SYNTHESES OF PH-RESPONSIVE POLYMERS VIA RAFT POLYMERIZATION
AND THEIR USE FOR PREPARATION OF PH-RESPONSIVE
MRI CONTRAST AGENTS

by

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A thesis submitted to the Faculty and the Board of Trustee of the Colorado School of Mines in partial fulfillment of the requirements for the degree of Doctor of Philosophy (Materials Science).

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ABSTRACT

Despite recent advances in the understanding of fundamental cancer biology, cancer remains the second most common cause of death in the United States. One of the primary factors indicative of high cancer morbidity and mortality and aggressive cancer phenotypes is tumors with a low extracellular pH (pHe). Thus, the ability to measure tumor pH in vivo using non-invasive and accurate techniques that also provide high spatiotemporal resolution has become increasingly important and is of great interest to both researchers and clinicians. Recently, the use of pH-responsive polymer systems in cancer diagnostics and treatment has received considerable attention due to their ability to undergo conformational changes in response to changes in the environmental pH. We have developed pH-responsive nanoscale contrast agents based on pH-responsive polymers and gadolinium (Gd) based nanoparticles for magnetic resonance imaging (MRI) that demonstrate large changes in relaxivity to improve both the selectivity and sensitivity of in vivo pH measurement.

A series of pH-responsive biocompatible block polymers with pKa ranging from 5.0 to 7.0 have been synthesized via reversible addition-fragmentation chain transfer (RAFT) polymerization. In this research, we chose three tertiary amine based methacrylates, including 2-(diisopropylamino)ethyl methacrylate (DPAEMA), 2-(dibutylamino)ethyl methacrylate (DBAEMA), and 2-(piperidino)ethyl methacrylate (PPDEMA), to prepare their homopolymers and block copolymers with poly(ethylene glycol) (PEG) maro-RAFT agents via RAFT
polymerizations and studied the polymerization kinetics of both. The pH-responsive properties of these block copolymers also has been studied through pKa titration and calculation, dynamic light scattering. These copolymers have then been used to modify Gd-based nanoparticles (GdNPs) via a grafting to method after reduction of the thiocarbonylthio end groups under basic conditions to thiolates. The performance of pH-responsive polymer modified Gd nanoparticles was then evaluated for potential use as pH-responsive MRI CAs via monitoring the relaxivity changes upon the environmental pH changing. Furthermore, the stability of the GdNPs in pH solutions has also been studied to ensure the potential use under different environmental pH. The results suggested that the pH-responsive polymers can be used to effectively modify the Gd nanoparticles surface to prepare a pH-responsive contrast agent for MRI, which will potentially allow for enhanced diagnostics in the treatment of cancer.
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The more I learn, the less I know.

“The more I learn, the more I realize how much I don’t know.”
— Albert Einstein

No pain, no gain.

Benjamin Franklin says,

“Industry need not wish, as Poor Richard says, and he that lives upon hope will die fasting.
There are no gains, without pains...”
— The Way to Wealth

Robert Herrick says,

“NO PAINS, NO GAINS.”
“If little labour, little are our gains: Man’s fate is according to his pains.”
— Hesperides 752

Rabbi Ben Hei says,

"According to the pain is the gain."
— Ethics of the Fathers (Pirkei Avot) 5:21
CHAPTER 1

INTRODUCTION

Despite recent advances in the understanding of fundamental cancer biology and developing novel and/or improving existing therapies for cancer, cancer remains the second most common cause of death in the United States. According to statistics from the International Agency for Research on Cancer (IARC), an estimated 14.1 million new cancer cases and 8.2 million cancer-related deaths occurred in 2012, compared with 12.7 million and 7.6 million, respectively, in 2008, and a predicted 19.3 million new cancer cases will be reached per year by 2025, due to growth and ageing of the global population.[1, 2] In some western countries, cancer mortality rates have recently started to show a declining trend, due to a reduction in smoking prevalence, improved early detection and advances in cancer therapy.[2] While the development of new treatment methodologies has been important in decreasing mortality rates, it is widely acknowledged that early detection protocols have played a critical role in battling cancer.[3] The prognosis of the disease is much better if treatment begins during a pre-invasive stage than if the cancer has become invasive. Thus, a major focus of cancer research today is the development of effective and affordable approaches for the early detection and diagnosis of cancer.

One such area is the measurement of the extracellular pH (pHe) in solid tumors. For many years researchers have known the importance of the pHe in relation to cancer morbidity and mortality.[4, 5] The physiological microenvironment of solid tumors is normally characterized
by poor perfusion and high metabolic rates. As a result, many regions within the tumor are chronically or transiently acidic and hypoxic. An increase in the glycolytic rate within tumors leads to the formation of lactate (the conjugate base of lactic acid). In conjunction to this, carbonic acid ($\text{H}_2\text{CO}_3$) is produced by hydration of carbon dioxide, which is formed by oxidation in the tricarboxylic acid cycle. The excess lactate and $\text{H}_2\text{CO}_3$ produced is removed from the cells via several transport systems, resulting in an intracellular pH (pHi) that is maintained at a neutral or alkaline pH when compared to normal cells. However, poor perfusion in tumors decreases the ability to remove the metabolic acids once they leave the cells and also results in regional hypoxia. The net result of these processes is a reduction of the pHe in solid tumors from 7.4 to between 6.8-6.2. Despite the acidic microenvironment in solid tumors, the tumor cells seem to be well suited to the environment and, in fact, in vitro studies have shown that tumor cells have maximum proliferation at a pHe of 6.8, in comparison to normal cells that require a pHe of 7.3. Ultimately, the tumor microenvironment leads to the development of tumor cells adapted to survive in an acidic microenvironment, where normal cells would die. As such, a low pHe has been identified as an important factor in producing more aggressive cancer phenotypes and causing metathesis of the primary carcinoma, both of which are leading causes of cancer morbidity and mortality.[6, 7]

Thus, the ability to measure the pHe of solid tumors using non-invasive and accurate techniques that also provide high spatiotemporal resolution has become increasingly important and is of great interest to clinicians. Despite this interest, there is currently no clinical method available for the in vivo determination of pHe in tumors. Therefore, the overall goal of this
research is to design and fabricate a pH-responsive magnetic resonance imaging (MRI) contrast agent (CA) that has the potential to image the pHe of solid tumors. Initial research in to pH responsive MRI CAs focused on gadolinium (Gd) based chelates where the hydration of the Gd complex, water accessibility and correlation times were pH dependent.[8-11] The main problems with these systems were related to the three main drawbacks of Gd chelates: 1) a low concentration of Gd per molecule; 2) non-specific targeting of the region of interest; and 3) fast elimination kinetics due to the dimensions of the complex. These factors have drawn an increasing focus on the use of targeted and smart CAs based on nanoparticles. Gd-based nanoparticles (GdNPs) demonstrate a series of advantages, such as longer retention time, high concentration of Gd per targeted molecule, and the ability to be surface modified to introduce further functionality.[12, 13] pH-Responsive polymers have been considered as a promising way of targeted cancer diagnosis and therapy.[14, 15] Therefore, our overall goal is to develop pH responsive nanoscale smart contrast agents based on GdNPs and pH-responsive polymers that demonstrate large changes in relaxivity upon environmental pH changing to improve both the accuracy and sensitivity of in vivo pHe measurement.

The relevant background needed to understand the concept and motivation for this work is presented in Chapter 2. First, an introduction of non-invasive molecular imaging techniques, the general theory and mechanisms behind MRI, the factors that affect MRI contrast agents, the concept of the pH-responsive MRI CAs, and the advantages of new developed Gd-based nanoparticles for use as CAs are provided. Then, the importance of pH-responsive polymers, their use in diagnosis and therapy in biomedical area, their chemical structure and properties
are discussed. Followed by an introduction of the synthesis techniques of pH-responsive polymers focused on reversible addition-fragmentation chain transfer (RAFT) polymerization. The final section provides a review on the pH-responsive polymers used in imaging pH in tumors, in particular with optical imaging and MRI/MRS techniques.

As discussed above, poor perfusion and high metabolic rates in solid tumors result in a reduction of the pH in solid tumors from 7.4 to between 6.8-6.2. Chapter 3 focuses on design and synthesis of pH-responsive polymers with pKa’s at relevant biological pH by RAFT polymerization. Among several pH-responsive polymer systems with a pKa above 6.0,[16-22] tertiary amine based methacrylate polymers are one of the most promising because their pKa can be tuned from approximately 4.5 to 8.5 depending on different substituent groups.[23] In this work, we have demonstrated that RAFT polymerization can be used to synthesize homopolymers and block copolymers of 2-(diisopropylamino)ethyl methacrylate (DPAEMA), 2-(dibutylamino)ethyl methacrylate (DBAEMA), and 2-(piperidino)ethyl methacrylate (PPDEMA) by appropriately choosing RAFT agents and solvent systems. The controlled character of the polymerizations was confirmed by kinetics studies. The pH-responsive properties of the homopolymers and block copolymers were investigated by pKa and dynamic light scattering (DLS) measurements.

Chapter 4 centers on the modification of GdNPs with the tertiary amine-based pH-responsive polymers discussed in Chapter 3 and evaluation of these systems as pH-responsive CAs in MRI using nuclear magnetic resonance (NMR) spectroscopy. Because the pH-responsive polymers will undergo a phase transition at different pH, the surface
modified GdNPs will be expected to exhibit changes in relaxivity based upon the environmental pH changing. Transmission electron microscopy (TEM) and fourier transform infrared (FTIR) spectroscopy were used to prove the successfully attachment of tertiary amine-based methacrylate pH-responsive polymers to the surface of the Gd NPs. Preliminary NMR results showed that the bulk water relaxation rates changes in response to different pHs in the presence of the pH-responsive polymer modified GdNPs.

As it has been reported that free Gd$^{3+}$ ion is toxic to humans, the stability of Gd-based CAs has recently been an important research area. Former work done by colleagues in our lab has suggested that the stability of GdNPs based on a metal organic framework (MOF) in aqueous and organic media is acceptable for both the in vitro and in vivo study. However, the stability of GdNPs in pH solutions hasn’t been studied yet. Therefore, Chapter 5 is used to study the stability of the nanoparticles in different pH solutions to determine whether the systems are stable when the relaxivity studies are performed.

Chapter 6 includes the main conclusions in this dissertation and recommended future work based on the achievements of this work. Appendix A and B include supporting information for the main chapters.
CHAPTER 2
RESEARCH BACKGROUND

2.1 Non-invasive molecular imaging techniques

Over the past few decades, medical imaging technologies have experienced dramatic growth and currently play a critical role in clinical oncology. With the advent of new and improved imaging techniques, clinicians will not only be able to see where a tumor is located in the body but also envisage biological processes and the expression and activity of specific molecules that influence the response to therapy and behavior of various tumors. Access to such knowledge is predicted to have a major impact on the detection and diagnosis of cancer, therapeutic development, and personalized treatment, in addition to dramatically improving researchers understanding of cancer.

Molecular imaging allows the visual representation, characterization and quantification of biological processes at the cellular and subcellular levels of intact living organisms by exploiting specific imaging reporter probes or contrast agents (CAs).[24, 25] As a powerful technique, molecular imaging has been applied to detect and characterize early stage disease and provide a rapid method for evaluating and optimizing treatment. Several diagnostic imaging methodologies have been invented and successfully applied to fields ranging from clinical diagnosis to research in cellular biology and drug discovery, including computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), single photon emission CT (SPECT), ultrasound (US) and optical imaging (OI). Each imaging
modality differs in terms of energy source, molecular probes or CAs needed, depth penetration, sensitivity, spatial and temporal resolution, and financial cost.[24, 26] Figure 2.1 shows typical molecular imaging instruments and images representative of each modality and Table 2.1 summarizes some of the general characteristics of various imaging modalities clinically available. As each imaging modality has relative advantages and disadvantages (Table 2.2), they are complementary to each other rather than competitive, which encouraged the concept of multimodality imaging, such as PET/CT, PET/MRI and MRI/CT.[24, 27] The choice of the appropriate molecular imaging modality mainly depends on the specific situations. Due to the ability in imaging or providing physiological or biochemical informations, PET, OI, magnetic resonance spectroscopy (MRS) and MRI have been used in an attempt to measure tissue pH in vivo.[28-31] Since the research presented here is focused on MRI, a brief discussion of MRI and MRI CAs/probes is presented below.

FIGURE 2.1 Typical molecular imaging instruments and their representative images.[26]
<table>
<thead>
<tr>
<th>Imaging Modality</th>
<th>Energy Source</th>
<th>Probe/CA type</th>
<th>Depth</th>
<th>Sensitivity (mol/L)</th>
<th>Spatial Resolution</th>
<th>Temporal Resolution</th>
<th>Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>X-rays</td>
<td>Heavy element, e.g. iodine</td>
<td>No limit</td>
<td>Not well characterized</td>
<td>50-200 μm</td>
<td>Minutes</td>
<td>$$</td>
</tr>
<tr>
<td>MRI</td>
<td>Radiowaves</td>
<td>Para- or superparamagnetic metals, e.g. Gd, Fe</td>
<td>No limit</td>
<td>$10^{-3}$ to $10^{-5}$</td>
<td>25-100 μm</td>
<td>Minutes to hours</td>
<td>$$$$$</td>
</tr>
<tr>
<td>PET</td>
<td>γrays</td>
<td>Radionuclides, e.g. $^{18}$F, $^{64}$Cu, $^{111}$In</td>
<td>No limit</td>
<td>$10^{-11}$ to $10^{-12}$</td>
<td>1-2 mm</td>
<td>10 sec to minutes</td>
<td>$$$$$</td>
</tr>
<tr>
<td>SPECT</td>
<td>γrays</td>
<td>Radionuclides, e.g. $^{18}$F, $^{64}$Cu, $^{111}$In</td>
<td>No limit</td>
<td>$10^{-10}$ to $10^{-11}$</td>
<td>1-2 mm</td>
<td>Seconds to minutes</td>
<td>$$$</td>
</tr>
<tr>
<td>US</td>
<td>High-frequency Sound</td>
<td>Gas filled microbubbles</td>
<td>mm to cm</td>
<td>Not well characterized</td>
<td>50-500 μm</td>
<td>Seconds to minutes</td>
<td>$</td>
</tr>
<tr>
<td>OI</td>
<td>Visible light</td>
<td>Fluorescent dyes, quantum dots</td>
<td>1-2 cm</td>
<td>$10^{-9}$ to $10^{-12}$, $10^{-15}$ to $10^{-17}$</td>
<td>2-5 mm</td>
<td>Seconds to minutes</td>
<td>$-$$$</td>
</tr>
</tbody>
</table>
TABLE 2.2 Advantages and disadvantages of various imaging modalities clinically available

<table>
<thead>
<tr>
<th>Imaging Modality</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>High spatial resolution</td>
<td>Require CAs for enhanced tissue contrast</td>
</tr>
<tr>
<td></td>
<td>Ability to differentiate between tissues</td>
<td>Radiation exposure</td>
</tr>
<tr>
<td></td>
<td>Good at bone and tumor imaging</td>
<td>Tissue non-specificity</td>
</tr>
<tr>
<td>MRI</td>
<td>High resolution</td>
<td>Long scan and post processing time</td>
</tr>
<tr>
<td></td>
<td>No ionizing radiation</td>
<td>Cannot be used in patients with metallic devices, e.g. pacemakers</td>
</tr>
<tr>
<td></td>
<td>Able to image physiological and anatomical details</td>
<td>Low sensitivity</td>
</tr>
<tr>
<td></td>
<td>Strong ability in distinguish soft tissues</td>
<td>High cost</td>
</tr>
<tr>
<td>PET &amp; SPECT</td>
<td>Ability to image biochemical processes</td>
<td>Radiation</td>
</tr>
<tr>
<td></td>
<td>High sensitivity</td>
<td>Low resolution</td>
</tr>
<tr>
<td></td>
<td>Reporter/gene expression</td>
<td>High cost</td>
</tr>
<tr>
<td>US</td>
<td>Ease of use, low cost</td>
<td>Low spatial resolution</td>
</tr>
<tr>
<td></td>
<td>No radiation exposure</td>
<td>Mostly morphological</td>
</tr>
<tr>
<td>OI</td>
<td>High sensitivity</td>
<td>Low spatial resolution</td>
</tr>
<tr>
<td></td>
<td>Provides functional information</td>
<td>Limited tissue penetration</td>
</tr>
<tr>
<td></td>
<td>No radiation exposure</td>
<td>Currently 2D imaging only</td>
</tr>
</tbody>
</table>

2.1.1 Imaging modality – MRI

MRI has become one of the most studied techniques due to a high spatial resolution and a so far unsurpassed ability in distinguishing soft tissues. It demonstrates a high potential for use in the accurate and precise non-invasive measurements of pH \textit{in vivo} with reasonable spatial resolution by exploiting new imaging probes or CAs.[6, 32-34]

An MR image is generated from the visualization of hydrogen protons in water and organic macromolecules (i.e., lipids and proteins) in the body. The MRI technique, based on the principles of nuclear magnetic resonance (NMR), is the precession of the body’s hydrogen atoms (protons) within an applied magnetic field that receives a pulse from a radio-frequency
(RF) transmitter. First, the static magnetic field causes the hydrogen nuclei to rotate and align themselves along the magnetic field to reach an equilibrium magnetization with a certain frequency which depends on the strength of the magnetic field. Then, a RF pulse at the same frequency is applied to and alters the alignment of the nuclei to result in a change in the net magnetization. After the RF pulse, the hydrogen atoms recover back to their original equilibrium state. This recovery of the system to thermal equilibrium is known as relaxation. With this relaxation process, $T_1$ is termed as the longitudinal relaxation time (or spin-lattice relaxation) and defines the time the system takes to relax back to its original longitudinal magnetic field, and $T_2$ is defined as the transverse relaxation time (or spin-spin relaxation), which describes the time the system takes to relax back to the transverse magnetization after an RF pulse. These changes are detected by the MRI instrument and translated into an MR signal to build three dimensional images of the body that includes $T_1$-weighted MRI or $T_2$-weighted MRI based on the different emphasis of $T_1$ or $T_2$.

