Next Generation Lanthanide – Based Contrast Agents for Applications in MRI, Multimodal Imaging, and Anti – Cancer Therapies

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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Abstract

A new class of polymer stabilized gadolinium trifluoride nanoparticles (NPs) have been developed as contrast agents for magnetic resonance imaging (MRI) and computed tomography (CT), with potential long term goals in targeted imaging and anti-cancer therapy. The NPs are comprised of a 90/10 mixture of GdF₃/EuF₃ and are coated with linear polyacrylic acid (PAA) chains consisting of 25 repeating units. The resulting aggregates are stable in serum and possess unprecedented mass relaxivities \[ i.e. \sim 100-200 \text{ s}^{-1} (\text{mg/mL})^{-1} \]. Electron microscopy images reveal various NP morphologies which depend on the exact synthesis protocol. These include highly cross-linked oblong clusters with 30-70 nm cross sections, extensively cross-linked aggregates with 100-300 nm cross sections, and distinct polymer stabilized nanocrystals with 50 nm diameters. Their application as contrast agents in \( T₁ \)-weighted MRI studies, CT imaging at various X-ray energies, and preliminary rat brain perfusion studies was also tested. NP contrast enhancement was compared to Gd-DPTA (Magnevist®) and iopramide (Ultravist 300®) to demonstrate their high contrasting properties and potential as multimodal contrast agents.
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# Table of Contents

Abstract .......................................................................................................................... ii
Acknowledgements ......................................................................................................... iii
Table of Contents ........................................................................................................... iv
List of Figures ................................................................................................................ vi
List of Tables ................................................................................................................... viii
List of Appendices .......................................................................................................... ix

Introduction .................................................................................................................... 1
Dendrimers ...................................................................................................................... 1
Micelles and Liposomes ................................................................................................. 2
Polysaccharides .............................................................................................................. 2
Polyamino Acids ............................................................................................................ 3
Zeolites ........................................................................................................................... 3
Metallofullerenes .......................................................................................................... 4
Superparamagnetic Iron Oxide Nanoparticles ......................................................... 4
Gadolinium-based Chelates ......................................................................................... 5
Gadolinium Oxide Nanoparticles .............................................................................. 5
Electronic Properties of Gadolinium ......................................................................... 7
Aim of Research ............................................................................................................. 7

Theory .............................................................................................................................. 13
Relaxivity ...................................................................................................................... 13
Magnetic Resonance Imaging (MRI) ........................................................................... 19
X-ray Scattering and Computed Tomography (CT) .................................................. 23
Zeta Potential .............................................................................................................. 28
Transmission Electron Microscopy (TEM) ............................................................... 29
**Materials and Methods**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthesis of Polymer-coated GdF₃/EuF₃ Nanoparticles</td>
<td>32</td>
</tr>
<tr>
<td>Zeta Potential Experiments</td>
<td>34</td>
</tr>
<tr>
<td>Magnetic Resonance Imaging (MRI) Experiments</td>
<td>34</td>
</tr>
<tr>
<td>Centrifugation and Staining</td>
<td>35</td>
</tr>
<tr>
<td>Transmission Electron Microscopy (TEM) Experiments</td>
<td>35</td>
</tr>
<tr>
<td>Rat Brain Perfusion Studies</td>
<td>35</td>
</tr>
<tr>
<td>X-ray Scattering and Computed Tomography (CT) Experiments</td>
<td>36</td>
</tr>
</tbody>
</table>

**Results and Discussion**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>The Use of Larger Polymers</td>
<td>37</td>
</tr>
<tr>
<td>The Synthesis in Detail</td>
<td>38</td>
</tr>
<tr>
<td>Properties of Lanthanides</td>
<td>40</td>
</tr>
<tr>
<td>Incorporation of Europium</td>
<td>41</td>
</tr>
<tr>
<td>Polymer Dynamics</td>
<td>42</td>
</tr>
<tr>
<td>Screening of Reaction Parameters</td>
<td>45</td>
</tr>
<tr>
<td>Quantification of Surface Charge</td>
<td>53</td>
</tr>
<tr>
<td>Proton Relaxation Enhancement</td>
<td>54</td>
</tr>
<tr>
<td>Dynamic Contrast Enhancement (DCE) in the Rat Brain</td>
<td>55</td>
</tr>
<tr>
<td>X-ray Scattering and Potential for CT</td>
<td>57</td>
</tr>
</tbody>
</table>

**Conclusions**

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>61</td>
</tr>
</tbody>
</table>

**References**

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>63</td>
</tr>
</tbody>
</table>

**Appendix A – Towards the synthesis of ¹³C-enriched para-fluorophenylalanine as a highly sensitive probe for studying protein dynamics**

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>67</td>
</tr>
</tbody>
</table>
List of Figures

Figure 1. Examples of Gd-based macromolecular complexes ........................................ 6
Figure 2. Schematic representation of polymer-coated NPs ........................................ 9
Figure 3. Comparison of relaxivities between polymer-coated NPs and recently published MRI contrast agents ................................................................. 10
Figure 4. Factors that influence relaxivity .................................................................. 14
Figure 5. Common geometries adopted by Ln$^{3+}$ ions in coordination ....................... 17
Figure 6. Examples of novel heptadentate ligands for coordination .............................. 18
Figure 7. Effect of an applied magnetic field on nuclear spins ..................................... 19
Figure 8. The inversion recovery pulse sequence ....................................................... 21
Figure 9. The relationship between magnetization and $T_1$ relaxation ......................... 22
Figure 10. The Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence ......................... 22
Figure 11. The relationship between magnetization and $T_2$ relaxation ....................... 23
Figure 12. X-ray scattering phenomena ..................................................................... 24
Figure 13. X-ray energy spectra and the use of filters ............................................... 25
Figure 14. X-ray energy profiles of Gd and I ............................................................... 27
Figure 15. Build-up of charge on a NP for measuring the zeta potential ....................... 29
Figure 16. Schematic representation of a transmission electron microscope ................ 30
Figure 17. Flow chart of NP synthesis protocol ......................................................... 33
Figure 18. TEM image of NPs synthesized with a larger polymer ............................... 38
Figure 19. Crystal growth of the NP core .................................................................. 41
Figure 20. Cross-linking of NPs ............................................................................... 43
Figure 21. Relaxivity of polymer-coated NPs as a function of stoichiometry ............... 47
Figure 22. Relaxivity of polymer-coated NPs as a function of reaction temperature .... 47
Figure 23. TEM images of NPs synthesized at lower pH and stoichiometric ratios ...... 48
Figure 24. TEM images of NPs synthesized at higher pH and stoichiometric ratios .... 48
Figure 25. $T_1$ relaxation rate as a function of NP concentration .................................. 50
Figure 26. TEM images of NPs synthesized with excess polymer .............................. 51
Figure 27. TEM image of NPs synthesized under dilute conditions ............................ 52
Figure 28. MR images of polymer-coated NPs and Gd-DTPA from a CPMG experiment...

Figure 29. DCE-MRI of a rat brain with administration of polymer-coated NPs...

Figure 30. Radiograph images of NPs, Gd-DTPA, and iopramide from X-ray scattering experiments...

Figure 31. Signal enhancement of NPs at various X-ray beam energies...

Figure 32. Signal enhancement of NPs, Gd-DTPA, and iopramide at various X-ray beam energies...
List of Tables

**Table 1.** The effect of salt stabilization on NP relaxivity .................................................. 45
**Table 2.** The effect of stoichiometry on NP relaxivity ......................................................... 49
**Table 3.** Zeta potentials of a few selected NPs .................................................................... 54
List of Appendices

Appendix A. Towards the synthesis of $^{13}$C-enriched para-fluorophenylalanine as a highly sensitive probe for studying protein dynamics .............................................. 67
Introduction

There are a host of macromolecules and nanoparticle complexes that incorporate gadolinium (III) (Gd$^{3+}$) ions into their design to help improve contrast in MRI studies by enhancing bulk water relaxation. Many of these complexes can also be functionalized or loaded with appropriate drugs to accomplish targeted imaging applications and drug delivery [1]. Such complexes include dendrimers, micelles, liposomes, polysaccharides, polyamino acids, zeolites, metallofullerenes, superparamagnetic iron oxide nanoparticles, Gd-chelates, and gadolinium oxide nanoparticles [1]. These complexes are briefly described below. In terms of contrast agents for MRI, Gd$^{3+}$ chelate complexes such as Gd-DTPA (Magnevist®) or Gd-DOTA (Dotarem®) are more commonly used in so-called $T_1$-weighted imaging, while iron-based contrast agents are more commonly used in $T_2$-weighted imaging.

Dendrimers

Dendrimers are a class of monodisperse macromolecules with highly branched, symmetric, three-dimensional architectures [1]. They consist of generations of branches that extend outward from a multifunctional core on which dendritic subunits are attached and can range from 10-100 nm in diameter [1]. Dendrimers can be tailored to achieve specificity by attaching drug molecules or targeting ligands to the surface while encapsulating drugs within the core. The periphery of dendrimers can be further conjugated with small molecules that can coordinate paramagnetic species. For example, diethylenetriaminepentaacetic acid (DTPA) can be conjugated to amine groups (e.g. polyamidoamine) on the surface of a dendrimer containing the amine functionality [2]. DTPA is able to coordinate Gd$^{3+}$ ions via chelation, resulting in dendritic gadolinium complexes that are now capable of enhancing solvent proton relaxation [2]. Figure 1A is a schematic representation of a macromolecular dendritic complex that can be used as a targeted imaging agent as well as a drug delivery agent [3]. Nevertheless, dendrimer chemistry is time consuming, labour-intensive, and becomes progressively difficult with each generation. Furthermore, by attaching targeting ligands and coordinating paramagnetic species, there may be increased peripheral hydrophobicity, aggregation, polydispersity and heterogeneity [1].
Micelles and Liposomes

Micellar and liposomal systems that incorporate Gd$^{3+}$ ions have also been explored [4]. Micelles are single layer detergent or phospholipid aggregates which spontaneously self-assemble due to the tendency of the hydrophobic tails to exclude water. Liposomes are bilayer phospholipid aggregates which also self-assemble but contain an aqueous interior in the centre. The driving force for self-assembly for such amphiphilic molecules is the hydrophobic associative interactions of the tails and the repulsive interactions between the hydrophilic headgroups in an aqueous environment [4]. There are several parameters such as the size and charge (cationic or anionic) of the headgroup, length of the chain, concentration, pH, and temperature that determine the structure, shape, and size of the aggregate [4]. Recent studies show that it is possible to complex Gd$^{3+}$ within a head group comprised of tetraazacyclododecanetetraacetic acid (DOTA) which is anchored via a carboxy linkage to an aliphatic, hydrophobic chain [5]. Figure 1B is a schematic representation of this complex [5]. Upon self assembly, each head group of the micelle contains a Gd$^{3+}$ ion which is chelated by DOTA [5]. Paramagnetic species can be anchored from liposomes via chelate-lipid molecule conjugation similar to the kind adopted for dendrimers [3]. Figure 1C is a schematic representation of such a liposomal design [3]. Gd-chelating lipids can also be incorporated into the lipid bilayer during liposome formulation [3]. However, only several Gd$^{3+}$ ions can be coordinated per micelle or liposome. Also, the circulation time in the blood pool may not be optimal for a micelle or liposome that contains paramagnetic species.

Polysaccharides

The conjugation of polysaccharides to Gd$^{3+}$ chelates has shown to result in water soluble contrast agents [6]. Since they are highly soluble in water, polysaccharides help increase the circulation time in the blood stream. Attaching dextran to Gd-DTPA, for example, would allow for the contrast agent to be retained in the blood stream for longer periods of time. Dextran is also able to coordinate many more Gd$^{3+}$ ions without intramolecular cross-linking [7]. Such a contrast agent is extremely advantageous for magnetic resonance blood pool imaging (figure 1D). It would be especially helpful in determining vasculature and tumor morphology [7]. Conjugation of natural polysaccharides to Gd-DTPA has also been explored [8]. Some examples include *Panax quinquefolium* (PQPS) which is
used for the treatment of high blood pressure and diabetes, and *Ganoderma applanatum pat* (GAPS) which possesses antitumor and immunological activities [8]. These have exhibited favourable proton relaxation enhancement in rat liver and kidney MRI [8].

**Polyamino Acids**

The attachment of polyamino acids to Gd$^{3+}$ chelates has applications in targeted imaging [9]. Some recent examples include polyornithine, polyarginine, and polylysine. Gd$^{3+}$ chelates containing pendant phosphonate and carboxylate groups were conjugated with the positively charged groups on the polyamino acids [9]. By chemically attaching the polyamino acid to the Gd$^{3+}$ chelate, the structure stays intact and the binding is strong enough to withstand the varying conditions of blood serum. In terms of applications, targeted imaging can also be achieved since positively charged polyamino acids selectively bind to tumor cells that have a surplus net negative charge as oppose to non-tumor cells [9]. Accumulation of the contrast agent at the tumor site can help determine extent of tumor growth and morphology.

**Zeolites**

Zeolites are porous, crystalline structures comprised of a combination of metals and non-metals – the most common form consisting of silicon, aluminum, and oxygen [10]. There are many naturally occurring and synthetically prepared zeolites. Certain zeolites that incorporate Gd$^{3+}$ ions into their matrix have been investigated as potential contrast agents. One specific example is of a sodium-yttrium zeolite doped with Gd$^{3+}$ ions and is roughly 80-100 nm in diameter [11]. Water molecules that coordinate with the immobilized Gd$^{3+}$ ions in the interior of the zeolite are in exchange with the bulk water outside the network (figure 1E). Bulk water relaxation can be achieved but it is limited by the rate of diffusion of water molecules through the pores of the zeolite channels [11]. Recent MRI applications involving the use of Gd-based zeolites include cardiovascular (Vasovist®) and gastrointestinal tract (Gadolite®) imaging [11].
Metallofullerenes

Gd-containing metallofullerenes represent a new class of MRI contrast agents that are still under study [12]. Metallofullerenes are spherical, cage-like structures that are comprised of carbon and encapsulate metal atoms [12]. Recent studies show that water-soluble, Gd-based metallofullerenes can significantly enhance proton relaxation [12, 13]. Water exchange between bulk water and water inside the cage depends on the porosity of the cage network and any functionalized groups that may be attached to it for solubility and tissue specificity purposes. An example of such a system is a 60 carbon atom cage, containing a Gd atom, and functionalized with carboxyl or hydroxyl groups on its exterior [13]. The aggregate cross-sections are on the 100 nm length scale. Uptake of hydroxylated, Gd-based metallofullerenes by the organs and tissues of the reticuloendothelial system (RES) in small animals has been observed in some studies [13].

Superparamagnetic Iron Oxide Nanoparticles

Although not Gd-based, superparamagnetic iron oxide nanoparticles (SPIONs) have also become more focused toward applications in diagnostic imaging. SPIONs can range from less than 50 nm (in which case they are referred to as ultra SPIONs) up to 500 nm in diameter [14]. The particle size dictates their physiochemical and pharmacokinetic properties and determines the appropriate tissue to target [14]. SPIONs can be functionalized with various coatings and targeting ligands, including polyamino acids, polysaccharides, and polymers. For example, a PEG group has been used to link folic acid to SPIONs in applications where the complex is intended to bind to cancer cells [15]. Depending on the coating and application, proton relaxation enhancement from SPIONs is comparable to that from many of the current Gd-based MRI contrast agents [16]. However, contrast is usually darkened in areas where targeted SPIONs accumulate. Hence, an extended image void is created which may obscure the surrounding anatomy and key features [16]. Thus, SPIONs are classified as negative contrast agents since they decrease contrast intensity in areas where they are concentrated.
**Gadolinium – Based Chelates**

$\text{Gd}^{3+}$ chelates are positive contrast agents because they increase contrast through $T_1$-weighted imaging in the area they accumulate. Most of the current $\text{Gd}^{3+}$ chelates are based on polyaminocarboxylate ligands which are either linear or macrocyclic molecules [17]. Such ligands form very stable complexes with $\text{Gd}^{3+}$ through a chelation effect, thereby minimizing leaching of $\text{Gd}^{3+}$ ions or dissociation of the complex and reducing toxicity as a result. Their stability arises from the thermodynamic and kinetic favorability of the complexation [17]. Two of the most common examples of $\text{Gd}^{3+}$ chelates are $\text{Gd-DTPA}$ (Magnevist®) and $\text{Gd-DOTA}$ (Dotarem®), which have been in clinical practice for many years (figure 1F) [18]. Many $\text{Gd}^{3+}$ chelates have also been successfully functionalized with targeting groups to achieve tissue specificity. The conjugation chemistry is relatively easy to accomplish and not too time consuming. Many different chelating ligands have been synthesized and tested for proton relaxation enhancement in an attempt to increase $T_1$ and $T_2$ relaxivities [17].

