coordination sphere ($q/q'$). The comparisons would lead to a relative estimation of the $q$ value of the synthesized Gd-DOTA-peptide conjugates based on the experimentally determined relaxivity ($r_1$). The synthesized contrast agents have electron donating atoms on the peptide sequences which could coordinate to the 9th coordination site of Gd-DOTA-peptide conjugate and decreasing the relaxivity. This coordination depend on the conformation of the peptide moiety around the Gd-DOTA moiety which could change upon phosphorylation catalyzed by ERK. Hence, relaxivity measurement before and after phosphorylation are important. Also, of paramount importance is to ascertain whether these synthesized Gd-DOTA-peptide conjugates are a substrate for ERK and, after phosphorylation, for Pin1. The structural changes associated with the isomerization of the phosphorylated peptide moiety by Pin1 is expected to have the most impact on relaxivity changes due it massive effect on the overall structure of the contrast agents as shown described in section 1.7.
7.2 RESULTS AND DISCUSSION

7.2a RELAXIVITY MEASUREMENTS FOR Gd-DOTA

Figure 7.1 Variation of longitudinal relaxation time of water ($T_1$) with varying [Gd-DOTA]

Figure 7.2 Variation of relaxation rates of water ($1/T_1$) with varying [Gd-DOTA]
7.2b Relaxivity for Measurements Gd-DOTA-PRTPGGRCKK

Figure 7.3 Variation of longitudinal relaxation time of water ($T_1$) with varying [Gd-DOTA-PRTPGGRCKK]

Figure 7.4 Variation of relaxation rates of water (1/$T_1$) with varying [Gd-DOTA-PRTPGGRCKK]
7.2c Relaxivity for Measurements Gd-DOTA-APRTPGGRCKK

Figure 7.5 Variation of longitudinal relaxation time of water ($T_1$) with varying [Gd-DOTA-APRTPGGRCKK]

Figure 7.6 Variation of relaxation rates of water ($1/T_1$) with varying [Gd-DOTA-APRTPGGRCKK]

\[ y = 4.01E-03x + 2.88E-04 \]
\[ R^2 = 9.98E-01 \]
7.2d Comparison of the Relaxivity Values (r1) of the Contrast Agents

The r1 values of the Gd-DOTA, Gd-DOTA-PRTPGGRCKK and Gd-DOTA-APRTPGGRCKK were 4.01 mM⁻¹ s⁻¹, 0.173 mM⁻¹ s⁻¹ and 4.01 mM⁻¹ s⁻¹ respectively. The low r1 of the Gd-DOTA-PRTPGGRCKK was promising since its relaxivity was close to systems with an inner sphere water coordination number of zero. This meant that the peptide chain was coordinating or blocking the coordination of water to the 9th coordination site of the Gd-complex according to our hypothetic scheme 1.2. To further elucidate the structure hyperchem calculations were done using CHARMM to show the PRTPGGRCKK moiety orientation around the Gd-DOTA moiety which is shown in figure 7.7.

Figure 7.7 Calculated structure for Gd-DOTA-PRTPGGRCKK
....The calculated structure shows that the proline and arginine residues hover over the 9th coordination site of the Gd(III) blocking bulk water to exchange with the coordinated water thereby causing low relaxivity. Other possibility is that if the nitrogens of the arginine moiety is are engaged in a direct coordination to Gd(III) which is not simulated by hyperchem but would lead to the lowering of relaxivity as well. The implications of the similar relaxivity of Gd-DOTA and Gd-DOTA-APRTPGGRCKK are discussed in section.

7.2E γ-33P-ATP based substrate assay for ERK1

During the phosphorylation event the terminal or γ-phosphate on the ATP is catalytically transferred to the substrate by ERK1 as shown in the scheme 1.1. The incorporation of a radioactive 33P isotope in the terminal phosphate allows the monitoring of the localization of the radioactive phosphoryl group. A series of concentrations of various substrates are allowed to react individually with the 33P-ATP in presence of ERK1 to get a correlation between the increasing substrate concentrations and the amount of radioactivity incorporated. For substrates which are phosphorylated an increase in the amount of radioactivity is expected with increase in substrate concentration since there is a greater amount of γ-33P phosphate incorporation on the substrate. For non-substrates, no change radioactivity is expected with change in concentration and most of the radioactivity contribution is from background radiation levels. This experiment could also show if there is any substrate inhibition at higher substrate concentrations of the substrate. In case of a substrate inhibition at high concentrations no increase in radioactivity is
expected at those concentrations leading to a decrease in slope. ATP is always in excess with respect to the substrate concentration so that there is no deceleration of the reaction due to lack of ATP. The isolation of the products was done by spotting the reaction mixture on to a P81 ion exchange paper prewetted with 1% phosphoric acid. The acidic P81 paper binds to the positively charged peptides and other reactants like excess radioactive ATP are washed away with dilute phosphoric acid. This leaves the substrates on a paper which can then be analyzed for radiation levels using a scintillation counter and counts per minute from individual substrates at various concentrations can be recorded. Washing is critical to remove all the unreacted $\gamma^{33}$P-ATP to avoid increase in background radioactivity.

Gd-DOTA-PRTPGGRCKK and Gd-DOTA-APRTPGGRCKK were tested for substrates of the enzyme ERK1. Blank samples were prepared by adding water instead of substrates. The radioactivity measured from the isolated products of the enzymatic phosphorylation was plotted with respect to concentration as shown in figure 7.8.
The phosphorylation product of Gd-DOTA-APRTPGGRCKK showed an increase in radioactivity with increasing Gd-DOTA-APRTPGGRCKK concentration. On the contrary, the phosphorylation product of Gd-DOTA-PRTPGGRCKK showed no increase in radioactivity and overlapped the radioactivity from the blank sample. The results suggest that the ERK1 recognizes Gd-DOTA-APRTPGGRCKK as a substrate and phosphorylates the threonine prior to proline. For Gd-DOTA-PRTPGGRCKK the PRTP moiety was not recognized by ERK1 and hence the threonine was not phosphorylated. The primary reason for this could be the proximity of the bulky Gd-DOTA moiety to the phosphorylation site which prevents the substrate from entering the active site of the enzyme to accept the phosphate from a molecule of ATP. It is interesting to note that the presence of only a single alanine molecule in between the Gd-DOTA
moiety and the PRTPGGRCKK moiety is enough to remove the steric limitation to substrate recognition. Increasing substrate to enzyme ratio did not affect the linearity of the plot which suggests that even at higher substrate concentrations the enzyme turns over all the substrate to product.

7.2f Large Scale Phosphorylation of Gd-DOTA-APRTPGGRCKK and Relaxivity Measurements

The enzymatic reaction conditions used for performing γ-33P-ATP based assay was scaled up to phosphorylate 2.8ml of 0.5mM Gd-DOTA-APRTPGGRC as shown in scheme 7.1. The molar ratio between Gd-DOTA-APRTPGGRC and ATP was kept constant with ATP being 70 times in excess. The cost of the ERK1 forced us to use 86 times more substrate than that was used for the assay. The final product obtained was used as is without purification. The blank sample was prepared without adding substrate to observe change in background levels of relaxivity.
Scheme 7.1 Large scale enzymatic phosphorylation of Gd-DOTA-APRTPGGRCKK (Peptide orientation around the Gd core is arbitrary)

Serial dilution was performed with phosphorylated Gd-DOTA-APRTPGGRCKK which was used to determine the $T_1$ of water in presence of the phosphorylated as before using the MRI simultaneously. The $1/T_1$ values were plotted against concentration of the phosphorylated Gd-DOTA-APRTPGGRCKK. The plot was superimposed on previously obtained $1/T_1$ versus concentration plots for Gd-DOTA-APRTPGGRCKK as shown in figure 7.8.
Figure 7.9 Comparison of relaxivity between phosphorylated and non-phosphorylated Gd-DOTA-APRTPGGRCKK with inset showing percent increase in contrast upon phosphorylation.

The slopes of the plots correspond to the relaxivity ($r_1$) which is a direct measure of the contrast enhancement of the water molecules exchanging with the molecules with respect to the bulk water. The relaxivity of phosphorylated Gd-DOTA-APRTPGGRCKK was 5.10 mM$^{-1}$ s$^{-1}$ and that of Gd-DOTA-APRTPGGRCKK was 4.01 mM$^{-1}$s$^{-1}$ which is a 28% increase in contrast. The blanks from the two separate experiments had same $1/T_1$ values signifying that the species present in the reaction mixture were not affecting the relaxation rates. Also, comparing the relaxivity for the phosphorylated Gd-DOTA-APRTPGGRCKK to the relaxivity of Gd-DOTA (4.01 mM$^{-1}$s$^{-1}$) interestingly suggests a contrast that is higher for the phosphorylated Gd-DOTA-
APRTPGGRCKK. This result shows that the contrast agent is turned on by phosphorylation catalyzed by ERK1. Gd-DOTA-APRTPGGRCKK is therefore a contrast agent which is a direct measure of ERK1 activity. This is contrary to what was hypothesized earlier where the contrast agent did not have an increase in contrast upon phosphorylation and the contrast agent was turned on after the isomerization triggered by Pin1. The relaxivity changes associated with isomerization of this phosphorylated Gd-DOTA-APRTPGGRCKK by Pin1 was monitored in subsequent experiments.