The amount of signal collected and the extent of observed contrast in MRI depends on factors such as the water proton density and the $T_1$ and $T_2$ of these protons. This is one of the main reasons that MRI has an excellent ability in distinguishing soft tissues, which usually contain a large amount of water. The values of these parameters differ between different tissues and cause contrast between tissues of various types for high quality MR images. However, some pathological conditions, such as inflammation or tumors, do not provide sufficient changes in the aforementioned parameters. Therefore, CAs or imaging probes are introduced
into the system to enhance the differentiation between tissues and provide information-rich images for better analysis of physiological information and diagnosis of diseases.

2.1.2 Imaging probes – MRI CAs

Typically, chemical agents that can change the MR signal intensity are used in MRI modality to enhance signal differences and further highlight abnormalities, such as tumors. These chemical agents are classified as either positive CAs or negative CAs. While magnetic nanoparticles of iron oxides have been reported as a typical negative contrast agent (CA), gadolinium (Gd) based CAs have been established as one of the best positive CAs and are the most commonly used in clinic.[35, 36] A negative CA typically contains iron oxide nanoparticles that are superparamagnetic and cause magnetic fields of inhomogeneities that decrease the $T_2$ relaxation time of the water protons that are near the iron oxide nanoparticles.[37] This permits negative contrast enhancement and thus, darkens images of the regions of interest. On the other hand, a positive CA usually contains paramagnetic molecules, such as Gd, that have a large magnetic moment and produces a large shortening of $T_1$ and $T_2$ of water protons that are near the molecule. Though Gd CAs decrease $T_1$ and $T_2$ by roughly similar amounts, the percentage change of $T_1$ in tissue is much greater than that of $T_2$.[36] Thus the Gd-based CA is best visualized using $T_1$-weighted images which provide brighter images.

Gd$^{3+}$ has been generally used for CAs because its physical properties lead it to be the most suitable candidate for efficiently reducing the $T_1$ and $T_2$ relaxation times.[36, 38, 39] Firstly, the seven unpaired electrons possessed by Gd$^{3+}$ is the maximum number of unpaired spins possible, at least for known elements, which provides strong dipolar interactions with water
protons.[39] Secondly, the symmetric S-state of Gd$^{3+}$ creates a more hospitable environment for electron spins, causing a much slower electronic relaxation rate that is more closely in tune with the proton’s frequency.[36] However, due to the high toxicity of free Gd$^{3+}$ ion, it has to coordinate with ligands to keep Gd$^{3+}$ completely bound in clinical use.[39] The higher the coordination numbers the metal can have the more possible open sites for inner-sphere water coordination. Due to the lanthanide contraction across the 4f row of the periodic table, the early lanthanides have higher coordination numbers than the lanthanides toward the end of the series.[39] Since Gd$^{3+}$ is situated in the middle of the row, it favors eight or nine coordination. While 6 to 8 coordination sites are usually used to complex with ligands, 1 to 3 open sites are reserved for inner-sphere water molecules, which is crucial for the contrast enhancement mechanism.[36]

The efficiency of CAs is evaluated by their relaxivity, including longitudinal ($r_1$) and transverse ($r_2$), which indicates how much the relaxation rates of water protons ($1/T_i$) are increased with increasing concentration of CAs. Both diamagnetic and paramagnetic species contribute to the observed relaxation rate of the water protons.[40] The former is the reciprocal of the water protons relaxation time in the absence of paramagnetic species ($[(1/T_i)_{\text{dia}}]$ and the latter is linearly related to the concentration of paramagnetic species presented in the solution [$(1/T_i)_{\text{obsd}}$]. The relaxivity ($r_i$, i=1,2) is defined as the slope of the plot of the relaxation rate in units of sec$^{-1}$ versus the concentration of the paramagnetic species in units of mM which is Gd$^{3+}$ in Gd-based CAs (Equation 2.1).[39]

$$\frac{1}{(1/T_i)_{\text{obsd}}} = \frac{1}{(1/T_i)_{\text{dia}}} + r_i[Gd^{3+}], i = 1, 2$$ (2.1)
The relaxivity enhancement of water protons in the presence of paramagnetic species arises from the dipolar interactions between the metal center and the proximate water protons.[39] Three factors contribute to this interaction: (1) the inner sphere contribution due to water molecules directly coordinated to the paramagnetic metal center, (2) the second sphere contribution due to the exchange of water molecules hydrogen bonded to polar groups on the paramagnetic species surface and/or the exchange of mobile hydrogen on the complexes, and (3) outer sphere contribution due to the diffusion around the paramagnetic species of the outer sphere water molecules.[40]

\[
\frac{1}{T_1} (\text{inner sphere}) = \frac{q [\text{Gd}^{3+}]}{55.56 (T_{1m} + \tau_m)} \quad \text{and} \quad T_{1m} = f(\tau_m, \tau_R, T_{ie}, r) \quad (2.2)
\]

The inner sphere contribution is well described by Solomon-Bloembergen-Morgan (SBM) paramagnetic theory (Equation 2.2), where \( q \) is the number of water molecules coordinated to \( \text{Gd}^{3+} \), \( \tau_m \) is the mean residence life time, and \( T_{1m} \) is the longitudinal relaxation time of the coordinated water protons, which is a function of several parameters and among them the most relevant ones are \( \tau_m \), \( \tau_R \), the rotational correlation time of the paramagnetic complexes, \( T_{ie} \), the longitudinal and transverse electron spin relaxation times, and \( r \), the distance between the \( \text{Gd}^{3+} \) center and the protons of the coordinated water molecules.[39, 41] The outer sphere relaxation is described by the Freed theory and is dependent on the distance of minimum approach between non-coordinated water and the \( \text{Gd}^{3+} \) ion and the sum of the water and the paramagnetic species diffusion coefficients.[40, 41] The second sphere contribution, which involves the hydrogen-bond, is an additional relaxation mechanism for the bulk water protons which are affected by engaging in a highly dynamic network of hydrogen bonds.[40]
Overall, the relaxivities can be enhanced by adjusting any of the aforementioned physical-chemical parameters. Among them, several parameters are of primary importance in the design of CAs and are the most studied in literature, including $q$, $\tau_m$, $\tau_R$ and interaction of the complex with water molecules in the second and outer spheres.[42] The typical relaxivity values of current clinically used CAs with chelating structure based on the polyaminocarboxylate scaffolds (Figure 2.2) are 4-5 mM$^{-1}$ s$^{-1}$ (1.5 T scanner) which are relatively small due to their inherent disadvantages, such as they only have one coordinated site for water molecules ($q=1$), their small size resulting in fast tumbling ($\tau_R$ is about 0.1 ns), and $\tau_m$ is more than 100 ns.[39, 42, 43] Thus, the traditional chelate-based CAs suffers from low sensitivity, which requires a high concentration of dosage to provide detectable signal, and lead to concerns over accumulation and toxicity.[42, 44, 45]

![Diagram of Gd(DTPA)(H$_2$O) and Gd(DOTA)(H$_2$O) complexes]

**FIGURE 2.2** Two mainly clinically used CAs based on polyaminocarboxylate scaffolds: Magnevist® [Gd(DTPA)(H$_2$O), DTPA = diethylenetriaminepentaacitic acid] and Datarem® [Gd(DOTA)(H$_2$O), DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid].

### 2.1.3 pH-Responsive MRI CAs

Research devoted to the design of new, effective CAs for MRI by optimizing these parameters has been extensively studied. Such research includes using different ligands instead
of polyaminocarboxylate to increase $q$, attachment of chelate-based CAs to macromolecules to increase $\tau_R$, and the development of responsive MRI CAs to improve the sensitivity.\[45-47\] The success of these studies has been extensively discussed in several review papers.\[36, 39, 45, 47-49\] Among these work, there has been considerable attention directed at the development of pH-responsive MRI CAs due to their potential in imaging the pHe of solid tumors. pH-Responsive MRI CAs are significantly different than conventional CAs in that, while high relaxivity values are desirable, the critical property of a pH-responsive MRI CA is the relative change in relaxivity on exposure to changes in the pH of the surrounding environment.\[45\] Despite a great deal of interest in developing pH-responsive MRI CAs,\[8, 10, 50-53\] the majority of research has focused on Gd based chelates where the hydration of the metal complex, water accessibility and correlation times were pH dependent.\[8-11\] The \textit{in vivo} application of these Gd-chelates based systems has been limited due to problems with low changes in relaxivity limiting their sensitivity, poor specificity due to lack of molecular targeting, solubility issues due to changes in the ligand structure, and difficulty in tuning the pH response to match tumors.\[11, 48\]

An ideal pH-responsive MRI CA should have large pH sensitivity with a pKa in the physiological range, metabolically stability and biocompatibility, and provide a high signal to noise ratio (SNR) within a short time period. By collecting the relaxivity change caused by pH-responsive agents, it is possible to determine the tissue pH. To date, no clinical method exists for the \textit{in vivo} measurement of pHe in solid tumors. The long-term goal of researchers in
this field is to develop new CA platforms containing the required chemistry and material properties to overcome all of these limitations.

With the tremendous recent advances in nanotechnology, nanoparticles have been introduced in the development of new types of CAs and the effort made so far in this area has been discussed in several reviews.[26, 54, 55] The properties of nanoparticle-based CAs are very different when compared to small chelates CAs. Firstly, the size of nanoparticles can vary from dozens of nanometers to hundreds of nanometers, which give these CAs a longer retention time in the body compared to small chelate-based CAs. Secondly, the increase in the surface area not only dramatically increases the concentration of Gd per molecule (or particle), but also provides the ability to functionalize the surface of the CAs, such as attaching targeting agents and responsive agents to make specific and stimuli-responsive CAs for use in both diagnosis and therapy for various diseases. Thus, development of Gd-based nanoparticles as CAs in MRI, including Gd₂O₃,[56, 57] GdF₃,[58] GdS:Eu³⁺,[59] Pyrene-Gd,[60] GdPO₄,[61-63] and Gd-metal organic frame (Gd-MOF) nanoparticles,[12] have become a research hotspot. Though nanoparticle-based imaging agents are still in the early stages of development, their emergence has opened an avenue for designing new MRI CA platforms to overcome non-specific, low relaxivity and sensitivity limitations.

The research presented in this thesis is focused on the development of a pH-responsive MRI CA based on Gd nanoparticles that can have been surface modified with pH-responsive polymers to introduce in the pH-responsiveness and biocompatibility. Therefore, the
importance of pH-responsive polymers, their chemical structure and properties, and the synthesis techniques of pH-responsive polymers are discussed in the following sections.

2.2 pH-Responsive polymer systems

FIGURE 2.3 Protonation changes of ionizable groups in typical anionic (PMMA) or cationic (PDPAEMA) pH-responsive polymers resulting in chain conformation, solubility, or volume changes in aqueous solutions.

Due to the different physiological microenvironments between normal tissue and cancerous tissue, pH-responsive polymer systems have been widely researched for targeted cancer diagnosis and therapy in biomedical applications.[64-66] These polymer systems demonstrate solubility, volume or chain conformation changes in aqueous solutions as a result
of environmental changes in pH. The chemical structures of pH-responsive polymers usually include ionizable groups in their backbone, side group, or end group that demonstrate pH-dependent physico-chemical properties. These ionizable functional groups are capable of releasing or accepting protons upon environmental pH changes. Thus, electrostatic interactions between charged polymer chains repulse each other, which cause the polymer chains to extend or collapse and show an alternation between hydrophobic and hydrophilic behavior (Figure 2.3).

2.2.1 Properties of pH-responsive polymers and physiological considerations

pH-Responsive polymer systems are usually classified as cationic, anionic or acid hydrolysis systems based on their responsive mechanisms in biomedical application.[65] Most of the cationic pH-responsive polymers contain amino or amine groups, such as poly(2-(diisopropylamino)ethyl methacrylate) (PDPAEMA)[67-69], poly(4-vinyl pyridine) (P4VP)[70], poly(histidine)[71, 72], poly(β-amino ester)[19, 73], in which the functional group will accept a proton and become hydrophilic when the environmental pH values are lower than their pKa values (Figure 2.3). In contrast, anionic pH-responsive polymers are polyacids, such as poly(methacrylic acid) (PMAA)[74-76], poly(aspartic acid) (PAsp)[77], and sulfonamide-based polymers[78], which will release protons and become soluble when the environmental pH values are higher than their pKa values (Figure 2.3). The acid hydrolysis systems are usually polymer-drug conjugates containing acid-labile chemical bonds, such as hydrazone and acetal bonds (Figure 2.4), which will be cleaved by hydrolysis under low pHs, less than 6.0, to release drug.
According to the pH profiles in the body and the properties of pH-responsive polymers, pH-responsive drug delivery systems are usually designed to target or be triggered at one of the following two sites: the extracellular matrix of solid tumor tissues, which has a pH of 6.2-6.8, or the intracellular endosomes or lysosomes of other cells, which have a pH of 4.5-6.5. The majority of reported pH-responsive delivery systems targeting a pH lower than 6.0, which are suitable only for intracellular targeting and delivery after cellular uptake, are acid-labile hydrolysis systems.[79-86] However, these intracellular pH-responsive systems are not specific to the cancer cells because normal cells also have the same endosomal and lysosomal acid pH and, in fact, many cancer cells have intracellular pH (pHi) values that are neutral or slightly alkaline.[4, 5] Consequently, designing new pH-responsive polymer systems to
specifically target the weakly acidic extracellular matrix microenvironment of solid tumors is important in designing pH-responsive imaging agents for the detection of tumors.

The pH-induced transitions of the pH-responsive polymers in chain conformation, solubility and volume depend on their pKa value, which is a function of their ionizable groups for anionic and cationic systems.[78, 87] While the pH transitions of the anionic pH-responsive polymers with weak acidic groups mostly occur in the range of pH 4 to 6 due to their intrinsic dissociation constants, the pH transitions of the cationic pH-responsive polymers, which contain weak base groups, have more potential to occur around pH of 7 by carefully selecting the ionizable functional groups.[78] Therefore, while the anionic pH-responsive polymer systems are more effective as intestinal and intracellular drug delivery systems, the cationic pH-responsive polymers have been extensively studied in tumor extracellular pH (pHe) targeting.[23, 66, 88, 89] The following sections will provide a discussion of the different cationic pH-responsive systems used as tumor targeting drug delivery systems, with focuses on the selection of pH-responsive units, the synthetic techniques used to prepare the polymers, as well as their self-assembling behaviors in the pH-responsive delivery.

2.2.2 Cationic polymer based pH-responsive systems

The pH-responsive systems for drug delivery based upon cationic polymers usually involve the formation of micelles or vesicles to facilitate encapsulation and release of drugs. Block copolymers are particularly interesting in designing the pH-responsive polymer systems as they offer the possibility to combine the hydrophobic and hydrophilic blocks in the same polymer, which are essential for formation of micelles. For cationic pH-responsive delivery
systems based upon block copolymers, the permanent hydrophilic blocks are usually also biocompatible due to the need for the optimum bio-distribution in vivo and enhancement of the in vivo circulation.[64, 66] The hydrophobic block is the pH-responsive block and responsible for the phase transition of polymers in aqueous solutions, which forms the core of the micelles under neutral or basic physiological pH and become hydrophilic once they are protonated at an acidic pH. Therefore, the hydrophobic chemotherapeutic drugs can be encapsulated in the hydrophobic core of the micelles under neutral or basic pH and will be released under acidic pH conditions as a result of the hydrophobic to hydrophilic transition of the pH-responsive block of the copolymer.[16, 19]

A reversible soluble-insoluble transition of the cationic pH-responsive polymer systems occurs due to the basic character of the ionizable amino or amine groups, which becomes ionized at pH values below the pKa and deionized at pH values above pKa. As the pKa value of pH-responsive polymers is a function of their ionizable groups, adjusting the nature of the functional groups, such as electronegativity, hybridization, resonance, induction and steric hindrance, can effectively modulate the pKa, and thus the pH-induced transitions can be adjusted to reach the requirements of specific application circumstances.[23, 87] Such pH-responsive polymer systems containing amino or amine groups have been extensively studied in literatures, including the imidazole groups in poly(histidine) (PHis),[16, 71, 90] amino groups in poly(β-amino ester),[19, 73, 91-95] and tertiary amine groups in methacrylate-based polymers.[67, 68, 96-98]
The imidazole groups in PHis can be protonated under their pKa to yield a positively charged block and thus cause the hydrophobic/hydrophilic transition (Figure 2.5a). Johnson et al. designed a pH-responsive block copolymer system that contained PHis as the pH-responsive block and poly(2-hydroxyethyl methacrylate) (PHEMA) as the biocompatible block.[71] The PHEMA was first synthesized by atom transfer radical polymerization (ATRP) and the subsequent bromine end groups were converted to amine groups which then acted as a macroinitiator for synthesis of polypeptide-containing block copolymers. Doxorubicin (Dox, an anti-cancer drug) loaded into the self-assembled micelles had an accelerated release rate at low pH 5.5 compared to pH 7.4, and the total amount of release after 100 h was found to be 60% at pH 7.4 while it reached to 90% at pH 5.5. Chang et al. also reported a histidine-based pH-responsive system.[16, 90] They synthesized a PLGA-PEG-PLGA (PLGA: poly(D,L-lactic acid-co-glycolic acid, PEG: poly(ethylene glycol)) triblock copolymer through ring-opening polymerization by using PEG as the macroinitiator and D,L-lactide and glycolide as monomers, which was then conjugated with the tert-butyloxycarbonyl protected histidine. The micelles formed in their system exhibited a faster in vitro release rate of Dox at pH 6.2, as compared to pH 7.4.

Poly(β-amino ester) (PAE) has been extensively studied as another pH-responsive block in pH-responsive polymers due to the tertiary amine groups in the back bone.[17-19] The PAE used in these systems are usually prepared via a Michael-type step polymerization with monomers of a diacrylate and a primary amine or a diamine compound (Figure 2.5b). Lee’s group reported that the block copolymer of PEG-PAE with a molecular weight of 17.4 kDa,
composed of mono-acrylate PEG, 1,6-hexanediol diacrylate and 4,4’-trimethylenedipiperidine, exhibited a pH transition between 6.4 and 6.8. It was demonstrated that the polymeric micelles started to be unassembled by lowering the pH below 6.8. A rapid drug release rate was observed up to 9 h at pH 6.4 and the cumulative drug release reached up to 66% while only 20% of drug was rapidly released in first 3 h and kept constant after 3 h at pH 7.4. They also studied the effect of the polymer molecular weight and the hydrophobic moiety on the pH-responsive micelle/demicellization transition. According to their results, they suggested that the greater the molecular weight of the pH-sensitive block, the lower the pH region of the transition.