**Gadolinium Oxide Nanoparticles**

Gadolinium oxide nanoparticles ($\text{Gd}_2\text{O}_3$ NPs) are an emerging class of contrast agents that aim to increase proton relaxation enhancement by encapsulating a high number of $\text{Gd}^{3+}$ ions within the core. A recent study reported pegylated $\text{Gd}_2\text{O}_3$ NPs with a single-crystal core having a mean diameter of 3 nm and consisting of approximately 200 Gd atoms per NP [19]. Possibilities of tailoring these NPs for biological labeling and targeting are currently under study [20]. At present, $\text{Gd}_2\text{O}_3$ NPs coated with polysiloxane and conjugated to PEG possess relaxivities two times higher than that of Gd-DTPA [20]. The potential for these NPs to be blood pool imaging agents has been demonstrated in some studies [19].
Figure 1. A) A dendritic complex that has a Gd$^{3+}$ contrast agent conjugated at the periphery along with additional multi-purpose functionalities [3]; B) A liposomal system containing a conjugated Gd$^{3+}$ contrast agent on the exterior [3]; C) A micellar structure containing Gd$^{3+}$ complexed in the head group of the lipid [5]; D) Gd$^{3+}$ chelated to DTPA which is attached to alternating glucose units of dextran [7]; E) A zeolite with Gd$^{3+}$ nested in the pocket with coordinated water molecules (A), uncoordinated water molecules in exchange with bulk water (B), and bulk water molecules (C) [13]; F) Two of the most commonly used commercialized contrast agents based on Gd$^{3+}$ chelates [18].
The quest for achieving the highest possible relaxivity with low toxicity and applicability in diagnostic imaging is still an ongoing, active area of research. Many of the current contrast agents provide proton relaxation enhancement that is good enough to differentiate between certain tissues. However, in areas where low concentrations of the contrast agent must be used, higher relaxivities become important. Areas where there is little vascular tissue density would benefit from high relaxivity contrast agents. Most of the contrast agents discussed above only incorporate one to several Gd\(^{3+}\) ions, while bound water exchange times are not optimized to affect the largest local changes in proton \(T_1\). The parameters important for optimizing relaxivity are discussed in the theory section.

**Electronic Properties of Gadolinium**

Gd\(^{3+}\) is an ideal paramagnetic relaxation agent because it has a large magnetic moment (7.98 BM, \(T=298\) K, seven unpaired electrons in the \(4f\) orbital, \(S=7/2\)) and nanosecond electronic spin relaxation time [21, 22]. Although dysprosium (Dy\(^{3+}\), 10.6 BM) and holmium (Ho\(^{3+}\), 10.9 BM) have larger magnetic moments than Gd\(^{3+}\), the asymmetry of their electronic states leads to very rapid electron spin relaxation [23]. Gd\(^{3+}\) has one electron in each of its \(f\) orbitals and is therefore electronically very stable. The \(4f\) orbitals are directly responsible for the magnetic and absorbance properties of the lanthanides. The lanthanides are not stabilized by ligand field stabilization energy (LFSE) because of reduced interactions with the \(4f\) orbitals [24]. They do not require specific geometries when forming complexes since coordination is generally determined by steric factors [24].

**Aim of Research**

Novel Gd-based contrast agents have been synthesized which possess characteristic properties suitable for MRI and CT imaging. Applications in radionuclide-based imaging (SPECT and PET) and anti-cancer therapies are long-term goals. This new class of contrast agents consists of lanthanide trifluoride nanoparticles (LnF\(_3\) NPs) that are typically comprised of a 90/10 mixture of GdF\(_3\) and EuF\(_3\) and are coated with polyacrylic acid (PAA), a linear polymer chain of 25 repeating units (PAA25). Figure 2 is a schematic representation of the design of these NPs which contain thousands of Gd\(^{3+}\) ions per NP, yet the majority of Gd\(^{3+}\) ions are located within a few Angstroms of the NP surface. Thus, a significant
enhancement in relaxivity is expected and indeed observed in comparison to some of the current organic chelates and even more exotic macromolecular contrast agents as discussed above. Figure 3 is a comparison of relaxivities (at 1.5 Tesla) among some recently published MRI contrast agents and polymer-coated GdF₃/EuF₃ NPs, which possess mass relaxivities that are much higher than their contenders [25]. Electron microscopy images reveal the NP morphology, which includes highly cross-linked oblong clusters with 30-70 nm cross sections, extensively cross-linked aggregates with 100-300 nm cross sections, and distinct polymer stabilized nanocrystals with 50 nm diameters. The morphology is strictly dictated by well-controlled reaction conditions. Parameters such as pH, temperature, solvent volume, and the stoichiometry between the lanthanide and polymer are of prime importance. Various combinations of these reaction parameters were tested to obtain the current optimal reaction conditions that produce NPs with the highest possible relaxivity, correct geometry, solubility in biological media, and potential for surface functionalization for tissue specificity.

The current synthesis of PAA25-coated GdF₃/EuF₃ NPs is an adaptation of a scheme described by van Veggel et al [21]. GdF₃/EuF₃ NPs coated with citrate (CIT) and aminoethyl phosphate (AEP) have been previously synthesized by the van Veggel group [21]. These NPs are 5 nm in diameter and possess relaxivities around 20 s⁻¹(mg/mL)⁻¹ but their solubility is limited to aqueous media. Several other combinations of lanthanides and coatings have also been synthesized by the van Veggel group. Many of these include LaVO₄, LaPO₄, and LaF₃ NPs coated with dithiophosphate alkyl groups, LaF₃ NPs coated with polyethylene glycol (PEG), AEP conjugated to biotin-avidin, and LaF₃ NPs doped with various other lanthanides such as Eu³⁺, Nd³⁺, Er³⁺, Pr³⁺, Ho³⁺, and Yb³⁺ [26, 27, 28, 29, 30]. PAA25-coated GdF₃/EuF₃ NPs possess relaxivities that are comparatively much higher than those reported by van Veggel et al for their Gd-based NPs [21]. Furthermore, these NPs are geared towards applications in multimodal imaging and anti-cancer therapy which can be achieved thanks to their stability in biological media.

Electron microscopy studies reveal a size range between 50-70 nm in diameter. Hence, PAA25-coated GdF₃/EuF₃ NPs are small enough for removal by macrophage extraction and ultimate clearance via bile pathways [31]. They are also small enough to penetrate through tissues and into the extracellular space of tumors but are sufficiently large to achieve contrast amplification [31, 32]. The multi-dentate carboxylate groups of the
polymer provide a strong interaction between each polymer molecule and the NP surface. PAA does not behave like DTPA because it is not a chelator. Rather, PAA is a polyelectrolyte that coordinates strongly with the NP surface, providing a high solubility in a range of organic solvents and biological media. During the synthesis, the electrostatic Coulomb force between PAA and the NP surface is weak enough to allow particle growth but strong enough to prevent particle coagulation. Since the NP system is colloidally stabilized, the polymer is also able to entrap a large number of water molecules while presumably slowing down the water exchange rate with the paramagnetic surface. This feature is a key factor in establishing high relaxivities.

Figure 2. A schematic representation of the design of polymer-coated GdF₃/EuF₃ NPs. The NP core clusters a high number of paramagnetic Gd³⁺ ions together along with dopant Eu³⁺ ions. The polymer entraps and coordinates as many water molecules as possible to the NP surface. X represents COOH groups of the polymer with the repeating unit shown on the right.
Figure 3. A comparison of $T_1$ relaxivities between polymer-coated GdF$_3$/EuF$_3$ NPs (last four bars on the right) and some recently published MRI contrast agents. Polymer-coated GdF$_3$/EuF$_3$ NPs possess unprecedented mass relaxivities that are comparatively higher than their contenders [25]. NPs synthesized with excess polymer (last three bars on the right) possess significantly higher relaxivities due to extensive nanoparticle cross-linking. Note that the first two bars on the left represent relaxivities of two common commercial agents – Gd-DTPA (Magnevist®) and Gd-DOTA (Dotarem®).

Perhaps one of the most exciting features of the polymer-coated GdF$_3$/EuF$_3$ NPs is their potential for multimodal imaging – namely MRI, CT, and with appropriate radionuclide doping, positron emission tomography (PET) and single photon emission computed tomography (SPECT). Phantom samples of PAA25-coated GdF$_3$/EuF$_3$ NPs prepared in water were used to carry out MRI and X-ray scattering experiments to test and prove their potential as multimodal contrast agents. The relaxivity of these NPs was compared to Gd-DTPA for MRI and the contrast was compared to a common iodinated contrast agent (iopamid, Ultravist 300®) for X-ray CT.

Targeted imaging can also be accomplished by functionalizing the polymer with targeting peptides or receptor substrates (e.g. integrins, folate, antibodies, fluorophores, etc.) to achieve tissue specificity. The folate receptor is often over-expressed in a wide range of
tumor cells in humans [15, 33]. By conjugating folic acid to the polymer via a peptide coupling reaction, the NP can be delivered to the specific site where it will accumulate and provide improved local contrast [15].

Polymer-coated GdF$_3$/EuF$_3$ NPs have the additional advantage that they can be doped with radionuclide versions of other lanthanides, thereby serving as cytotoxic agents for applications in anti-cancer therapies. Radionuclides such as $^{177}$Lu or $^{18}$F can be incorporated into the lanthanide matrix during the synthesis as Na$^{18}$F and Lu(NO$_3$)$_3$, respectively. $^{177}$Lu has a half-life of 161 hours and emits $\beta^-$ particles with maximum energies of 498 keV when it decays [34]. $^{177}$Lu also produces gamma rays with energies of 113 keV and 208 keV and Auger electrons with energies between 42-46 keV and 51-56 keV upon secondary collisions of $\beta^-$ particles with electrons [34, 35]. $^{18}$F has a half-life of 2 hours and emits $\beta^+$ (positron) particles with energies of 511 keV along with gamma rays when it decays [34]. By doping NPs with $^{177}$Lu, the presence of high atomic number species like Gd and Eu in the immediate vicinity of the radionuclide will aid in the overall radiation. Although $\beta^-$ particles have stopping distances between 2-12 mm in tissue, $^{177}$Lu $\beta^-$ particles are such that their average path distance is reduced to about 0.28 mm in tissue [35, 36, 37]. In order to confine these $\beta^-$ particles within a few cell diameters, a large fraction of $\beta^-$ particles emitted from $^{177}$Lu will encounter the Gd-rich matrix and additional Auger electrons (which travel a few microns) and gamma ray photons will be produced [38]. The stopping distance of $\beta^-$ particles will also be reduced as a result of the intrinsic collisions. Hence, the extent of exposure will be localized to the site where $^{177}$Lu-loaded NPs accumulate and local cell damage will be amplified with secondary radiation. In this case, the NP formulation is quite useful since it already contains heavy metals and radionuclide within one macromolecular bundle. The incorporation of $^{18}$F into the NP matrix would allow for applications in SPECT and PET. SPECT is based on the emission of a single photon with an energy of 140 keV whereas in PET, the emission of two photons of 511 keV each occurs [39]. In this case, PET is superior to SPECT because of its greater sensitivity and better resolution. When positrons encounter electrons of high atomic number elements such as Gd$^{3+}$ and Eu$^{3+}$, gamma ray photons are produced [38]. With the use of an appropriate gamma ray counter, a three-dimensional image of the distribution of the radionuclide can be obtained. It is important to achieve tissue-specific targeting with GdF$_3$/EuF$_3$ NPs before they can be used as tracking agents for
SPECT or PET. GdF₃/EuF₃ NPs are ideal candidates for such applications because a high number of gamma ray emitters can be clustered together in a single aggregate, providing greater sensitivity. Radionuclides can be incorporated into the lanthanide matrix quite easily. Neutron capture therapy (NCT) can also be made possible with a NP matrix that is already rich with Gd³⁺ [40]. NCT can be used as a cancer therapeutic modality [41]. ¹⁵⁷Gd, which is a stable, non-radioactive nuclide, can be delivered to the target site and irradiated with thermal or epithermal neutrons. ¹⁵⁷Gd is known to have the largest neutron capture cross-section of 254,000 barn, which is 66 times larger than that of boron, ¹⁰B [41]. Upon irradiation, ¹⁵⁷Gd produces long-range gamma rays (> 100 μm) with energies of 7.94 MeV and Auger electrons with maximum energies of 41 keV [42]. Such energetic photons and electrons are toxic enough to destroy cellular matter within close proximity.
Theory

Relaxivity

Gd$^{3+}$ is an ideal paramagnetic relaxation agent because it has a large magnetic moment and nanosecond spin relaxation time [21]. Hence, it can significantly affect its surrounding environment by relaxing local proton nuclei very quickly. The contrast enhancing efficiency is expressed in terms of relaxivity ($R$), which is the increase in the solvent proton spin-lattice ($T_1$) and spin-spin ($T_2$) relaxation rates per unit concentration of contrast agent [17]. This can be obtained from the slope of a plot of relaxation rate versus contrast agent concentration (equation 1).

$$R = \frac{1}{T_{1,2}} \left( \frac{1}{[CA]} \right)$$

(1)

A reduction in $T_1$ and $T_2$ results in the increase of relaxation rates (i.e. $1/T_1$, $1/T_2$) and the corresponding relaxivities ($R_1$, $R_2$) for a given concentration of the contrast agent. The relaxivity of water protons in the presence of paramagnetic species arises from the dipolar coupling interaction between the electron magnetic moment of the metal ion and the nuclear magnetic moment of the solvent proton nuclei [17, 43]. Water molecules can either be directly coordinated to the metal ion (inner sphere, IS), diffuse in close proximity of the metal ion (outer sphere, OS), or can be involved in hydrogen-bonding interactions with polar groups of the ligand (second sphere, SS). As a result, the total relaxivity is the sum of these three contributions [17].

$$R = R^{IS} + R^{OS} + R^{SS}$$

(2)

Water molecules that are hydrogen-bonded to polar groups of the ligand provide an additional relaxation mechanism for the bulk water protons. They also affect the relaxivity because they engage in a highly dynamic network of hydrogen bonds in the second coordination sphere of the NP. Three main factors that affect the interaction between solvent protons and the paramagnetic metal ion are: the rotational correlation time of the aggregate
\((\tau_R)\), the residence lifetime of the bound water \((\tau_M)\), and the paramagnetic relaxation time \((T_1\) and \(T_2)\) (figure 4) [43]. Equation 3 gives the inner sphere \(T_1\) relaxivity where \(q\) is the number of water molecules bound to the metal surface, \(\tau_M\) is their mean residence lifetime, \([CA]\) is the concentration of the contrast agent, and \(T_{1M}\) is the spin-lattice relaxation time of the water protons [17].

\[
R_{i}^{IS} = \frac{[CA]q}{55.6 \ T_{1M} + \tau_M}
\]  

Figure 4. Relaxivity of a contrast agent depends on the molar concentration of the paramagnetic species, the number of coordinating water molecules, the residence time of bound water \((\tau_M)\), the rotational correlation time of the complex \((\tau_R)\), and the relaxation times of bound water proton nuclei \((T_1\) and \(T_2)\) [43].