7.2G Monitoring Pin1 catalyzed isomerization of phosphorylated Gd-DOTA-APRTPGGRCKK using CD spectroscopy

Isomerization of 200 µL of 0.02 mM phosphorylated Gd-DOTA-APRTPGGRCKK was performed using 1.1 µL of 1 µg/µL Pin1 in a 1mm path length cuvette and monitored over time in-situ by CD spectroscopy. Higher concentrations of the substrate could not be used due to limitations of the maximum voltage allowed for the detector. A blank sample was run using the same components without the use of substrate to remove the background signal from enzymes and buffers present in the system. The blank ellipticity was subtracted from the sample ellipticity and plotted for the range of 190 nm to 275 nm as shown in the figure 7.9.
Figure 7.10 Monitoring Pin1 catalyzed isomerization of Gd-DOTA-APRT(Phospho)PGGRCKK using CD spectroscopy over time

Primary changes in absorption are observed around 200nm which is expected for a non-aromatic peptide chain. The signal absorbing group here is primarily the peptide bond where
there is $n\rightarrow\pi^*$ and $\pi\rightarrow\pi^*$ transition resulting in a broad absorption. At zero minutes the shape of the CD spectra for the phosphorylated Gd-DOTA-APRTPGGRRC corresponds to the CD spectra of a peptide chain in a random coil or irregular structure which is also expected for such a small peptide. Upon the addition of Pin1 CD spectra is taken at regular intervals over a period of 300 minutes. The gradual decrease in the initial absorption in the presence of Pin1 suggests that Pin1 recognizes Gd-DOTA-APRT(phospho)PGGRCKK as a substrate and isomerizes the peptide bond between phosphorylated threonine and proline which in turn changes the overall secondary structure of the peptide. The isomerization of the phosphorylated Gd-DOTA-APRTPGGRCKK by Pin1 leads us to perform experiments to monitor relaxivity changes associated with it. There could be an enhancement is contrast where the peptide chain of phosphorylated Gd-DOTA-APRTPGGRCKK could be isomerized to a structure which results in enhanced water exchange which increases relaxivity as discussed later. On the contrary, there could be negative impact on the contrast if the peptide isomerization leads to a peptide folded in such a way that the peptide chain blocks the 9th coordination of the Gd(III) ion in the complex which leads to decrease in contrast. Also, there could be no effect of the isomerization of the peptide on the contrast if the peptide structural changes do not affect water relaxation rates.

**7.2h Time based measurement of relaxivity changes associated with Pin1 catalyzed isomerization of phosphorylated Gd-DOTA-APRTPGGRCKK**

To investigate the contrast changes associated with Pin1 catalyzed isomerization, $1/T_1$ of phosphorylated Gd-DOTA-APRTPGGRCKK was monitored over a period of 631 minutes where the measurements were started as soon as Pin1 was added to the reaction mixture. $T_1$
measurements were done while the Gd-DOTA-APRT(Phospho)PGGRCKK underwent isomerization by Pin shown in the figure 7.10. Control for this experiment were chosen to be Gd-DOTA-APRT(Phospho)PGGRCKK without Pin1 and Gd-DOTA-APRTPGGRCKK.

Figure 7.11 Time based measurement of relaxivity changes associated with Pin1 catalyzed isomerization of Gd-DOTA-APRT(Phospho)PGGRCKK

The 1/T₁ values during the Pin1 catalyzed isomerization of phosphorylated Gd-DOTA-APRTPGGRCKK did not change overtime relative to the 1/T₁ values of that of the non-isomerized phosphorylated Gd-DOTA-APRTPGGRCKK. Also, the 1/T₁ values of Gd-DOTA-APRRTPGGRCKK maintained a consistent difference from the Gd-DOTA-APRRT(Phospho)PGGRCKK(with or without Pin1) for a particular time point signifying that
the phosphorylation induced increase in contrast is maintained. The data shows that the structural changes of the peptide in the Gd-DOTA-APRT(Phospho)PGGRCKK does not affect water exchange rates around the Gd(III) ion. This result is contrary to the original hypothesis where the contrast agent is turned on after the activity of Pin1 on the contrast agent. The contrast agent Gd-DOTA-APRTPGGRCKK reaches its maximum contrast upon phosphorylation by ERK1 and is unchanged after Pin1 activity.

7.2 Monitoring Pin1 Catalyzed Isomerization of Gd-DOTA-APRTPGGRCKK Using CD Spectroscopy

Gd-DOTA-APRTPGGRCKK was treated with the isomerization enzyme Pin1 to study structural changes in the peptide moiety. It is known that the isomerization rates of peptides with non-phosphorylated threonine prior to proline is very slow and hence there should not be any structural changes in Gd-DOTA-APRTPGGRCKK.
Figure 7.12 Monitoring Pin1 catalyzed isomerization of Gd-DOTA-APRTPGGRCKK using CD spectroscopy over time.

The CD spectra shown in figure 7.11 did not have any ordered structural change over time. This is expected since non-phosphorylated Gd-DOTA-APRTPGGRCKK is not a substrate for Pin1. Also, comparing figures 7.9 and 7.11 it is observed that the CD spectra for the Gd-DOTA-APRT(Phospho)PGGRCKK seems to have fewer fluctuations which is consistent with earlier findings which have shown that the isomerization of phosphorylated Thr/Ser-Pro bond is 8 times slower than non-phosphorylated Thr/Ser-Pro bond. The phosphorylation thus renders a greater structural rigidity to Gd-DOTA-APRT(Phospho)PGGRCKK and is a more direct regulator of peptide backbone dynamics. This rigidity of the phosphorylated peptide around the Gd-DOTA moiety might have effects on the inner/outer sphere water exchange rates as discussed later.
7.2j Time based measurement of relaxivity changes associated with Pin1 catalyzed isomerization of Gd-DOTA-APRTPGGRCKK

Although there was no noticeable structural change induced by Pin1, relaxation time measurements of Gd-DOTA-APRTPGGRCKK treated with Pin1 and untreated Gd-DOTA-APRTPGGRCKK were plotted along the course of the reaction and plotted in figure 7.12.

![Graph showing relaxation time measurements](image)

Figure 7.13 Time based measurement of relaxivity changes associated with Pin1 catalyzed isomerization of Gd-DOTA-APRTPGGRCKK

As expected there was no observed relaxation time changes of Pin1 treated Gd-DOTA-APRTPGGRCKK. All data points overlapped measured relaxation times of the untreated Gd-DOTA-APRTPGGRCKK suggesting that Pin1 had no effect on the relaxation rates of the water
molecules surrounding the Gd(III) center. These results are consistent with the CD spectroscopy studies which also showed no structural changes of the peptide in Gd-DOTA-APRTPGGRCKK upon treating with Pin1.

7.3 Introduction—MAP Kinases Associated with Stress and Inflammatory Stimuli

c-jun N-terminal kinases (JNK1/2/3) and the p38α/β/γ/δ (also known as CSBP, RK, MPK2 and HOG1) are other two types of MAP kinases apart from ERK. All the MAP kinases are activated by phosphorylation by their upstream MAPK kinase on both the threonine and the tyrosine in a Thr-X-Tyr motif. This motif is present in the activation loop close to the ATP and substrate binding sites. The X is Glu for ERKs, Pro for JNKs and Gly for p38 kinases. Due to these differences in the phosphorylation site the MAP kinases are activated by specific upstream kinases as shown in figure 7.13. This specificity allows the JNKs and p38 kinases to be activated by stress and inflammatory stimuli whereas the ERKs are activated by mitogen based stimuli.
7.3A $\gamma^{\text{32P}}$-ATP BASED SUBSTRATE ASSAY FOR JNK1 AND p38

The radioactive assays were performed exactly the way described in section 7.2e. Gd-DOTA-PRTPGGRCKK and Gd-DOTA-APRTPGGRCKK were tested for substrates of the enzymes JNK1 and p38. Blank samples were prepared by adding water instead of substrates. The radioactivity measured from the isolated products of the enzymatic phosphorylation was plotted with respect to concentration as shown in figure 7.15.
The results showed that high specificity of ERK1 for Gd-DOTA-APRTPGGRCKK. JNK1 and p38 has no activity on Gd-DOTA-PRTPGGRCKK which was similar to that shown by ERK1. JNK1 showed no activity towards Gd-APRTPGGRCKK but there was a very slight activity of p38 towards phosphorylating Gd-DOTA-APRTPGGRCKK at higher substrate concentrations. A positive control was added which had Gd-DOTA-APRTPGGRCKK as substrate with ERK1 and the activity of the phosphorylated Gd-DOTA-APRTPGGRCKK.
matched that found in figure 7.8. Both the radioactivity studies were done when the $^{33}$P-ATP has the same activity so that the data could be compared consistently.

### 7.4 Conclusion

Gd-DOTA-APRTPGGRCKK was specifically phosphorylated by ERK1 and not by other MAPKs like JNK1 and p38. Phosphorylation increased contrast by 28% which is referred to a switching ON event. Pin1 catalyzed isomerization changed the peptide structure as observed by CD spectroscopy, but MRI studies did not show any change is relaxivity associated with the isomerization. Hence, we have successfully demonstrated in vitro activation of a contrast agent by phosphorylation independent of Pin1 isomerization. Our initial hypothesis shown in scheme 1.2 did not work, but now we have a more direct way to monitor ERK activity. The Gd-DOTA-PRTPGGRCKK was not a substrate for ERK1, JNK1 and p38. Even though it had the ideal characteristics of an OFF contrast agent; i.e, a very low relaxivity, it was not recognized as a substrate by any of the MAPKs.
8.1 INTRODUCTION

As introduced briefly in section 1.5, the inner sphere relaxivity of the contrast agent is directly proportional to the number of water molecules in the inner sphere coordination (q) and is inversely proportional to the residence lifetime of the water molecule in the inner sphere (τ_M) and longitudinal relaxation time of the bound water (T_{1M}) of the water in the inner coordination sphere according to equation 8.1.

\[
\frac{1}{T_1} = \frac{P_M q}{T_{1M} + \tau_M}
\]

(8.1)

The T_{1M} is given by the Solomon-Bloembergen equation 8.2 which incorporates the sum of interaction which occur through space and through bond where γ_1 is the proton gyromagnetic ratio, g is the electronic g-factor, S is the total electron spin of the metal ion, β is the Bohr magneton, r is the proton-metal ion distance, (A/ℏ) is the electronic-nuclear hyperfine coupling constant, τ_c is the correlation time due to dipolar interaction, τ_e is the correlation time for scalar relaxation, ω_S and ω_I are the electronic and proton Larmor precession frequencies, respectively.
The contributions to dipolar relaxation rate (1/τ<sub>c</sub>) and scalar relaxation rate (1/τ<sub>e</sub>) are expressed by the equations 8.3 and 8.4 where T<sub>1e</sub> is the longitudinal electron spin relaxation time, τ<sub>R</sub> is the rotational correlation time. The dipole-dipole relaxation can be modulated by electronic relaxation time, correlation time for bound water and correlation time for rotation whereas the scalar relaxation is independent of correlation time for rotation. The smallest denominator on the right hand side of these equations governs the major contributing factor.

\[
\frac{1}{T_{1M}} = \frac{2}{15} \frac{\gamma^2 g^2 S(S+1) \beta^2}{r^6} \left[ \frac{7 \tau_c}{1 + \omega_S^2 \tau_c^2} + \frac{3 \tau_c}{1 + \omega_I^2 \tau_c^2} \right] + \frac{2}{3} S(S + 1) \left( \frac{\alpha}{\hbar} \right)^2 \left[ \frac{\tau_e}{1 + \omega_S^2 \tau_e^2} \right]
\]

(8.2)

At lower field strengths (ν<sub>H</sub> < 15 T), the zero field splitting of Gd(III) ion is negligible and electronic relaxation contribution is low and can its contribution to overall relaxivity can be ignored. At higher fields modified Solomon-Bloembergen-Morgan (MSBM) equation (eq 8.5) is

\[
\frac{1}{T_{1e}} = \frac{1}{T_{1e}} + \frac{1}{\tau_M} + \frac{1}{\tau_R}
\]

(8.3)

\[
\frac{1}{\tau_e} = \frac{1}{T_{1e}} + \frac{1}{\tau_M}
\]

(8.4)

\[
\frac{1}{T_{1e}} = \frac{\Delta^2 [4S(S+1)]}{25} \left[ \frac{\tau_v}{1 + \omega_S^2 \tau_v^2} + \frac{4 \tau_v}{1 + 4\omega_S^2 \tau_v^2} \right]
\]

(8.5)
used to express 1/T$_{1e}$ which accounts for transient zero-field splitting (ZFS) which is caused by collision of solvent molecules with the Gd(III) complex. Electron relaxation is enhanced due to this ZFS modulation and is a function of the correlation time ($\tau_v$) characteristic of these fluctuations.