By increasing the molecular weight from 6905 to 13460 g/mol, the starting pH of demicellization was decreased from 7.8 to 7.27. The effect of the hydrophobic moiety was studied by introducing hydrophobic groups, such as deoxycholic acid, into the side chain. In this case, PAE was prepared first with 3-amino 1-propanol and 1,4-butanediol diacrylate, and then the PEG-COOH and deoxycholic acid were sequentially grafted to the hydroxyl groups on the side chains of PAE (Figure 2.5c). The prepared PAE-g-PEG was reported to have a pH responsive demicellization transition at pH 6.8, which is different from the aforementioned PEG-PAE system. The introduced deoxycholic acid significantly improves the micelle stability due to the hydrophobic interaction in the micellar core, which causes the disruption of micelle to occur at a lower pH. While a higher amount substitution of deoxycholic acid in PAE-g-PEG decreased the pH transition to pH 5.8, a lower amount also shifted it to pH 6.2.
Tertiary amine-based methacrylate polymers, which undergo a hydrophilic/hydrophobic pH-induced transition within a physiologically relevant pH window, have also been studied extensively and reported on the context of pH-responsive therapeutic delivery systems (Figure 2.5d). [67, 69, 96, 97, 99-101] Car et al. discussed the use of poly(dimethylsiloxane)-b-poly(2-dimethylamino)ethyl methacrylate) (PDMS-b-PDMAEMA) block copolymers synthesized via ATRP for intracellular anticancer drug delivery.[97] The DOX loaded in the micelles of PDMS-b-PDMAEMA remained at 80 wt% of the drug for 3 days at pH 7.4, while more than 80 wt% of the DOX was released within 48 h at pH 5.5. However, they didn’t report the pKa value of the system and the release data at other physiological relevant pHs, such as any pH between 6.0-7.4. Butun and coworkers synthesized a series of tertiary amine methacrylate pH-responsive homopolymers via group transfer polymerization, including PDMAEMA, poly(2-diethylamino)ethyl methacrylate) (PDEAEMA), DPAEMA, and poly(2-(N-morpholino)ethyl methacrylate) (PMEMA) (Figure 2.5d).[102] Their results demonstrated that different tertiary amine groups render different pKa values for the conjugate acid forms of the polymer system. According to the titration curves, pKa values of 7.0, 7.3, 6.0 and 4.9 were given for DMAEMA, DEAEMA, DPAEMA and MEMA homopolymers.

Based on this previous research, the tertiary amine based methacrylate monomers were selected to synthesize pH-responsive polymers for the preparation of pH-responsive MRI CAs via reversible addition-fragmentation chain transfer (RAFT) polymerization in the work
discussed in this thesis. As such, the advantages and mechanism of RAFT polymerization as a living radical polymerization will be introduced in the following section.

(a) L-histidine (His)

(b) monomers used for synthesis of poly(β-amino ester)

1,6-hexanediol diacylate
4,4'-trimethylenedipiperidine

1,4-butanediol diacylate
3-amino 1-propanol

(c) PAE-g-PEG

(d) tertiary amine groups (TAG) in methacrylates

FIGURE 2.5 Summary of the different chemical structures that have been discussed in cationic pH-responsive polymer systems
2.3 Synthesis techniques for pH-responsive block copolymers

The use of pH-responsive polymer systems in biomedical applications usually requires that they have both biocompatible and pH-responsive properties. As a result, much of the research in this area has utilized block copolymers where one block contains the pH-responsive properties and the other block is biocompatible. Most of the pH-responsive systems contain two or three blocks to form AB-type diblock or ABA- or BAB-type triblock systems, and the hydrophilicity/hydrophobicity or the pH-responsiveness can be manipulated by modulating the molecular weight, composition or functionality of each block.[65, 103]

2.3.1 Living radical polymerization

Living radical polymerization (LRP) is well known for preparing well-defined block copolymers with precisely controlled architectures, which is one of the main driving forces for the rapid expansion of the LRP techniques in recent years.[104, 105] Although ‘living’ polymerization was introduced into polymer chemistry by Michael Szwarz in 1950s[106] and started from anionic polymerization[107] to cationic polymerization[108] and coordination polymerization[109], living radical polymerization has become one of the most developed areas and has been used to prepare many novel, previously inaccessible polymeric materials with precisely controlled composition and architecture in last two decades.[110-112]

Conventional radical polymerization is the most widely used process in industry for the polymerization of a tremendous amount of different monomers in an array of different compositions and architectures, because it is relatively easy to perform and much less sensitive to the effect of impurities.[113] It has been successfully used to prepare many commercially
important polymers, such as polyethylene (PE), polystyrene (PS), polytetrafluoroethylene (PTFE), poly(vinyl chloride) (PVC), poly(methyl methacrylate) (PMMA), and polyacrylonitrile (PAN). However, it is a formidable challenge for conventional radical polymerization to control molecular weight (MW), provide narrow molecular weight distributions (MWDs) and to yield block copolymers due to the very short life time of the growing chains and uncontrolled nature of the polymerization. The advent of LRP has ushered radical polymerization into a new era, providing all the benefits of radical polymerization along with predictable molecular weights, narrow MWDs, controlled end group functionality, and the ability to make block copolymers and other complex architectures.[110]

![Scheme 2.1 General mechanism of LRP](image)

A very important difference between conventional radical polymerization and LRP is the presence of a reversible activation and deactivation process which permits controlled growth from a fast initiation, relative to propagation and also minimizes termination reactions.[110] Instead of quickly and irreversibly deactivating to dead polymers, the generated radical end deactivates to a so-called dormant species which possesses appropriate functional groups at the polymer end for radical reactivation (Scheme 2.1). This deactivation of the polymer end radicals to the dormant species minimizes termination by decreasing the concentration of radical species. The key to control in LRP is to establish a dynamic equilibrium between
propagating radicals and various dormant species. The deactivation needs to be fast enough to assure that all polymer chains have equal probability of adding monomer, whereas the activation needs to be sufficiently high to progress the polymerization.[104, 110]

There are three main approaches to control this exchange process, including stable free radical polymerization (SFRP), ATRP and the degenerative chain transfer (DT) mechanism.[110] SFRP, exemplified typically by nitroxide-mediated polymerization (NMP), involves deactivation by reversible coupling with a persistent radical and, typically, a unimolecular activation process.[114] ATRP involves deactivation by reversible atom or group transfer and demonstrates a bimolecular activation process, in which the deactivator is a transition metal complex with the metal in a higher oxidation state and the activator is the transition metal complex in a lower oxidation state and can be considered to be a catalyst for the process.[115, 116] The DT mechanism involves simultaneous deactivation and activation by reversible (degenerative) chain transfer.[109] The best known of this class is RAFT polymerization with thiocarbonylthio compounds, which will be discussed in more detail in the following section since the synthesis work in this thesis will utilize this technique.

2.3.2 Reversible addition-fragmentation chain transfer polymerization

RAFT polymerization, which proceeds via the DT mechanism, is arguably the most effective and versatile LRP process due to its tolerance to a wide variety of reaction conditions and functionalities when compared to the other LRP techniques.[117-119] Especially with the scope of this work, the use of thiocarbonylthio compounds as the chain transfer agents allows
the polymers prepared by RAFT polymerization to be ideal candidates for surface functionalization of a wide range of surfaces and structures.[120-124]

**SCHEME 2.2 General mechanism for RAFT polymerization.**

The generally accepted mechanism for RAFT polymerization is presented in Scheme 2.2.[117] After a radical is generated from decomposition of a conventional radical initiator, it will react with a monomer to form a propagating oligomeric radical and then react with the RAFT agent 1 to form an intermediate radical 2 that can fragment to yield an oligomeric RAFT agent 3 and a reinitiating R radical or fragment back to the original RAFT agent 1 and the oligomeric radical. Due to the highly active C=S bond of the RAFT agent, the radical addition to the RAFT agent or an oligomeric RAFT agent 3 is favored over the addition to the double bond on the monomer. This process will continue until all of the R groups are fragmented from the RAFT agent and have reacted with monomer to form propagating oligomeric radicals. After this, the polymerization reaches the main equilibrium stage, in which the active
propagating radicals, $P_m^*$ and $P_n^*$, and the oligomeric RAFT agent 3 and 5, which are dormant species in the RAFT mechanism, quickly exchange to provide an equal probability for all chains to grow. Although irreversible termination in RAFT polymerization cannot be completely eliminated, it is minimized by maintaining a small concentration of radicals that prefer to undergo the reversible addition-fragmentation process. Therefore, most of the polymer chains retain the thiocarbonylthio end groups when the monomer is fully consumed or the polymerization is stopped.[117] Due to a high fraction of polymer chains initiated by RAFT agents and limited termination reactions, the number of polymer chains formed will be a constant and the average degree of polymerization ($DP_n$) is then given by equation 2.3, where $[M]_0$ and $[RAFT]_0$ are the initial concentrations of monomer and the RAFT agent, respectively, and $p$ is the fractional conversion of monomer.

$$DP_n = \frac{p[M]_0}{[RAFT]_0} \quad (2.3)$$

The effectiveness of a thiocarbonylthio RAFT agent 1 on controlling RAFT polymerization depends on the structures of the R and Z groups.[125] Most of the monomers that are polymerized via conventional radical polymerization can be polymerized with the RAFT process by carefully selecting the RAFT agent. The structure of R should assure it is a good reinitiating group and a better leaving group than the propagating oligomeric radical. The structure of Z should ensure activation (or at least not deactivation) of the C=S bond toward radical addition and provide minimal stabilization of the intermediate radical to prevent the formation of a more reactive intermediate radical species. The leaving/reinitiating ability of the R group is mostly affected by steric factors, radical stability and polar effects. A common
strategy for selection of the R group is to design it to be structurally similar to the monomer being polymerized, which allows the R group to have similar structural and electronic properties to the propagating radical, thus increasing reinitiation ability.[117] While most monomers are more resilient with respect to the R group, the Z group is highly influential in determining the reactivity of a RAFT agent and consequently its effectiveness at mediating the polymerization.

![Diagram of xanthates and dithiocarbamates and their canonical forms](image)

**FIGURE 2.6** Xanthates and dithiocarbamates and their canonical forms

According to the different choices of the Z group, the most widely utilized thiocarbonylthio RAFT agents 1 include dithioesters (Z = alkyl or aryl), trithiocarbonates (Z = SR’), xanthates (Z = OR’, 6), and dithiocarbamates (Z = NR’R”, 7). While the dithioesters and trithiocarbonates are the most reactive RAFT agents, the xanthates and dithiocarbonates have dramatically lower reactivity toward radical addition due to the zwitterionic canonical forms 8 and 9 (Figure 2.6).[125] The choice of Z group is tightly connected with the monomers in the polymerization system. Monomers are categorized to be the ‘more activated’ monomers (MAMs) and the ‘less activated’ monomers (LAMs) based on the adjacent groups to the double bond.[125] The MAMs have adjacent groups that can stabilize the propagating radicals, such as aromatic ring (styrene, vinylpyridine), a carbonyl group (methyl methacrylate, acrylamide), or a nitrile (acrylonitrile). The LAMs includes those where the double bond is conjugated to a saturated
carbon, oxygen or nitrogen lone pair (vinyl acetate or vinylpyrrolidone) or the heteroatom of a hetreooromatic ring (N-vinylcarbazole).

For the MAMs, the propagating radicals are relatively good hemolytic leaving groups and, thus, are less reactive in radical addition.[125] Therefore the more active RAFT agent such as dithioesters and trithiocarbonates are required to control these polymerization systems. For LAMs with higher rates of propagation ($k_p$) and addition ($k_{add}$), which are highly reactive in radical addition, the polymerization can be better controlled by RAFT agents with a less activating effect on the thiocarbonyl group and a greater destabilization effect on the intermediate radical to favor fragmentation.[125] Considering the monomers used in this research are methacrylates, which generates the bulky tertiary propagating radicals, the more active RAFT agents will allow the preparation of polymers with narrow MWDs. 4-cyanopentanoic acid dithiobenzoate (CPAD), a typical dithioester, and s-1-dodecyl s’-(α,α-dimethylacetic acid) trithio-carbonate (DATC), a widely used trithiocarbonate, are chosen to mediate the polymerizations of the pH-responsive monomers discussed in this thesis.

2.4 pH-Responsive polymers for imaging pHe in tumors

As mentioned previously, numerous biomedical imaging modalities have been investigated for pHe imaging in solid tumors. However, in the cases where pH-responsive polymers have been the critical component of the imaging agent, research has centered on either OI via fluorescent imaging or MRS/MRI.
2.4.1 Optical imaging

By taking advantages of variations in the fluorescent properties of probes in response to the local pH, optical measurements can be converted to pH distribution data and used to measure the pH of tumors \textit{in vivo}.[126] Fluorescence-based pH sensing has been demonstrated using a variety of different materials, including quantum dots, fluorescent proteins and other small molecule dyes, utilizing intramolecular or intermolecular energy transfer mechanisms.[127] Recently there has been increasing interest in the use of polymeric fluorescent probes due to their increased retention times \textit{in vivo}, optical signal amplification, and the ability to be functionalized with targeting moieties and biocompatible segments.[87, 127-129] 

Gao and coworkers have recently demonstrated the ability to prepare tunable, pH-activatable micellar nanoparticles based on the supramolecular self-assembly of ionizable block copolymers (Figure 2.7a).[87] The pH-responsive segment of the polymer utilized various amino groups to provide tunable hydrophobic groups in the physiological pH range of interest (5.0-7.0). In addition, a poly(ethylene oxide) (PEO) block was incorporated to stabilize the micelles and improve the biocompatibility of the nanoparticles. In order to introduce OI capabilities, the pH-insensitive dye tetramethyl rhodamine (TMR) was conjugated to the amino containing block. At higher pH values, the amino blocks are hydrophobic and self-assemble to form the hydrophobic cores of the micelle nanoparticles. This results in aggregation of the dye and quenching of the fluorescent signal via the Föster resonance energy transfer (FRET) and photoinduced electron transfer (PeT) mechanisms.[130] Conversely, at lower pH values, the amino blocks become protonated and hydrophilic, leading to the micelle
disassembling and a large increase in the fluorescence emission (up to 55 times more intense).

In essence, this produced an OI imaging agent based on a nonlinear optical responsive nanosystem that is controlled by changes in physiological pH.

![Diagram](image)

**FIGURE 2.7** Summary of the different pH-responsive block copolymers that have been used to prepare micelles as nanoscale pH-responsive imaging agents.

In an alternative approach, that also utilized small molecule dyes, Wolfbeis and coworkers used both a pH-sensitive dye, fluorescein isothiocyanate (FTIC) and a pH-insensitive dye, tetrakis(pentafluorophenyl) porphyrin (TFPP), in conjunction with the commercially available triblock copolymer Pluronic F-127.[128] This copolymer is a nonionic, surfactant composed of
a central hydrophobic block of poly(propylene oxide) (PPO) and two hydrophilic outer blocks of PEO, that forms micelle structures in aqueous solution. The FITC dye was attached to the end groups of the copolymer so that it was present on the surface of the micelle and, thus, exposed to the external environment, while the hydrophobic TFPP dye was located in the hydrophobic core of the micelle and used as a reference signal. These micelles demonstrated a large change in the green luminescence signal, increasing between pH 5 and 8, due to the pKa value of 6.4 for the FTIC dye, while the red luminescence signal from the TFPP dye is not sensitive to pH. As a result, the system demonstrated a distinct color change from red to green as the pH was increased from 3.0 to 9.0.[128]

In another study, Wang and co-workers took advantage of the pH dependent change in the redox state of dopamine to prepare a pH-responsive conjugated polymer.[127] In aqueous media, dopamine has been shown to reversibly convert between hydroquinone (reduced state) and quinone (oxidized state) with changes in pH.[127] By attaching dopamine to a polyfluorene derivative, the fluorescence of the conjugated polymer backbone can be activated by change in pH. In acidic environments, the dopamine primarily exists as the hydroquinone form, which lacks the ability to quench the polymer fluorescence, due to the absence of electron transfer from the polymer main chain to the hydroquinone. However, under basic conditions the dopamine is predominantly in the quinone form, which does allow for efficient electron transfer and quenching of the fluorescence of the polymer main chain. Using this system, Wang and coworkers demonstrated a linear correlation between the maximum fluorescence intensity of the polymer and the solution pH over the pH range of 5.0 – 9.0.
Despite these reported encouraging and promising research work in the development of imaging agents to assist pH imaging, the success is limited due to the inherent disadvantages of OI modality, such as poor tissue penetration (1-2 cm), low spatial resolution and noisy background from tissue auto-fluorescence and light absorption.

2.4.2 Magnetic resonance imaging/spectroscopy

The most studied techniques for the non-invasive measurement of pH and pHi are based on magnetic resonance (MR). Both endogenous and exogenous low molecular weight MR active compounds have been used to measure pH in vivo.[131] MRS methods are typically based on the difference in chemical shift between pH dependent and independent resonances. The use of MRS for pH measurement is centered on the concept that protonation reactions are in fast exchange when they are in same compartment, allowing for chemical shifts to be used to predict pH.