Contrast in MRI can be greatly enhanced by clustering a high number of paramagnetic species within a small enough aggregate. The aggregate should be small enough so that the surface area-to-volume ratio is relatively large in order to ensure that the highest fraction of \(\text{Gd}^{3+}\) ions is available for coordination on the surface. An increase in the molar fraction of water protons interacting with the paramagnetic surface directly contributes to the overall relaxivity. Hence, a surface ligand which entraps a large number of water molecules, such as a multi-dentate ligand containing several pendant carboxylate groups like PAA, is preferred. Higher relaxivities are indeed observed since the polymer creates a large network of cross-linking and hydrogen bonding with water molecules. Water molecules travel through the network and are brought into close proximity of the paramagnetic surface of the NP. The exchange of water protons hydrogen-bonded to the carboxylate groups would dominate in the second sphere of the aggregate [17, 18, 44].
The rotational correlation time ($\tau_R$) of the aggregate should be long enough (longer than nanoseconds) in order to increase relaxivity [17]. This can be achieved by increasing the size of the macromolecular substrate, thereby increasing the rotational diffusion correlation time to a point where tumbling can be neglected as a source of relaxation [17, 44]. However, the aggregate must remain small enough to ensure most of the Gd$^{3+}$ ions are available at the surface. The pharmacokinetic behaviour of the aggregate must also be considered and often holds higher precedence than its relaxometric properties [17, 43]. Unlike the hydration parameter, the correlation time becomes independent of the aggregate size at some point where the relaxivity depends only on the parameters in equation 3 — $T_{IM}$, $q$, $\tau_M$, and $[CA]$ [17]. The paramagnetic centre must also be accessible by the solvent protons; otherwise it may be buried under a large coating system that may deplete its interaction with the solvent environment; thus reducing the relaxivity. When $\tau_M \ll T_{IM}$ in equation 3, the relaxivity depends on the relaxation rate of the coordinated solvent molecules [18]. When the water exchange rate is fast enough, the bulk solvent protons will relax as fast as the coordinated solvent protons. However, if $\tau_M$ is too short, it will begin to influence $T_{IM}$ in the inner sphere [45].

$$\frac{1}{T_{im}} = \frac{1}{T_{i}^{DD}} + \frac{1}{T_{i}^{SC}} \quad i = 1, 2$$

$$\frac{1}{T_{1}^{DD}} = \frac{2}{15} \gamma^2 g^2 \mu_B^2 S(S+1) \left( \frac{3}{1 + \omega_f^2 \tau_e} + \frac{7}{1 + \omega_s^2 \tau_e^2} \right)$$

$$\frac{1}{T_{1}^{SC}} = \frac{2}{3} S(S+1) \left( \frac{A}{\hbar} \right)^2 \left( \frac{\tau_e}{1 + \omega_s^2 \tau_e^2} \right)$$

$$\frac{1}{T_{2}^{DD}} = \frac{1}{15} \gamma^2 g^2 \mu_B^2 S(S+1) \left[ \frac{3}{1 + \omega_f^2 \tau_e} + \frac{13}{1 + \omega_s^2 \tau_e^2} + 4 \tau_e \right]$$

$$\frac{1}{T_{2}^{SC}} = \frac{1}{3} S(S+1) \left( \frac{A}{\hbar} \right)^2 \left( \frac{\tau_e}{1 + \omega_s^2 \tau_e^2} + \tau_e \right)$$
Equation 4 shows that the overall relaxivity in the inner sphere ($T_{im}$) is a sum of the relaxivities resulting from dipole-dipole (DD) and scalar (SC) relaxation. Equations 5a and 5b describe the $T_1$ relaxivity and equations 6a and 6b describe the $T_2$ relaxivity [18]. Here, $S$ is the electron spin quantum number (Gd$^{3+}$ $S = 7/2$), $\gamma_I$ is the nuclear gyromagnetic ratio for spin $I$, $g$ is an electronic factor, $\mu_B$ is the Bohr magneton, $r$ is the electron spin-solvent nuclear spin distance, $\tau_{ci}$ is correlation time associated with dipole-dipole relaxation, $\tau_{ei}$ is the correlation time associated with scalar relaxation, $\omega_S$ and $\omega_I$ are the electron and nuclear Larmor frequencies, respectively, and $A/\hbar$ is the scalar (hyperfine) coupling constant between the electron of the paramagnet and the proton of the coordinated water [18]. These equations show that the relaxation rate is inversely proportional to the distance between the paramagnetic species and water molecules and is directly proportional to the gyromagnetic ratio of the solvent proton, the magnetic moment, and spin quantum number of Gd$^{3+}$. The characteristic correlation times depend on the dynamic processes that occur at the molecular level. This may include molecular interactions through space and contact with the paramagnetic surface. These equations are known as the Solomon-Bloembergen-Morgan equations and are used to describe inner sphere relaxation, assuming isotropic reorientation, which results from Gd$^{3+}$-based contrast agents [17, 18, 44, 45].

$$
\left( \frac{1}{T_{1e}} \right)^{ZFS} = \frac{1}{25} \tau_v^2 \left\{ 4S(S+1) - 3 \right\} \left[ \frac{1}{(1 + \omega_S^2 \tau_v^2)} + \frac{4}{(1 + 4\omega_S^2 \tau_v^2)} \right] 
$$

Equations 7 and 8 describe the relaxivity of Gd$^{3+}$ complexes as a function of the magnetic field [17]. They show that the $T_1$ and $T_2$ relaxation rates are frequency dependent (recall that the nuclear or electron Larmor frequency is related to the magnetic field, $B$, by the gyromagnetic ratio, $\gamma$, $\omega = \gamma B$) [17, 18]. The $\tau_v$ parameter is the correlation time of the modulation of the zero-field splitting (ZFS) energy [17]. ZFS refers to the removal of spin degeneracy as a result of solvent collisions or molecular or electronic vibrations [18]. The gyromagnetic ratio of an electron is much larger than that of a proton ($\gamma_S/\gamma_H = 658$); thus the $\omega_S^2 \tau_v^2$ term becomes much greater than 1 at lower magnetic fields. The bracketed terms in
equations 7 and 8 become very small with a large $\tau_v$. Hence, equations 7 and 8 are only valid when $\omega_S^2 \tau_v^2 \ll 1$ (the extreme narrowing condition) so that the electronic relaxation can be considered a mono-exponential process [18].

The hydration number ($q$) significantly affects the inner sphere relaxivity (equation 3). Lanthanide ions can have coordination numbers of six, seven, eight, or nine of which the most common is eight or nine [24]. When Gd$^{3+}$ is nine-coordinate (CN=9), the water exchange process has been observed to occur through an eight-coordinated transition state [17]. Hence, the water residence lifetime is related to the free-energy difference between the transition and ground states. The coordination geometry around the Gd$^{3+}$ can either be a tri-capped trigonal prism (TTP) or a mono-capped square antiprism (MSA) with possible interconversion between the two [18]. The TTP geometry is sterically more favourable than the MSA geometry. Figure 5A illustrates these geometries and figure 5B is an example of how DTPA fits the coordination scheme for TTP geometry.

![Figure 5. A) Common geometries adopted by Ln$^{3+}$ ions in coordination; B) An example of the trigonal prism geometry with Gd-DTPA. The ninth coordination site is occupied by one water molecule.](image)

In these cases, only one water molecule is directly coordinated to Gd$^{3+}$. An increase in the relaxivity is expected if hepta- or hexa-dentate ligands are used for coordination, thereby providing coordination sites for additional water molecules. But this may also lead to a decrease in the thermodynamic stability and an increase in the toxicity of the Gd$^{3+}$ complex since water molecules are easily replaceable with donors that have a higher affinity for Gd$^{3+}$. There are some examples of ligands that chelate with two inner sphere water molecules and form relatively stable complexes (figure 6) [44]. HOPO is a heptadentate ligand and its coordinating geometry is such that it prevents the replacement of the two water molecules.
The exchange rate of the water molecules is comparable and the electronic relaxation is also slow enough to attain high relaxivities [44]. PCP2A and AAZTA are also heptadentate ligands. The Gd$^{3+}$-AAZTA complex has been characterized to have a relaxivity of 7.1 mM$^{-1}$s$^{-1}$ (at 20 MHz, 298 K), high thermodynamic stability in aqueous solution, and complete inertness towards bidentate anions [44].

![Figure 6. Examples of some new heptadentate ligands that allow two coordinating water molecules in the inner sphere of a Gd$^{3+}$ complex [44].](image)

Some other approaches that are worth mentioning are Gd$^{3+}$ complexes that use human serum albumin (HSA) as the interacting substrate and pH-sensitive agents [44, 45]. Gd$^{3+}$ complexes that have a high binding affinity for HSA exhibit higher intravascular retention time, thereby rendering them as good blood pool imaging agents [44]. Protonation/deprotonation of chelate moieties, formation of intramolecular hydrogen bonds, and multiple spheres of hydration are strongly influenced by changes in pH and can be tailored to increase the relaxivity of pH-sensitive agents.

The exchange between free and bound water must be slow enough to maximally contribute to the nuclear spin relaxation process at MRI field strengths. By slowing down the exchange, water molecules have a longer time span to coordinate with the paramagnetic surface. A ligand that can entrap a large number of water molecules would also slow down the exchange between free and bound water. A second sphere of hydration would help amplify this effect. The relaxivity of PAA25-coated GdF$_3$/EuF$_3$ NPs reveals that they possess most of the inherent properties required for a highly effective contrast agent as discussed above.
Magnetic Resonance Imaging (MRI)

MRI is one of the most commonly used diagnostic imaging tools because it provides precise anatomical information and is quick and easy to do [46]. It is based on the interaction between an external magnetic field and the nuclear spin angular momentum of NMR-active nuclei. The overall spin of the atom provides information on the possible number of orientations. For example, a nucleus with a spin of 1/2 (e.g. $^1$H nuclei) can have two orientations: a positive spin of +1/2 and a negative spin of -1/2 which can be in random motion. In the absence of a magnetic field, these spins are of equal energy but when placed between the poles of a strong magnet, the nuclear spins adopt specific orientations (i.e. either parallel or anti-parallel) along the applied magnetic field [46]. As a result, the magnetic field causes the energy levels to split and each level is denoted with a magnetic quantum number ($m$). The parallel orientation is slightly lower in energy than the anti-parallel orientation therefore it is slightly more favourable. According to a Boltzmann distribution, the lower energy orientation has a slightly higher proportion of the nuclei population than the higher energy orientation. The energy difference ($\Delta E$) between these two orientations depends on the strength of the applied field ($B_0$) [47]. Figure 7A illustrates the concept of energy level splitting of a nucleus.

![Diagram A](image1.png)

**Figure 7.** A) Applying a magnetic field splits the degeneracy of the nucleus according to a Boltzmann distribution. The lower energy state contains slightly more nuclear spins than the higher energy state. The difference in the two energy states is proportional to $B_0$; B) The Cartesian coordinate system is used to describe nuclei precession in terms of vectors. The nuclei precess about the magnetic field $B_0$ [46, 47].
In the presence of a magnetic field, individual proton nuclei precess about the magnetic field. They are tilted slightly away from the axis of the magnetic field, but the axis of rotation remains parallel to $B_0$ (figure 7B) [47]. The stronger the $B_0$, the greater the energy difference between the two spin states and therefore a higher frequency (higher energy) radiation is required for a spin-flip to occur [47]. The frequency of precession is proportional to the strength of the magnetic field and is expressed by equation 9 where $\nu_0$ is the Larmor frequency in MHz, $B_0$ is the magnetic field strength in Tesla (T) experienced by the proton nuclei, and $\gamma$ is the gyromagnetic ratio of the nuclei ($\gamma = 2.675 \times 10^8 \text{ s}^{-1}\text{T}^{-1}$ for $^1\text{H}$ nuclei).

$$\nu_0 = \frac{\gamma B_0}{2\pi} \quad (9)$$

When the oriented nuclei are irradiated with radiofrequency (rf) pulses at the Larmor frequency, the energy is absorbed by the nucleus and causes the angle of precession to change [46]. For example, for a low energy (+1/2) spin state, absorption of resonant radiation energy flips the orientation to the higher energy state. As a result, the nuclei now exist in the excited state which has a finite lifetime; thus relaxation processes follow.

Relaxation does not occur by emission of radiation because the probability of re-emitting photons at radiofrequencies is very low; hence non-radiative relaxation processes take place [47]. Non-radiative relaxation occurs by either spin-lattice ($T_1$) or spin-spin ($T_2$) relaxation. Vibrating and rotating nuclei generate a magnetic field which is referred to as the lattice field. Some of the high-energy nuclei will have vibrational/rotational frequencies that are equivalent to and in-phase with the Larmor frequency [47]. This energy match causes the high-energy nuclei to spin-flip to the low-energy state. These nuclei regain their equilibrium position by returning to the low-energy state and the excess energy is lost to the lattice in the form of heat ($T_1$ process) [47]. The amount of time it takes for the excited state to return to 63% of its original state is denoted $T_1$. $T_2$ relaxation occurs through the exchange of energy between the two energy levels. It describes the interaction between neighboring nuclei which have the same vibrational/rotational frequencies [46, 47]. Nuclei can exchange their quantum states and the nucleus in the low-energy state becomes excited while the high-energy nucleus relaxes to the low-energy state. *$T_2$ is the total loss of transverse coherence
which includes the $T_2$ relaxation process as described above and loss of transverse coherence due to a fluctuating local magnetic field around each proton nuclei [46]. $T_1$ is the time it takes for the magnetization to return to equilibrium along the $z$ axis whereas $T_2$ is the decay of magnetization along the $x$ and $y$ axes to their equilibrium states so that zero net macroscopic magnetization can be restored.

In MRI, the magnetic field is made spatially dependent by applying linear magnetic field gradients [46]. A gradient along each axis of the coordinate system is applied to obtain an image through slice selection, frequency encoding, and phase encoding [46, 48]. Protons, either in fat or water, will resonate at a unique frequency that depends on their exact position within the gradient field. The resulting image is a frequency map of the protons with respect to their position and differing magnetic fields [48]. Pixel intensity is proportional to the number of protons contained within a voxel (volume element) with respect to the $T_1$ and $T_2$ relaxation times [46, 48]. Gradient pulses produce less than 1% of field distortion, therefore they cause relatively small perturbations to the main magnetic field $B_0$ [46].

The proton $T_1$ relaxation time is measured using an inversion recovery (IR) pulse sequence which is shown in figure 8 [49]. The IR sequence consists of two radiofrequency (rf) pulses. The first 180° pulse along the $x$-axis flips the magnetization along the $-z$-axis and is allowed to recover for a period $\tau$. During the recovery period, the magnetization returns to equilibrium along the $z$-axis. The value of the magnetization remaining after the delay time $\tau$ is then measured by applying a 90° pulse along the $y$-axis followed by acquisition. The magnetization along the $z$-axis must be fully restored to its equilibrium state before repeating the sequence with a new $\tau$.

![Figure 8](image)

**Figure 8.** The inversion recovery pulse sequence is commonly used to measure the $T_1$ relaxation time.

By varying $\tau$, a plot of intensity as a function of $\tau$ (in seconds) can be obtained (figure 9A). By integrating the exponential growth curve (figure 9A), a linear relationship can be obtained
from which the slope provides the value of $T_1$ (figure 9B). The inversion recovery sequence must be repeated for a series of $\tau$ values in order to fit the intensity data to the integrated Bloch equation (equation 10) that describes the relationship between $T_1$ and the magnetization.

$$M_y = M_o \left[1 - 2 \exp(-\tau / T_1)\right]$$

\[(10)\]

**Figure 9.** A) An exponential growth curve for measuring the $T_1$ relaxation time [49]. This is obtained by varying $\tau$ and is described by equation 10; B) Integration of the curve in A gives a linear relationship from which the $T_1$ value can be extracted.

Figure 10 is the Carr-Purcell-Meiboom-Gill (CPMG) sequence (a spin echo sequence) which is used to measure the proton $T_2$ relaxation time [50]. For MRI experiments, this sequence consisted of 96 echoes in total, therefore the $180^\circ$ pulse was applied 96 times.

**Figure 10.** A CPMG sequence measures $T_2$ [50]. The dashed line indicates the repetition of the $180^\circ$ pulse for a total of 96 echoes.