The contribution of outer sphere relaxation rate (1/T$_{1'}$) can be expressed by equation 8.6 which has a form similar to equation 3 where q' is the number of water molecules in outer sphere coordination, T$_{1M}'$ the relaxation rate of these water molecules and $\tau'_M$ is the residence lifetime.

$$\frac{1}{T_{1'}} = \frac{P_M q'}{T_{1M}' + \tau'_M}$$  \hspace{1cm} (8.6)

The influence of molecular parameters on the inner and outer sphere relaxivity is summarized in figure 8.1.
The increase in the number of water molecules in the inner or outer coordination sphere increases the relaxation rates. The rate of exchange of outer sphere water \( (1/\tau'_{M}) \) is generally too high for contrast agents with a hydrophobic exterior. The water molecule does not spend enough time in the outer sphere to get complete relaxation and hence the outer sphere relaxation is ineffective. Inner sphere relaxation rates have higher contribution since the distance of the inner sphere water proton \( (r) \) is less than that of outer sphere water proton \( (r') \); the dependence being a function of \( 1/r^6 \). The water exchange rates for inner sphere water \( (1/\tau_M) \) and rate of tumbling \( (1/\tau_R) \) for the contrast agent are interrelated though the equations 8.1 – 8.5. The interdependence of these parameters can be understood through simulations where these parameters are varied using known constraints of a Gd-based contrast agent as shown in figure 8.2.\textsuperscript{63}
Figure 8.2 Interdependence of inner sphere water residence lifetime ($\tau_M$) and rotational correlation time ($\tau_R$) in maximum achievable inner sphere relaxivity.\textsuperscript{64} (Caravan et. al, Contrast Media Mol. Imaging, 2009, 4, 89–100)
Figure 8.2 shows that at higher fields there is more available maneuverability in parameters to attain maximum relaxivity. Bulkier contrast agents, like those bound to proteins, with larger rotational correlation time had a preference for water residence lifetime depending upon field used. Smaller contrast agents, like Gd-DOTA or Gd-DTPA, whose rotational correlation times are in the range of ~ 0.1ns, show no dependence on water residence lifetime at fields lower than 3T. Also, change in relaxivity for varying the water exchange rate \( (k_{\text{exchange}} = 1/\tau_M) \) for a molecule with a particular rotational correlation time is maximum at a particular water residence lifetime.\(^6\) Hence, depending on the rotational correlation time of the contrast agent and the residence lifetime of the water in the tissue environment the contrast agent is localized the appropriate field should be used for MRI measurements. Due to poor understanding of the outer sphere relaxation mechanisms similar simulations to understand the interplay between outer sphere parameters have not been reported in the literature.

8.2 RESULTS AND DISCUSSION

8.2A RATIONALE FOR INCREASE IN RELAXIVITY UPON PHOSPHORYLATION

To understand the mechanism of activation of Gd-DOTA-APRTPGGRCKK computational studies were done using hyperchem to understand the structural changes of the peptide backbone geometry associated with phosphorylation. The structures of Gd-DOTA-APRTPGGRCKK and Gd-DOTA-APRT(Phospho)PGGRCKK were optimized individually and was expressed in the figure before and after phosphorylation by ERK1.
The calculations showed a very unique activation mechanism of structural changes associated with phosphorylation. The calculated structure of the non-phosphorylated contrast agent has a proline residue hovering over the 9th coordination site of the Gd(III) complex. The calculated distance between the Gd center and plane of proline’s five membered ring was 5.841 Å. This distance is long enough for water molecules which are directly bound to the Gd(III) ion to relax with bulk water but it does create a hydrophobic hindrance which increases the residence lifetime of bound water. This phenomenon of increasing residence lifetime of bound water may increase or decrease depending on the field strength used and the rotational correlation time of the molecule. All MRI measurements were carried out at 7T and hence, plot D in figure 8.2 can be used to explain the data for relaxivity changes. Gd-DOTA has a residence lifetime of about 100
ns and rotational correlation time of 0.1 ns. Addition of a peptide moiety to the Gd-DOTA increases the size of the molecule which increases the rotational correlation time of the molecule which increases the relaxivity of the molecule according to plot D. Although this was not observed in our experiments since the r1 for Gd-DOTA and Gd-DOTA-APRTPGGRCKK were exactly the same. This can be explained by increase in the residence lifetime value of Gd-DOTA-APRTPGGRCKK causing the relaxivity to remain in the same percent of maximum relaxivity. In this case, the relaxivity of Gd-DOTA-APRTPGGRCKK can be decreased by reducing the distance between proline and Gd(III) so as to decrease the residence lifetime of the coordinated water. This would lead to a decrease in the percent of maximum relaxivity with respect to figure D which could potentially bring because a similar relaxivity of the two compounds.

On the contrary, if the actual structure of the Gd-DOTA-APRTPGGRCKK is not predicted correctly by hyperchem and there is not a proline hovering over the ninth coordination of Gd(III) complex, the presence of hydrogen bonding NH and/or OH groups of the peptide chain close to the Gd(III) center could increase the residence time of the water molecules to a point where it again falls in the same percent of maximum relaxivity as that of Gd(DOTA). This shows the importance of the optimization of the water exchange rates in achieving the desired relaxivity for a particular contrast agent.

The calculated structure for Gd-DOTA-APRT(Phospho)PGGRCKK shows that the phosphorylation event had caused a dramatic change in the peptide orientation around the Gd-DOTA moiety. This causes free access of the water molecule to the 9th coordination site of Gd(III) in the complex. This contributes partially of the increase in the contrast. The negatively
charged oxygen on the phosphate group did not cause any change to the inner sphere water coordination of Gd(III). Part of the increase in relaxivity for Gd-DOTA-APRT(Phospho)PGGRCKK was due to outer sphere coordination sphere. Although it is impossible to predict the magnitude of outer sphere relaxivity, looking at known compounds with known contributions from inner and outer sphere coordination gave some idea about the relaxation contribution can be obtained. The parameters that govern the outer sphere relaxivity have some similarity to those that contribute towards the inner sphere relaxivity like distance of the water proton from the Gd(III) (r’) and residence lifetime of the water (τ’m)around the Gd(III) complex. Generally the residence lifetime of outer sphere water molecules are much smaller than that of the inner sphere water molecules. The small residence lifetime of the outer sphere coordination water molecules causes the water molecules to not be in contact with the Gd(III) complex long enough for it to relax completely. The residence lifetime of outer sphere water molecules have been shown to be increased by using hydrogen bonding interactions which hold the outer sphere water molecules long enough for them to relax completely. The increase in the organizational rigidity of the outer sphere water can also be caused by phosphate groups. The negative charge on the phosphate is very efficient in associating with water protons allowing a fast relaxation pathway and hence increasing relaxation rates. The organizational rigidity around the Gd-complex was shown by CD spectroscopy measurements in section 7.2g.

Structures of Gd-DOTA, Gd-DO3A, Gd-DOTP, Gd-DO3PR and Gd-DO3AP are shown in figure 8.4. Inner sphere water is absent in Gd-DOTP due to the steric blockage from the four phosphates and hence it shuttions the contribution of inner sphere relaxivity to the overall
relaxivity. Comparison of the relaxivity of Gd-DOTA and Gd-DOTP in figure 8.5 shows that the contribution from inner sphere coordination to relaxivity in Gd-DOTA is almost equal to the contribution from outer sphere relaxivity in Gd-DOTP. Gd-DO3PR has three phosphates thereby releasing some of the steric hindrance and allowing water in the inner coordination. This allows Gd-DO3PR to have contributions from both the inner sphere water and the outer sphere water which is held by the hydrogen bonding from the three phosphate groups resulting in the highest relaxivity shown in figure 8.5.

![Chemical Structures](image)

R₁ = R₂ = CH₂COO⁻         Gd-DOTA
R₁ = CH₂COO⁻ R₂ = H         Gd-DO3A
R₁ = R₂ = CH₂PO₃⁻²         Gd-DOTP
R₁ = CH₂PO₃⁻² R₂ = -COOEt     Gd-DO3PR
R₁ = CH₂COO⁻ R₂ = CH₂PO₃⁻²     Gd-DO3AP

Figure 8.4 Comparison of structures of Gd-DOTA, Gd-DO3A, Gd-DOTP, Gd-DO3PR and Gd-DO3AP⁶⁵,⁶⁶
From the previous example the contribution from the three phosphates can now be compared to a molecule with only one phosphate group replacing one of the acetate arms on DOTA (Gd-D03AP) which resulted in 28% increase in relaxivity compared to Gd-DOTA. This contribution could only be explained from outer sphere contributions. The similarity in local environment of Gd-D03AP to Gd-DOTA APRT(Phospho)PGGRCKK allows us to conclude that the basis of increase in relaxivity from Gd-DOTA-APRTPGGRCKK to phosphorylated Gd-DOTA-APRTPGGRCKK is primarily due to the increase in outer sphere relaxation from incorporation of a phosphate group near the Gd(III).