Alternatively, as discussed above an MR image is generated from the nuclear magnetic resonance (NMR) of water protons. Typically CAs are used in MRI to aid in diagnostic imaging by increasing the contrast between the particular organ or tissue of interest and the surrounding tissues in the body.[132] Recently, there has been considerable research into the development of low molecular weight pH-responsive MRI CAs.[29, 36, 133, 134] pH-Responsive MRI CAs are significantly different than conventional MRI CAs in that, while high relaxivity values are desirable, the critical property of a pH responsive MRI CA is the relative change in relaxivity on exposure to changes in the pH of the surrounding environment.[45] Typically pH-responsive MRI CAs function as a result of either water
accessibility and correlation times that are pH dependent or via pH sensitive chemical exchange saturation transfer (CEST).[134, 135]

Lee and coworkers were one of the first groups to prepare a pH-responsive MRI CA based on a pH responsive polymer.[136] In this case, a diblock copolymer consisting of PEO as the hydrophilic, biocompatible segment and a PAE as the pH-sensitive segment, was used to form micelle nanoparticles (Figure 2.7b). To enable their use as an MRI CA, iron oxide nanoparticles were encapsulated in the hydrophobic core of the micelle. The pH-sensitive nature of the MRI CAs was demonstrated by monitoring $T_2$ while changing the pH from 7.6 to 6.2. The results demonstrated that the transverse relaxivity increased when the pH was lower than 6.8 due to the release of the iron oxide nanoparticles from the core of the micelles. This release was driven by the change of the PAE from hydrophobic to hydrophilic, and thus dissolution of the micelles, at a pH of 6.8. In addition to the in vitro studies, this is one of the few systems to have been tested in an animal model. Using a mouse model with a subcutaneous tumor, the authors demonstrated that the $T_2$-weighted MRI images of the pH responsive systems show an increased signal in the tumor over a 24-hour period while a pH-insensitive system demonstrated no change in signal.[136]

A recent report from Kikuchi and co-workers used a different approach in the development of a pH-responsive MRI CA.[31] In this study, the authors took advantage of the morphological changes in both a pH responsive polymer and a pH-responsive cross-linked polymer nanoparticle to create a pH-responsive MRI CA (Figure 2.8). These materials were synthesized using methacrylic acid as the monomer and $N,N'$-methylenebisacrylamide as the
cross-linker. An amine-modified Gd chelate was then attached via the carboxylic acid functionality to produce the pH-responsive MRI CA. In each case, MRI studies indicated that the longitudinal relaxivities of the CAs increased with a decreasing pH. This change can be attributed to a decrease in the molecular tumbling of the water molecules as a result of the conformation change in the polymer with pH. The results also demonstrated that the cross-linked systems provided the largest changes in relaxivity with pH and, thus, had the most potential for application as a pH-responsive MRI CA.

![Diagram](image)

**FIGURE 2.8** Scheme proposed by Kikuchi and co-workers for pH-based switch of an MRI CA.

Bae and co-workers have also recently reported a pH-responsive MRS probe.[137] Once again; this system was based upon the pH dependent formation and dissolution of polymeric micelles. However, in this case the pH-induced transformation of the micelles allowed for pH imaging by a pH-induced ‘on/off’ sensing of NMR and MRS. The pH-responsive block copolymers in this study contained a hydrophilic and biocompatible PEO block and either a PHis or poly(L-lactic acid) (PLLA) block (Figure 2.7c). PEO-b-PHis has been shown to form pH-sensitive micelles in basic solutions but physically dissociated in acidic conditions.[137] The authors demonstrated that by incorporating PEO-b-PLLA in to this system to produce a mixed micelle allows for some degree of control over the pH at which the micelles dissociate.
$^1$H-MRS was then used to show a pH-dependence on the chemical shift of protons on the PHis block of the copolymer.

Although this pioneering work in development of pH-responsive MRI CAs has been investigated and showed potential in pH imaging, these systems are either based on iron oxide nanoparticles, which can only be used as a negative CA, or have a pH transition different from the physiological tumor pH. However, to the best of our knowledge the successful development of a pH-responsive positive CA based on pH-responsive polymers and nanoparticles hasn’t been reported yet.

2.4.3 Conclusions

To summarize, there is a clinical need for the development of new techniques that enable the non-invasive in vivo determination of tumor pH due to the correlation between low tumor pH and increased cancer morbidity and mortality. While there has been a significant amount of research into the measurement of pH in vivo using low molecular weight materials, to date all of the techniques developed suffer from significant limitations that have precluded their use clinically. The use of pH-responsive polymers for the preparation of imaging agents offers tremendous potential in the area of pH imaging. Therefore, in this research, pH-responsive polymers will be used for the preparation of a pH-responsive MRI CA based on Gd nanoparticles to realize the determination of tumor pH via MRI.
CHAPTER 3
SYNTHESIS OF TERTIARY AMINE-BASED PH-RESPONSIVE POLYMERS BY RAFT POLYMERIZATION

3.1 Introduction

Over the past few decades, polymer-based pH-responsive materials have received considerable attention for enhanced diagnostics and therapeutic efficacy in the treatment of a wide range of diseases due to their ability to convert environmental pH stimulus to an observable change, such as solubility, volume or chain conformation in aqueous solutions. [15, 66, 138] One area where pH-responsive polymers have potential application is in the diagnosis and treatment of some cancers. For many years researchers have known the importance of the extracellular pH (pHe) of some tumors in relation to cancer morbidity and mortality. [4, 5] A low pHe has been identified as an important factor in producing more aggressive cancer phenotypes and causing metathesis of the primary carcinoma, both of which are leading causes of cancer morbidity and mortality. [6, 7] As such, there has been much interest in developing pH-responsive drug delivery systems to target or be triggered in the extracellular matrix of solid tumor tissues, which have a pH of 6.2–6.8. However, many of the reported pH-responsive delivery systems have pKa values lower than 6.0, including the acid-labile pH-responsive polymer systems, [79-82, 85, 139] some histidine systems, [71] and 2-aminoethyl methacrylamide [140] (AEMA) based systems, which are suitable only for intracellular targeting and delivery after cellular uptake in non-cancerous cells. These intracellular
pH-responsive systems are not useful for cancer cells because the intracellular pH (pHi) of many cancer cells is neutral or slightly alkaline relative to normal cells, which have intracellular endosomes or lysosomes with a pH of 4.5-6.5.[7] Thus considering this pH gradient difference between normal tissue and tumor tissue pHe, well-defined pH-responsive polymer systems with a pKa of 6.2-6.8 need to be developed to specifically target the weakly acidic extracellular matrix microenvironment of solid tumors for enhanced diagnostics and/or therapeutic efficacy in the treatment of cancer.

The chemical structures of pH-responsive polymers usually include ionizable groups in their backbone, side group, or end groups that demonstrate pH-dependent physicochemical properties. It has been documented that the nature of these functional groups can be adjusted to effectively modulate the pKa of the final polymer. Among the pH-responsive polymer systems with a pKa above 6.0,[16-22] tertiary amine-based methacrylate polymers have been recognized as one of the most promising groups because their pKa can be tuned from approximately 4.5 to 8.5 depending on different substituent groups.[23] When the solution pH is less than the polymer pKa, the tertiary amine groups are protonated and the polymers become soluble in aqueous media. When the solution pH is higher than the polymer pKa, the polymer will precipitate out of aqueous solution, as the polymer becomes hydrophobic due to deprotonation, or form micelles, if they were designed to be block copolymers with a permanent hydrophilic block.

Several pH-responsive homopolymer and block copolymer systems containing tertiary amine-based methacrylate monomers have been well studied, including
2-(dimethylamino)ethyl methacrylate (DMAEMA),[99, 101, 141] 2-(diethylamino)-ethyl methacrylate (DEAEMA),[98, 142] 2-(diisopropylamino)ethyl methacrylate (DPAEMA),[67-69, 143] 2-(dibutylamino)ethyl methacrylate (DBAEMA), 2-(tetramethyleneimino)ethyl methacrylate (TMIEMA), 2-(piperidino)ethyl methacrylate (PPDEMA).[23, 67-69, 98, 99, 101, 102, 142, 143] The majority of the research in this area has been focused on DMAEMA, DEAEMA and DPAEMA due to their commercial availability. In addition, a number of different polymerization techniques, including conventional radical polymerization, group transfer polymerization, atom transfer radical polymerization (ATRP) and reversible addition-fragmentation chain transfer (RAFT) polymerization, have been used to synthesize homopolymer or block copolymers that incorporate at least one of these tertiary amine-based monomers. Among these techniques, ATRP and RAFT polymerization, as living radical polymerization techniques (LRPs), are arguably the most useful techniques since they provide controlled chain lengths, narrow molecular weight distributions, and well-defined end groups, which allows for the formation of block copolymers.

Recently, Gao’s research group have synthesized and studied the properties of block copolymers containing a variety of different tertiary amine-based methacrylate monomers.[87, 130, 144] The block copolymers were synthesized via ATRP and the pH-responsive block copolymers were used to form micellar nanoparticles that were based on the supramolecular self-assembly of the block copolymers. The incorporation of fluorophores in to the polymer allowed for the potential use of the nanoparticles as nonlinear on/off nanosystems for diagnostic imaging. However, when ATRP is compared to RAFT polymerization, RAFT is
widely considered to be more suitable for biomedical applications as it does not require a metal catalyst.[122, 145] In addition, polymers prepared by RAFT polymerization contain thiocarbonylthio groups that can be easily reduced to a thiol, which makes RAFT polymers ideal candidates for surface functionalization of a wide range of nanoparticles.[120-124] Despite the potential application of well-defined tertiary amine-based methacrylate polymers as pH-responsive polymers in the diagnosis and treatment of cancer, to the best of our knowledge, there are no reports that present a detailed kinetic study on the application of RAFT polymerization to prepare tertiary amine-based methacrylate homopolymers and block copolymers.

SCHEME 3.1 RAFT polymerization of tertiary amine-based methacrylate pH-responsive monomers
Herein, we describe the preparation of homopolymers and block copolymers containing different tertiary amine-based methacrylate monomers and present a detailed study of the polymerization kinetics for each of these systems. Furthermore, the pH-responsive properties of these homopolymers (Scheme 3.1) and block copolymers (Scheme 3.2) were also studied by determining the pKa values using titration-based techniques and also monitoring the hydrodynamic volume and zeta potential of the polymers in solution via dynamic light scattering (DLS).

**SCHEME 3.2 RAFT polymerization of tertiary amine-based methacrylate monomers for the preparation of pH-responsive block copolymers using PEG-CPAD as a macro-RAFT agent.**

### 3.2 Experimental

The following sections introduced materials used in this Chapter and described the relevant experiments and characterization techniques used.

#### 3.2.1 Materials

Sulfur (reagent grade), sodium methoxide (25% methanol solution), benzyl chloride (reagent plus, 99%), dodecanethiol (≥98%), tetrabutyl ammonium bromide (reagent plus, 99%), 2,2’-azobisisobutyronitrile (AIBN) (98%), 4,4’-azobis(4-cyanovaleric acid) (ACVA) (≥98%), methacryloyl chloride (stabilized with ~200ppm monomethyl ether hydroquinone) (97%), triethylamine (≥99.5%), 2-(piperidino)ethanol (PPDE) (99%), 2-(dibutylamino)ethanol (DBAE) (99%), 2-(diisopropyl amino)ethyl methacrylate (DPAEMA) (stabilized with ~100ppm monomethyl ether hydroquinone) (97%), and 1,4-dioxane (99%).
ppm monomethyl ether hydroquinone) (97%), anhydrous dichloromethane (DCM) (≥99.8%), anhydrous 1,4-dioxane (99.8%), anhydrous N,N-dimethylformamide (99.8%), anhydrous 2-propanol (99.5%) and poly(ethylene glycol) methyl ether (PEG) (average Mₙ 5000) were purchased from Sigma-Aldrich. 4-dimethylaminopyridine (DMAP) (99%) and N,N’-dicyclohexylcarbodiimide (DCC) (99%) were purchased from Acros Organics. Sodium sulfate (anhydrous, certified ACS, granular, 99.7%), potassium ferricyanide (certified ACS, 99.4%), hydroquinone (crystalline/laboratory), carbon disulfide (certified ACS, ≥99.9%) and ethyl ether (anhydrous, BHT stabilized, 99.9%) were purchased from Fisher Scientific. Toluene (ChromAR) and tetrahydrofuran (THF) (ChromAR) were purchased from Macron Fine Chemicals and were purified through Innovative Technology’s Pure-Solv System before use. DPAEMA used as a monomer was purified by distillation under reduced pressure to remove inhibitor. AIBN was purified by recrystallization from methanol twice. All other solvents and reagents were used as received.

S-1-dodecyl S’-(α,α-dimethylacetic acid) trithio-carbonate (DATC) was prepared following the procedure reported in literature.[146, 147] Yield: 44%. ¹H NMR (500 MHz, CDCl₃, δ): 0.86 (t, 3H, -CH₂CH₃), 1.24-1.37 (m, 18H, -CH₂(CH₂)₉CH₃), 1.66 (m, 2H, -CS-CH₂-CH₂-), 1.71 (s, 6H, -C(CH₃)₂-SC), 3.27 (t, 2H, -CS-CH₂-CH₂-), 11.71 (s, 1H, -COOH). mp 61-62 °C (lit. 62 °C).

4-Cyanopentanoic acid dithiobenzoate (CPAD) was synthesized according to a previously described procedure (yield, 49%).[148] ¹H NMR (500 MHz, CDCl₃, δ): 1.93 (s, 3H, -C-CH₃),
2.46-2.78 (m, 4H, -C-(CH\(_2\))\(_2\)-COOH), 7.38 (t, 2H, H3 in phenyl), 7.55 (t, 1H, H4 in phenyl), 7.88 (d, 2H, H2 in phenyl). mp 97-99 °C (lit. 97-99 °C).

3.2.2 Instrumentation and characterization

\(^1\)H NMR spectra were obtained on a JEOL ECA 500 liquid state NMR spectrometer and data obtained was processed with MestReNova software. Molecular weight and molecular weight distributions of the homopolymer and block copolymer samples were determined by gel permeation chromatography (GPC) using stabilized tetrahydrofuran as the eluent with a flow rate of 1.0 mL/min (Viscotek GPC pump; PLgel 5 μm MIXED-C and MIXED-D columns: molecular weight range 200-2,000,000 and 200-400,000 g/mol (PS equiv), respectively). Dynamic light scattering (DLS) measurements were performed on a Malvern Zetasizer Nano ZS at a scattering angle of 173° and analyzed by Malvern Zetasizer Software version 6.20. The viscosity (0.88 mPa·s) and the refractive index (1.33) of distilled water at room temperature was used for data analysis.

3.2.3 Synthesis of PEG macro-RAFT agent (PEG-CPAD)

PEG-CPAD was prepared from PEG and CPAD by a Steglich esterification following a procedure modified from literature.[149] PEG (10 g, 2.0 mmol) was first dried by azeotropic distillation with anhydrous toluene. After that, a solution of dried PEG, CPAD (1.37 g, 5.0 mmol) and DMAP (60 mg, 0.50 mmol) in 100 mL anhydrous DCM was prepared. After 10 min of stirring in an ice bath, a solution of DCC (1.15 g, 5.5 mmol) in 5.0 mL DCM was added dropwise. The reaction mixture was stirred at 0 °C for 2 hrs and then 20 hrs at room temperature. The solid was filtered off, and the filtrate was dried with sodium sulfate overnight.
After filtration, the DCM was removed using a rotary evaporator and the residue was precipitated to a large excess of ethyl ether three times and dried under vacuum for 24 hrs to give a pink product (yield, 88%). $^1$H NMR (500 MHz, CDCl$_3$, δ): 1.91 (s, 3H, CH$_3$), 2.43-2.69 (m, 4H, C-CH$_2$-CH$_2$-CO), 3.36 (s, 3H, -O-CH$_3$), 3.48-3.76 (m, 452H, -(OCH$_2$CH$_2$)$_n$O-), 4.25 (t, 2H, -COO-CH$_2$-CH$_2$-O-), 7.39 (t, 2H, H3 in phenyl), 7.55 (t, 1H, H4 in phenyl), 7.89 (d, 2H, H2 in phenyl). mp 68-70 °C.

3.2.4 Synthesis of tertiary amine-based methacrylate monomers

Three methacrylate monomers containing different tertiary amine substituent groups were used in this research. While the monomer DPAEMA was purchased, the monomers DBAEMA and PPPDEMA were synthesized by the reaction of methacryloyl chloride with corresponding tertiary amino ethanol compound using a previously reported method with slight modification.[87] As an example, the synthesis of DBAEMA was achieved as follows, to a solution of DBAE (18.92 g, 22.0 mL, 0.11 mol), triethylamine (14.3 mL, 0.10 mol) and hydroquinone (0.10 g) in THF (100 mL), a methacryloyl chloride (10.7 g, 10.0 mL, 0.10 mol) and THF (10 mL) mixture was added dropwise at room temperature. The resulting solution was refluxed for 2 hrs and then filtered to remove the white solid (triethylamine-hydrochloric salt), which was rinsed with THF. The THF was removed from the filtrate using a rotary evaporator and the resulting residue was diluted with chloroform and washed with water, 10 wt.% sodium carbonate solution, saturated NaCl solution, and finally with water. The organic layer was collected and dried with sodium sulfate overnight. After filtration, the chloroform was removed using a rotary evaporator and the residue was distilled under reduced pressure to give
pure DBAEMA as a transparent colorless liquid (yield, 66%). $^1$H NMR (500 MHz, CDCl$_3$, $\delta$): 0.87 (t, 6H, -CH$_2$CH$_3$), 1.25-1.37 (m, 8H, -CH$_2$CH$_2$CH$_3$), 1.90 (s, 3H, CH$_3$C=), 2.41 (t, 4H, -NCH$_2$CH$_2$-), 2.70 (t, 2H, -CH$_2$CH$_2$N-), 4.15 (t, 2H, -OCH$_2$CH$_2$N-), 5.52 (s, 1H, CH$_2$=C), 6.06 (s, 1H, CH$_2$=C). The following is a typical recipe for synthesis of PPDEMA: PPDE (14.6 g, 15.0 mL, 0.11 mol), triethylamine (15.7 mL, 0.11 mol), hydroquinone (0.12 g), methacryloyl chloride (11.77 g, 11.0 mL, 0.11 mol), THF (110 mL), with a yield of 80%. $^1$H NMR (500 MHz, CDCl$_3$, $\delta$): 1.31 (m, 2H, -NCH$_2$CH$_2$CH$_2$-), 1.46 (m, 4H, -NCH$_2$CH$_2$CH$_2$-), 1.82 (s, 3H, CH$_3$C=), 2.35 (t, 4H, -NCH$_2$CH$_2$CH$_2$-), 2.54 (t, 2H, -OCH$_2$CH$_2$N-), 4.16 (t, 2H, -OCH$_2$CH$_2$N-), 5.44 (s, 1H, CH$_2$=C), 5.98 (s, 1H, CH$_2$=C).