The initial $90^\circ$ pulse flips the magnetization onto the $y$-axis and over a period of $\tau$, the nuclear spins begin to precess in the $xy$ plane. The $180^\circ$ inverts the sign of the magnetization vector but not the direction of precession. After some time $\tau$, the spins
coincide and a spin echo results as they precess in opposite directions. After a period of $\tau$, the $180^\circ$ pulse is applied again to detect the spin echo. This process was repeated 96 times to monitor the decay of the spin echo with time. Equation 11 describes an exponential decay relationship between $T_2$ and the magnetization. An exponential decay curve is obtained with a CPMG sequence which is then integrated to obtain a linear relationship. The value of $T_2$ is obtained from the slope.

$$M_y = M_o \exp\left[-\left(\frac{\tau}{T_2}\right)\right]$$

(11)

**Figure 11.** **A)** An exponential decay of a spin echo measures the $T_2$ value for a given number of spin echoes according to equation 11; **B)** Integration of the decay curve in A provides a linear relationship from which the $T_2$ value can be obtained from the slope. A time of $2\tau$ accounts for the total time between two consecutive $180^\circ$ pulses.

The echo time ($TE$) is the time from the excitation pulse to the echo signal maximum. $TE$ determines the amount of $T_2$ weighting for spin-echo images. The repetition time ($TR$) is the time between successive excitation pulses. $TR$ determines the amount of $T_1$ weighting contributing to the image contrast. The $T_1$ inversion time (or the delay time, $\tau$) is the time between the $180^\circ$ and $90^\circ$ pulses in the IR sequence. The specific values of these parameters that were used in the MRI experiments are explained in the method section.

**X-ray Scattering and Computed Tomography (CT)**

CT provides distinct information about the anatomical features of bones, tissues, and muscle. Contrast in CT is achieved through differential attenuation of the incident X-rays. X-rays are a form of high-energy electromagnetic radiation and have wavelengths ranging from 10 to 0.01 nm [51]. They are generated when accelerated electrons from the cathode of
an X-ray tube interact with the atoms of an anode target [52]. Intense heating of the cathode filament releases electrons by thermionic emission. Electrons accumulate at the surface of the filament and results in a large build-up of negative charge [51]. A high voltage is then applied across the cathode and anode and the electrons are immediately accelerated toward the positive anode. Each electron possesses a kinetic energy (in keV) equal to the applied tube voltage [51].

Figure 12. Electrons 1, 2, and 3 collide, decelerate, and experience a change in their momentum with the emission of a continuous energy spectrum of X-ray photons. Electron 4 collides with an electron in the K shell of the atom and produces characteristic radiation. The energy of the incoming photon must be greater than the K-shell electron binding energy to displace it. An electron in a higher shell replaces the vacant spot in the K shell and emits X-rays that are characteristic of that specific atom [51].

For CT, the energy range is 120 – 140 kV (50 – 70 keV) depending on the examination [51]. Fast moving electrons rapidly decelerate upon colliding with the target nuclei at the anode (e.g. rhodium anode). Kinetic energy lost by the electrons is converted into X-ray photons which generate a continuum of X-rays (the Bremsstrahlung spectrum). Closer interactions with the nucleus cause greater deceleration of electrons and results in higher X-ray photon energies. The probability of this occurrence decreases with increasing distance from the nucleus [51, 52]. When an incident photon displaces a K-shell electron upon collision, an electron from a higher energy level fills the vacant spot. The electron that fills the vacant spot emits an X-ray with energy equal to the difference in the binding energies of the
electrons in the higher shell and K shell, which is characteristic of that specific atom. Figure 12 illustrates this concept. The energy for this transition is very high because it is based on the displacement of an electron from the closest energy level (the K shell) to the nucleus of the atom. An X-ray spectrum usually contains sharp characteristic peaks of maximum energies (in keV) for specific L → K electron transitions (figure 13A) [53]. These peaks are characteristic because each element has different electron binding energies. Figure 13B is example of an X-ray spectrum obtained using a rhodium anode and a rhodium filter which cuts off all X-rays greater than 23.22 keV [53]. A rhodium anode and filter were used when signal enhancement with respect to water was measured for NPs, Gd-DTPA, and iopramide at 25, 35, and 45 kVp. A copper filter was used when the experiment was carried out at 49 kVp.

**Figure 13.** A) An example of an X-ray energy spectrum obtained at 80 kVp illustrating the Bremsstrahlung spectrum and the characteristic radiation peaks; B) A rhodium filter removes all X-rays energies greater than 23.22 keV. The characteristic energy peaks of rhodium appear at 20.3 and 22.7 keV. The molybdenum peaks are shown for comparison purposes [53].

Other than photoelectric absorption as described above, X-ray emission can also occur through pair production, Rayleigh scattering, and Compton scattering [52, 54]. Pair production is the displacement of an electron and generation of a positron. This can occur if the incident X-ray photon has energy greater than 1.02 MeV [54]. Any excess energy is converted into the kinetic energy of both particulates and is split equally [54]. Ionization of the atom does not occur. Such high-energy X-rays are more commonly used in PET; thus the probability of pair production in CT is very low. Rayleigh scattering occurs when the incident photon is deflected by an electron with no loss of energy [52, 54]. The energy of the
electron is temporarily increased and since it is not enough for complete removal, it returns to its normal energy state by emitting an X-ray photon equal to the initial energy increase and travels in a slightly different direction [52]. This scattering event has very low probability of occurring in soft tissue because of low atomic number elements being present. Compton scattering occurs when an incident X-ray photon interacts with a valence shell electron which is not tightly bound to the nucleus [55]. Energy of the incoming photon is much greater than the binding energy of the electron. Upon collision, only a part of the photon energy is transferred to the electron. The photon continues to travel but with reduced energy and the electron is ejected from its shell [54]. Compton scattering dominates at higher X-ray energies. The probability of photoelectron absorption occurring decreases as the incident X-ray photon energy increases. This relationship is represented by equation 12 where $\mu_{\text{photoelectron}}$ is the X-ray attenuation factor and $E$ is energy of the incident X-ray photon energy beam [52].

$$\mu_{\text{photoelectron}} \propto \frac{1}{E^3}$$  \hspace{1cm} (12)

Tissues in the human body contain mostly low atomic number elements therefore they have low K-shell binding energies and less prominent characteristic X-ray production [52]. In order to image with greater contrast, the use of high atomic number elements such as iodine (I, $Z=53$) and Gd (Z=64) would be beneficial. The K-shell binding energies of I and Gd are 33.2 and 50.2 keV, respectively [22, 52]. The probability of photoelectric absorption increases with increasing atomic number (equation 13).

$$\mu_{\text{photoelectron}} \propto Z^3$$  \hspace{1cm} (13)

In summary, attenuation of X-rays is greater when working with higher atomic number elements and lower X-ray energies. However, in cases where differentiation between fat and muscle is necessary, high-energy X-rays are required. For such purposes, the use of Gd-based contrast agents designed to selectively partition into one of the two tissues would be highly advantageous.
In order to compare contrast and test the potential of NPs as CT agents, X-ray scattering experiments were performed on PAA25-coated GdF$_3$/EuF$_3$ NPs, Gd-DTPA, and iopramide (IP) in water. To achieve maximal contrast, imaging should be performed at X-ray energies where the linear attenuation is highest according to figure 14. A sudden increase in the attenuation of X-rays indicates the incident photon energy required to displace an electron from the K shell of that specific atom. This energy is characteristic of photoelectric absorption as discussed above. The K-edge energy is usually slightly more than the binding energy of the K-shell electron so that the incident photon can be absorbed. For maximum contrast between tissue and Gd-enhanced areas, imaging should be performed at either low energies or right at the K-edge of Gd [56]. Low energy X-rays are used to image soft tissues whereas high energy X-rays (55-70 keV) are used to image bone. It is easy to differentiate between soft tissue and bone but not soft tissue and muscle. At lower energies (15-25 keV), greater contrast can be achieved because the NPs will partition into one of the two tissues and attenuate X-rays more than iodinated contrast agents.

![Figure 14. The X-ray energy profile of Gd and I. A sudden increase in the linear attenuation indicates the amount of X-ray energy required to displace an electron in the K shell of that specific atom for photoelectric absorption to occur [56].](image-url)
Zeta Potential

Zeta potential experiments were performed on PAA25-coated GdF$_3$/EuF$_3$ NPs in order to measure the amount of surface charge from the negative polymer coating. Zeta potential is the electrical potential that exists at the shear plane of a particle that carries a surface charge [57]. It is measured at a specific distance from the particle surface and accounts for the amount of charge that resides on it. This distance is essentially the width of the diffuse double layer that forms on the charged particle surface and is determined by the electrophoretic mobility of the particle in an applied electric field [57].

The mobility of a particle depends on its ionic charge and any frictional forces present in the buffer it is dissolved in. The electrophoretic mobility refers to the migration of charged particles towards the electrode of opposite charge [57]. Build-up of a net charge on the particle surface due to attractions between positive and negative ions results in the formation of an electrical double layer. The region of the layer closest to the particle surface (the Stern layer) contains counter ions which are strongly bound to the particle surface ions. The outer region (the diffuse layer) contains counter ions which are less firmly attached. Figure 15 illustrates the concept of the diffuse double layer build-up on a negatively charged particle surface. The interface between the diffuse layer surface and the surrounding environment experiences a hydrodynamic shear effect and this interface is referred to as the slipping plane [57]. The potential that exists at this interface is the zeta potential. The mobility of a particle is determined by measuring the average velocity per electric field unit.

The magnitude of the zeta potential provides information about the potential stability of the particle. If the zeta potential is large (for either negatively or positively charged particles), then the particles have a sufficient amount of surface charge to keep them well separated and prevent coagulation. Typically, particles with zeta potentials whose magnitude is greater than $+30$ mV are considered stable colloid species [57].

The velocity of a particle depends on the strength of the applied electric field, the dielectric constant of the medium, the viscosity of the medium, and the zeta potential [57]. The stronger the electric field, the faster the particle will travel because of a stronger electrical force that acts upon it. If the medium is viscous, then the particles will travel more slowly. There may even be a solvating effect on the particles if the conditions are too dilute.
The zeta potential also depends on the pH of the solution, the ionic strength of the medium, and particle concentration.

![Diagram showing electrical double layer](image_url)

*Figure 15.* Counter ions tend to collect on the particle surface and aid in the formation of an electrical double layer. The inner region of the double layer (the Stern layer) is where the electrostatic attraction is the strongest whereas the outer region (the diffuse layer) is where the counter ions are less associated. The zeta potential is a measure of charge build-up at the surface of the electrical double layer [57].

**Transmission Electron Microscopy (TEM)**

TEM is a highly sensitive characterization technique that employs the interactions between electrons and matter to directly image spatial topography at the nanometer scale [58]. The maximum resolution that can be achieved with a TEM is 0.1-0.2 nm [58].

TEM utilizes a highly energized (>100 kV) beam of electrons which interacts with the sample. The electron source is a thermionic electron gun that is usually made of tungsten. The tungsten filament (the cathode) is heated until a stream of electrons is produced [58]. The electrons are accelerated toward the sample by applying an electric potential. The entire microscope column is operated under vacuum in order to avoid collisions between the electrons and stray molecules. Stray molecules can cause excess scattering of electrons or volatilization of organic molecules that may be present. This can contaminate the microscope column at the joints and decrease the accuracy of the aperture.
The sample is evaporated onto a carbon-coated copper grid and is completely dried of water. It has to be as thin as possible to ensure electron transmittance.

Figure 16. A schematic representation of a transmission electron microscope illustrating the sequence of optical components and the ray path of the electron beam [59].

Figure 16 illustrates the fundamental design of a transmission electron microscope [59]. The first lens is a condenser lens which focuses the electron beam from the filament onto the sample [58]. The objective lens forms the initial enlarged image. The aperture removes any electrons that may have been scattered and only allows electrons that scatter parallel to the direction of the beam to come through. Hence, any electrons that may have deviated from the optical path (including the ones that back-scatter) will not interfere with the image magnification. Generally, a smaller aperture is able to remove more scattered electrons and increase the resolution. Working with a higher energy electron beam will also help increase the resolution. The next two projector lenses further enlarge the image to the desired magnification. The total magnification is a product of the enlargement by the objective and projector lenses [58].
The magnified image is created by the interaction between electrons and the sample via electron absorption or scattering. Electrons that interact with the sample form the image and those that are stopped or deflected are subtracted from image [58]. High atomic number elements such as Gd and Eu deflect a large fraction of electrons because of their large electron shells. Thus, they create dark voids of space which define the size and morphology of the particle they comprise [58]. As a result, black and white contrast defines the image and this depends on the orientation of the crystal core with respect to the direction of the incoming electron beam [58]. Individual crystal morphology can be differentiated since randomly oriented crystals will have their own grey-level contrast. Since TEM is a high resolution technique, atomic rearrangements in crystalline structures can also be imaged in great detail. The magnified image is detected on either a fluorescent screen, photographic plate, or a light-sensitive sensor camera and is displayed on a monitor [58].

Electrons can scatter forward or backward when they encounter atoms [58]. In TEM, forward scattering is considered to occur in many different ways of which elastic (no loss of energy), inelastic (some loss of energy), diffraction, and refraction scattering are the most common forms. In inelastic scattering, X-rays can be generated, Auger electrons can be dislodged, and sputtering of valence shell electrons can also occur [58].
Materials and Methods

The following chemicals were obtained from Sigma-Aldrich (Mississauga, Ontario): Gd(NO$_3$)$_3$•6H$_2$O, Eu(NO$_3$)$_3$•6H$_2$O, NaF, and polyacrylic acid (PAA25, average molecular weight = 1800 Da corresponding to 25 units).

**Synthesis of Polymer-coated 90/10 GdF$_3$/EuF$_3$ Nanoparticles**

A solution of 0.05 g of PAA25 in 20 mL of distilled water was prepared in a 100 mL round bottom flask. The pH of the solution was adjusted to approximately 10 with 6 M NH$_4$OH$(_{aq})$ and verified with pH paper. To this solution, 0.029 g of NaF was added and the resulting solution was heated to 85 °C. A 0.65 M stock solution consisting of 7.92 g of Gd(NO$_3$)$_3$•6H$_2$O and 0.83 g of Eu(NO$_3$)$_3$•6H$_2$O was prepared in 30 mL of distilled water. A 1 mL aliquot of the stock solution was diluted to 10 mL with distilled water to obtain a 0.065 M solution of which, 3.55 mL was added in drop-wise increments to the reaction mixture at 85 °C with vigorous stirring. The resulting reaction mixture was stirred for 3-4 hours at 85 °C and then concentrated to a volume of approximately 3-5 mL by rotary evaporation. The concentrated reaction mixture was filtered using a 25 mm syringe filter with a 0.45 μm pore size membrane (VWR, USA). The filtered reaction mixture was then transferred to a 50 mL centrifuge tube and approximately 45 mL of acetone was added to precipitate the NPs. The NPs were then centrifuged at 5000 rpm for 2 minutes and the supernatant was poured off. The precipitate was re-dissolved in 2-3 mL of distilled water, re-precipitated with acetone, and centrifuged again. The resulting NPs were dried under reduced pressure overnight in a dessicator containing drying agent (Drierite®, 98% CaSO$_4$, 2% CoCl$_2$). The synthesis protocol is outlined in figure 17 below.
Figure 17. A flow chart of the synthesis of polymer-coated 90/10 GdF₃/EuF₃ NPs as described above but in terms of molar amounts of the reagents used and the sequence of their addition.
Zeta Potential Experiments

Zeta potential experiments were performed on PAA25-coated GdF$_3$/EuF$_3$ NPs to quantify the surface charge of the polymer coating. The experiments were performed using the ZetaSizer 3000HS (Malvern Instruments, Westborough, MA, USA). The NP sample was prepared in water at a concentration of 7.5 mg/mL. Approximately 5 mL of the sample was injected into the sample chamber using a 5 mL syringe. The average zeta potential was determined based on five runs for each sample.