Another parameter which could have some contribution to the increase in the relaxivity upon phosphorylation of Gd-DOTA-APRTPGGRCKK is if there was some additional strain to the complex geometry. Electron relaxation time under zero field splitting (ZFS) conditions is given
by the correlation time ($\tau_{S0}$) which is inversely proportional to the square of zero field splitting energy ($\Delta$). Lowering this correlation time increases relaxivity. In symmetric molecules like Gd-DOTA, there is very low zero field splitting whereas as soon as asymmetry is introduced in the electronic relaxation pathway gains importance. The phosphate incorporation could add strain to the complex leading to a greater ZFS value than the initial Gd-DOTA APRTPGGRCKK causing decrease in the correlation time leading to increase in relaxivity.

8.2b CLINICAL APPLICABILITY WITH 28% INCREASE IN CONTRAST

The factors discussed earlier could be optimized to get a higher change in relaxivity which can be clinically applied. The required change is relaxation rate is approximately 5 s$^{-1}$ and dosage as high as 0.3 mmol/kg of Gd have been administered to patients for extracellular contrast agents like Gd-DOTA. Relaxivity of Gd-DOTA being 4.01 mM$^{-1}$ s$^{-1}$ (figure 7.2) would require a concentration of 125 μM in the body where the detection limit is 30 μM. For a relaxivity change of 1.1 mM$^{-1}$ s$^{-1}$ due to phosphorylation of Gd-DOTA-APRTPGGRCKK would require a concentration of 456 μM to achieve the same level of contrast. The LD50 values of Gd-DOTA-APRTPGGRCKK being unknown the efficacy of this dosage cannot be attested. Increasing in the change of relaxivity due to phosphorylation would require lower dosage of the activatable contrast agent to the patient which is preferred. The possible molecular modifications to Gd-DOTA-APRTPGGRCKK described below might change the substrate recognition by ERK1 and hence the radioactive assays would have to be repeated with the modified molecules.
8.2c Optimizable Parameters for Contrast Agent

Parameters that are not changing during the phosphorylation are not contributing to the increase in relaxivity. These parameters which contribute towards the background relaxivity, can be manipulated to get an OFF state for the contrast agent which has a lower relaxivity than what is obtained for Gd-DOTA-APRTPGGRCKK. The primary contributing factor for relaxivity in the OFF contrast agent is the water in the inner sphere coordination. Although the exchange times of that might be changing upon phosphorylation and it might be contributing to the increase in relaxivity, further experiments are necessary to ascertain its contributions. Also, the relaxation rate of this coordinated water might be increased with phosphorylation which also needs to be experimentally verified. Increase in molecular size increases the rotational correlation time which in turn increases the relaxivity as seen before in figure 8.2. Since phosphorylation introduces a phosphate moiety in the peptide chain of the contrast agent increasing its size this effect contributes to increasing relaxivity and can be amplified further by introducing more phosphorylation sites near the Gd(III). The maximum number of peptide strands possible for the DOTA is four since it has four functionalizable carboxylic groups. Also, introducing four peptide strands might could lead to a very sterically hindered 9th coordination site for Gd(III). These strands with hydrophobic amino acids like proline could agglomerate together to completely block the 9th coordination site for the off contrast agent. Upon phosphorylation, due to favorable hydrogen bonding interactions from the phosphates, these strands could get freely dispersed in the hydrophilic environments opening the 9th coordination for water to coordinate. This would lead to a huge change in relaxivity since the number of water
molecules in the inner sphere coordination is changing which according to equation is one of the prime factors for relaxivity changes. The increased number of phosphates would also bring rigidity to the outer sphere water due to the negatively charged phosphate groups causing much higher changes in outer sphere relaxation than what we observed. The increase in percent size of the molecule can be amplified by using minimal number of amino acids in the peptide chain. Our results show the importance of the APRTP moiety for substrate recognition and KK moiety in increasing the hydrophilic properties of the contrast agent but the importance of the rest of the residues is unknown.

8.3 CONCLUDING REMARKS

In conclusion, we have developed an activatable Gd-based MRI contrast agent which is enzymatically turned on by phosphorylation of the threonine residue by ERK1. The activation is thought to be primarily due to the increase in outer sphere relaxation rates of water. To our best knowledge this is the first activatable contrast agent triggered by a phosphorylation event. Since, the phosphorylation and hence the activation of the contrast agent is a direct measure of enzymatic activity we envision that this would be useful for distinguishing normal from cancer cells which not only have overexpressed ERK1 but their activity is also greatly heightened. Although we observed contrasts changes which are high enough to be visualized in mouse tumor cells we could make plenty of modifications in the contrast agent structure that would enhance the increase in contrast.
PART I EXPERIMENTAL PROCEDURES

1. Materials

1,4,7,10-Tetraazacyclododecane (I) and Gadolinium (III) (1,4,7,10-tetraazacyclododecane)-1,4,7,10-tetraacetate (Gd-DOTA) were purchased from Macrocyclics. HATU was purchased from Chempep. Fmoc-Arg(Pbf)-OH and Fmoc-Lys(Boc)-OH were purchased from AAPPTEC. All other Fmoc protected amino acids and Rink resin HS, 100-200 mesh, 1%DVB (0.8 mmol/g) were purchased from Advanced Chemtech. Chelex 100 resin (Na⁺ form) was purchased from Biorad. Recombinant full-length active tag free human ERK1, ATP stock solution, Kinase Dilution Buffer III, Kinase Assay Buffer I were purchased from SignalChem. Radioactive P33 was purchased from PerkinElmer. All other chemicals and solvents were purchased from Sigma Aldrich. ¹H (400MHz) and ¹³C (100MHz) NMR spectra was recorded on a Varian 400 spectrometer with 5mm OD sample tubes from Wilmad. Analytical purification was performed using LCMS/MS which was an assembly of Varian ESI 500-MS, Varian 212 LC pumps, Varian 410 autosampler used with a Polaris 5 C18-A 150x2.0mm column. Peak homogeneity of the LCMS chromatograms at different elution times were measured and checked for the correct species for all reported data. Semi-preparative purification was performed using Varian Prostar HPLC with UV/VIS detector. Gadolinium chelates were characterized using ABI QSTAR Elite nanospray QTOF (HR) MS in the APCI mode using direct infusion (nanospray). Pharmacia Wallac 1410 Liquid Scintillation Counter was used for radioactive assays. Jasco J-710 Spectropolarimeter was used for circular dichroism measurements. An electron multiplied charge coupled (EMCCD) camera was used to determine the size of the beads. Each pixel had a length.
and width of 20 μm. Solid phase peptide synthesis was performed on fluorinated fritted Buchner funnels. Completion of the reaction was tested using standard Ninhydrin test for primary amines and Chloranil test for secondary amines. Presence of free Gd$^{3+}$ ions was tested by Xylenol Orange test. All synthesis was carried out under nitrogen.

2. Synthesis

1,4,7-Tris(tert-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane.HBr (2). In a 500 ml round bottomed flask 1,4,7,10-Tetraazacyclododecane (1) (5 g, 29 mmol), sodium hydrogen carbonate (8 g, 95 mmol) were mixed in 170 ml dry acetonitrile and cooled to 0 °C. tert-Butyl bromoacetate was added dropwise at a rate of 16 ml/hr. The reaction was allowed to stir at room temperature for 48 h. Any acetonitrile loss due to evaporation was compensated for. The reaction mixture was filtered; filtrate was rotary evaporated to get an off white solid which was recrystallized from 150 ml toluene to get the title compound as a white solid (7.273 g, 42 %).$^1$H NMR (CDCl$_3$) δ 1.47 (27H, s, tert-Bu), 2.89 (4H, s, 8,12-CH$_2$), 2.93 (8H, s, 2,3,5,6-CH$_2$), 3.10 (4H, s, 9,11-CH$_2$NH), 3.30(2H, s, 4-CH$_2$CO$_2$), 3.38 (4H, s, 1,7-CH$_2$CO$_2$), 10.03 (2H, br s, NH.HBr); $^{13}$C (CDCl$_3$) δ 28.5, 47.8, 51.7, 58.5, 82.0,170.0, 170.8; ESI LCMS 515.4 m/z [M-Br]$^+$

1,4,7-Tris(tert-butoxycarbonylmethyl)-10-(benzyloxy carbonylmethyl)-1,4,7,10-tetraazacyclododecane (3). In a 50ml round bottomed flask (2) (500 mg, 0.8 mmol) and sodium bicarbonate (354 mg, 4.2 mmol) were mixed in 20ml anhydrous acetonitrile and cooled to 0 °C. Benzyl bromoacetate was added dropwise and after addition was complete and refluxed for 24 h.
LCMS showed pure product. Recrystallized from ethyl acetate to get title compound as a white solid (273.5 mg, 52%). $^1$H NMR (CDCl$_3$) $\delta$ 1.46 (27H, s, tert-Bu), 2.41-3.50 (24H, br), 5.13 (2H, s), 7.30-7.36 (5H, 5H), ESI LCMS 663.4 m/z [M+H]$^+$ 10.1021/ja077058z

$^{1,4,7}$-Tris(tert-butoxycarbonylmethyl)-$^{1,4,7,10}$-tetraazacyclododecane-$^{10}$-acetic acid (4). In a Parr instrument compound (3) (273 mg, 0.5 mmol) was mixed with Pd/C (10 mg) in 10 ml methanol with a drop of ethanol and stirred at 80 psi of hydrogen overnight. Reaction mixture was passed through celite and product rotary evaporated to get title compound as white solid. ESI LCMS 595.3 m/z [M+Na]$^+$

$^{1,4,7,10}$-Tetraazacyclododecane-$^{1,4,7}$-tris(t-butyl acetate)-$^{10}$-acetate mono (N-hydroxysuccinimide ester) (5). Compound (4) (200 mg, 0.35 mmol) N-hydroxysuccinimide (45.2 mg, 0.39 mmol) and Obenzotriazol-1-yl-$N,N,N',N'$-tetramethyluronium hexafluorophosphate [HBTU] (148 mg, 0.39 mmol) were dissolved in 10 mL of acetonitrile. The reaction mixture was stirred at room temperature for 24 hr. Crude was dissolved in dichloromethane and washed with water. Dichloromethane layer was dried over sodium sulphate, dichloromethane rotary evaporated to get pale yellow solid. Recrystallization from ethyl acetate resulted in white crystalline solid. ESI LCMS 692.4 m/z [M+Na]$^+$