3.2.5 General synthesis of tertiary amine-based methacrylate homopolymers via RAFT polymerization

All polymerizations were carried out using a similar technique. A representative example for poly(DBAEMA) is as follows: DBAEMA (2.04 g, 8.7 x 10$^{-3}$ mol), CPAD (0.0238 g, 8.7 x 10$^{-5}$ mol, and anhydrous 1,4-dioxane (2.0 mL) were added to a Schlenk flask equipped with a stir bar. The flask was sealed with a rubber septum, then purged with high-purity nitrogen for 45 min and subsequently left under a nitrogen atmosphere. AIBN (0.0028 g, 1.7 x 10$^{-5}$ mol) was added to a second Schlenk flask equipped with a stir bar. The flask was sealed with septum and vacuum purged 3 times using high-purity nitrogen. Then the monomer solution was transferred via cannula to the initiator flask. The polymerizations were stirred at 75 °C for 10 hrs. After the polymerization time had been reached, the reaction mixture was diluted with THF and precipitated in methanol; the resulting polymer was collected and washed with water,
and then dried under vacuum at 50 °C overnight. For poly(DPAEMA) and poly(PPDEMA), the techniques used were similar except the reaction mixture was precipitated in a methanol and 0.2 M KOH solution with a 4:3 volume ratio. To follow the kinetics of the polymerization, samples (1.5-5.0 mL) were withdrawn from the reaction flask using a needle and purged syringe at specific time intervals. For the determination of the monomer conversion, the quenched reaction mixture was analyzed by $^1$H NMR spectroscopy in CDCl$_3$ at room temperature directly after it was collected from the polymerization. The integration of the CH$_2$ protons on the side chains of the polymer was compared with the monomer CH$_2$=C resonance (1H). A typical recipe for poly(DPAEMA) was: DPAEMA (1.64 g, 1.82 mL, $7.7 \times 10^{-3}$ mol), CPAD (0.0211 g, $7.7 \times 10^{-5}$ mol, AIBN (0.0025 g, $1.5 \times 10^{-5}$ mol), and anhydrous 1,4-dioxane (2.0 mL). A typical recipe for poly(PPDEMA) was: PPDEMA (1.98 g, 2 mL, $1.0 \times 10^{-3}$ mol), CPAD (0.0276 g, $1.0 \times 10^{-4}$ mol, AIBN (0.0033g, $2.0 \times 10^{-5}$ mol), and anhydrous 1,4-dioxane (3.0 mL).

3.2.6 General synthesis of pH-responsive block copolymers via RAFT polymerization

The pH-responsive block copolymers were prepared using techniques that were similar to the synthesis of the homopolymers. A representative example of the synthesis of the pH-responsive block copolymers is the synthesis of PEG-b-PDBAEMA: DBAEMA (7.12g, 7.7 mL, 0.029 mol) and anhydrous 1,4-dioxane (7.0 mL) were added to a Schlenk flask equipped with a stir bar. The flask was sealed with a rubber septum and purged with high-purity nitrogen for 45 min and subsequently left under a nitrogen atmosphere. AIBN (0.0054g, $3.3 \times 10^{-5}$ mol) and PEG-CPAD (1.73g, $3.3 \times 10^{-4}$ mol) were added to a second
Schlenk flask equipped with a stir bar. The flask was sealed with septum and vacuum purged 3 times using high-purity nitrogen. The monomer solution was then transferred via cannula to the initiator flask. The polymerizations were stirred at 80 °C for 12 hrs. After the polymerization time had been reached, the reaction mixture was diluted in THF and the polymer was then precipitated in ethyl ether. The resulting polymer was then isolated by centrifugation and dried in a vacuum oven under room temperature overnight to give PEG-b-PDBAEMA. To follow the kinetics of the polymerization, samples (1.5-5.0 mL) were withdrawn from the reaction flask using a needle and purged syringe at specific time intervals. For the determination of the monomer conversion, the quenched reaction mixture was analyzed by $^1$H NMR spectroscopy in CDCl$_3$ at room temperature directly after it was collected from the polymerization. The integration of the CH$_2$ protons on the side chains of the polymers was compared with the monomer CH$_2$=C resonance. Similar techniques were used to prepare PEG-b-PDPAEMA and PEG-b-PPPDEMA. A typical recipe for PEG-b-PDPAEMA was: DPAEMA (8.43 g, 9.2 mL, 4.0 × $10^{-2}$ mol), PEG-CPAD (2.08 g, 4.0 × $10^{-4}$ mol, AIBN (0.0065 g, 4.0 × $10^{-5}$ mol), and anhydrous 1,4-dioxane (10.0 mL). A typical recipe for PEG-b-PPPDEMA was: PPDEMA (8.12 g, 8.2 mL, 4.1 × $10^{-2}$ mol), PEG-CPAD (2.41 g, 4.6 × $10^{-4}$ mol, AIBN (0.0075 g, 4.6 × $10^{-5}$ mol), and anhydrous 1,4-dioxane (12.3 mL).

3.2.7 pH transition of RAFT prepared homopolymers and block copolymers

A small amount of polymer was dissolved in HCl solution (~0.1 M) to reach a concentration of 2-4 mg/mL. Then, NaOH solution (0.1 M) was used to titrate the polymer
solutions until the pH was above 11. Samples were taken at different pH and then the hydrodynamic diameter and zeta potential of the polymer chains were measured by DLS.

3.3 Results and Discussion

The tertiary amine-based methacrylate monomers were chosen in this research such that their pKa values are close to the literature reported values of the pH in various cancers and include the monomers DBAEMA, DPAEMA and PPDEMA. Homopolymers of each of these monomers were prepared using either a dithioester or trithiocarbonate based RAFT agent (Scheme 3.1). The block copolymers were prepared with a synthesized poly(ethylene glycol) (PEG) macro-RAFT agent (Scheme 3.2). A detailed study of the polymerization kinetics for each of these systems was performed and the pH-responsive properties of these homopolymers and block copolymers were also studied.

3.3.1 RAFT polymerization for the synthesis of pH-responsive homopolymers

RAFT polymerization is arguably the most versatile LRP technique with respect to the variety of monomers that can be controllably polymerized and the tolerance for functional groups.[117, 119] RAFT has been used by several groups to (co)polymerize a variety of tertiary-amine based methacrylate monomers,[69, 98, 99, 142, 150, 151] with CPAD and DATC being two commonly used RAFT agents. Also, pH-responsive block copolymers have been synthesized by using the related homopolymers as macro-RAFT agents.[98, 150, 152] However, this previous work has mainly focused on three specific tertiary amine-based monomers, DMAEMA, DEAEMA and DPAEMA, and there has been limited research comparing the RAFT polymerization of different tertiary amine-based methacrylate monomers.
and investigating the kinetics of these systems except for research investigating the use of two different RAFT agents for polymerization of DEAEMA by Liu’s group[142] and the comparison of the RAFT polymerization of DPAEMA and DEAEMA by Lee’s group[151].

Also, with respect to pH-responsive systems for cancer diagnostics and therapeutics, the polymers derived from DMAEMA (pKa ~ 8.0) and DEAEMA (pKa ~ 7.2) have pKa values that are too high when targeting the extracellular matrix of solid tumor tissues, which has a pH of 6.2–6.8. Therefore, it is important to identify techniques to produce well-defined homopolymers and block copolymers that exhibit pH-responsive behavior at values close to the pHε of solid tumors.

The tertiary amine-based methacrylate monomers DBAEMA, DPAEMA and PPDEMA were chosen because their pKa values are close to the literature reported values of the pHε in various cancers and there is little to no detailed information on the use of RAFT polymerization to prepare homopolymers and block copolymers from these monomers. To establish suitable conditions for the synthesis of PDBAEMA, PDPAEMA and PPPDEMA using RAFT polymerization, polymerizations of the different monomers were performed under various conditions to investigate the influence of two different RAFT agents (CPAD and DATC) and three different solvents (1,4-dioxane, DMF/2-propanol or acidic buffer solution) on the control of the polymerization (Table 3.1). The solvents were chosen based on previous literature reports for the polymerization of monomers containing tertiary amine groups.[87, 99, 153]
TABLE 3.1 Polymerizations for the preparation of pH-responsive homopolymers under different conditions

<table>
<thead>
<tr>
<th>Polymer Entries</th>
<th>Solvent System</th>
<th>RAFT Agents(^c)</th>
<th>Initiator</th>
<th>Time, h</th>
<th>Conv. % (^e)</th>
<th>Theor. (M_n, \text{Da})</th>
<th>GPC Results (M_n, \text{Da})</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>N/A</td>
<td>AIBN</td>
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<td>81</td>
<td>N/A</td>
<td>132000</td>
<td>2.90</td>
</tr>
<tr>
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<td>AIBN</td>
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<td>51000</td>
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</tr>
<tr>
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<td>22000</td>
<td>25400</td>
<td>1.14</td>
</tr>
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<td>DMF/2-propanol(^a)</td>
<td>CPAD</td>
<td>AIBN</td>
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<td>19500</td>
<td>21800</td>
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</tr>
<tr>
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<td>acidic buffer(^b)</td>
<td>CPAD</td>
<td>ACVA</td>
<td>10</td>
<td>92</td>
<td>20100</td>
<td>25800</td>
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<tr>
<td>1</td>
<td>1,4-dioxane</td>
<td>N/A</td>
<td>AIBN</td>
<td>5(^d)</td>
<td>88</td>
<td>N/A</td>
<td>40500</td>
<td>1.42</td>
</tr>
<tr>
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<td>AIBN</td>
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<td>98</td>
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<td>60100</td>
<td>1.84</td>
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<td>CPAD</td>
<td>AIBN</td>
<td>20</td>
<td>95</td>
<td>19000</td>
<td>21600</td>
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<tr>
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<td>DMF/2-propanol</td>
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<tr>
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<td>20</td>
<td>95</td>
<td>19000</td>
<td>22200</td>
<td>1.13</td>
</tr>
</tbody>
</table>

\(^a\) DMF/2-propanol = 1:1 (volume ratio). \(^b\) acidic buffer: 3mL DIUF water with 0.02mol acetic acid and 0.002mol sodium acetate. \(^c\) \([\text{Monomer}] = 2.0 \text{ M}, \text{ and } [\text{Monomer}]_0/[\text{RAFT Agent}]_0/[\text{Initiator}]_0 = 100:1:0.2\) for all RAFT polymerizations. For conventional radical polymerization, all the conditions are same with entry 3 in each set except no RAFT agent was added. \(^d\) Polymerization were quenched earlier because of stirring issue due to high viscosity of the reaction mixture. \(^e\) Conversion were calculated from integral the areas under the peaks of the characteristic signals from monomer and polymer in \(^1\)H NMR. Entry 5 in each set was calculated by weighing reaction mixture and dried polymer samples. \(^f\) Theoretical molecular weight were calculated based on the conversion which were calculated from NMR. It is defined as follows:

\[
M_n(\text{theory}) = \frac{[\text{Monomer}]_0}{[\text{RAFT agent}]_0} \times M_{\text{monomer}} \times \text{Conversion} + M_{\text{RAFT agent}},
\]

where \(M_{\text{monomer}}\) and \(M_{\text{RAFT agent}}\) are the molecular weights of monomer and RAFT agent, and \([\text{Monomer}]_0\) and \([\text{RAFT agent}]_0\) are the initial concentrations of monomer and RAFT agent.

It has been reported that dithiobenzoates are the best RAFT agents for controlling the polymerization of methacrylates, because a strongly stabilizing Z group is required in order to favor the bulky tertiary propagating radical addition to the thiocarbonyl bond.[117] Compared to DATC, the phenyl group as the Z group in CPAD can better stabilize the intermediate
radicals than the dodecyl group in DATC. Thus, most of the reported RAFT polymerizations of tertiary amine based methacrylates have used CPAD as the RAFT agent.[98, 142, 151, 152] However, DATC also has been reported to be used in the RAFT polymerization of DEAEMA.[98] To investigate the effect of the RAFT agent on the polymerization of the different monomers used in this study, polymerizations were carried out using either DATC, a trithiocarbonate based RAFT agent, and CPAD, a dithioester based RAFT agent. Under each of the conditions, homopolymer was produced for all of the monomers. Example $^1$H NMR spectra of the different homopolymers, prepared using the RAFT agent CPAD and the solvent 1,4-dioxane are shown in Figure 3.1. The $^1$H NMR spectra for the polymers prepared under the other conditions were identical. All of the peaks correspond to the specific protons present in each of the polymers. The protons from the CPAD RAFT agent, which is present as the end groups of the polymers, were not detected due to the relatively high molecular weight of the polymers; however, each of the polymers had a pinkish color, which suggests the presence of the CPAD end groups. With DATC as the RAFT agent, the polydispersity index (PDI) of PDPAEMA and PPPDEMA were relatively high and close to the polymers formed using conventional radical polymerization under similar conditions. Also the experimental molecular weights were significantly higher than the theoretical molecular weights. These results suggest that in most of these cases the DATC RAFT agent was not providing the required control to give well-defined homopolymers. However, the PDI of PDBAEMA decreased to be 1.34 which suggests that DATC provided some control for polymerization of DBAEMA. Even though the difference in chemical structure between these tertiary amine based methacrylates is
subtle; significant differences in their RAFT polymerizations were observed. Overall, the polymerization of all three monomers were well controlled by using CPAD as the RAFT agent and 1,4-dioxane as the solvent, as low PDIs were observed (1.10-1.19) and experimental molecular weights closely matched the theoretical molecular weights.

FIGURE 3.1 ¹H NMR spectra of homopolymers.

3.3.2 Kinetics study of the formation of pH-responsive homopolymers via RAFT polymerization

To fully determine the effectiveness of a RAFT polymerization and to take advantage of the versatility of these systems it is important to evaluate the kinetics. As such, the RAFT polymerization of the monomers DBAEMA, DPAEMA and PPDEMA, using CPAD as RAFT agent in the presence of AIBN in 1,4-dioxane at 75 °C, was monitored over time and, hence, conversion (Table 3.2). The semilogarithmic plots of conversion versus time for the preparation of the different homopolymers are shown in Figure 3.2. Most of these plots demonstrates a relatively linear relationship for ln([M]₀/ [M]ₜ) versus time, indicating a constant
concentration of active species across the course of the polymerization, with the plot for PPDEMA having the largest slope indicating it has the fastest rate of polymerization.

TABLE 3.2 Synthesis of pH-responsive homo-polymers by RAFT polymerization of tertiary amine-based methacrylate monomers using CPAD as the RAFT agent in 1,4-dioxane at 75 °C

<table>
<thead>
<tr>
<th>No.</th>
<th>Time, h</th>
<th>Conv.%</th>
<th>( M_n, \text{ Theor.} )</th>
<th>( M_n, \text{ GPC} )</th>
<th>PDI, GPC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PDBAEMA</strong></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>K1</td>
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<td>8720</td>
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</tr>
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<td>83.3</td>
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<td>20500</td>
<td>1.14</td>
</tr>
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</table>

\[ \text{[Monomer]}_0 = 2.0 \text{ M}, \frac{\text{[Monomer]}_0}{\text{[CPAD]}_0} = 90/1 \text{ for DBAEMA and 100/1 for DPAEMA and PPDEMA. [CPAD]}/([AIBN])_0 = 10/1 \text{ in all cases.} \]

The semilogarithmic plot of conversion versus time for the polymerization of DBAEMA (Figure 3.2a) demonstrates a deviation from the expected trend. Figure 3.2a shows a decreasing slope in the semilogarithmic plot accompanied by a relatively high initial PDI of 1.32 (Figure 3.2d). These results suggest that in the early stages of the polymerization, the control of the polymerization is poor. A possible explanation for this is the butyl groups attached to the
nitrogen in the monomer structure of DBAEMA. The proposed RAFT polymerization
mechanism[117, 119] demonstrates that to achieve a narrow molecular weight distribution, all
of the R groups (in the case of CPAD, the 4-cyanopentanoic acid group) on the RAFT agent
should undergo fragmentation and reaction with monomer very early in the polymerization,
ideally before significant amounts of propagation have occurred. This requires favorable
fragmentation of the R group over the growing polymer chain in the intermediate radical
involved in the pre-equilibrium stage of the polymerization. In the case of DBAEMA, the size
of the two n-butyl groups may result in the pre-equilibrium intermediate radical preferentially
fragmenting to the growing polymer chain. This would result in a broader molecular weight
distribution, as the polymer chains would be initiated at different times. In addition, the
semilogarithmic plot for DBAEMA appears to experience increased termination at higher
conversions, as seen by the decreasing slope.

In addition to the semilogarithmic plots of conversion versus time, another critical
component of an effective RAFT polymerization is a linear relationship between the number
average molecular weight ($M_n$) and conversion (Figure 3.2). For each of the monomers
polymerized in this study the $M_n$ versus conversion plot is linear and, in addition, the PDI
values for the polymers are typically below 1.2. Figures 3.2 d, e and f show that the $M_n$ values
determined by GPC were higher than the predicted $M_n$ values. This is most likely due to a
lower initiation efficiency resulting in fewer chains than predicted. Figures 1e and 1f also show
a relatively constant PDI value during the polymerization, which is expected in a RAFT
polymerization system. However, Figure 1d shows a decrease in the PDI with conversion.
Once again, this is most likely due to slow initiation of the R group in the initial stage of the polymerization, as discussed above.

FIGURE 3.2 (a-c) First-order kinetics plots for polymerization of tertiary amine based methacrylate monomers (a-DBAEMA, b-DPAEMA, c-PPDEMA) with AIBN in the presence of CPAD as RAFT agent in 1,4-dioxane at 75 °C. (d-f) Number-average molecular weight ($M_n$) and PDI as a function of conversion (d-PDBAEMA, e-PDPAEMA, f-PDDEMA).