Magnetic Resonance Imaging (MRI) Experiments

The proton $T_1$ and $T_2$ relaxation times of water in presence of paramagnetic NPs were measured on the 1.5 T (63.5 MHz) Signa MRI scanner (GE Healthcare, Wakesha, Wisconsin, USA) located at Sunnybrook and Women’s College Health Sciences Centre (North York, Toronto). Imaging of phantom samples was performed using an axial slice of 4 mm thickness. The proton relaxation times were measured for a dilution series consisting of 0.2, 0.15, 0.1, 0.05 mg/mL. The NP samples were placed on a multiple sample holder which was centered inside a receiver coil chamber. The chamber, which was placed on the moveable examination table, was plugged into the console system and moved into the centre of the cylindrical magnet. The proton $T_1$ was determined using the inversion recovery pulse sequence and the proton $T_2$ was determined using the Carr-Purcell-Meiboom-Gill (CPMG) sequence as shown in figures 8 and 10, respectively. Proton $T_1$ was measured using a repetition time ($TR$) of 2500 ms, an echo time ($TE$) of 9 ms, and the experiment was repeated for several different $T_1$ mixing times (50, 100, 200, 300, 500, 700, 900, 1500 ms). A $TR$ of 2500 ms and a $TE$ of 10 ms with a total of 96 echoes were used to measure the $T_2$ in the CPMG experiments. Hence, images of the phantom samples were acquired every 10 ms for 960 ms.
Centrifugation and Staining

In order to test removal by centrifugation, a 7.5 mg/mL sample of NPs prepared in distilled water was centrifuged at 10 000 rpm. A 0.04% w/v solution of bromocresol green (Sigma-Aldrich, Mississauga, Ontario) was prepared in water. Approximately 5 drops of the indicator solution was added to the supernatant to observe a colour change from colourless to yellow. A yellow coloration indicated the presence of PAA25 in solution with NPs in the pellet at the bottom of the microfuge tube.

Transmission Electron Microscopy (TEM) Experiments

TEM was used to characterize the NPs for their size and shape morphology. A Hitachi H-7000 microscope (Pleasanton, California) was operated at 100 kV. A NP sample of approximately 1-2 mg was dispersed in 5 mL of water and a drop of this mixture was evaporated on carbon-coated 300 mesh copper grids. Samples in powder form were sent to the van Veggel lab at the University of Victoria, B.C. where TEM was performed.

Rat Brain Perfusion Studies

PAA25-coated 90/10 GdF₃/EuF₃ NPs were prepared at a concentration of 18.58 mg/mL and administered to a rat brain with an injection dose of 0.4 mL. The NPs possessed $T_1$ and $T_2$ relaxivities of 66.1 and 76.3 s⁻¹(mg/mL)⁻¹, respectively. For comparison, the typical dose of Gd-DTPA in a rat is 0.4 mL of a 469 mg/mL solution. Dynamic contrast-enhanced MRI (DCI-MRI) was used to observe the behaviour of the NP contrast in the rat brain for 36 minutes and 40 seconds (110 time points, with 20 s between each time point). Dynamic scanning was used to monitor the passage of the contrast agent by repeatedly acquiring axial $T_1$-weighted MRI scans every 20 s. The behaviour of the average signal within several regions of interest over the duration of the experiment was plotted against time to obtain enhancement curves. The rat did not exhibit any obvious adverse reaction to the contrast, its heart rate was stable throughout the experiment, and its anesthetic recovery time appeared to be normal.
X-ray Scattering and Computed Tomography (CT) Experiments

A dilution series consisting of 20, 30, 40, and 50 mg/mL was prepared for one NP sample, iopramide, and Gd-DTPA in order to compare contrast enhancement with reference to the water signal. The Senographe 2000D full field digital mammography system (GE Medical Systems, Waukesha, Wisconsin, USA) was used to carry out the X-ray CT experiments on the phantom samples. The detector was comprised of a layer of thallium-activated CsI phosphor deposited on an array of photodiodes and thin film transistor readout switches, which were arranged as a matrix on a flat panel. The detector pixel pitch was 100 μm. The GE 2000D is equipped with both molybdenum and rhodium anodes with a choice of molybdenum, rhodium, or copper filters. Imaging of the phantom samples was performed at four different X-ray beam energies: 25, 35, 45, and 49 kVp. Imaging at 25, 35, and 45 kVp was performed using a rhodium anode and a rhodium filter whereas imaging at 49 kVp was performed using a rhodium anode and a copper filter. The region of interest (ROI) for determining signal enhancement of each sample tube was chosen to be a circle with a 5 mm diameter.
Results and Discussion

Novel Gd-based contrast agents comprised of a 90/10 mixture of GdF₃/EuF₃ and coated with PAA25 have been synthesized. NPs synthesized at the optimal reaction conditions (3.55 mL Ln salt solution, 0.05 g PAA25, 20 mL H₂O, starting pH ~ 10, 85 °C) possess relaxivities of $R_1, R_2 = 82.3 \pm 8.4, 123.3 \pm 13.7 \text{ s}^{-1} \text{ (mg/mL)}^{-1}$. Electron microscopy images reveal that these NPs are fairly monodisperse. The current synthesis of PAA25-coated GdF₃/EuF₃ NPs is an adaptation of a scheme described by van Veggel et al [21]. Van Veggel’s NPs are 5-10 nm in diameter, coated with citric acid, and possess relaxivities around 20 s⁻¹ (mg/mL)⁻¹ [21]. Citric acid is a weak acid and is a tri-dentate ligand since it contains three carboxylate groups (pKa = 3.15, 4.77, and 5.19). The carboxylate groups covalently interact with the NP surface by weakly coordinating with surface Gd³⁺ ions. However, this coordination is not strong enough to stabilize the NPs in anything but aqueous conditions. In order to render GdF₃/EuF₃ NPs as multimodal imaging and anti-cancer agents, they must be stable in biological media and withstand various conditions (i.e. saline conditions, pH, temperature, etc.) PAA was used to encapsulate the NP core because it is able to coordinate more strongly than citrate due to multiple carboxylate groups that can entrap a large fraction of water molecules close to the NP surface.

The Use of Larger Polymers

Initially, the synthesis was carried out using a 43-unit linear chain (PAA43) and a 450-unit star-shaped polymer (PAA450) of PAA (courtesy of the Winnik group). Although the stoichiometric mole ratio between the lanthanide and each of the two types of polymer was not optimized at the time, the synthesis was performed using the procedure described in the method section. The resulting aggregates were quite large (400-500 nm in diameter) and had very low dispersibility in aqueous media. A TEM image of NPs synthesized with PAA43 is shown in figure 18. Dynamic light scattering (DLS) experiments were also performed to determine the hydrodynamic radii of the individual NPs but the extensively cross-linked polymer network considerably skewed the measurements. DLS results indicated NP hydrodynamic radii between 700 and 1200 nm. These measurements were disregarded when TEM of the same NPs were obtained which indicated the aggregates to be much smaller. Figure 18 shows NPs clustered in some areas of the polymer network with most of
the aggregate consisting of the polymer. The aggregates were much larger for the PAA450-coated NPs and had even lower dispersibility than that of PAA43-coated NPs. This was most probably because the star-shaped polymer created a much more cross-linked network of greater complexity. Despite the dispersibility issue, the relaxivity of both types of aggregates was measured on the 1.5T MRI scanner. Both possessed significantly high relaxivities which lead to the possibility of tailoring and optimizing the synthesis of PAA-coated NPs.

![Figure 18](image.png)

**Figure 18.** A TEM image of PAA43-coated 90/10 GdF3/EuF3 NPs synthesized at a starting pH ~ 8 and 75 °C with empirical Ln:PAA43 ~ 4. These NPs were not synthesized at the current optimal reaction conditions which are employed for PAA25. For this particular batch of NPs $R_1, R_2 = 94.9, 102.7 \text{ s}^{-1} (\text{mg/mL})^{-1}$.

**The Synthesis in Detail**

The synthesis of polymer-coated NPs was continued with the 25-unit linear chain polymer since it is readily available from commercial suppliers. The larger polymers have to be synthesized via controlled radical polymerization [60]. More importantly, coating NPs with the shorter polymer would help reduce cross-linking and allow for particles to be better separated. PAA is a polyelectrolyte and its characteristic features arise from the electrostatic repulsion between the chains as opposed to the entangled nature of their uncharged counter parts. Each unit of PAA contains one carboxylate group; hence the 25-mer contains 25 carboxylate groups and provides an overall negative surface charge. The $pK_a$ of PAA is
approximately 4.8 but the multiple carboxylate groups cause the actual pKₐ to be a broadened range around 4.8. In order to ensure all of the carboxylate groups are deprotonated, the pH of the reaction mixture must be well above 4.8. An aqueous solution of PAA was initially neutralized with 6M NH₄OH to ensure all carboxylate groups were ionized. Sodium fluoride (NaF) was used as a source of water-soluble F⁻. The counter ions, Na⁺ and NH₄⁺, coordinate with the negatively charged carboxylate groups. At this point, the solution was heated to allow the polymer to attach onto the NP exterior and this step is important for rendering NPs with a high dispersibility in organic solvents [21]. The Ln salt solution was added drop-wise to allow complete dissolution of each drop before adding the next one. Adding the solution too quickly or all at once resulted in a thick white precipitate that was difficult to dissolve. If NPs coated with PAA actually form, they should dissolve within some amount of time since the reaction is carried out in water. Hence, it was experimentally determined that the rate of addition of the Ln salt solution is a crucial step in making PAA-coated NPs. By making the Ln salt solution ten times more dilute, each drop added to the solution was less concentrated and resulted in a fine suspension of the initial precipitate which aided in speeding up the dissolution step. The amount of precipitate that forms with each drop of the Ln salt solution has a lower particle density; thus it is able to dissolve quickly. The initial precipitate that forms eventually dissolves after 3-4 hours of vigorous stirring and this probably occurs because the polymer gradually binds to the surface. Once the NP surface is completely covered with the polymer, the whole colloid system dissolves. The lanthanide core is insoluble in aqueous conditions on its own. A complex matrix consisting of cross linkages between the lanthanide and fluorine ions allows for their growth until the polymer begins to attach to the NP surface. This growth is thought to be controlled by the affinity of the anionic polymer for the hydrophobic NP surface although a mechanism explaining this phenomenon has not been investigated. Van Veggel et al suggest that an organic ligand, such as a dithiophosphate, coordinates weakly to the lanthanide ions allowing for growth of NPs but coordinates strongly enough to prevent particle coagulation [27].
Properties of Lanthanides

Lanthanides behave as hard acids, *i.e.* they are small in size, have a relatively high charge and charge density, and are weakly polarizable [61, 62]. Hence, their charge-to-size ratio is relatively high. Since hard acids prefer to bind with hard bases, lanthanides prefer to bind with F and O donor ligands [62]. The formation of GdF3/EuF3 NPs is based on a fast ligand exchange between the nitrate and water ligands in Ln(NO3)3•xH2O and the free fluorine ions in solution. Lanthanides are not stabilized by ligand field stabilization energy (LFSE) and there are minimal interactions with the 4f orbitals because they are buried under the filled 5s and 5p orbitals; thus they do not require specific geometries when forming complexes [61]. This is one of the key reasons why a fast ligand exchange takes place since the affinity between lanthanides and fluorides allows the exchange to be thermodynamically favourable (figure 19). The geometric preference is more related to steric effects which may arise when larger ligands try to bind to the lanthanide atom, for example DTPA. Fluorine is a small, accessible, and mobile ligand that can bind very quickly. Fluorine complexes are generally more stable in water because fluorine is more electronegative than nitrate and water ligands. Once the crystal grows to an optimum size under the particular reactions conditions, the polymer is able to electrostatically bind to the surface of the NPs. It is evident from figure 19 that sharing of fluorine atoms promotes the coalescence of the single crystal units to form the NP core. Although the growth of the NP is highly sensitive to the type of surface ligand present and reaction parameters such as pH, temperature, and stoichiometry, the extent of fluorine sharing also plays a key role. A recent study of LaF3 NPs based on solid-state NMR characterization of bulk crystalline LaF3 shows that the fluoride ions undergo dynamic exchange within the NP core [63]. Based on the TTP geometry, which is sterically more favoured, there are three different fluorine environments in a single crystal unit. The single crystal unit geometry is discussed in greater detail in the theory section.
Figure 19. Gd is a nine-coordinate complex when it is in the hydrated salt form and most probably adopts a trigonal prismatic geometry. F\(^-\) ions instantly replace all nine positions because of a fast ligand exchange. The NP is comprised of single crystal units and grows to a certain size before the polymer attaches onto its surface.

**Incorporation of Europium (Eu\(^{3+}\))**

The NP core is doped with 10% Eu\(^{3+}\) in order to help improve NP solubility. Since solubility typically decreases across the lanthanide series, Eu\(^{3+}\) is more soluble than Gd\(^{3+}\). NPs doped with La\(^{3+}\) were also synthesized but their solubility was not comparable to the ones doped with Eu\(^{3+}\). This may be because the effective ionic radius of La\(^{3+}\) (1.18 Å) is larger than that of Gd\(^{3+}\) (1.06 Å) which may cause the NP core to be an amorphous mixture of the two with possible geometric distortions [22]. The ionic radii of Eu\(^{3+}\) (1.07 Å) and Gd\(^{3+}\) are nearly identical therefore the coordinating geometry involved in the growth of the crystal is virtually identical for both ions [22]. Hence, the GdF\(_3\)/EuF\(_3\) core most probably consists of a statistical mixture of both single units and adopts a more homogeneous, crystalline structure. The GdF\(_3\)/LaF\(_3\) NPs were also less crystalline based on physical appearance when compared to the GdF\(_3\)/EuF\(_3\) NPs which further infers the possibility of a different lanthanide matrix structure. However, further characterization of GdF\(_3\)/EuF\(_3\) NPs is required to confirm this inference. The amount of doping of Eu\(^{3+}\) is not significant enough to cause major distortions in the crystal structure.
Of the lanthanides, Eu$^{3+}$ is known to have the most intense fluorescence properties with absorption at 393 nm and emission at 591 nm and 615 nm [29]. Europium is the most unique of the lanthanides because it has excited states above the triplet excited state. The main route of emission for Eu$^{3+}$ occurs via $^5D_0 \rightarrow ^7F_n$ ($n= 0, 1, 2$) where the excited $^5D_0$ state is non-degenerate [24]. This means that the excited $^5D_0$ state exists above the triplet state and its individual energy levels are of unequal energy; hence they can be split by a ligand field [24]. The excited $^5D_0$ state is of a 4$f$ electron shell and has a relatively slow radiative decay rate ($100 \text{ s}^{-1}$); hence non-radiative routes are preferred for relaxation and emission [24]. These routes include complex electronic energy conversion processes that allow excited electrons to convert to a singlet excited state first and then relax to the ground state via fluorescence. This conversion occurs via electric or magnetic dipole transitions [24]. Hence, a reliable signal at 620 nm makes Eu$^{3+}$ a great optical probe for detecting and studying NP adherence to cells.

**Polymer Dynamics**

The dynamic behaviour of the polymer is crucial for the growth of NPs. The polymer does not interfere in the growth of the NPs because the exchange rate is very fast and the affinity is very high for fluoride ions. During synthesis, the electrostatic Coulomb force between PAA and the LnF$_3$ surface is weak enough to allow particle growth but strong enough to prevent particle coagulation. The NP system is essentially a colloid whose formation depends on kinetic parameters such as the polymer cohesive energy, NP surface energy, and the segmental mobility of the polymer chains [64]. PAA physically adsorbs to the NP surface which is induced by electrostatic forces between the NP surface and the negatively charged carboxylate groups. This interaction is heavily controlled by the pH of the solution, especially in the case of PAA as discussed above. Adsorption is a slow process and can depend on the diffusion of the polymer to the NP surface and/or the time it takes to reach an equilibrium state of the polymer in the adsorption layer [64]. The adsorption rate depends on the NP concentration, molecular mass of the polymer, time and intensity of mixing, and viscosity of the medium [64]. Since the PAA chain is relatively long and flexible, it adheres to the NP surface in loops with multiple contact points. The conformation of the adsorbed polymer chains varies constantly on the NP surface. The polymer shell may
also consist of a first dense layer and a second loose, more dynamic layer of polymer chains. The first layer contains a large number of contact points with the NP surface whereas the second layer contains scattering loops of the chains. This concept is in agreement with the inner and outer spheres of hydration as well.