**Typical Synthesis for DOTA-Peptide Conjugate**

Rink resin (1 g, 0.8 mmol) was taken in a fluorinated fritted Buchner funnel and soaked overnight in dimethylformamide under a purge of nitrogen from under the frit. The Fmoc
protecting group was removed using 20% piperidine in dimethylformamide (3 x 5 ml) for 30 min, washed with (3 x 5 ml) DMF for 10 min. Fmoc protected amino acid (0.88 mmol), (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate [PyBOP] (413 mg, 0.88 mmol) and N-Hydroxybenzotriazole monohydrate [HOBT] (119 mg, 0.88 mmol) was dissolved in 8 ml dimethylformamide and added to the resin. N,N-Diisopropylethylamine (418 µL, 2.4 mmol) was added directly to the reaction mixture. After 3 h the reaction mixture was washed with dimethylformamide and few resin beads were tested with standard free amine test. If test results showed colored beads coupling was repeated with same stoichiometry. This cycle of deprotection and coupling was repeated for corresponding amino acids. After the required amino acid sequence was achieved, compound 4 (590 mg, 0.88 mmol), HOBT (119 mg, 0.88 mmol), N,N-Diisopropylethylamine (418 µL, 2.4 mmol) and 2-(1H-7-Azabenzotriazol-1-yl)--1,1,3,3-tetramethyl uronium hexafluorophosphat Methanaminium [HATU] (305 mg, 0.8 mmol) were dissolved in 8 ml dimethylformamide and added to the Fmoc deprotected resin-peptide conjugate. After 3 h resin was washed with dimethylformamide and free amine test was performed. Coupling protocol repeated if required. The resin was washed with dimethylformamide (3 x 5 ml), dichloromethane (3 x 5 ml) and dried under suction in air. Resins were then treated with cleavage cocktail (3 x 10 ml) consisting of trifluoroacetic acid 95%, triisopropylsilane 2.5% and water 2.5% for 45 min each. The filtered cleavage cocktail were combined and rotary evaporated to remove volatiles. They are purified using semi-prep HPLC and characterized using QTOF MS.
(DOTA)-A-P-R-T-P-G-G-R-C-K-K-NH₂ (8)

Yielded 327mg (26%) Calculated mass [M+2H]²⁺ 778.4m/z, Observed 778.5m/z

(DOTA)-P-R-T-P-G-G-R-C-K-K-NH₂ (10)

Yielded 499 mg (42 %). ESI LCMS 845.5 m/z [M+2HBr+2Na]²⁺

Complexation with Gd³⁺

DOTA-peptide conjugate was dissolved in water and pH was adjusted to 6.5. GdCl₃ was added in to the solution and stirred for 1 week. The reaction mixture was rotary evaporated and purified with HPLC. The purified products were tested for free Gd³⁺ using Xylenol orange test and characterized using QTOF MS.

Gd-(DOTA)-A-P-R-T-P-G-G-R-C-K-K-NH₂ (9)

Yielded 28mg (8%) Calculated mass for [M+3H]³⁺ was 570.58 m/z; observed with the required isotopic distribution 570.271 m/z.

Gd-(DOTA)-P-R-T-P-G-G-R-C-K-K-NH₂ (11)

Yielded 80 mg (14%) Calculated mass for [M+HCOOH+3H]³⁺ 563.237 m/z; observed with the required isotopic distribution 563.663 m/z.
3. Biological Studies

**P33 Radioactive assay**

10 µL $^{33}$P-ATP (3000 Ci/mmol, 10 mCi/ml), 25 µL ATP stock solution (10 mM) was mixed with 95 µL Kinase Assay Buffer I to get the $^{33}$P-ATP cocktail. Serial substrate concentrations of 9 and 11 were 0.01 mM, 0.03 mM, 0.06 mM, 0.125 mM, 0.25 mM, 0.50 mM. ERK1 (0.1 µg/µL) was mixed with 95 µL Kinase Dilution Buffer III to get the ERK1 cocktail. All the cocktails and substrates were stored in on ice prior to use. Distilled water was stored at 4 °C. The reaction mixtures was prepared by mixing 10 µL of the ERK1 cocktail, 5µL of the substrate, 5µL of the distilled water and adding the $^{33}$P-ATP cocktail at last to initiate the reaction. The blank was prepared by mixing 10 µL of the ERK1 cocktail, 10µL of the distilled water and $^{33}$P-ATP cocktail. The reactions were run at 35 °C for 1 h. The reaction was terminated my spotting 5µL of each reaction mixture into individual precut phosphocellulose P81 paper. The P81 papers were air dried and washed with (3 x 10 ml) 1% phosphoric acid solution for 10 min with gentle stirring. The P81 papers were thoroughly dried using blotting paper and under air. Each of the dried P81 paper were put in a separate with X ml scintillation liquid. The scintillation vials were placed in the scintillation counter and program for $^{33}$P isotope was used to get the counts per minute (CPM).
Large Scale Phosphorylation of Gd-(DOTA)-P-R-T-P-G-R-C-K-K-NH₂ (9) using ERK1.

Compound 9 (2.8 mL, 0.5 mM), ATP (400 µL, 10 mM) mixed with 1 ml Kinase Assay Buffer I, ERK1 (90 µL, 0.1 µg/µL) mixed with 2.814 ml Dilution Assay Buffer III were combined and stirred at 35 °C. Relaxation rate of phosphorylated 9 was measured.

CD studies with Pin1 isomerization.

Blank was prepared by mixing 80 µL of Kinase Dilution Buffer III, 28.5 µL of Kinase Assay Buffer I, 94.4 µL water and 1.1 µL DTT. Sample was prepared by mixing 204 µL of phosphorylated 9, 408 µL of water and 5 µL Pin1 (1.0 mg/ml) which gave a high tension on the CD of <500. Reaction monitored for 24 h.

Relaxivity measurements of Pin1 isomerization.

Relaxivity measurements were carried out simultaneously with water as blank, Gd(DOTA), compound 9, phosphorylated compound 9 and phosphorylated compound 9 with Pin1 enzyme with Gd concentration is all solutions at 0.0837 mM. The relaxivity was measured over a period of 800 min.
PART II ROLE OF NATURAL BITE ANGLE AND ELECTRONIC FACTORS OF A LIGAND ON THE REGIOSELECTIVITY OF Rh(I) CATALYZED HYDROFORMYLATION REACTION
CHAPTER 9 ROLE OF ELECTRONEGATIVITY IN DETERMINING REGIOSELECTIVITY OF HYDROFORMYLATION REACTION

9.1 INTRODUCTION

Hydroformylation was discovered by Otto Roelen in 1938 where he added a formyl group and hydrogen atom across a carbon-carbon bond as shown in scheme 9.1. He is considered a pioneer in industrial homogeneous catalysis for his contributions.\textsuperscript{69} Initial catalysis was performed with HCo(CO)\textsubscript{4} which was eventually replaced by rhodium based catalyst with triphenylphosphine ligands which was found to be much more active in 1968.\textsuperscript{70} Since then the reaction has undergone tremendous improvements in terms of reaction scope, product selectivity and higher yields at milder conditions. Reaction temperatures have been lowered from 170 °C to 50 °C and pressure requirements have been lowered from 300 bar to 10 bar whereas the activity has been increased $10^4$ times to that was initially observed with cobalt catalyst. The primary application of this reaction is to make long chain alcohols which serve as intermediates for lubricants, plasticizers and detergents. To date this is one of the most important homogeneously catalyzed reaction and more than 9 million tons of alcohol and aldehyde are made annually using this reaction.\textsuperscript{71}

Research in this field mainly revolves around designing new ligands with systematic tuning of their steric and electronic properties to gain insight into the regioselectivity, chemoselectivity yields and rate of reaction. The general reaction mechanism proposed by Heck and Breslow can be shown in scheme 9.2.\textsuperscript{72}
Scheme 9.1 Hydroformylation reaction

Scheme 9.2 General hydroformylation reaction mechanism proposed by Heck and Breslow

The catalytically active catalyst is generated by dissociation of a CO molecule from the dicarbonyl metal complex. The molecular geometry for catalytically active species is trigonal bipyramidal which can have two isomeric forms one where the two phosphines are equatorially present (ee isomer) and the other where the one is axial and the other is equatorial (ea isomer). The bulkier phosphine ligands are assumed to prefer the ee isomer configuration since the equatorial positions have less steric interactions than the ea isomer. A dynamic equilibrium exists between these two isomers as shown in the scheme 9.3.

![Scheme 9.3 Dynamic equilibrium between ee and ea isomers](image)

The regioselectivity is set by the largely irreversible hydride addition to the coordinated alkene. To define the steric bulk and phosphine basicity Tolman introduced the terms cone angle ($\theta$) and electronic parameter ($\chi$) respectively as shown in figure 9.1.73

![Figure 9.1 Schematic representations of electronic and steric effects.](image)
The concept of natural bite angle ($\beta_n$) was developed by Casey and Whiteker where the phosphorus to metal to phosphorus (P-M-P) angle of a bidentate phosphine ligand was used to correlate the regiochemistry and wide bite angled ligands. The natural bite angle was calculated by reducing the P-M-P bending force constant to 0 kcal mol$^{-1}$ rad$^2$, reducing the metal to a dummy metal and metal phosphorus bond length was fixed to 2.315 Å.$^{74}$

```
BISBI  T-BDCP  DIOP  DIPHOS
```

Figure 9.2 Structural comparisons of diphosphine ligands with vary bite angle

BISBI was a diphosphine ligand which showed excellent selectivity towards linear aldehyde and hence to investigate the rationale some other diphosphine ligands (structures shown in figure 9.2) were chosen with different bite angles to study the effect of varying bite angle the results of which is summarized in table 9.1. Good correlation between selectivity for the linear aldehyde and bite angle of the diphosphine ligands was observed. The high bite angle of BISBI was attributed to result in greater amount of ee isomer which was considered to be the reason for greater amount of linear aldehyde.$^{74}$
<table>
<thead>
<tr>
<th>Diphosphine</th>
<th>TOF</th>
<th>l:b</th>
<th>Calculated Bite angle</th>
</tr>
</thead>
<tbody>
<tr>
<td>BISBI</td>
<td>29.4</td>
<td>66.5</td>
<td>112.6</td>
</tr>
<tr>
<td>T-BDCP</td>
<td>3.7</td>
<td>12.1</td>
<td>106.6</td>
</tr>
<tr>
<td>DIOP</td>
<td>6.4</td>
<td>8.5</td>
<td>102.2</td>
</tr>
<tr>
<td>DIPHOS</td>
<td>1.1</td>
<td>2.1</td>
<td>84.5</td>
</tr>
</tbody>
</table>

Table 9.1 Change is turn over frequency (TOF), linear to branched ratio (l:b) and calculated bite angle with varying diphosphine ligands.