Finally, all the GPC traces obtained for the polymers (Figure 3.3) were unimodal, even the GPC traces for the polymerization of DBAEMA were unimodal, despite the suggested preference of preferential fragmentation in the initial stages of the reaction. Therefore, the above results suggested that the RAFT agent CPAD and a solvent system of 1,4-dioxane can be used to synthesize well defined homopolymers via RAFT polymerization of the proposed tertiary amine-based methacrylate monomers.
3.3.3 Kinetics study of the formation of pH-responsive block copolymers via RAFT polymerization

The use of pH-responsive polymers for biomedical applications typically requires the formation of block copolymers, where one block demonstrates the desired pH-response and the other block is permanently hydrophilic and, usually, biocompatible. This allows the pH-responsive polymers to form micelles when they undergo their hydrophilic to hydrophobic transition, rather than precipitate out of solution. To demonstrate the ability to prepare pH-responsive block copolymers with DBAEMA, DPAEMA, and PPDEMA, a PEG-CPAD macro-RAFT agent was synthesized and used to polymerize each of the monomers (Table 3.3).
TABLE 3.3 Synthesis of block copolymers by RAFT polymerization of tertiary amine-based methacrylate monomers using PEG-CPAD as a macro-RAFT agent in 1,4-dioxane

<table>
<thead>
<tr>
<th>No.</th>
<th>Time, h</th>
<th>Conv.%</th>
<th>Mn, Theor.</th>
<th>Mn, GPC</th>
<th>PDI, GPC</th>
</tr>
</thead>
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<tr>
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<td>67.7</td>
<td>20000</td>
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<td>1.39</td>
</tr>
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</table>

PEG-b-PDPAEMA

<table>
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<tr>
<th>No.</th>
<th>Time, h</th>
<th>Conv.%</th>
<th>Mn, Theor.</th>
<th>Mn, GPC</th>
<th>PDI, GPC</th>
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PEG-b-PPPDEMA

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<th>Conv.%</th>
<th>Mn, Theor.</th>
<th>Mn, GPC</th>
<th>PDI, GPC</th>
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<td>24050</td>
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</table>

*Polymerization temperature: 80 °C for DBAEMA and PPDEMA and 75 °C for DPAEMA, [Monomer]₀=2.0mol/L, [Monomer]₀/[PEG-CPAD]₀=100/1 for DPAEMA and 90/1 for DBAEMA and PPDEMA. [PEG-CPAD] /[AIBN]₀ = 10/1 in all cases.

The pH-responsive block copolymers were synthesized using similar conditions to the pH-responsive homopolymers. These conditions were chosen as the PEG-CPAD macro-RAFT agent contains the CPAD that proved successful in the preparation of the homopolymers. However, the temperature was increased slightly to accommodate the increase in viscosity when using a macro-RAFT agent. In each case, RAFT polymerization of the tertiary amine-based methacrylate monomers using the PEG-CPAD macro-RAFT agent, successively produced block copolymers. The 1H NMR spectra of the pH-responsive block copolymers are shown in Figure 3.4. All the peaks present correspond to the specific protons present in each of...
the block copolymers. Once again, the protons from the CPAD moiety were not detected due to the relatively high molecular weight of the block copolymers. However, the protons from the PEG were seen in each case.

FIGURE 3.4 $^1$H NMR spectra of the pH-responsive block copolymers.

The semilogarithmic plots of conversion versus time for the preparation of the different block copolymers are shown in Figure 3.5. In each case, there is a high rate of polymerization initially, followed by a relatively linear relationship between $\ln([M]_0/[M]_t)$ versus time. As discussed above, this is believed to be due to termination at the later stages of the polymerization. Once again, the slope of the $\ln([M]_0/[M]_t)$ versus time was largest for the polymerization of PPDEMA with the macro-RAFT agent, indicating this monomer has the highest rate of polymerization. For each of the monomers polymerized using the macro-RAFT agent, the $M_n$ versus conversion plots is relatively linear (Figure 3.5).
FIGURE 3.5 (a, b, c) First-order kinetics plots for polymerization of tertiary amine-based methacrylate monomers (a-DBAEMA, b-DPAEMA, c-PPDEMA) with AIBN in the presence of PEG-CPAD as RAFT agent in 1,4-dioxane (d, e, f) $M_n$ and PDI of the block copolymers (d, PEG-b-PDBAEMA; e, PEG-b-PDPAEMA; f, PEG-b-PPPDEMA) as a function of conversion.

For the preparation of the block copolymers with DBMEMA and DPAEMA, the $M_n$ values determined by GPC matched well with the predicted $M_n$ values. For the block copolymer prepared with PPDEMA, the experimental $M_n$ values are initially close to the predicted values, but deviated to higher values at high conversion. This is most likely due to a termination, via combination, at conversions above 80%. Figure 3.5f shows relatively low PDI values, which are expected in a RAFT polymerization system, although the values are slightly higher than values obtained in the homopoly-merization of PPDEMA. However, Figures 3.5 d and e shows the block copolymers produced from DBAEMA and DPAEMA have relatively high PDI values (1.3-1.4) initially, which tend to decrease in the PDI with conversion. This is most likely
due to a combination of slow fragmentation of the R group in the initial stage of the polymerization and, possibly, a small amount of termination during this stage of the polymerization.

FIGURE 3.6 GPC traces of pH-responsive block copolymers: (a) PEG-b-PDBAEMA, (b) PEG-b-PDPAEMA, (c) PEG-b-PPPDEMA.

Finally, while the GPC traces obtained for the different polymerizations clearly showed a peak shift to higher molecular weight with conversion and were unimodal (Figure 3.6), each of the traces show with a low molecular weight tail. These results suggest that there is a small amount of PEG homopolymer present that does not contain the CPAD RAFT agent. Preparation of the PEG-CPAD macro-RAFT agent included attempts to remove any unreacted PEG; however, as reported in literature, it is extremely difficult to remove the entire untreated
PEG and the end-group functionality is often around 85-90%, [125, 149, 154-156] which is comparable to what we have achieved a 85-95% end-group functionality according to $^1$H NMR (Figure AA. 3). Despite this, overall these results suggested that the PEG-CPAD macro-RAFT agent was successful in preparing tertiary amine-based methacrylate block copolymers by the RAFT process.

3.3.4 pH-Responsive properties of the block copolymer

The homopolymers and block copolymers prepared from tertiary amine-based methacrylate monomers are classified as cationic pH-responsive polymers because the tertiary amine functional group will accept a proton and become hydrophilic when the environmental pH value is lower than their pKa value. As such, the pKa value is a critical parameter for pH-responsive polymers and can be calculated via pH titration experiments. To achieve this, a 0.1 M NaOH solution was used to titrate polymer solutions made by dissolving a small amount of polymer in HCl solution to produce a sample that was 2-4 mg/mL of polymer. Figure 3.7 shows typical titration curves for the homopolymer and block copolymers of PPDEMA. These titration curves show a number of different features. Initially, the solution pH increases from the low pH of the HCl polymer solution with the addition of NaOH as the free protons in solution are neutralized. As the pKa value of the PPPDEMA is approached, the solution pH increases rapidly until it plateaus. This plateau is due to the tertiary amine groups becoming deprotonated and corresponds to the pKa transition for the polymers. Once the polymer is fully deprotonated, the solution pH continues to increase with the addition of NaOH. The pKa value was calculated at the 50% titration point for the plateau; that is where the volume of added
NaOH solution is half of the second equivalence point volume minus the first equivalence point volume. Similar results were seen for PDBAEMA and PDPAEMA (Figure AA. 4 and 5 in Appendix A).

FIGURE 3.7 Titration curves for PPPDEMA and PEG-b-PPPDEMA.

The experimental pKa values for PDBAEMA and PEG-b-PDBAEMA were approximately 5.1, for PDPAEMA and PEG-b-PDPAEMA were approximately 6.4, and for PPPDEMA and PEG-b-PPPDEMA were approximately 6.8. It is expected that the pKa values for homopolymers and their resultant block copolymers are similar as the type of ionizable functional group present determines the pKa value and is the same for the different homopolymer/block copolymer combinations. While the pH transition for homopolymer or block copolymers with different molecular weights occurred at a similar pH, the plateau for block copolymer with a higher molecular weight was longer than the lower one due to the higher molecular weight one having more tertiary amine groups compared to the lower one. Similar results were observed in homopolymers compared to block copolymers with similar molecular weights. In addition, the pKa values reported here are comparable to literature values.[87]
TABLE 3.4 pKa, hydrodynamic diameter and zeta potential of the pH-responsive block copolymers

<table>
<thead>
<tr>
<th>M_n, Da</th>
<th>Hydrodynamic diameter, nm</th>
<th>Zeta Potential, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PEG-b-PDBAEMA, pKa = 5.1 ± 0.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>11450</td>
</tr>
<tr>
<td>19500</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PEG-b-PDPAEMA, pKa = 6.4 ± 0.1</th>
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</thead>
<tbody>
<tr>
<td>11400</td>
</tr>
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</tr>
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</table>

<table>
<thead>
<tr>
<th>PEG-b-PPPDEMA, pKa = 6.8 ± 0.1</th>
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<tbody>
<tr>
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<td>15400</td>
</tr>
<tr>
<td>18100</td>
</tr>
<tr>
<td>23000</td>
</tr>
</tbody>
</table>

In the case of the pH-responsive block copolymers, the polymer solutions will be able to form micelles when the solution pH is above pKa, with the pH-responsive block in its hydrophobic state being the core of the micelle and the hydrophilic PEG block forming the corona. To determine the formation and properties of these polymer micelles in solution, DLS was used to determine the hydrodynamic diameter and zeta potential of the polymer chains in solution (Table 3.4 and Figure 3.8). As can be seen from Figure 3.8, when the solution pH was above the pKa of each pH-responsive block copolymer, the polymer chains formed micelles. The size of the formed micelles increased with the increasing molecular weight of the pH-responsive block for the same type of block copolymers. For example, for the PEG-b-PPPDEMA copolymer the micelle size increased from 24nm to 32nm when the molecular weight increased from 12400 g/mol to 23000 g/mol. This result was expected, as the
increase in molecular weight of the block copolymer is due to an increase in the pH responsive block since the PEG block was fixed at 5000 g/mol.

In addition, the micelle size increased for the different block copolymers with similar overall molecular weight, for example, the micelle size was 24nm for PEG-b-PDBAEMA of 19500 g/mol, 26nm for PEG-b-PDPAEMA of 17500 g/mol and 26nm for PEG-b-PPPDEMA of 15400 g/mol. It is proposed that this is due to differences in the flexibility of the groups attached to the nitrogen in the monomers. The monomers DBAEMA, DPAEMA, and

FIGURE 3.8 The hydrodynamic diameter (D_h, nm) (solid line) and zeta potential (mV) (dot line) of PEG-b-PPPDEMA (a & b) and PEG-b-PDPAEMA (c) and PEG-b-PDBAEMA (d) with different M_n as a function of solution pH.
PPDEMA have the dibutylamino, diisopropyl-amino and piperidino groups attached, respectively. The relatively flexibility of these groups decrease from dibutyl to diisopropyl to piperidine, which in turn increases the size of the hydrophobic core of the micelles for similar molecular weights.

The zeta potential of the polymers was also measured by DLS and used to evaluate the protonated/deprotonated status of the different pH-responsive block copolymers. The zeta potential of the tertiary amine based methacrylate block will be positive when the block is protonated at low pH and will be close to zero after the block becomes deprotonated when the solution pH is above pKa value for that block copolymer. As can be seen from Figure 3.8, the zeta potential decreased from a positive value to approximately zero with increasing pH. The zeta potential of PEG-b-PPPDEMA polymer chains demonstrated a sharp decrease when the solution pH approached the pKa value, while the zeta potential for both the PEG-b-PDBAEMA and PEG-b-PDPAEMA showed a more gradual decrease with increasing solution pH. This is most likely due to their different pKa values, as they will have different dissociation constants.

While PEG-b-PDAEMA, with the highest dissociation constant, will dissociate more and have the lowest concentration of the protonated tertiary amine groups, PEG-b-PPPDEMA, with the lowest dissociation constant, will have the highest concentration of the protonated groups. This is also evidenced by the lower starting zeta potential for both PEG-b-PDBAEMA and PEG-b-PDPAEMA (Figure 3.8). With a lower starting zeta potential, the overall decrease to zero at the pKa will be less and is observed by a more gradual transition, rather than the sharp
transition seen at the high starting zeta potential for PEG-b-PPPDEMA. Furthermore, the molecular weight of the DBAEMA and DPAEMA monomers are larger than molecular weight of the PPDEMA monomer. Therefore, at a fixed mass of block copolymers with the same overall molecular weight, there are a lower number of moles of the pH-responsive monomer for PEG-b-PDBAEMA and PEG-b-PDPAEMA compared to PEG-b-PPPDEMA. Regardless of the magnitude of the transition, the decrease in zeta potential for each of the pH-responsive block copolymers corresponded to an increase in hydrodynamic volume of the chains and both occurred around the pKa value for the specific pH-responsive polymer.

3.4 Conclusions

We have demonstrated that CPAD and PEG-CPAD can be used as RAFT agents to mediate the polymerization of DBAEMA, DPAEMA and PPDEMA by a RAFT process. The controlled character of the polymerizations was confirmed by kinetics studies and GPC results. The pH-responsive properties of the homopolymers and block copolymers were investigated by pKa determination and DLS measurements. The results suggest that the pKa, as a critical value to characterize the dissociation ability of the different tertiary amine functional groups, keeps constant for the polymers prepared from the same pH-responsive monomer within a certain range of molecular weight. The DLS results proved that the pH-responsive block copolymers will undergo a transition from unimers to micelles in aqueous media in responses to changes in solution pH. By tuning the pH-response of these well-defined block copolymers to that seen in the pH of tumors, we believe the prepared polymers may be useful in the design of new diagnostic and therapeutic devices for the treatment of cancer.
CHAPTER 4

MODIFICATION OF GD-MOF NANOPARTICLES WITH TERTIARY AMINE-BASED PH-RESPONSIVE BLOCK COPOLYMERS FOR USE AS PH-RESPONSIVE MRI CAS

4.1 Introduction

According to statistics from the International Agency for Research on Cancer (IARC), an estimated 14.1 million new cancer cases and 8.2 million cancer-related deaths occurred worldwide in 2012, compared with 12.7 million and 7.6 million, respectively, in 2008.[1, 2] However, with remarkable breakthroughs in the understanding of fundamental cancer biology, improved early detection and advances in cancer therapy, cancer mortality rates have recently started to show a declining trend in some more developed western countries.[2] As the importance of treatment is widely recognized, there has been an increasing focus on early detection and diagnosis in the battle against cancer. One of the most promising developments in detection and diagnosis has been the development of molecular imaging.

Molecular imaging, as a powerful imaging technique, allows the visual representation, characterization and quantification of biological processes at the cellular and subcellular levels in intact living organisms. While there are many different biological processes that have been the focus of different molecular imaging technologies, the measurement of pH \textit{in vivo} has received considerable attention. Due to the correlation between a low extracellular pH (pHe) in solid tumors and increased cancer morbidity and mortality (Figure 4.1), the ability to measure the pHe of solid tumors provides potential not only for early detection and diagnosis of tumors,
but also to monitor the effectiveness of the treatment plan.[6, 7] Over the past few decades, several molecular imaging techniques, including computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), single photon emission CT (SPECT), ultrasound (US) and optical imaging (OI), have been successfully applied to fields ranging from clinical oncology to research in cellular biology and drug discovery.[24, 26] Among them, MRI is one of the most widely available, clinical diagnostic tools, and also the most studied technique due to a high spatial resolution and an, so far, unsurpassed ability in distinguishing soft tissues. By exploiting new imaging probes or contrast agents (CAs) that can exhibit a difference in relaxation time upon pH change, MRI offers great potential for accurate and precise, non-invasive measurement of pH in vivo with excellent spatial resolution.[6, 32-34]

These pH-responsive MRI CAs are significantly different than conventional MRI CAs in that, while high relaxivity values are desirable, the critical property of a pH responsive MRI CA is the relative change in relaxivity on exposure to changes in the pH of the surrounding environment (Figure 4.1). Typically pH-responsive MRI CAs function as a result of either water accessibility or correlation times that are pH dependent, which are two key factors to determine the relaxivities of gadolinium(Gd)-based CAs.[134, 135] An ideal pH-responsive MRI CAs should have large pH sensitivity with a pKa in the physiological range, be metabolically stable and biocompatible, and provide a high signal to noise ratio (SNR) within a short time period. Initial research in developing pH-responsive CAs to assist MRI in pH measurement focused on Gd-based chelates, which are the most widely used positive contrast
agents in the clinic.[8-10] However, the main problems with these systems are related to the three main drawbacks of Gd chelates, low concentration of Gd per molecule, non-specific uptake and fast elimination kinetics due to the dimensions of the complex.

FIGURE 4.1 General scheme for the imaging of extracellular pH in tumors using pH-responsive imaging agent. A low pHe is normally characterized by rapid proliferation and poor perfusion of tumor cells. While the tumor cells have adapted to survive in an acidic microenvironment, normal cells will die. Thus the cancer morbidity and mortality increases with the decreasing pHe. The pH-responsive imaging agents can be accumulated in the tumor tissue via the enhanced permeability and retention (EPR) effect through the leaky blood vessels. Due to the acidic microenvironment, the accumulated pH-responsive imaging agents will be triggered to enhance the relaxivity in response to pHe.