When the polymer chain is long and flexible, cross-linking of NPs occurs almost certainly at the synthesis stage. More than one particle can be bound by a single polymer chain and there is no uniform wrapping of the polymer around a single NP. The resulting aggregate looks like a network or web with NPs concentrated in some areas and separated in others. Figure 20 illustrates this concept. This phenomenon causes the actual aggregate sizes to be larger even though the individual NPs are much smaller. When the inner adsorbed polymer layer of one particle approaches another, the layers may also superimpose and the loops may interact and entangle with each other [64]. This may cause some NPs to exist in clusters within a polymer network. This may also lead to the loss of the conformational entropy of the polymer on the surface of a NP and growth of the entire aggregate’s free energy [64]. This may be the case when NPs were synthesized with excess polymer which resulted in an extensively cross-linked system of greater complexity. However, cross-linking presumably slows down the water exchange rate with the paramagnetic surface and this is a key factor in establishing large relaxivities.

![Figure 20](image.png)

**Figure 20.** A cross-linking network of the polymer binds multiple NPs together. The inner layer of the polymer adsorbs onto the NP surface whereas the outer layer consists of loose polymer chains.

Variation in the polymer-solvent interaction can generate local osmotic pressure, which was observed in dialysis experiments. PAA25-coated GdF$_3$/EuF$_3$ NPs were dissolved in water and saline solution (100 mM NaCl) at various concentrations ranging from 10
mg/mL to 1 g/mL and dialyzed against water and 100 mM NaCl in 15,000 and 35,000 molecular weight cut-off (MWCO) dialysis filters. In all cases, the NP solution in the dialysis tubing became a gel after some time which inferred that once the water equilibrium was disturbed, the polymer may have been displaced from the NP surface or it intertwined to a greater complexity with possible NP aggregation. In order to render polymer-coated GdF₃/EuF₃ NPs as contrast agents for applications in multimodal imaging, the polymer must be able to entrap a large number of water molecules and coordinate them close to the NP surface to achieve maximal proton relaxation. PAA is excellent at achieving this because it stabilizes the NPs against aggregation, makes them highly dispersible in aqueous solution, and provides a tool for further functionalization. Since lanthanides have a high affinity for oxygen and phosphorus containing ligands, presence of phosphates in biological media for example, generates the possibility of NP-phosphate complexes to precipitate. Hence, the surface coating must be strong enough to withstand such conditions. PAA has proven to adsorb strongly enough to the NP surface to prevent such precipitates from forming, especially in biological media. Solubility tests in phosphate buffer saline (PBS, pH=7.4), 1 mg/mL bovine serum albumin (BSA), and 100 mM NaCl were performed to ensure that precipitation of NPs does not occur. The aggregates in PBS and serum were found to be stable for one to two weeks after which some sedimentation was observed. This most probably occurs because of slow displacement of the polymer from the NP surface and eventual collapse of the colloidal system. The collapse of the colloidal system was also observed when NPs dispersed in water were centrifuged at 10,000 rpm. The insoluble NP core deposited at the bottom whereas the polymer remained in solution. With the addition of bromocresol green, an acid-specific (COOH) indicator, the supernatant changed colour from colourless to pale yellow, which indicated that the polymer was indeed removed from the NP surface.

In order to prevent a rigid rod-like behaviour of the polymer, counter ions in the form of NaCl were introduced into the reaction to force the polymer to bend when it attaches onto the NP surface. Counter ions coordinate with the carboxylate groups and screen neighboring negative charges from each other. Consequently, the polyelectrolyte tends to curl and behaves more like a flexible chain as oppose to a rigid rod. Relaxivity measurements of the resultant NPs showed a decrease ($R_1$, $R_2 = 54.3, 80.7 \text{s}^{-1}(\text{mg/mL})^{-1}$) which indicated that Na⁺
screened the interaction between the carboxylate groups of the polymer and the NP surface. In another case, the relaxivity of NPs was measured in 100 mM NaCl and it decreased drastically due to Coulombic screening of the carboxylate groups by Na\(^+\) \((R_1, R_2 = 12.3, 20.9 \text{ s}^{-1}\text{(mg/mL)}^{-1})\) when compared to relaxivities of the same NPs in water \((R_1, R_2 = 82.3, 123.3 \text{ s}^{-1}\text{(mg/mL)}^{-1})\). Table 1 summarizes these results below. A smaller decrease in relaxivity is expected if the NPs were dispersed in a 50 mM NaCl solution (physiological salt concentration) but this is yet to be confirmed.

<table>
<thead>
<tr>
<th>Reaction Conditions</th>
<th>Relaxivity (s^{-1}\text{(mg/mL)}^{-1})</th>
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</thead>
<tbody>
<tr>
<td>(3.50 \text{ mL Ln, 0.05g P25, 10 mL H}_2\text{O, pH ~ 10, 85 °C} ) 7mL 100mM NaCl added before neutralization of PAA25</td>
<td>(R_1) (R_2)</td>
</tr>
<tr>
<td></td>
<td>54.3 ± 4.9 (80.7 ± 8.5)</td>
</tr>
<tr>
<td>(3.50 \text{ mL Ln, 0.05g P25, 10 mL H}_2\text{O, pH ~ 10, 85 °C} ) NP samples prepared in 100mM NaCl solution (NPs from 2(^{nd}) reaction in this table)</td>
<td>(R_1) (R_2)</td>
</tr>
<tr>
<td></td>
<td>12.3 ± 4.3 (20.9 ± 8.4)</td>
</tr>
</tbody>
</table>

Table 1. The effect of salt stabilization on relaxivity. Screening of the carboxylate groups by excess Na\(^+\) ions reduces the interaction between the polymer and the NP surface.

**Screening of Reaction Parameters**

The synthesis of polymer-coated GdF\(_3\)/EuF\(_3\) NPs was originally performed with larger polymers as discussed above. Optimization of the synthesis was later switched to PAA25 since it is readily available from commercial suppliers. The optimization involved screening many combinations of pH, temperature, Ln:PAA25 empirical mole ratio, and the solvent volume. The relaxivity of almost every batch of NPs synthesized at the various reaction conditions was measured. Relaxivity measurements were performed to monitor the effect of parameter variation and observe which combinations of the reaction parameters succeeded in increasing proton relaxation. Figure 21 shows \(R_1\) and \(R_2\) relaxivities as a function of the volume (mL) of 65 mM Ln solution added to the reaction mixture. It is evident that maximal relaxivities are obtained when approximately 3.55 mL of the Ln salt solution (at the specified conditions) is used at which point the empirical Ln:PAA25 mole ratio is 8:1. This mole ratio is an empirical relationship; hence it is not indicative of the number of polymer molecules per NP. Further characterization is needed to confirm the number of Ln atoms and polymer molecules per NP. In this case, the empirical
stoichiometric ratio between Ln and PAA25 was established based on relaxivity measurements, crystallinity, and dispersibility of the resultant NPs in water, PBS, and BSA.

The NP synthesis was carried out at various reaction temperatures and figure 22 illustrates the effect of temperature on the relaxivity of polymer-coated GdF$_3$/EuF$_3$ NPs. The temperature parameter was screened much earlier; hence other reaction parameters were not optimized at the time (see figure 22 caption for specific parameters). The reaction vessel was placed in an oil bath and gradually heated to the desired temperature before the Ln solution was added. At least one to two hours are required for the temperature to equilibrate between the oil bath and the reaction solution (both were monitored with separate thermometers). Once the temperature was stable for at least half an hour, the Ln salt solution was added drop-wise. Although a higher relaxivity resulted for NPs synthesized at 90 °C, subsequent reactions were performed at 85 °C instead (± 2 °C). A reaction temperature of 90 °C was thought to be too close to the boiling point of water which may cause the solvent system to fluctuate. Too high of a temperature may also inhibit particle growth. If the rate of the reaction is increased with increased temperature, the polymer may attach faster to the NP surface; thus limiting the growth of the particle. The polymer chain may also be more dynamic at higher temperatures which may cause more intertwining amongst minimal particle growth. A large mesh containing very small NPs may form as a result. It may also be that the carboxylate groups get dehydrated at higher temperatures, resulting in reduced adsorption.

The Ln salt solution is comprised of a 90/10 mole percent Gd(NO$_3$)$_3$•6H$_2$O / Eu(NO$_3$)$_3$•6H$_2$O which is relatively acidic. Initially, the NPs were synthesized at a starting pH of approximately 8 in order to ensure all carboxylate groups were deprotonated. However, with the addition of the Ln salt solution, the pH of the reaction mixture dropped by almost 3 units. This indicated that at the time of adsorption, some or most of the carboxylate groups may have been uncharged since the pK$_A$ of the polymer is a broaden region around 4.8. Instead of adding a buffer solution which may cause side reactions or dispersibility issues, the starting pH was increased to 10 so that even with the addition of the acidic Ln salt solution, the reaction pH will remain well above the pK$_A$ range of the polymer. The pH still dropped approximately 3 units but remained around 7 so that the polymer would be fully
charged at the time of adsorption. The pH was adjusted using 6M NH₄OH and was never increased beyond 10 since a pH close to 12 may risk the oxidation of Gd³⁺ to Gd⁴⁺.

Figure 21. $R_1$ and $R_2$ relaxivities of PAA25-coated GdF₃/EuF₃ NPs as a function of stoichiometry between Ln and PAA25. Each point represents a reaction performed at 85 °C, at a starting pH ~ 10, with 0.05g PAA25 while varying the volume of the Ln salt solution. Optimal relaxivities are achieved when approximately 3.55 mL of a 65 mM Ln solution is added, at which point the empirical Ln:PAA25 mole ratio is approximately 8:1.

Figure 22. $R_1$ and $R_2$ relaxivities of PAA25-coated GdF₃/EuF₃ NPs as a function of reaction temperature. Each point represents a reaction performed with 0.05 g PAA25, 3 mL of a 65 mM Ln solution, and starting pH ~ 8. These reactions were performed before determining the current optimal conditions. The empirical Ln:PAA25 mole ratio for these reactions is approximately 7:1.
Figure 23. TEM images of PAA25-coated 90/10 GdF3/EuF3 NPs synthesized at a starting pH ~ 8 and 85 °C with empirical Ln:PAA25 ~ 2.3. The aggregates are spindle-shaped and appear to be comprised of much smaller NPs clustered together since the aggregates are not solid crystal cores. These particular NPs were synthesized at a lower pH and Ln:PAA25 ratio. 
\[ R_1, R_2 = 49.1 \pm 4.9, 58.3 \pm 5.9 \text{ s}^{-1}\text{(mg/mL)}^{-1} \]

Figure 24. TEM images of PAA25-coated 90/10 GdF3/EuF3 NPs synthesized at a starting pH ~ 10 and 85 °C with empirical Ln:PAA25 ~ 9.3. The aggregates appear to be more spherical in shape and uniform in size. These particular NPs were synthesized at a higher pH and Ln:PAA25 mole ratio. 
\[ R_1, R_2 = 82.3 \pm 8.4, 123.3 \pm 13.7 \text{ s}^{-1}\text{(mg/mL)}^{-1} \]
Figures 23 and 24 are TEM images NPs synthesized at two different pH levels and Ln:PAA25 mole ratios. Figure 23 shows NPs synthesized at a lower pH (~8) and Ln:PAA25 (~2.3) mole ratio and do not consist of solid crystal cores. Instead, they appear to be aggregates of very small NPs (<10 nm) clustered together by a tight polymer network in the shape of a spindle. These particular NPs possess relaxivities of $R_1, R_2 = 49.1 \pm 4.9, 58.3 \pm 5.9 \text{ s}^{-1}(\text{mg/mL})^{-1}$. Figure 24 shows NPs synthesized at a higher pH (~10) and Ln:PAA25 (~9.3) mole ratio. These particular NPs contain solid crystal cores of relatively uniform shape and size and possess relaxivities of $R_1, R_2 = 82.3 \pm 8.4, 123.3 \pm 13.7 \text{ s}^{-1}(\text{mg/mL})^{-1}$. Polymer can be seen in the background which appears to have NPs aggregated in some areas more than others. Evidently, the growth of the NP core and morphology highly depends on the pH at the time of polymer adsorption.

Even though the empirical stoichiometry between Ln and PAA25 was already established, the use of excess polymer in the synthesis of PAA25-coated GdF$_3$/EuF$_3$ NPs was explored to observe its effect on relaxivity. Table 2 summarizes the reaction conditions and corresponding relaxivities for some of the reactions that were performed using excess polymer. The first three reactions are shown for comparison purposes. The last three reactions were performed with two, four, and six times the original polymer amount (0.05 g) and the relaxivity increased dramatically as a result (between 100-200 s$^{-1}$(mg/mL)$^{-1}$).

<table>
<thead>
<tr>
<th>Reaction Conditions</th>
<th>Relaxivity $s^{-1}(mg/mL)^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R_1$</td>
</tr>
<tr>
<td><strong>3.40 mL Ln, 0.05g P25, 10 mL H$_2$O, pH ~ 10, 85 °C</strong></td>
<td>43.9 ± 6.1</td>
</tr>
<tr>
<td><strong>3.50 mL Ln, 0.05g P25, 10 mL H$_2$O, pH ~ 10, 85 °C</strong></td>
<td>82.3 ± 8.4</td>
</tr>
<tr>
<td><strong>3.60 mL Ln, 0.05g P25, 10 mL H$_2$O, pH ~ 10, 85 °C</strong></td>
<td>61.7 ± 5.5</td>
</tr>
<tr>
<td>3.55 mL Ln, <strong>0.1g P25</strong>, 20 mL H$_2$O, pH ~ 10, 85 °C</td>
<td>138.8 ± 54.9</td>
</tr>
<tr>
<td>3.55 mL Ln, <strong>0.2g P25</strong>, 20 mL H$_2$O, pH ~ 10, 85 °C</td>
<td>213.5 ± 58.3</td>
</tr>
<tr>
<td>3.55 mL Ln, <strong>0.3g P25</strong>, 20 mL H$_2$O, pH ~ 10, 85 °C</td>
<td>231.8 ± 17.8</td>
</tr>
</tbody>
</table>

**Table 2.** For the first three reactions, the volume of the 65 mM Ln salt solution was varied to determine the optimal Ln:PAA25 mole ratio. For the last three reaction conditions, the amount of polymer was varied to observe the effect on relaxivity (TEM images in figure 26).
Figure 25 shows the proton $T_1$ relaxation of PAA25-coated GdF$_3$/EuF$_3$ NPs as a function of sample concentration for the reactions listed in table 2. A steeper slope results in higher relaxivities and this is observed for the NPs synthesized with excess polymer. The goal to achieve higher relaxivities would be especially beneficial in cases where low concentrations of the contrast agent must be delivered to the site of interest. Areas where there is little vascular tissue density would also benefit from high relaxivity contrast agents. On the other hand, aggregates must remain small enough to penetrate through tissues and pass through vascular capillaries.

**Figure 25.** $T_1$ relaxation rate as a function of NP concentration for selected PAA25-coated GdF$_3$/EuF$_3$ NPs synthesized at the reaction conditions specified in table 1. The slope of the linear relationship provides the relaxivity ($R_1$).

Figures 26 A, B, and C are TEM images of NPs synthesized using two, four, and six times the original polymer amount, respectively. In all cases, the use of excess polymer creates a large, extensively cross-linked network that is able to entrap many more water molecules with possible multiple spheres of hydration. A larger network is able to uptake many more water molecules and coordinate them to the paramagnetic NP surface. It may also inhibit particle growth due to steric factors that may arise because of high polymer density in the immediate vicinity of the NP. From the TEM images in figure 26, the NPs appear to be much smaller in size (<10 nm). Too much polymer most probably restricts the space required for NP growth since a high number of carboxylate groups are available for adsorption. Upon introducing the Ln salt solution into the reaction mixture at the specific
temperature, the particles may not have enough space or time to grow before the polymer begins to attach to its surface. However, with an increased surface area-to-volume ratio, more Gd$^{3+}$ ions are available on the NP surface. Hence, with a large network coordinating many more water molecules with smaller NPs, the resulting relaxivities are expected and indeed observed to be significantly high (last three reactions listed in table 2).