To isolate the electronic effects from the steric effects Leeuwen et al. synthesized a series of diphosphines which had similar bite angles and only changed the electronic parameter as shown in figure 9.3. The hydroformylation results shown in table 9.2 indicates that even though the ee isomer increased in the equilibrium, there was no change observed in selectivity of the linear isomer which were all between 92 and 93%. The finding led to the conclusion that regioselectivity was not affected by phosphine basicity and might be solely dependent on steric factors. The regioselectivity did not correlate to the coordination geometry (ee or ea) of the active catalyst complex. Rate of hydroformylation was observed to increase with increasing acidity of the ligand. Speculations were made whether the (diphosphine)Rh(CO)₂H complex geometry was changing upon formation of (diphosphine)Rh(CO)H(alkene) complex which
would mean that focus has to be shifted to study the later species rather than the former complex.  

<table>
<thead>
<tr>
<th>Ligand</th>
<th>R</th>
<th>Calculated Bite Angle</th>
<th>Flexibility range(deg)</th>
<th>Electronic Parameter $\chi$</th>
<th>% ee:ea</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N(CH$_3$)$_2$</td>
<td>109.1</td>
<td>92-124</td>
<td>1.8</td>
<td>47</td>
</tr>
<tr>
<td>2</td>
<td>OCH$_3$</td>
<td>106.9</td>
<td>91-123</td>
<td>3.4</td>
<td>59</td>
</tr>
<tr>
<td>3</td>
<td>CH$_3$</td>
<td>106.7</td>
<td>91-125</td>
<td>3.5</td>
<td>66</td>
</tr>
<tr>
<td>4</td>
<td>H</td>
<td>106.4</td>
<td>91-127</td>
<td>4.3</td>
<td>72</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>106.6</td>
<td>92-128</td>
<td>5.0</td>
<td>79</td>
</tr>
<tr>
<td>6</td>
<td>Cl</td>
<td>107.8</td>
<td>91-126</td>
<td>5.6</td>
<td>84</td>
</tr>
<tr>
<td>7</td>
<td>CF$_3$</td>
<td>109.3</td>
<td>92-128</td>
<td>6.6</td>
<td>92</td>
</tr>
</tbody>
</table>

Figure 9.3 Isolating electronic effects from steric effects. Electronic parameter $\chi$ directly relates to electronegativity of the group R, % ee:ea refers to the ration of the ee to the ea isomer expressed as a percentage.  

140
<table>
<thead>
<tr>
<th>Ligand</th>
<th>R</th>
<th>l:b ratio</th>
<th>% Selectivity for Linear</th>
<th>%Isomer</th>
<th>TOF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N(CH₃)₂</td>
<td>44.6</td>
<td>93.1</td>
<td>4.8</td>
<td>28</td>
</tr>
<tr>
<td>2</td>
<td>OCH₃</td>
<td>36.9</td>
<td>92.1</td>
<td>5.3</td>
<td>45</td>
</tr>
<tr>
<td>3</td>
<td>CH₃</td>
<td>44.4</td>
<td>93.2</td>
<td>4.7</td>
<td>78</td>
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<tr>
<td>4</td>
<td>H</td>
<td>50.0</td>
<td>93.2</td>
<td>4.9</td>
<td>110</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>51.5</td>
<td>92.5</td>
<td>5.7</td>
<td>75</td>
</tr>
<tr>
<td>6</td>
<td>Cl</td>
<td>67.5</td>
<td>91.7</td>
<td>6.9</td>
<td>66</td>
</tr>
<tr>
<td>7</td>
<td>CF₃</td>
<td>86.5</td>
<td>92.1</td>
<td>6.8</td>
<td>158</td>
</tr>
</tbody>
</table>

Table 9.2 Results of hydroformylation of 637 mM 1-Octene at 80 °C, $P$(CO/H₂) of 20 bar at 1:1 pressure, using 1 mM Rh catalyst using the ligand shown below with varying R group as shown in figure 9.6. l:b isomer refers to the ratio of linear to branched aldehyde from the hydroformylation reaction. %Isomer refers to the amount of isomerization product with respect to the overall products. ⁷⁶

The increase is l:b ratio upon increasing the electronegativity of the ligand was due to the facilitation of the increased formation of the isomerization product from the branched product.
which is shown by the % isomer column in table 9.2. The possible routes for the various product formation is shown in scheme 9.4.

Scheme 9.4 Possible routes for product formation from the hydroformylation reaction.\(^{76}\)

It is hence important to show how much of the isomerization is occurring to get an idea of the actual linear to branched ratio of products formed.
9.2 Revisiting the role of electronegativity and natural bite angle in determining regioselectivity of hydroformylation reaction

The goal of our project was to explore phosphine based ligands with nitrogen backbone and study regioselectivity of hydroformylation. Also our focus was towards diphosphine ligands with small natural bite angle since they have been rarely studied. It is envisioned that the small bite angle will favor a coordination geometry where the ligand in a ea conformation rather than a ee conformation in the trigonal bipyramidal geometry of the active catalyst. This is because the small bite angle ligand with a phosphorus-nitrogen-phosphorus (PNP) type backbone favours the low angle requirements in the ea conformation. Advantage of nitrogen backbone over a carbon backbone is that it is more electronegative and hence reduces $\pi$-backbonding to the CO facilitating the dissociation for the activation step of the pre catalyst to form the active catalyst causing shorter reaction times. Some of metal complexes diphenylhydrazine ligands are shown in figure 9.4 showing octahedral, square planar and trigonal bipyramidal geometry.\textsuperscript{77-79}

![Figure 9.4 Metal complexes diphenylhydrazine ligands](image-url)
9.3 RESULTS AND DISCUSSION

9.3a NATURAL BITE ANGLE FOR BIS-DIPHENYLPHOSPHINOHYDRAZINE (BDPH)

The natural bite angle for BDPH was calculated using MM2 simulation in hyperchem which gave a natural bite angle of 80° which is almost equal to that of diphenylphosninoethane (DPPE) as shown in figure 9.5. Hence, comparison of regioselectivity of products obtained from hydroformylation reactions using BDPH and DPPE as ligands would give us a correlation between backbone electronegativity without any interference from steric factors.

Figure 9.5 Hyperchem simulated structure of Bis-Diphenylphosphinohydrazine coordinated to Rh(I)
9.3B SYNTHESIS OF BIS-DIPHENYLPHOSPHINOHYDRAZINE (BDPH)

The synthesis was done according to scheme 9.5 in two steps slightly modifying procedure from Woolins et al.\textsuperscript{80} Slow addition of PCl\textsubscript{3} at room temperature to a finely ground powder of 1,2-diethylhydrazine dihydrochloride resulted in the formation of a viscous orange suspension.

\[
\begin{array}{cccc}
\text{NH}_2 \cdot 2\text{HCl} & \text{neat PCl}_3 & \text{Reflux} & 96 \text{ h} \\
\text{NH} & \text{Cl} & \text{N} \quad \text{P} \quad \text{Cl} & \text{N} \quad \text{P} \quad \text{Cl} \\
\text{Cl} & \text{N} \quad \text{P} \quad \text{Cl} & 4\text{eqv. PhMgBr} & \text{Et}_2\text{O} \\
& & & 25 \degree\text{C} \\
& & & 24 \text{h} \\
\text{Ph} & \text{Ph} & \text{Ph} & \text{BDPH} \\
\end{array}
\]

Scheme 9.5 Synthesis of Bis-Diphenylphosphinohydrazine

The use of PCl\textsubscript{3} meant that the reaction setup could not be done using plastic clamps as they would degrade upon exposure to PCl\textsubscript{3}. Instead metal clamps were used for the whole setup. The reaction mixture was then refluxed for 96 hours in neat PCl\textsubscript{3}. This resulted in orange slurry which had excess PCl\textsubscript{3} which was removed by Kugelrohr distillation which left the crude product in the reaction vessel. This was filtered to remove the orange impurities to get oil with yellowish tint. The compound purity was checked with $^{31}$P-{$^1$H}$\textsuperscript{1981}$ NMR which showed a singlet at 156 ppm which matched the reported values for Cl\textsubscript{2}PN(Et)N(Et)PCl\textsubscript{2}. $^1$H and $^{13}$C NMR also showed absence of impurities. This tetrachloro compound was the precursor for the next step where 4 equivalents of phenyl magnesium bromide were used to do a Grignard addition. Our results greatly varied from the published results. Crude from the reaction mixture suggested that the
reaction went to completion after 24 h as suggested by the $^{31}$P-$^1$H NMR which showed a single peak at 64 ppm. Quenching the Grignard reaction with water led to immediate decomposition of the product. The product was isolated by rotary evaporating the crude reaction mixture and the desired product was extracted using dichloromethane. The product crystallized from the dichloromethane extract upon concentrating it and which was stored in the glove box for future use.

9.3c Attempted BDPH Synthesis using Ph$_2$PCl with Different Reaction Conditions.

A more direct approach for the synthesis of the ligand was tried as shown in scheme 9.6. Although the reactions in the scheme seemed to be straightforward none of them resulted in product formation. $^{31}$P-$^1$H NMR of the crude from the reaction carried out without the addition of a base showed unidentified peaks of 42 ppm and 27 ppm along with peak for unreacted Ph$_2$PCl at 82 ppm after 18 h. The reaction carried out in the presence of Et$_3$N showed peaks for 24 ppm which did not correspond to the product or starting material. The reaction using BuLi as a base resulted in a large number of peaks ranging from 15 ppm to 40 ppm which were not identified. The two later reactions were used by Rosenthal et al. to derivatize 1,2-diphenyl hydrazine, both of which worked probably due the stabilizing effect of the phenyls$^{82}$ For the reaction of diethylhydrazine with Ph$_2$PCl it is possible that the reaction intermediate is unstable leading to decomposition before the required product is formed. Failure to find a more direct way to synthesize the BDPH ligand led us to keep using a dual step reaction as shown in scheme 9.6.
Scheme 9.6 Alternate attempted routes to synthesis of BDPH ligand using Ph\textsubscript{2}PCl

9.3d **SYNTHESIS OF Rh(acac)(CO)\textsubscript{2}**

The synthesis of the precatalyst was done using literature procedure using dimethyl formamide as a source for CO with simultaneous reduction of Rh(III) to Rh(I) as shown in scheme 9.7.
The produced dimethyl amine acts as a base to deprotonate the acetylacetone for it to coordinate to Rh(I). Upon removal of organic solvent shiny greenish blue crystals were obtained which showed the required $^1$H and $^{13}$C NMR spectrum for Rh(acac)(CO)$_2$.