These factors have drawn an increasing focus on developing new pH-responsive CAs based on nanoparticles. With the tremendous advances in nanotechnology, nanoparticles have been widely studied in the development of new types of CAs and the effort made so far in this area has been discussed in several reviews.[26, 54, 55] The properties of nanoparticles are very different from small chelates. Firstly, the size of nanoparticles can vary from several nanometers to hundreds of nanometers, which give CAs a longer retention time in body
compared to small chelates. Secondly, the increase in the surface area of nanoparticles not only dramatically increases the concentration of Gd per molecule (particle), but also provides opportunities for functionalizing the CAs, such as attaching targeting agents and molecular responsive agents to make specific and smart CAs possible in both diagnosis and therapy for various diseases. Specifically for MRI, the development of Gd-based nanoparticles for use as CAs in MRI has become a research hotspot. One such candidates is Gd$_2$O$_3$ nanoparticles that have been synthesized and are typically modified with D-glucuronic acid or poly(ethylene glycol) (PEG) to provide stability and biocompatibility.\cite{56, 57, 157, 158} The Gd$_2$O$_3$ nanoparticles were reported to have a size range between 2-10 nm and a higher longitudinal relaxivity ($r_1$), ranging from 5.7 to 22.8 s$^{-1}$ mM$^{-1}$, compared to the commercially available Gd-DTPA (DTPA=diethylenetriaminepentaacetic acid) chelates which have previously been reported to have a $r_1$ between 4 to 5 s$^{-1}$ mM$^{-1}$ (1.5 T).\cite{39} GdPO$_4$ nanoparticles have been studied by Suzuki’s group and Talham’s group.\cite{61-63} While the former reported the use of GdPO$_4$ nanoparticles as a positive CA with a size of 23 nm and a $r_1$ of 13.9 s$^{-1}$ mM$^{-1}$ at low field (0.47 T), the latter reported use as a negative CA with a size of 50 nm in length and 10 nm in width and a $r_1$ of 0.2 s$^{-1}$ mM$^{-1}$ and a transverse relaxivity ($r_2$) of 12.8 s$^{-1}$ mM$^{-1}$ at high field (14.1 T). GdF$_3$,\cite{58} GdS:Eu$^{3+}$,\cite{59} Pyrene-Gd,\cite{60} and Gd-metal organic frame (Gd-MOF)\cite{12} nanoparticles have also been synthesized and reported to have a larger $r_1$ values than Gd-chelates. Although nanoparticle-based imaging agents are still in their early stages of development, their emergence have opened an avenue for designing new MRI CAs platforms.
to overcome the non-specific uptake, low relaxivity and sensitivity limitations of small molecule chelates.

While the development of pH-responsive MRI CAs based on Gd-nanoparticles represents one of the most exciting research areas in this field, the use of pH-responsive polymers has been considered as one of the most promising ways for responsive tumor diagnosis and therapy due to their solubility or chain conformation changes in aqueous solutions as a result of environmental pH changes.[88, 159] Lee and coworkers were one of the first groups to prepare a pH-responsive MRI CA by modification of iron oxide nanoparticles with a pH responsive diblock copolymer, consisting of poly(ethylene oxide) (PEO) as the hydrophilic, biocompatible segment and a poly(β-amino ester) (PAE) as the pH-sensitive segment.[136] The pH-sensitive nature of the MRI CAs was demonstrated by monitoring the transverse relaxation time ($T_2$) while changing the pH from 7.6 to 6.2. The results demonstrated that $r_2$ increased when the pH was lower than 6.8 due to the release of the iron oxide nanoparticles from the core of the micelles of the pH-responsive polymers. Kikuchi and co-workers developed a different approach to prepare a pH-responsive MRI CA by attaching Gd-chelates to a pH-responsive cross-linked polymer system, which were synthesized using methacrylic acid as the monomer and $N,N'$-methylenbisacrylamide as the cross-linker.[31, 160] MRI studies indicated that $r_1$ of the CAs in a cross-linked polymer system increased from 13.6 to $28.0 \text{ s}^{-1}\text{ mM}^{-1}(0.47 \text{ T})$ when the environmental pH decreased from 7 to 4. However, the dramatic change in their system occurred when pH was lower than 5 due to the pKa of the poly(methacrylic acid) (PMAA) used in their system is around 4.5.[161]
In order to take advantages of both pH-responsive polymers and Gd nanoparticles, we designed a pH-responsive MRI CA based on Gd nanoparticles and pH-responsive polymers to show relaxivity change with the pH change in the physiological range. The Gd-MOF nanoparticles (GdNPs) were synthesized and subsequently surface-modified with well-defined tertiary amine-based pH-responsive block copolymers to produce a pH-responsive MRI CA. The successful modification of GdNPs with pH-responsive polymers was demonstrated and the performance of the pH-responsive CAs was evaluated.

4.2 Experimental

The following sections introduced materials used in this Chapter and described the relevant experiments and characterization techniques used.

4.2.1 Materials

Cetyltrimethylammonium bromide (CTAB), gadolinium chloride hexahydrate (GdCl$_3$·6H$_2$O), terephthalic acid, 1-hexanol, methylamine (40 wt% in water), sodium salicylate (NaSal), hexylamine, 4-morpholineethanesulfonic acid (MES hydrate), 4-morpholinepropanesulfonic acid (MOPS) were purchased from Aldrich and used without further purification. Heptane and ethanol (EtOH) were purchased from Macron Fine Chemicals. Tetrahydrofuran (THF) (ChromAR) was purchased from Macron Fine Chemicals and purified through Innovative Technology’s Pure-Solv System before use.

The tertiary amine-based pH-responsive polymers used in this chapter were synthesized via reversible addition-fragmentation chain transfer (RAFT) polymerization and the detailed synthesis and characterization information was included in Chapter 3. Sample K1 with a
number average molecular weight ($M_n$) of 9600 g/mol and K3 with a $M_n$ of 13,900 g/mol of PEG-b-PDPAEMA, sample K2 with a $M_n$ of 15,400 g/mol and K4 with a $M_n$ of 23,000 g/mol of PEG-b-PPPDEMA were used in this chapter.

### 4.2.2 Instrumentation and Characterization

Transmission electron microscopy (TEM) was performed on a Philips CM200 with an accelerating voltage of 120kV for both the unmodified and polymer modified GdNPs. Nanoparticle dispersions were diluted and thoroughly dispersed in EtOH via sonication. A drop of the dispersion was then placed on a TEM grid and allowed to air dry. A Keen View Soft Imaging System coupled to iTEM Universal TEM Imaging Platform Software was utilized to acquire images. Fourier transform infrared (FTIR) spectra were collected using a Nicolet iS50 FT-IR spectrometer (Thermo Scientific) operating with a liquid-nitrogen-cooled mercury cadmium telluride detector. Thermogravimetric analysis (TGA) was performed using a TA Q500 equipped with a platinum pan and heated at a rate of 10 °C/ min under air. Powder X-ray diffraction (XRD) intensity data was collected on a Bruker D2 Phaser diffractometer using CuKα radiation. Zeta potential was measured by dynamic light scattering (DLS) on a Malvern Zetasizer Nano ZS at a scattering angle of 173° and analyzed by Malvern Zetasizer Software version 6.20. The concentration of Gd$^{3+}$ in modified and unmodified GdNP solutions were acquired on a Perkin Elmer NexION 300D with an S10 auto-sampler. Samples were diluted in 2% nitric acid solution and run against an internal quality control indium standard. After standard optimization, linear calibration of Gd$^{3+}$ signal intensities versus mass concentrations
was performed with a 10 \mu g/L indium (In) internal standard. Sample mass concentrations were then calculated based on the calibration of gadolinium standard from High Purity Standards.

4.2.3 Synthesis of 1,4-benzenedicarboxylate methyl ammonium salt (1,4-BDC salt).

Terephthalic acid (2.0 g, 1.2 × 10^{-4} mol) was dissolved in 5 mL methyamine (40 wt% in water). 1,4-BDC salt was isolated via solvent removal under reduced pressure and dried in vac. oven at 50 °C for 24 hr.

4.2.4 Synthesis of the Gd MOF nanoparticles (GdNPs) with sodium salicylate.

GdNPs were synthesized via a reverse microemulsion system developed by Reiter and coworkers and modified by our group.[13, 162] A representative example of synthesis of one 800 mL batch of GdNPs is as follows: 1,4-BDC salt (0.0856 g, 3.8 × 10^{-4} mol) was dissolved in 5 mL of DIUF water to produce a 0.075 M solution. Dissolve GdCl_3·6H_2O (0.0929 g, 2.5 × 10^{-4} mol) in 5mL of DIUF water to produce a 0.05M solution of GdCl_3. CTAB (14.58 g, 4.0 × 10^{-2} mol), NaSal (0.0352 g, 2.2 × 10^{-4} mol), hexanol (78.4 mL), and heptane (721.6 mL) were added to a 1 L Pyrex bottle equipped with a stir bar. The mixture was stirred vigorously for 10 min. Then, the 0.075 M 1,4-BDC salt solution (3.6 mL, 2.0 × 10^{-1} mol of water) and the 0.05 M GdCl_3 solution (3.6 mL, 2.0 × 10^{-1} mol of water) were added into the bottle. The bottle was sealed and stirred vigorously overnight. After 24 h, 800 mL of the resulting micro-emulsion was centrifuged at 5000 rpm to remove the supernatant. Then the particles were separated into four 50 mL centrifuge tubes, washed with EtOH (30 mL), sonicated, and then re-centrifuged for 20 min. The supernatant was discarded. The particles underwent an additional cycle of re-dispersement in EtOH (30 mL), sonication, and centrifugation, followed
by re-dispersement in deionized water (30 mL), sonication, and centrifugation to remove any excess reactants. The particles collected were defined as one 800 mL batch of GdNPs, and stored in DIUF water until further use or characterization.

4.2.5 Grafting of tertiary amine-based pH-responsive block copolymers onto Gd nanoparticles.

All deposition reactions were carried out using a similar technique. A representative example for grafting PEG-b-PPPDEMA is as follows: Sample K4 of PEG-b-PPPDEMA (0.30 g, $1.3 \times 10^{-5}$ mol) was dissolved in anhydrous THF (100 mL) in a 250 mL Schlenk flask equipped with a stir bar and then sealed with a rubber septum. The solution was purged with high purity nitrogen and subsequently left under a nitrogen atmosphere. Hexylamine (0.4 mL, $3.0 \times 10^{-3}$ mol) was added to the polymer solution to reduce the thiocarbonylthio end groups of polymer to a thiolate via aminolysis. To prepare the nanoparticles solution, one 800 mL batch of GdNPs was dispersed in 5 mL of DIUF water and 2 mL of the dispersed nanoparticle solution was separated from water by centrifugation and re-dispersed in THF (25 mL) in a second 100 mL Schlenk flask equipped with a stir bar. The nanoparticle solution was then purged with high purity nitrogen for 30 min with the flask in an ice bath and was subsequently left under a nitrogen atmosphere. After 2 h, to allow for the completion of reduction of polymer end groups, the GdNP solution was transferred via cannula to the polymer solution. The resulting solution was allowed to stir for 24 h at room temperature under a nitrogen atmosphere. After reaction, the free polymer was removed from the polymer modified GdNPs via repeated centrifugation and re-dispersion in THF (2x) and EtOH (1x). In each case, the supernatant was
decanted and the polymer modified GdNPs were re-dispersed in 25 mL of solvent prior to sonication. The washed polymer modified GdNPs were stored in DIUF water until further use and characterized by FTIR, TGA and TEM.

4.2.6 pH-Responsive experiments of block copolymers modified GdNPs.

A small amount of unmodified or polymer modified GdNPs were dispersed in DIUF (pH ~6.5). HCl (0.1 M) and NaOH (0.1 M) solutions were then used to adjust the pH of nanoparticles solution to reach a pH range of 5-8. Samples were taken at different pH values and were used to determine the zeta potential of the nanoparticles by DLS. According to the results from Chapter 3, the PEG-b-PPDEMA modified GdNPs were chosen as a representative sample to prove the pH-responsive nature due to the higher zeta potential of the protonated PEG-b-PPPDEMA polymer chain and the dramatic change in zeta potential of free PEG-b-PPPDEMA in solution upon pH changing, compared to PEG-b-PDPAEMA.

4.2.7 NMR experiments for measurement of $T_1$ relaxation.

The longitudinal relaxation time ($T_1$) was measured on a Bruker AVANCE III 400 MHz solid state NMR using the inversion-recovery sequence. Parameters were as follows: the radio frequency field is 50 KHz (90 degree pulse is 5 μs), the delay times, $\tau$, are as follows: 50, 80, 120, 200, 300, 400, 500, 600, 700, 800, 1000, 1500, 2000, 3000, 5000 and 8000 ms. Data was processed with Bruker Topspin 3.0 software according to the equation: $y = A + C(1 - e^{-\frac{\tau}{T_1}})$. NMR samples were prepared by dispersing unmodified GdNPs or polymer modified GdNPs in different pH buffer solutions in the range of 5.9-7.3 with different concentrations in the approximate range of 0-0.1mM of Gd. The buffer solutions were prepared by dissolving MES
or MOPS in DIUF water, titrated with 1 M NaOH solution to adjust to the targeted pH, and then diluted to a concentration of 0.01 M.

4.3 Results and Discussion

The use of both pH-responsive polymers and Gd nanoparticles in the preparation of pH-responsive MRI CAs has tremendous potential to make an effective pH-responsive MRI CA due to several advantages. Firstly, the pH-responsive polymer can stabilize the Gd nanoparticles and provide the biocompatibility by properly designing the copolymer attached to the nanoparticle. Secondly, the water accessibility can be changed with a transition between hydrophilic and hydrophobic of the pH-responsive polymer chain upon the physiological pH changing. Thirdly, while the tumbling rate of nanoparticles is constant due to the relatively large size, the tumbling rate of the water molecules can be affected by both the random coil structure of the polymer and the conformational change in the polymer with pH changes. Lastly, but importantly, the surface of the nanoparticles can also be modified to provide further functionality, such as targeting agents, or fluorescent agents, to make targeted or multimodal imaging agents, respectively. By observing the relaxivity changes caused by pH-responsive CAs, it is possible to determine the tissue pH via MRI. Herein, the tertiary amine-based methacrylate pH-responsive polymers were chosen due to their pKas are reported to be in the range of 6.0 to 7.0 which are close to the slightly acidic microenvironment of tumors.[87] The GdNPs were chosen in this research because they have been reported to have much high $r_1$ values (a typical $r_1$ is 35.8 s$^{-1}$ mM$^{-1}$ with a size of 100 nm in length and 40 nm in width at 3 T MRI scanner) than both clinically used Gd-chelates and the aforementioned Gd-based
nanoparticles.[162] Despite the reported research work in the areas of tertiary amine-based pH-responsive polymers and GdNPs,[12, 23, 87, 163] to the best of our knowledge the combination of these systems to produce a pH-responsive MRI CA has not been reported in literature.

4.3.1 Synthesis of tertiary amine-based pH-responsive block copolymers

pH-Responsive polymers have the potential to be invaluable in targeted anticancer drug delivery and diagnosis based on the intrinsic pH differences between pathological tissues and the surrounding normal tissues.[15, 70, 72, 164, 165] In our research, block copolymers consisting of a biocompatible block, PEG, and a pH-responsive block, tertiary amine-based methacrylates, were prepared. Initially, a PEG macro-RAFT agent was made via an esterification reaction between mono-hydroxyl functional PEG and the RAFT agent 4-cyanopentanoic acid dithiobenzoate (CPAD). Then, well-defined tertiary amine-based pH-responsive block copolymers were synthesized via RAFT polymerization using the PEG-CPAD macro-RAFT agent. $^1$H NMR and gel permeation chromatography (GPC) results confirmed the successful synthesis of these block copolymers. The pKa titration experiments demonstrated that the pKa value for PEG-b-PDPAEMA was approximately 6.4 and approximately 6.8 for PEG-b-PPPDEMA. The DLS investigation showed that, as expected, all of the block copolymers underwent a sharp transition from unimers to micelles around their pKa. In this chapter, PEG-b-PPPDEMA and PEG-b-PDPAEMA were chosen to modify the surface of the GdNPs due to their pKa values providing the optimal overlap with the
documented tumor pH range of 6.2-6.8.[4, 5] Detailed synthesis and characterization information for these block copolymers can be found in Chapter 3.

SCHEME 4.1 General synthesis route of pH-responsive polymer modified GdNPs and the predicted conformational changes of pH-responsive block copolymer chains on the surface of the GdNPs upon the pH changing.

The use of RAFT polymerization in this research not only provides the advantages of controlled molecular weights and narrow molecular weight distributions, but also results in the synthesized polymers having a thiocarbonylthio end group, which can be used for surface modification of nanoparticles, upon reduction to a thiolate end group.[120-124] Using the PEG-CPAD macro-RAFT agent to prepare the pH-responsive block copolymers resulted in the thiocarbonylthio group attached to the end of the pH-responsive block (Scheme 4.1). Therefore, when these polymers are used for surface modification of nanoparticles, the pH-responsive block will be located closer to the surface of the particles. This allows the PEG block to stabilize the particles in aqueous solution, while the hydrophilic to hydrophobic transition of the pH-responsive block can be used to manipulate the surface properties.
4.3.2 Surface modification of GdNPs with pH-responsive block copolymers

The Gd nanoparticles used in this research were synthesized via a reverse microemulsion procedure developed by Reiter and coworkers and were reported to have a nanoscale metal-organic framework (MOF) structure with the general composition of Gd(1,4-BDC)$_{1.5}$(H$_2$O)$_2$.[12, 162] Synthesis conditions were chosen based on the research work previously performed by our group[13] to provide GdNPs with an average length of 136±30 nm and width of 31±7 nm, as determined from TEM images (Figure 4.2). GdNPs with this rod-like shape and size were chosen as it has been demonstrated that nanoparticles with these properties are taken up preferentially through the enhanced permeation and retention (EPR) effect by many tumors.[122, 166, 167] The FTIR spectrum of the unmodified GdNPs (Figure 4.3) showed that the nanoparticle structure consisted of the Gd$^{3+}$ ions bridged by 1,4-BDC, as evidenced by the characteristic stretches of the carboxylate and aromatic ring of the 1,4-BDC bridging ligand at 1395 cm$^{-1}$, 1538 cm$^{-1}$, 3065 cm$^{-1}$, and also the coordinate water at 3460 cm$^{-1}$. TGA and XRD results also confirmed the Gd MOF structure as they corresponded well to the literature reported data (Appendix B Figure 1 and 2).[145, 162]

![FIGURE 4.2 TEM images of GdNPs with the general composition of Gd(1,4-BDC)$_{1.5}$(H$_2$O)$_2$ at different scales.](image)
FIGURE 4.3 FTIR spectra of the GdNPs, free PEG-b-PDPAEMA, and the PEG-b-PDPAEMA modified GdNPs. The black arrows indicate the carbonyl stretch at 1730 cm$^{-1}$ and methylene stretches from 2810-3000 cm$^{-1}$ which are attributed to PEG-b-PDPAEMA attached to the GdNPs.