**Figure 26.** TEM images of PAA25-coated 90/10 GdF$_3$/EuF$_3$ NPs synthesized at a starting pH ~ 10 and 85 °C with: A) Two times the polymer (0.1g) with an empirical Ln:PAA25 ~ 4.2 and $R_1$, $R_2$ = 165, 234 s$^{-1}$(mg/mL)$^{-1}$; B) Four times the polymer (0.2g) with empirical Ln:PAA25 ~ 2.1 and $R_1$, $R_2$ = 213.5, 239.2 s$^{-1}$(mg/mL)$^{-1}$; C) Six times the polymer (0.3g) with empirical Ln:PAA25 ~ 1.4 and $R_1$, $R_2$ = 231.8, 222.2 s$^{-1}$(mg/mL)$^{-1}$. All of the images show an extensively cross-linked network containing very small NPs (<10 nm, dark spots) that possess significantly high relaxivities.
In order to reduce cross-linking between NPs, the synthesis was also performed under very dilute conditions. Optimal reaction conditions were employed except for the solvent volume, which was arbitrarily chosen to be 500 mL. Figure 27 is a TEM image of NPs synthesized under dilute conditions. Individual particles can be seen but they are coagulated with minimal polymer coating surrounding each of them. Some polymer coating can be seen in the background and around an entire coagulated system. The synthesis was repeated three times and in all three cases, the NPs were insoluble in water, PBS, and BSA. The TEM image in figure 27 indicates that carrying out the synthesis under dilute conditions causes particle coagulation. This most probably occurs because the polymer is widely dispersed in solution and is not in close proximity at the time of adsorption. Before the polymer can coat the surface, the particles coagulate. Since the resulting aggregates were insoluble in water, this most probably means that the polymer was unable to completely solubilize the aggregate itself. The aggregates are also quite large (300-500 nm) in diameter. Performing the synthesis under dilute conditions did not help reduce cross-linking. Instead, it promoted particle coagulation which resulted in virtually no dispersibility in aqueous media.

![TEM image of PAA-coated 90/10 GdF₃/EuF₃ NPs synthesized at a starting pH ~ 10, 85 °C, empirical Ln:PAA25 ~ 8.1, and solvent (water) volume of 500 mL. These NPs were synthesized in dilute conditions (as oppose to using 20 mL of water) in the attempt to reduce cross-linking. However, the NPs appear to be agglomerated and have minimal polymer coating. These particular NPs had very low solubility in water which further indicated that the polymer was not attached and the relaxivity could not be measured as a result.](image)

**Figure 27.** A TEM image of PAA-coated 90/10 GdF₃/EuF₃ NPs synthesized at a starting pH ~ 10, 85 °C, empirical Ln:PAA25 ~ 8.1, and solvent (water) volume of 500 mL. These NPs were synthesized in dilute conditions (as oppose to using 20 mL of water) in the attempt to reduce cross-linking. However, the NPs appear to be agglomerated and have minimal polymer coating. These particular NPs had very low solubility in water which further indicated that the polymer was not attached and the relaxivity could not be measured as a result.
The use of excess polymer to reduce cross-linking and stabilize the NPs was also tested. An aqueous solution of the NPs was heated to 40 °C and 0.5 g of PAA25 was added to the mixture. The resulting solution was vigorously stirred for 3-4 hours and the NPs were collected via precipitation as described in the original procedure in the method section. By increasing the negative charge on the surface of the NP, the repulsion between each NP would be greater, thereby increasing their dispersibility. However, the relaxivity of excess polymer-stabilized NPs increased along with more cross-linking \( R_1, R_2 = 123.4 \pm 11.6, 143.7 \pm 15.6 \text{s}^{-1}\text{(mg/mL)}^{-1} \). Another approach to reduce cross-linking was to stabilize the polymer-coated NPs with a linker such as ethylenediamine (H2NCH2CH2NH2). However, this would have resulted in a polymerization reaction with the carboxylate groups of the polymer, which would generate more complex dispersibility issues. Hence, this approach was not pursued.

**Quantification of Surface Charge**

Zeta potential experiments were performed on a few selected PAA25-coated GdF3/EuF3 NPs and table 3 summarizes the results below. The first reaction in table 3 is of NPs synthesized at almost optimal conditions (these were synthesized earlier) whereas the last two reactions are of NPs synthesized using excess polymer. For the first reaction, the zeta potential is relatively large (-56.0 ± 1.6 mV) but this number is not truly indicative of the amount of surface charge per NP because the NPs are most probably aggregated. Thus, the measured zeta potentials are of the amount of charge that resides on the surface of the entire aggregate. Presence of counter ions would deplete some of the surface charge as well. Species are considered stable if their zeta potential is at least -30 mV or more [57]. If the zeta potential is large, then the particles will not coagulate because of large repulsions. But in the case of cross-linked NPs, even if there are individual particle repulsions, their mobility will be limited to some degree, depending on the extent of the polymer network. However, because the measured zeta potentials are well above the threshold, they do indicate stable aggregate species that have a sufficient amount of surface charge. The stability of these aggregates most probably arises from the highly cross-linked polymer network. In the case of NPs synthesized with excess polymer, the zeta potentials are smaller, indicating that the aggregate size is probably smaller.
<table>
<thead>
<tr>
<th>Reaction Conditions</th>
<th>$R_1$ [s$^{-1}/$(mg/mL)$^{-1}$]</th>
<th>$R_2$ [s$^{-1}/$(mg/mL)$^{-1}$]</th>
<th>Zeta* (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5 mL Ln, 0.05g P25, 10 mL H$_2$O, pH ~ 10, 85 °C</td>
<td>82.3 ± 8.4</td>
<td>123.3 ± 13.7</td>
<td>-56.0 ± 1.6</td>
</tr>
<tr>
<td>3.55 mL Ln, 0.1 g P25, 20 mL H$_2$O, pH ~ 10, 85 °C</td>
<td>138.8 ± 54.9</td>
<td>212.2 ± 29.2</td>
<td>-38.0 ± 3.7</td>
</tr>
<tr>
<td>3.55 mL Ln, 0.2 g P25, 20 mL H$_2$O, pH ~ 10, 85 °C</td>
<td>273.5 ± 25.2</td>
<td>244.8 ± 28.3</td>
<td>-40.0 ± 3.6</td>
</tr>
</tbody>
</table>

* Sample concentration ~ 30 mg / 4 mL H$_2$O = 7.5 mg/mL

**Table 3.** Zeta potentials were measured for a few selected samples of PAA25-coated GdF$_3$/EuF$_3$ NPs. The last two reactions are of GdF$_3$/EuF$_3$ NPs synthesized with excess polymer in a larger solvent volume. The corresponding $R_1$ and $R_2$ relaxivities are provided for comparison.

**Proton Relaxation Enhancement**

Figure 28 is a series of MR images acquired during a CPMG experiment for measuring the $T_2$ relaxation time of PAA25-coated GdF$_3$/EuF$_3$ NPs and Gd-DTPA of equivalent mass concentrations. The images were acquired every 1.3 ms and the intensity of the samples is relative to the water signal. The NPs used for this particular experiment possessed relaxivities of $R_1, R_2 = 194.5 \pm 32.4, 348.4 \pm 39.6$ s$^{-1}$(mg/mL)$^{-1}$. These relaxivities were measured on the 1.5 T MRI scanner for a dilution series consisting of 20, 30, 40, and 50 mg/mL. From the intensity of the phantom samples, the signal decays much faster for the NPs compared to Gd-DTPA; hence they relax much faster. With reduced relaxation times, the signal intensity of NPs is greater and the images appear brighter in an MR image and this is exactly what is observed in figure 28. NPs possess their own relaxivities which differ from batch to batch even if they are all synthesized in the exact same way. The synthesis is highly sensitive to the rate of addition of the Ln salt solution, size of each drop, degree of temperature fluctuation, the exact polymer and NaF amount in solution as oppose to what is measured, and the length of time for drying the final product. All of these factors vary slightly for every synthesis because these are technical aspects that depend on the individual. Recently, it was discovered that the length of time used to dry NPs after re-precipitation affects the morphology to a certain degree. Hence, drying the NPs overnight or over a drying agent generates conditions which are too harsh. For further syntheses, the NPs will only be dried in an empty dessicator under vacuum for a few hours.
Figure 28. MR images of PAA25-coated GdF\(_3\)/EuF\(_3\) NPs and Gd-DTPA illustrating the signal decay during a CPMG experiment (TE = 10 ms, TR = 2.5 s) performed on the 1.5 T MRI scanner. The samples were prepared in water with equal mass concentrations. A greater decrease in the signal intensity is indicative of a faster spin-spin (\(T_2\)) relaxation. The NPs relax much faster than Gd-DTPA since their signal intensity is much less than that of Gd-DTPA at any given time in the CPMG experiment. At 9.1 ms, there is virtually no signal from the NPs. For this particular batch of NPs \(R_1, R_2 = 194.5 \pm 32.4, 348.4 \pm 39.6 \text{ s}^{-1} (\text{mg/mL})^{-1}\).

Dynamic Contrast Enhancement (DCE) in the Rat Brain

In order to demonstrate the potential of PAA25-coated GdF\(_3\)/EuF\(_3\) NPs as contrast agents for DCE-MRI, NPs were administered to a rat brain. Vascular perfusion of the contrast agent was monitored based on the average signal intensity within several regions of interest over the duration of the experiment. Perfusion weighted imaging (PWI) is based on the passage of intravascular traces like Gd-DTPA through capillaries [65]. DCI-MRI is performed just like traditional MRI but the contrast agent is monitored from the moment it is administered.

The average signal intensity was plotted against time to obtain enhancement curves as shown in figure 29. Figure 29A is an example of an axial \(T_1\)-weighted image which was obtained every 20 seconds following the injection of NPs. Figures 29 B, C, and D are enhancement curves for muscle, cerebral blood vessel, and brain, respectively. The contrast agent appeared to have the fastest clearance rate in the cerebral blood vessel with an
approximate clearance time of 70 minutes. The NPs used for this study possessed \( R_1 \) and \( R_2 \) relaxivities of 66.1 and 76.3 s\(^{-1}\)(mg/mL\(^{-1}\)), respectively. The typical dose of Gd-DTPA in a rat is 0.4 mL of a 469 mg/mL solution whereas the NP dose was 0.4 mL of an 18.58 mg/mL solution. The rat did not exhibit any obvious adverse reaction to the contrast agent, its heart rate was stable throughout the experiment and its anesthetic recovery time appeared to be normal. This preliminary study demonstrates that signal enhancement is possible with PAA25-coated GdF\(_3\)/EuF\(_3\) NPs and at doses almost 10 times smaller than standard Gd-DTPA chelates.

![Figure 29](image.png)

**Figure 29.** PAA25-coated 90/10 GdF\(_3\)/EuF\(_3\) NPs were prepared at a concentration of 18.58 mg/mL and administered to a rat brain with an injection dose of 0.4 mL. For this particular NP batch \( R_1, R_2 = 66.1, 76.3 \) s\(^{-1}\)(mg/mL\(^{-1}\)). Dynamic contrast enhancement (DCE) MRI was performed on a 1.5 T scanner. a) Axial \( T_1 \)-weighted images were obtained every 20 seconds following injection for a period of 35 minutes. Enhancement curves are shown for (b) muscle, (c) cerebral blood vessel and (d) brain. DCE-MRI demonstrates feasibility of using NPs as perfusion contrast agents at doses 10 times smaller than standard Gd-DTPA chelates.
X-ray Scattering and Potential for CT

X-ray scattering experiments of PAA25-coated GdF₃/EuF₃ NPs reveal their potential as multimodal imaging agents. Figure 30 is a set of radiograph images of phantom samples of iopramide (IP), NPs, and Gd-DTPA obtained at four different X-ray beam energies. Iopramide (figure 30E) is a substitute for Ultravist 300®, which is a common iodinated contrast agent used for CT (figure 30F). All samples were prepared in terms of mass concentrations in water and water was used as the control. The signal intensity (i.e. brightness with respect to water) of the NP samples is relatively greater than that of iopramide and Gd-DTPA in figures 30 A, B, and C. The signal intensity is greater for iopramide in figure 30D compared to that of NPs which was acquired at 49 kVp, 25 mAs, using a rhodium anode and copper filter. Figure 30D shows what imaging contrast looks like when the X-ray beam is optimized for iodine (I) imaging. Imaging with I is performed at X-ray energies between 33-50 keV where the attenuation of X-rays is greater with I than Gd according to figure 14. For the CT technique, the mean energy of the X-ray beam should be about 22 keV using a rhodium anode and an aluminum filter. Figure 30B closely matches these imaging conditions where the mean X-ray beam energy was 19.25 keV. Hence, for CT imaging, Gd proves to better at attenuating X-rays than I and gives rise to greater contrast. However, in cases where differentiation between fat and muscle is necessary, high-energy X-rays are required. Greater contrast with Gd-rich NPs can be achieved when imaging at X-ray energies between 50-75 keV, allowing for better differentiation between fat and muscle. Imaging can also be performed at low energies (<30 keV) if soft tissue enriched with Gd-based NPs is being studied. More importantly, these preliminary experiments prove that X-ray imaging can performed at lower energies and a sufficient amount contrast can still be achieved. This would be especially beneficial to patients since the exposure to high energy X-rays would be reduced or low doses of NPs would be required to achieve sufficient contrast comparable to that from iodinated CT agents.
Figure 30. Radiograph images of phantom samples prepared in water with equal mass concentrations for PAA25-coated 90/10 GdF₃/EuF₃ NPs, Gd-DTPA, and iopramide (IP). Images were acquired at X-ray energies of: A) 25 kVp, Rh anode, Rh filter; B) 35 kVp, Rh anode, Rh filter; C) 45 kVp, Rh anode, Rh filter; D) 49 kVp, Rh anode, Cu filter; E) Structure of iopramide which was used as a substitute for F) Ultravist 300®, a common iodinated CT agent.
Figures 31 and 32 illustrate signal intensity as a function of mass concentration for the phantom samples that were imaged with X-rays in figure 30. Figure 31 shows a decrease in the signal intensity as the energy of the X-ray beam is increased for PAA25-coated GdF₃/EuF₃ NPs. Since this is in the low-energy regime of the X-ray profile for Gd (figure 14), the linear attenuation decreases as the K-edge of Gd is approached. In figures 32 A, B, and C, signal enhancement by the NPs is greater than that of iopramide and Gd-DTPA since imaging was performed at X-ray energies where the linear attenuation of Gd is greater than I. In figure 32D, imaging was performed at 49 kVp, an X-ray energy where the linear attenuation of I is greater than Gd and thus, signal enhancement by iopramide is greater. However, in all four cases, signal enhancement by NPs is greater than Gd-DTPA and this is primarily because there are thousands of Gd³⁺ ions per NP compared to a single Gd³⁺ ion per Gd-DTPA complex. Maximum signal enhancement in X-ray CT depends on which energy regime imaging is performed at. Signal enhancement is greatest beyond the K-edge of Gd (55-70 keV) and at energies lower than 30 keV.

![Figure 31](image)

**Figure 31.** Signal enhancement (*i.e.* intensity with respect to water) as a function of mass concentration of PAA25-coated 90/10 GdF₃/EuF₃ NPs at various X-ray beam energies. Maximal signal enhancement depends on which energy regime imaging is performed in. Signal enhancement is greater at the K-edge of Gd and at lower energies.
Figure 32. Signal enhancement (i.e. intensity with respect to water) as a function of mass concentration for PAA25-coated 90/10 GdF$_3$/EuF$_3$ NPs, Gd-DTPA, and IP (iopramide) obtained at X-rays energies of A) 25 kVp, B) 35 kVp, C) 45 kVp, and D) 49 kVp. Imaging was performed using a rhodium anode and rhodium filter for plots A, B, and C. Plot D was obtained using a rhodium anode and copper filter.
Conclusions

A novel paramagnetic NP formulation consisting of 90/10 GdF$_3$/EuF$_3$ and coated with PAA25 has been presented for purposes in MRI and CT with potential applications in radionuclide-based imaging (PET and SPECT) and anti-cancer therapy. These NPs possess high contrasting properties with low toxicity, high solubility in aqueous and biological media, and potential for surface functionalization with a variety of targeting molecules.