**Scheme 9.7 Synthesis of Rh(acac)(CO)$_2$**

**9.3E Hydroformylation using Bis-Diphenylphosphinohydrazine (BDPH)**

Hydroformylation of 1-octene was performed using Rh(acac)(CO)$_2$ as the pre catalyst in a Parr low pressure reaction vessel as shown in scheme 9.8. Hydrogen and carbon monoxide were maintained in equimolar amounts to give a total pressure of 130.5 psi at 80 °C. The active catalyst was generated *in-situ* after coordination with the ligand. Conditions for hydroformylation of styrene were kept the same. Results from the hydroformylation reaction for BDPH and DPPE ligands using 1-octene and styrene as substrates are shown in table 9.3.
Scheme 9.8 Typical conditions for hydroformylation reactions.

![Scheme 9.8](image)

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Substrate</th>
<th>Conversion</th>
<th>((\text{Linear Aldehyde}))</th>
<th>((\text{Branched Aldehyde}))</th>
<th>Aldehyde : Internal Olefin</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDPH</td>
<td>1-Octene</td>
<td>100%</td>
<td>2.56</td>
<td></td>
<td>4.5</td>
</tr>
<tr>
<td>BDPH</td>
<td>Styrene</td>
<td>100%</td>
<td>0.19</td>
<td></td>
<td>n/a</td>
</tr>
<tr>
<td>DPPE</td>
<td>1-Octene</td>
<td>100%</td>
<td>0.52</td>
<td></td>
<td>1.98</td>
</tr>
<tr>
<td>DPPE</td>
<td>Styrene</td>
<td>100%</td>
<td>0.41</td>
<td></td>
<td>n/a</td>
</tr>
</tbody>
</table>

Table 9.3 Results from the hydroformylation reaction for BDPH and DPPE ligands using 1-octene and styrene as substrates.

The ratio between the linear aldehyde and branched aldehyde was 5 times higher for BDPH ligand than that of DPPE which was a much larger change in regioselectivity than that was previously expected. Previously it was shown that changing electronics of the ligand alone does not affect regioselectivity, as discussed in the introduction. Here we have evidence that just
changing the electronics of the ligand can change the regioselectivity. The ligand BDPH was specifically chosen over 1,2-Bis(diphenylphosphino)-1,2-diphenylhydrazine to remove any steric effects from the phenyls on the nitrogen. Also a very low isomerization of the 1-octene was observed for BDPH than DPPE resulting in purer aldehyde conversion. The results with styrene hydroformylation were showed opposite selectivity in terms of regioselectivity. The linear to branched ratio for the aldehyde formed from styrene seemed was lower for BDPH which shows it prefers the formation of the branched aldehyde. Although, in general, the overall regioselectivity for styrene was lower than 1-octene. The bite angle for BDPH and DPPE being very close, 80° and 79° respectively, clearly show that there is no steric effect involved and these regioselectivities were solely based on electronic factors.

9.3F UNDERSTANDING THE STRUCTURE OF THE ACTIVE CATALYST

The huge change in regioselectivity based on electronic factors alone intrigued us to study the structure of the active catalyst in depth. Initial attempts to isolate the active catalyst by reacting Rh(acac)(CO)₂ with BDPH under hydroformylation led to decomposition before isolation. The protocol was hence modified by using HRh(CO)(PPh₃)₃ as a precursor for generating the active catalyst which already had a hydride and a carbonyl as ligand with labile triphenylphosphine groups for facile substitution by the bidentate ligand.
9.3G SYNTHESIS OF trans-HRh(CO)(PPh$_3$)$_3$

The yellow complex trans-HRh(CO)(PPh$_3$)$_3$ was prepared according to published methods as shown in scheme 9.9. The complex had the characteristic absorption of 1962 cm$^{-1}$ for CO in IR and a doublet for the three phosphines coupling with Rh at 40.5 ppm ($J_{\text{Rh-P}} = 155.5$ Hz) in $^{31}$P $^1$H NMR. The hydride in $^1$H NMR showed up as a broad singlet around 9.33 ppm. This complex has been known to catalyze hydroformylation reactions under mild conditions and the replacement of two equatorial phosphines with a bidentate phosphine directly results in the formation of the required active catalyst.

\[
\text{RhCl}_3.3\text{H}_2\text{O} + 3\text{PPh}_3 \xrightarrow{\text{KOH}} \xrightarrow{\text{HCHO}} \text{Ph}_3\text{P}-\text{Rh-PPh}_3
\]

Scheme 9.9 Synthesis of trans-HRh(CO)(PPh$_3$)$_3$

9.3H IN-SITU SYNTHESIS OF THE ACTIVE CATALYSTS FOR DPPE

Reaction between HRh(CO)(PPh$_3$)$_3$ and DPPE was performed in a NMR tube to check the efficacy of formation of the desired product as shown in scheme 9.10.

\[
\text{Ph}_3\text{P}-\text{Rh-PPh}_3 + \xrightarrow{\text{DCM}} \xrightarrow{\text{RT}} \text{Ph}_3\text{P}-\text{Rh-Ph}_2
\]

Scheme 9.10 Synthesis of the active catalyst for DPPE
The $^{31}$P ($^1$H) NMR showed two doublets at 57.4 ppm ($J_{\text{Rh-P}} = 132.7$ Hz) and 40.4 ppm ($J_{\text{Rh-P}} = 152.1$ Hz). The $^1$H NMR showed the hydride peak shift upfield to 9.7 ppm. The presence of two peaks for the complex shows that there are two types of phosphines. The peak at 40.4 ppm corresponds to coordinated PPh$_3$ and the one at 57.4 ppm to that of coordinated DPPE.

### 9.3i Attempted In-situ Synthesis of the Active Catalysts for BDPH

Reaction between HRh(CO)(PPh$_3$)$_3$ and BDPH was performed in a NMR tube as shown in scheme 9.11.

![Scheme 9.11 Synthesis of the active catalyst for BDPH](image)

Initially the reaction was performed under nitrogen and was monitored using $^{31}$P ($^1$H) NMR. Apart from unreacted BDPH ligand at 63.4 ppm a doublet at 123.2 ppm ($J_{\text{Rh-P}} = 144.5$ Hz) was observed which corresponded to the BDPH complex. It was surprising to see just one peak for the phosphine since there are two types present in the product complex. The reaction mixture produced effervescence during the course of the reaction which indicated that the increased electronegativity of the ligand increased the $\pi$-backbonding capability to the phosphines of the
BDPH ligand and decreased the backdonation to the CO which lead to its release from the complex. This could have also resulted in the dissociation of the coordinated PPh₃. No hydride peak was observed.

There was evidence for the formation of the active catalytic complexes of Rh(I) with DPPE. The HRh(CO)(PPh₃)(DPPE) was formed under nitrogen and didn’t show any evidence of decomposition under those conditions. Using BDPH as a ligand resulted in a very electron deficient Rh(I) complex which lead to release of CO and PPh₃ during the reaction. It is possible that the penta-coordinated active catalyst might be favorable under hydroformylation conditions, where the CO pressure was high enough to prevent dissociation of the CO moiety from the active catalyst when the electron deficient ligands like BDPH are coordinated to Rh(I). Hence doing these reactions in a NMR tube under hydroformylation conditions at would likely increase the lifetime of these complexes in solution. The active catalyst structure with DPPE was an ee isomer since both the phosphines of DPPE after coordination showed a single type of phosphine. A statement about the conformation of the active catalyst for BDPH could not be made since it could not be stabilized in a penta-coordinated form under nitrogen.

9.4 Conclusion

The BDPE ligand was successfully synthesized using a slightly different procedure and other efficient synthetic routes were explored. The routes using Ph₂PCl to functionalize the hydrazine did not result in the expected product which is probably due to the inherent instability of the intermediates formed during the reaction. Comparison of the linear to branched aldehyde molar
ratios formed during hydroformylation reaction with DPPE and BDPH ligand showed a much higher preference for linear aldehyde than branched in case of BDPH as a ligand. Since the ligands are sterically very similar with almost the same natural bite angle it the regioselectivity is attributed to electronic factors. Since the backbone of BDPH has nitrogens instead of carbons, as in case of DPPE, the electronegativity of the BDPH ligand is much higher which results a greater preference for linear aldehydes and also reduces isomerization reaction which lead to internal alkenes. These results contradict previous experiments which have shown electronegativity to play no role in regioselectivity and sterics to be the determining factor. NMR studies to elucidate the exact structure of the active catalyst did not result in a clear answer and further studies with high pressure NMR tubes are necessary.
CHAPTER 10 ROLE OF LEWIS ACID IN DETERMINING REGIOSELECTIVITY OF HYDROFORMYLATION REACTION USING PNP TYPE LIGANDS

10.1 INTRODUCTION

Natural bite angle plays an important role in activity, stability and selectivity of the catalyst in a hydroformylation reaction. Small bite angle ligands like PNP type backbone are interesting ligands with one atom determining the flexibility or the natural bite angle as shown in figure.

![Natural Bite Angle controlling group X](image)

Figure 10.1 One atom controlling the natural bite angle of a PNP type ligand

PNP type ligands have been used for Pt/Pd catalyzed hydroformylation but its application for Rh catalyzed hydroformylation has not been examined. Apart from hydroformylation these ligands have been used for hydrogenation and C-C bond forming reactions.