The synthesis of polymer-coated GdF$_3$/EuF$_3$ NPs was optimized to achieve the highest possible relaxivities by screening various combinations of the reaction parameters. At the current optimal reaction conditions, NPs that are not extensively cross-linked possess mass relaxivities between 80-120 s$^{-1}$ (mg/mL)$^{-1}$. Electron microscopy images show polymer stabilized nanocrystals with 50-70 nm diameters and highly cross-linked aggregates with 100-300 nm cross-sections. The size and morphology is strictly dictated by the reaction conditions. Highly cross-linked aggregates give rise to mass relaxivities between 100-250 s$^{-1}$ (mg/mL)$^{-1}$. A large polymer network with extensive cross-linking entraps a higher proportion of water molecules while presumably slowing down the water exchange rate with the paramagnetic surface. Such high relaxivities provide significant contrast enhancement for $T_1$-weighted imaging in MRI.

The potential of polymer-coated GdF$_3$/EuF$_3$ NPs as contrast agents for DCI-MRI and X-ray CT imaging was also tested. DCI-MRI of a rat brain demonstrated that NPs can be used to study vascular perfusion with doses 10 times smaller than standard Gd-DTPA chelates. The NPs were observed to have the fastest clearance rate in the cerebral blood vessel with an approximate clearance time of 70 minutes. Measurement of signal enhancement based on various X-ray energies was compared amongst NPs, iopramide (a common iodinated contrast agent), and Gd-DTPA. X-ray contrast enhancement by Gd-rich NPs can be achieved at either low energies (<30 keV) or at higher energies (50-75 keV) where the linear attenuation of Gd is greater than I. Gd-rich NPs maximize contrast while in some cases minimize exposure to radiation.
Future prospects of utilizing polymer-coated GdF$_3$/EuF$_3$ NPs include the attachment of targeting molecules such as folic acid, integrin peptides, and fluorophores. *In vitro* studies based on human prostate (PC3) cancer cell lines will be investigated by first, functionalizing the NPs with folic acid, and then detecting fluorescence signals from Eu to measure cell adherence. Synthesis of radionuclide-doped (*e.g.* $^{177}$Lu and $^{18}$F) NPs will allow for applications in anti-cancer therapies.
References


13. Sitharaman, B.; Bolskar, R. D.; Rusakova, I.; Wilson, L. J., Gd@C60(COOH)2\textsubscript{10} and Gd@C60(OH)\textsubscript{10}: Nanoscale aggregation studies of two metallofullerene MRI contrast agents in aqueous solution. *Nano Letters* 2004, 4, (12), 2373-2378.


Appendix A

Towards the Synthesis of $^{13}$C-Enriched Para-fluorophenylalanine as a Highly Sensitive Probe for Studying Protein Dynamics
Abstract

Fluorinated amino acids can be used as biomarkers for studying topological and behavioural features of intrinsically disordered proteins (IDPs) and membrane proteins. Once incorporated into protein models, fluorinated amino acids can help detect $^{19}$F – $^{19}$F and $^{19}$F – $^1$H interactions with good peak separation and well resolved spectra. A modified version of the Balz-Schiemann mechanism for aromatic fluorination was used to synthesize non $^{13}$C-enriched para-fluorophenylalanine (p-FPhe) from para-aminophenylalanine (p-NH$_2$Phe). The crude yield of p-FPhe was determined to be approximately 6% from absorbance measurements. A 1D $^{19}$F NMR spectrum of the crude reaction mixture verified a characteristic peak for p-FPhe at -115 ppm. Once reproducibility, sufficient yield, and product purity is achieved, the synthesis of $^{13}$C-enriched p-FPhe starting with phenylalanine (Phe) will be possible.
Introduction

Background of $^{19}$F NMR

$^{19}$F NMR has long been used in the study of structural features of proteins [1]. Its sensitivity is 83% that of $^1$H NMR while there is generally no background $^{19}$F NMR signal. $^{19}$F NMR chemical shifts are extremely sensitive to changes in local van der Waals and electrostatic environments; hence they can provide specific information of local topology, clustering, and dynamics with improved peak assignment, resolution, and sensitivity [2]. Although site-directed mutagenesis has been extensively used for characterizing proteins, the incorporation of fluorinated amino acids proves to be more advantageous primarily because of strong C-F bonds that are resistant to metabolic transformations, large $^{19}$F-$^1$H coupling constants which give rise to increased sensitivity, relatively similar reactivity as non-fluorinated amino acids, and similarity in size between $^{19}$F and $^1$H nuclei [3].

Phenylalanine (Phe) is one of the aromatic amino acids and is generally found in the hydrophobic regions of proteins. It serves as an excellent probe for studying protein folding and unfolding. The incorporation of $p$-FPhe also exhibits excellent dispersion in biosynthetically labeled proteins and does not significantly alter or disturb their conformation or behaviour. Prominent chemical shift anisotropy (CSA) effects give rise to line broadening when $p$-FPhe is incorporated into larger, immobile proteins [4]. Line broadening effects result when a large protein system tumbles more slowly. Short spin-spin ($T_2$) relaxation times due to long correlation times cause resonance lines to broaden. The line width ($\Delta\nu_{1/2}$) is inversely proportional to $T_2$ according to equation A-1.

$$\Delta\nu_{1/2} = \frac{1}{\pi T_2} \quad (A-1)$$

The CSA arises from the inhomogeneity due to the orientation and resonant frequencies of nuclei that are a part of specific functional groups and may be shielded by the local electronic environment [4]. The CSA contributes to nuclear spin relaxation mechanisms and depends on the applied magnetic field [4]. By utilizing $^{13}$C-enriched $p$-FPhe with deuterated aromatic proton nuclei, line broadening effects due to the CSA are expected to be reduced, allowing for better-resolved spectra. $P$-FPhe can also be used as a probe for studying membrane
protein topology in which case, helix-helix interactions can be studied by measuring $^{19}$F–$^{19}$F distances.

Towards the Synthesis of $^{13}$C-enriched $p$-FPhe

Although $p$-FPhe is readily available from commercial suppliers, fluorination of the aromatic ring at the para position must be achieved in order to synthesize a $^{13}$C-enriched version starting with Phe. Figure A-1 is the reaction scheme that is yet to be performed in its entirety, although the fluorination step has been performed several times with non $^{13}$C-enriched $p$-NH$_2$Phe.

![Reaction Scheme](image)

**Figure A-1.** The reaction scheme for synthesizing $p$-FPhe is based on the Balz-Schiemann mechanism for aromatic fluorination. Boc-protected $p$-NO$_2$Phe is used as the starting material since it is commercially available and must be hydrogenated to obtain boc-protected $p$-NH$_2$Phe to carry out the fluorination reaction.

Since only $^{13}$C-enriched Phe is commercially available, boc protection and nitration of the aromatic ring will also have to be performed once the fluorination step has been reproduced with non $^{13}$C-enriched material. The fluorination reaction begins with a diazotization of an aniline-type compound which results in the formation of an aryl diazonium salt. A possible mechanism for this step is that the aryl amine nitrogen electrons attack the nitrosonium (+N=O) nitrogen, followed by two proton transfers to the oxygen atom to release water and form Ar – N≡N. The tetrafluoroborate counter ion (BF$_4^-$) is thought to behave as a Lewis
acid and presumably donates fluoride ions [5]. However, there may be the possibility that the fluoride ions remain in equilibrium with BF$_4^-$ to some degree during the attachment to the aromatic ring [5]. Upon thermal decomposition, de-diazotization takes place with liberation of N$_2$ gas and results in the aryl fluoride [6]. This procedure is a modification of the Balz-Schiemann mechanism for aromatic fluorination and was first reported by Milner [7, 8].
Materials and Methods

The following chemicals were obtained from Sigma-Aldrich (Mississauga, Ontario): p-NH₂Phe, p-FPhe, nitrosonium tetrafluoroborate (NOBF₄), and o-dichlorobenzene (o-DCB).

Synthesis of p-fluorophenylalanine (p-FPhe)

A modified version of the Balz-Shiemann mechanism as described by Milner was employed for synthesizing p-FPhe [8]. A mixture of 0.07 g of NOBF₄ (10% molar excess than the arylamine) in 20 mL of CH₂Cl₂ was cooled in an ice bath while being stirred vigorously at room temperature. Approximately 0.1 g of p-NH₂Phe was added to the mixture and stirred vigorously overnight to allow the completion of diazotization. Approximately 20 mL of o-DCB was added and the mixture was heated in an oil bath. A distillation apparatus was setup to remove CH₂Cl₂ (b.p. 40 °C). The decomposition of the diazonium salt occurred with the liberation of N₂ gas which was detected with a bubbler. The decomposition temperature was observed to be between 110-120 °C. The solvent was poured off to obtain the decomposed product which was insoluble in o-DCB. The decomposed product was dissolved in water, gravity filtered, and extracted from fresh o-DCB to remove any residual amounts of the organic solvent in the aqueous layer. 1D ¹⁹F NMR spectra were acquired for 500 μL samples of the organic (NMR solvent: CDCl₃) and aqueous (NMR solvent: 10% D₂O) layers to detect the presence of p-FPhe. The spectra were acquired using a 600 MHz NMR spectrometer (Varian Inc., Palo Alto, CA, USA). Absorbance measurements using the internal standard method with pure p-FPhe were performed to determine the amount of p-FPhe in the aqueous mixture. The measurements were made on an Ultrospec 3100 pro UV/Vis spectrometer (Biochrom Ltd., Cambridge, UK). Thin layer chromatography (TLC) was also performed to verify the presence of the product in the aqueous mixture. A 2:1 acetonitrile:water (CH₃CN:H₂O) reverse-phase solvent system was used to develop the silica plate which was spotted with the aqueous mixture and standard solutions of p-FPhe and p-NH₂Phe prepared in water. The TLC plate was then sprayed with a 0.25% w/v ninhydrin solution in acetone and placed in an oven for a few minutes for a pink colouration of the spots to develop [9].
Results

Figure A-2. A 1D $^{19}$F NMR spectrum of the crude solid product dissolved in water. The peak at -115 ppm is characteristic for $p$-FPhe and the other peaks are of unreacted NOBF$_4$ (-130, -143, -150 ppm). The spectrum was acquired on the 600 MHz spectrometer with 10% D$_2$O.

Figure A-3. 1D $^{19}$F NMR spectra of A) pure $p$-FPhe and B) pure NOBF$_4$ both acquired on the 600 MHz spectrometer with 10% D$_2$O. The characteristic peak for $p$-FPhe appears at -114 ppm and for NOBF$_4$ at -130, -143, and -150 ppm.
Figure A-4. Standard addition plot for determining the amount of $p$-FPhe present in the aqueous mixture.

### Table A-1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R_f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$p$-NH$_2$Phe</td>
<td>0.44</td>
</tr>
<tr>
<td>$p$-FPhe</td>
<td>0.60</td>
</tr>
</tbody>
</table>

Table A-1. Retention factors for $p$-NH$_2$Phe and $p$-FPhe from TLC experiments. The TLC plate was developed in a reverse-phase 2:1 CH$_3$CN:H$_2$O solvent system.
Discussion

The fluorinated aromatic amino acid is formed after the thermal decomposition of the diazonium salt. The decomposition temperature was determined to be between 110 and 120 °C after which point the reaction was stopped and the o-DCB was poured off. The solid decomposed product was insoluble in the organic solvent and was removed from the flask by dissolving it distilled water. Figure A-2 is a 1D $^{19}$F NMR spectrum of the crude mixture dissolved in water which shows the characteristic peak at -115 ppm for p-FPhe. 1D $^{19}$F NMR spectra of standard p-FPhe and NOBF$_4$ were also acquired to verify the characteristic chemical shifts and are shown in figures A-3 A and B, respectively. From these preliminary results, it is evident that p-FPhe was indeed synthesized. From the spectrum in figure A-2, however, it is also evident that there is a considerable amount of unreacted fluorinating agent in the aqueous mixture. A 1D $^1$H NMR spectrum of the reaction mixture was not acquired at the time.

The standard addition method was used to determine the amount of p-FPhe present in the aqueous mixture via absorbance measurements. The wavelengths at which maximum absorbance of p-NH$_2$Phe and p-FPhe occur were determined by acquiring wave scans of standard solutions. Maximum absorbance of p-NH$_2$Phe and p-FPhe occurs at 237 nm and 264 nm, respectively. A calibration curve was constructed to find the linear range of concentration that can be used to do the standard addition. Figure A-4 is a standard addition plot for one of the aqueous mixtures. In this case, the crude yield was determined to be 6 mg starting with 100 mg of p-NH$_2$Phe with the assumption that p-NH$_2$Phe and p-FPhe are in a 1:1 mole ratio. Since the fluorination step was repeated several times, the crude yield was determined each time and was found to vary between 2-6 mg.

TLC was also performed with the aqueous mixture to verify the presence of p-FPhe. A reverse-phase 2:1 CH$_3$CN:H$_2$O solvent system was utilized to develop the plate containing standard solutions of p-NH$_2$Phe and p-FPhe and the aqueous mixture. TLC results confirmed the presence of both compounds and the retention factors of each are summarized in table A-1. Since it was a reverse-phase system, the more non-polar p-FPhe travelled faster and farther than the more polar p-NH$_2$Phe. Hence, the R$_f$ value of p-NH$_2$Phe is lower than that of p-FPhe. From the preliminary characterization data discussed above, it was concluded that
the aqueous mixture contained \( p \)-FPhe in very low amounts along with unreacted \( p \)-NH\(_2\)Phe and NOBF\(_4\).

During the fluorinating step, \( p \)-NH\(_2\)Phe was found to be sparingly soluble in CH\(_2\)Cl\(_2\) which generated dispersibility issues. Upon introducing \( p \)-NH\(_2\)Phe to the slurry of NOBF\(_4\) and CH\(_2\)Cl\(_2\), a thick residue formed which never completely dissolved. Hence, the resulting solution was a heterogeneous mixture. This most probably hindered the fluorination of \( p \)-NH\(_2\)Phe since some of it might have been solution and some remained in the solid phase. In order to prevent this heterogeneous mixture from forming, a more non-polar version of \( p \)-NH\(_2\)Phe should be used so it can dissolve in CH\(_2\)Cl\(_2\). Figure A-1 shows the reaction scheme with \textit{boc}-protected \( p \)-NH\(_2\)Phe as the starting material. \textit{Boc}-protected \( p \)-NO\(_2\)Phe, which is commercially available, was hydrogenated to obtain \textit{boc}-protected \( p \)-NH\(_2\)Phe and the product was verified with a Waters Micromass ZQ LC-MS equipped with a photodiode array detector (Waters Corp., Milford, MA, USA). However, the fluorination step with \textit{boc}-protected \( p \)-NH\(_2\)Phe is yet to be performed. Once each step in figure A-1 has been reproduced, including the \textit{boc} protection and nitration of Phe (which is not shown in figure A-1), the \(^{13}\)C-enriched version of \( p \)-FPhe can be synthesized. For now, reproducibility of the fluorination reaction with sufficient yield must be achieved.
Conclusions

Non $^{13}$C-enriched $p$-FPhe was synthesized from $p$-NH$_2$Phe with a crude yield of 6% as determined from absorbance measurements. A 1D $^{19}$F NMR spectrum of the crude aqueous reaction mixture confirmed the presence of $p$-FPhe with a characteristic peak at -115 ppm and was also verified with TLC. The fluorination step results in very low yields of $p$-FPhe because it is performed in heterogeneous conditions. Hence, it needs to be repeated with the more non-polar boc-protected $p$-NH$_2$Phe to increase its solubility in the reaction solvent (CH$_2$Cl$_2$). The complete synthesis of $p$-FPhe beginning with Phe is yet to be performed.
References