Studies in chapter 9 showed that the regioselectivity of a hydroformylation reaction varied with changing electronegativity of the ligand backbone. This change in regioselectivity is based on varying electronegativity is a phenomenon which could be used to devise a ligand with switchable characteristics. The electronegativity of a ligand with nitrogen backbone can be
increased using by protonation using a Lewis acid. This increased electronegativity of the ligand due to the additive, could change the regioselectivity leading to applications in tandem catalysis.\textsuperscript{88} Also, a chiral Lewis acid could impart chiral information to the product leading to stereoselective product. The hypothesis is illustrated in scheme

\begin{center}
\begin{tikzpicture}
  \node (catalyst_x) at (0,0) {Catalyst X};
  \node (reactant) at (0,-1) {Reactant};
  \node (product_i) at (1,-1) {Product I};
  \node (catalyst_x_prime) at (2,0) {Catalyst X'};
  \node (reactant_prime) at (2,-1) {Reactant};
  \node (product_ii) at (3,-1) {Product II};
  \draw[->] (catalyst_x) -- (reactant);
  \draw[->] (reactant) -- (product_i);
  \draw[->] (product_i) -- (catalyst_x_prime);
  \draw[->] (catalyst_x_prime) -- (reactant_prime);
  \draw[->] (reactant_prime) -- (product_ii);
  \draw[->] (product_ii) -- (catalyst_x);
\end{tikzpicture}
\end{center}

Scheme 10.1 Lewis acid triggered tandem catalysis.

\textbf{10.2 RESULTS AND DISCUSSION}

The functionalization of primary amine to synthesize PNP ligands was done in a via aminophosphines or directly as shown in scheme 10.2 and 10.3 respectively.

\textbf{10.2A SYNTHESIS OF AMINOPHOSPHINES}

\[
\begin{align*}
\text{R} \text{NH}_2 + \text{Ph}_2 \text{PCl} & \xrightarrow{\text{neat}} \text{R} \text{NHPPh}_2 \\
\text{(excess)} & \quad \quad \text{Aminophosphine} \\
& \quad \quad \text{R = n-Propyl} \\
& \quad \quad = \text{Benzyl} \\
& \quad \quad = \text{Phenyl}
\end{align*}
\]

Scheme 10.2 Aminophosphine synthesis using primary amine.

The diphenylphosphine chloride was used in a stepwise fashion for the incorporation for the diphenylphosphine moiety. The aminophosphines formed in the presence of excess amine had characteristic $^{31}\text{P} \{^{1}\text{H}\}$ NMR singlets at 41 ppm, 42 ppm and 28 ppm for $n$-propyl, phenyl and
benzyl amines respectively. $^1$H NMR showed the single amine proton as a broad doublet with $^2$J$_{H,P}$ of 6-7 Hz. The n-propylamino phosphine and phenylamino phosphine were isolated. The n-propylamino showed $^2$J$_{C,P}$ of 14 Hz and $^3$J$_{C,P}$ of 6 Hz which for phenylamino phosphine were 17 Hz and 12 Hz respectively in $^{13}$C { $^1$H} NMR. The lower boiling amines were easily removed using a rotary evaporator while distillation under low vacuum had to be performed to remove the higher boiling amines. The phenylamino phosphine was able to be crystallized from ethanol while the rest were oily liquid. Using one equivalent of amine, diphenylphosphine chloride and base always resulted in the formation of the PNP type ligand directly and was not dependent on the temperature at which the diphenylphosphine chloride was added.

10.2b SYNTHESIS OF PNP TYPE LIGANDS

The PNP type ligands were synthesized directly from the amine or aminophosphine using two equivalents or one equivalent of diphenylphosphine chloride respectively, and base as shown in the scheme 10.3. This reaction worked best with polar solvents like dichloromethane. The removal of the triethylamine hydrochloride salt was performed by extracting the product from the dried reaction mixture using a non-polar solvent like benzene.

Scheme 10.3 Synthesis of PNP type ligands from primary amines
The \(^{31}\)P \(^{1}\)H NMR showed a singlet peak for phosphine at 62 ppm and 60 ppm for PNP ligands from \(n\)-propylamine and benzylamine respectively which matched literature values.\(^{81}\!^{89}\)

The PNP type ligands were used to for hydroformylation reaction using the same conditions as shown in scheme 9.4. The PNP ligand, Rh(acac)(CO)\(_2\) and the additive added were dissolved in dichloromethane and was stirred under pressurized H\(_2\)/CO mixture for an hour for formation of the active catalyst. After an hour, the Parr low pressure reactor was depressurized, and the 1-octene was added. The reaction product was analyzed using GCMS. The data obtained was tabulated in the table 10.1.

<table>
<thead>
<tr>
<th>(R_1)</th>
<th>Additive</th>
<th>Substrate</th>
<th>Conversion</th>
<th>Linear : Branched</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propyl</td>
<td>-</td>
<td>1-Octene</td>
<td>100%</td>
<td>1.32</td>
</tr>
<tr>
<td>Benzyl</td>
<td>-</td>
<td>1-Octene</td>
<td>100%</td>
<td>1.27</td>
</tr>
<tr>
<td>Benzyl</td>
<td>9-BBN</td>
<td>1-Octene</td>
<td>100%</td>
<td>1.39</td>
</tr>
<tr>
<td>Benzyl</td>
<td>(+)-CSA</td>
<td>1-Octene</td>
<td>100%</td>
<td>1.13</td>
</tr>
<tr>
<td>Benzyl</td>
<td>B(C(_6)F(_5))(_3)</td>
<td>1-Octene</td>
<td>100%</td>
<td>1.27</td>
</tr>
</tbody>
</table>

Table 10.1 Regioselectivity of hydroformylation reaction with \(R_1N(PPh_2)_2\) ligand (PNP ligand) with various additives at 9 bars of 1:1 H\(_2\) and CO and stirred at 80 °C for 24 h.

The hydroformylation reactions were performed using various additives which were added in equimolar amounts with the PNP ligand. The PNP ligand was added in twice the molarity of Rh(acac)(CO)\(_2\). The 9-BBN and B(C\(_6\)F\(_5\))\(_3\) were added to see the effect of adduct formation with one of the phosphines of the PNP ligand leaving only one phosphine for coordination to the...
There was no change in regioselectivity due to the addition of B(C₆F₅)₃. There was an increase in the l:b ratio when 9-BBN was used. These results lead us to questions whether both the phosphines on the PNP ligand are actually coordinating to the Rh(I). It is possible that due to the immensely small bite angle of PNP it was unable to act like a bidentate ligand and is acting like a monodentate ligand. The adduct formation with one of the phosphines with B(C₆F₅)₃ hence, does not change the regioselectivity of the product. When 9-BBN is used as an additive, the binding with the phosphine occurs as before but due to steric hindrance from the formed adduct, the linear product is favored giving a higher l:b ratio of 1.39.

The addition of camphor sulphonic acid (CSA) surprisingly decreased the l:b ratio which is contrary to what was expected from previous results. The increasing acidity of the PNP ligand upon protonation of the nitrogen should have increased the l:b ratio. The decrease in the l:b ratio indicates that there are other factors at play here. Upon protonation of the nitrogen lone pair of the PNP ligand the natural bite angle increases due conversion of the lone pair to a bond pair resulting in decrease of its steric requirements. This increase in the natural bite angle of the PNP ligand is probably the cause of the decrease in the l:b ratio. The branched product was not tested for stereoselectivity but the proximity of the chiral Lewis acid to the metal center could induce chiral information on the branched product and requires further experimentation.

### 10.3 Conclusion

Our results suggest that there was a substantial change in regioselectivity upon protonation of the nitrogen backbone of the PNP ligand. Electronic effects of the protonation seemed to have
been overridden by the steric effects due to the increase in the natural bite angle of the PNP ligand which lead to an increased branched product. This phenomenon can be optimized for larger changes in regioselectivity which could lead to a better switchable ligand for tandem catalysis. Also optimizations could be performed on the Lewis acid to obtain a greater stereoselectivity of the branched product.
PART II EXPERIMENTAL PROCEDURES

1. Materials and Methods

Unless stated otherwise, all synthetic reactions were carried out under an inert atmosphere of nitrogen using standard Schlenk techniques. Benzene, toluene, and DCM were all distilled from CaH$_2$ under nitrogen. Solvents were further degassed for reactions involving phosphine chemistry and hydroformylation reactions. $^1$H, $^{13}$C and $^{31}$P NMR spectra were recorded on a Varian MR 400 (400 MHz, 100.47 and 161 MHz, respectively). All NMR spectra were recorded at room temperature unless otherwise noted and were indirectly referenced to residual solvent signals or TMS as internal standards. $^{31}$P was referenced to an external reference sample of HMPA. Hydroformylation reaction was carried out in a Parr low pressure reactor with a 300 ml reaction vessel. Varian 500 MS was used for ESI MS. A Varian 3900 GC which had a Varian VF-1ms (dimethylpolysiloxane) 30 m x 0.25 mm GC column coupled to a Saturn 2100T MS was used to analyze hydroformylation reaction mixtures. Hyperchem 8.0.3 was used for calculating structures. Synthesis of Bis-diphenylphosphinohydrazine, Rh(acac)(CO)$_2$, trans-HRh(CO)(PPh$_3$)$_3$, aminophosphines and PNP type ligands were done according to literature methods.

2. General Hydroformylation Condition

In a low pressure Parr reactor, toluene (10 ml), substrate (1-octene or styrene) (66 µL, 420 mmol), ligand (63 mmol), Rh(acac)(CO)$_2$ (5.6 mg, 21.7 mmol) were added and purged with
nitrogen, purged with CO and then purged with H₂/CO mixture. After purge was satisfactory the reaction vessel was pressurized to 9 bars of 1:1 H₂ and CO and stirred at 80 °C for 18 h.

3. General Hydroformylation Condition using Additives

In a low pressure Parr reactor, toluene (10 ml), ligand (23.8 mg, 0.111 mmol), Rh(acac)(CO)₂ (14 mg, 0.056 mmol) and additive (0.111 mmol) were added and purged with nitrogen, purged with CO and then purged with H₂/CO mixture. After purge was satisfactory the reaction vessel was pressurized to 9 bars of 1:1 H₂ and CO and stirred at 80 °C for 1 h. After preforming the catalyst, the reactor vessel was depressurized and substrate (1-octene or styrene) (17.4 mL, 111.2 mmol) was added through an injection port in the reactor. The vessel was again pressurized to 9 bars of 1:1 H₂ and CO and stirred at 80 °C and stirred for 24 hours.

4. GCMS Analysis of Products from Catalysis

The reaction mixture was analyzed using GCMS where the concentration of the starting materials and the products were determined by using previously obtained calibration plots from pure compounds. All chromatogram peaks from different isomerization products of 1-octene hydroformylation were quantified using the calibration curves for 2-octene.
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