FIGURE 4.4 FTIR spectra of the unmodified and polymer modified GdNPs
FIGURE 4.5 TEM images of PEG-b-PDP-M-GdNPs (a&amp;b), PEG-b-PPD-M-GdNPs (c&amp;d), and Combined-polymer-M-GdNPs (e&amp;f) at different scales.
In an effort to develop a pH-responsive MRI CAs, the pH-responsive block copolymers were used to modify the GdNPs (Scheme 4.1). Three polymer modified GdNPs samples were prepared, including PEG-b-PDPAEMA modified GdNPs (PEG-b-PDP-M-GdNPs), PEG-b-PPPDEMA modified GdNPs (PEG-b-PPD-M-GdNPs), and GdNPs modified with both PEG-b-PDPAEMA and PEG-b-PPPDEMA combined at a mass ratio of 1:1 (Combined-polymer-M-GdNPs). The successful modification of the GdNPs was demonstrated by four different techniques. Firstly, qualitatively, better dispersibility of the GdNPs in aqueous solution was observed after polymer modification. This is believed to be due to the existence of the hydrophilic PEG block on the surface of the GdNPs. Secondly, by comparing the FTIR spectra of the polymer modified and unmodified GdNPs (Figure 4.3), it was concluded that polymer was attached to the Gd NPs after the reduction of thiocarbonylthio end groups of the polymer to a thiolate. In the FTIR spectrum of the polymer modified GdNPs, a carbonyl stretch at 1730 cm\(^{-1}\) and methylene stretches from 2810-3000 cm\(^{-1}\) are attributed to the attached PEG-b-PDPAEMA, as they correspond to the same peaks in the free polymer. Similar results were observed with the other two polymer modified GdNPs samples (Figure 4.4). Thirdly, from TEM images (Figure 4.5), the existence of polymer on the surface of the GdNPs was observed, although it is hard to clearly distinguish the boundaries between the polymer and the GdNP due to their similar electron densities. Finally, TGA results showed a higher percent of weight loss for the polymer modified GdNPs between 200 °C and 475 °C when compared to the unmodified GdNPs (Figure 4.6). It is believed that this is due to the
attached polymer as it is the typical weight loss range for the polymers in TGA (Appendix B Figure 3).

FIGURE 4.6 TGA curve of unmodified and the polymer modified GdNPs

The grafting density is a critical parameter to determine the amount of polymer attached to the surface and, thus, the surface coverage of the polymer. As previously demonstrated by our group,[145] a relatively high grafting density can be reached by using RAFT polymers to modify the surface of the GdNPs, when compared to other ‘grafting to’ methods, which mostly generate values less than 0.1 chains nm\(^{-2}\). The grafting density (\(\sigma, \text{chain nm}^{-2}\)) for the different samples in this research were determined to be, 0.1 chains nm\(^{-2}\) for the PEG-b-PDP-M-GdNPs, 0.2 chains nm\(^{-2}\) for the PEG-b-PPD-M-GdNPs and 0.3 chains nm\(^{-2}\) for the Combined-polymer-M-GdNPs. They were calculated using equation 4.1,[168] where \(N_A\) is Avagadro’s number, \(m_{\text{polymer}}\) is the mass of polymer determined by TGA, \(M_{n,\text{polymer}}\) is the molecular weight of polymer determined by gel permeation chromatography (GPC),

\[ \sigma = \frac{m_{\text{polymer}}}{M_{n,\text{polymer}} N_A} \]
\( \rho_{\text{GdNPs}} \) is the bulk density of the GdNPs which was determined to be 2.529 g cm\(^{-3} \).[145] \( m_{\text{GdNPs}} \) is the mass of the GdNPs determined by TGA, \( V_{\text{GdNPs}} \) is the volume of an average GdNPs, and \( S_{\text{GdNPs}} \) is the average surface area of a GdNP. \( V_{\text{GdNPs}} \) and \( S_{\text{GdNPs}} \) were calculated based on a ‘rod-like’ GdNP with an average diameter of 31 nm and length of 136 nm, as determined by TEM, in this research.

\[
\sigma = \frac{m_{\text{polymer}} \cdot N_A}{M_n_{\text{polymer}}} \cdot \frac{\rho_{\text{GdNPs}} \cdot V_{\text{GdNPs}}}{m_{\text{GdNPs}} \cdot S_{\text{GdNPs}}}
\]  

(4.1)

<table>
<thead>
<tr>
<th>No.</th>
<th>Block Copolymer</th>
<th>( M_n ), g/mol</th>
<th>( \sigma ), chain/nm(^2)</th>
<th>Relaxivity ( (r_1) ), s(^{-1}) mM(^{-1}) under differ. pH</th>
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<tbody>
<tr>
<td>1</td>
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<td>N/A</td>
<td>0</td>
<td>9.0, 8.7, N/A, 8.7, 8.7</td>
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<td>0.1</td>
<td>9.7, 9.6, 7.9, 7.4, N/A</td>
</tr>
<tr>
<td>3</td>
<td>PEG-b-PPPDEMA</td>
<td>23000</td>
<td>0.2</td>
<td>11.3, 10.9, 10.7, 7.6, 7.4</td>
</tr>
<tr>
<td>4</td>
<td>PEG-b-PDPAEMA</td>
<td>13900</td>
<td>0.3</td>
<td>11.9, 11.7, 9.0, 8.3, 6.6</td>
</tr>
<tr>
<td></td>
<td>PEG-b-PPPDEMA</td>
<td>15400</td>
<td></td>
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</table>

In order to further confirm the successful polymer modification of the GdNPs, the zeta potential of the unmodified GdNPs and the polymer modified GdNPs was measured by DLS to evaluate the charges on the particles surface. As seen in Figure 4.7, the zeta potential of the unmodified GdNPs was positive, mainly due to the Gd\(^{3+} \) at the surface of the nanoparticles. The positive charges had a slightly increase when the pH increased from 5.0 to 8.1, although this may be within error of the measurement. However, the zeta potential of PEG-b-PPPDEMA modified GdNPs had a much higher value than the unmodified GdNPs at low pH and the zeta potential decreased with increasing pH, which was expected and was evidence for the existence of PEG-b-PPPDEMA on the surface of the GdNPs. At low pH, the
tertiary amine groups of the PEG-b-PPPDEMA will be protonated and therefore the surface of the GdNPs is more positively charged. As the pH is increased, the tertiary amine groups of the block copolymer will start to be deprotonated and the surface of the GdNPs become less positively charged. A large decrease of zeta potential, from 21.1 mV to 10.5 mV, was observed for the PEG-b-PPD-M-GdNPs when the pH increased from 6.9 to 7.3. This corresponds well with the calculated pKa of PEG-b-PPPDEMA, which was 6.8±0.1. By summarizing the results of FTIR, TEM, TGA and zeta potential, we were certain that the GdNPs were successfully modified with tertiary amine-based pH-responsive block copolymers. The performance of these polymer modified GdNPs for use as pH-responsive MRI CA will now be evaluated in the following section.

FIGURE 4.7 Zeta potential of the unmodified GdNPs and the PEG-b-PPPDEMA modified GdNPs as a function of solution pH.
4.3.3 Evaluation of pH-responsive polymer modified GdNPs for use as MRI CAs using NMR experiments

The relaxivity of a pH-responsive MRI CA should change as the environmental pH changes. Ideally, the MRI CAs can be ‘switched on’ by lowering the pH below the pKa of the pH responsive block and ‘switched off’ by raising the pH above the pKa (as illustrated in Scheme 4.1) and this pH transition corresponds to physiological relevant pH values. In our case, the degree of ionization of the pH-responsive block increases by lowering the pH below the pKa and the polymer chain becomes hydrophilic. This will allow more water molecules to access the surface of the GdNPs, resulting in enhanced relaxivity and better performance as a MRI CA. In contrast, when raising the pH above the pKa, the pH-responsive block of the copolymer will become hydrophobic on the surface of the GdNP and hinder water access to the surface. This results in less water interacting with the Gd and the relaxivity of the CA decreases, along with the performance of the system as a MRI CA. Therefore, when pH-responsive CAs are accumulated in the tumor tissue with a low pH, which is comparable to the pKa of the pH-responsive polymers, they will be ‘switched on’ and result in a higher relaxivity value. In contrast, when the pH-responsive CAs are circulating in the blood stream or interact with normal tissues, in which typical pH value is approximately 7.4, they are ‘switched off’, which results in a lower relaxivity value.

The longitudinal relaxivity of a CA is defined as the slope of a plot of \((1/T_1)_{\text{obs}}\) versus concentration of CA, and indicates how much the relaxation rate of water protons is increased with the increasing concentration of the CA. In order to determine the relaxivity of the polymer
modified and unmodified GdNPs at different pH values, nanoparticles were dispersed in MES or MOPS buffer solutions with different pH to reach a concentration in the range of 0-0.1mM of Gd. The MES buffer solutions, with a buffer region of 5.5-6.7, and the MOPS buffer solutions, with a buffer region of 6.5-7.9, are members of Good’s buffers, and are widely used in biochemical and biological research because their pKa is close to physiological pH and they have especially low affinities for metal ions.[169-171]

FIGURE 4.8 TEM images of unmodified GdNPs (a&b) and Combined-polymer-M-GdNPs (c&d) in MES 5.9 (a&c) and MOPS 7.1 (b&d) buffer solutions.

It is important to note that a critical factor in the *in vivo* use of any nanoparticle system is the chemical stability of the nanoparticle. This was the primary reason that the MES and MOPS buffer systems were chosen, as they should prevent coordination between the Gd$^{3+}$ ions and the buffer, enhancing the stability of the GdNPs. To verify this, TEM was used to monitor the stability of the GdNPs in the buffer solutions. As can be seen from Figure 4.8, no significant
changes were observed for the GdNPs, both polymer modified and unmodified, after being suspended in buffer solution for 72 h. Due to the importance of this topic, the stability of the GdNPs in different pH solutions will be discussed more in Chapter 5.

To determine the $r_1$ values for the unmodified and polymer modified GdNPs at different pH values, the longitudinal relaxation times of the water protons in each sample were measured on a 400 MHz (9.4 T) NMR and demonstrated a decreasing trend with increasing GdNPs concentration. The $r_1$ values of each sample were collected from the slopes of a linear fit of the different sets data (Table 4.1 and Figure 4.9). A typical set of relaxation times for unmodified GdNPs in MES at pH 5.9 with different concentrations is as follows: 1986 ms for 0.014 mM, 1396 ms for 0.036 mM, and 1126 ms for 0.057 mM, and were used to determine the $r_1$ value of the unmodified GdNPs at pH 5.9 to be 9.0 s$^{-1}$ mM$^{-1}$. As seen from Figure 4.9, while the $r_1$ values of the unmodified GdNPs showed no significant change with increasing pH, the $r_1$ values of polymer modified GdNPs all had a decreasing trend with increasing pH, with the most dramatic decrease after their pKa. For example, for the PEG-b-PDPAEMA, with a pKa of 6.4±0.1, modified GdNPs, the $r_1$ decreased by 1.5 s$^{-1}$ mM$^{-1}$ when the pH increased from 6.6 to 6.8, but barely decreased when pH increased from 6.8 to 7.1. This can be attributed to the hydrophilic to hydrophobic change in the polymer chains of pH-responsive block. With the increasing pH, the tertiary amine groups on the polymer chains become deprotonated, which eventually result in the polymer chain becoming hydrophobic and collapsing on the surface of the GdNPs. Furthermore, while the $r_1$ values of the PEG-b-PDP-M-GdNPs and PEG-b-PPP-M-GdNPs only showed one large change, the $r_1$ values of the
Combined-polymer-M-GdNPs had two significant decreases. The decreased relaxivity in the pH range of 6.6 to 6.8 is mostly attributed to the hydrophilic to hydrophobic change from PEG-b-PDPAEMA and the decrease in the pH range of 6.8 to 7.3 is mostly attributed to the conformation change of PEG-b-PPPDEMA. As discussed in Chapter 3, in solution the free polymer chains formed micelles when the solution pH was above their pKa due to formation of a hydrophobic block. However, the result obtained here suggested that the sharp change of relaxivity caused by the conformation change was slightly delayed when the polymer was attached to the nanoparticles, if considering the pKa of PEG-b-PDPAEMA is 6.4±0.1 and the largest change in relaxivity for GdNPs modified with this polymer was observed between 6.6 and 6.8. A similar tendency was observed for the PEG-b-PPD-M-GdNPs, in which the relaxivity dramatically decreased when pH increased from 6.8 to 7.1, but decreased more from 7.1 to 7.3. We propose two possible explanations for these observations: The first one is that the dissociation constant of the polymer chain has changed because one end of the polymer chain is tethered on the surface of the GdNPs, resulting in a slightly higher pKa due to a localized high concentration of chains at the surface of the nanoparticles. The second possible explanation is that the polymer chains undergo a different conformational change on the surface right after the pKa due to the high grafting density and interactions between the chains on the surface. Further investigation is required to differentiate between these explanations or determine other possible explanations and to fully understand these observations.
FIGURE 4.9 The longitudinal relaxivity ($r_1$) of the unmodified GdNPs and polymer modified GdNPs as a function of solution pH.

As mentioned previously, pH-responsive MRI CA are significantly different than conventional MRI CAs in that, while high relaxivity values are desirable, the critical property of a pH-responsive MRI CA is the relative change in relaxivity on exposure to changes in the pH of the surrounding environment. It can be observed from Figure 4.9 that the relaxivities for all of the polymer modified GdNPs at low pH has a higher value than unmodified GdNPs. Further, when comparing the polymer modified GdNPs, the relaxivity increased with the increasing of the graft density (Table 4.1 and Figure 4.9). Both of these results were expected in this case because, at low pH, the pH-responsive block copolymers are completely hydrophilic, which, as previously reported, increased the water retention time on the surface of the GdNPs due to changes in the tumbling rate of water in the polymer chain.[145] The increased water retention and change in tumbling rate allow for faster exchanging between the
excited water protons and relaxed water protons. In contrast, when the solution pH increased above the pKa of the pH-responsive block copolymers, the pH-responsive polymer chain directly tethered on the surface of GdNPs becomes hydrophobic, which will decrease the water retention time and the concentration of water at the surface. However, due to the effects of the surface immobilization of polymers on their pKa mentioned above, this effect is more complex in this case and the relaxivity values at high pH do not drop to zero.

Finally, it should be noted that T₁ values for MRI CAs have been reported to have a high sensitivity to the field strength and that the proton relaxation rates inherently decrease with increasing magnetic field strength.[39] In this work, the relaxation time was collected on a 400 MHz (9.4 Telsa) NMR, which is significantly higher than most clinically used MRI scanners (0.47-4.7 T). Therefore it is reasonable to expect that these modified GdNPs will give a higher relaxivity and provide larger differences under a lower magnetic field. Further study to confirm this will be continued in our group.

4.4 Conclusions

Due to the lack of clinical methods for the in vivo measurement of pH in solid tumors, new MRI CA platforms containing the required chemistry and material properties need to be developed. In this research, both of the advantages of RAFT synthesized pH-responsive polymers and GdNPs with a high relaxivity value are taken to design a new pH-responsive MRI CA that is able to exhibit relaxivity changes upon changes in the pH at the physiological values. By reducing the RAFT agent functional end group of synthesized polymers to a thiolate, well-defined tertiary amine-based pH-responsive block copolymers were successfully grafted
to the GdNPs with an average length of 136 nm and width of 31 nm. The successful modification of the GdNPs with pH-responsive polymers was demonstrated using TEM, FTIR, TGA and DLS. The relaxation times of water protons in the presence of the pH-responsive polymer modified GdNPs were measured by NMR to study the relaxivity changes with changes in the environmental pH. Results showed that the relaxivity decreased with increasing solution pH due to the conformation changes of the pH-responsive block copolymers. By increasing the grafting density on the surface of GdNPs, the relaxivity was enhanced due to the increasing water retention and changes in the water tumbling rate. These results suggest that it is possible to use these polymers modified GdNPs as pH-responsive MRI CAs to build a relationship between relaxivity and environmental pH.
CHAPTER 5

STABILITY STUDY OF GDNPS IN PH SOLUTIONS

5.1 Introduction

Gadolinium(Gd)-based contrast agents (CAs) have received considerable research attention since they are the most widely used clinical positive contrast agents in magnetic resonance imaging (MRI), which is one of the most important medical imaging techniques in cancer diagnosis.[36, 46, 48, 49] Gd-based CAs can produce a large shortening of the longitudinal relaxation time ($T_1$) and a high longitudinal relaxivity ($r_1$) because the Gd$^{3+}$ ion has a high magnetic moment and a long electronic relaxation time.[36] However, due to the high toxicity of Gd$^{3+}$, Gd ions must be chelated for use in humans. Since 1988, five different Gd chelates have been approved for use as MRI CAs by the Food and Drug Administration (FDA) in the US.[39, 42] These Gd-based CAs were proved to be safe and well-tolerated in patients by numerous clinical cases and extensive clinical experiments. However, in 2006, Grobner was the first to propose a possible correlation between the administrations of Gd chelates and nephrogenic systemic fibrosis (NSF), which occurs in patients with severe renal impairment.[172] A prevailing theory for this correlation proposes that the free Gd$^{3+}$, released from the CAs by dissociation of the Gd-based chelate, is a trigger for NSF.[173] Justifiably, the potential correlation between Gd-based CAs and the development of NSF in patients with end stage renal disease (ESRD) is a serious concern and has resulted in significant research in this area in recent years[173-176]. This research has highlighted two critical findings that must be
considered in the clinical use of Gd-based CAs and for potential research in this area. Firstly, the published literature clearly documents that patients with ESRD are at the highest risk to develop NSF.[173] Approximately 80% of patients that have developed NSF have ESRD with the remainder having stage 5 renal failure. It is critical to note that there have been no instances of NSF in patients without renal disease nor with patients with stages 1 through 3 renal disease.[177] The second critical finding is the development of NSF after exposure to Gd-based CAs is dependent on the chelating ligand.[174, 178-182] With related clinical cases and studies reported, the FDA issued a public health advisory to warn patients and physicians that some Gd chelates had the potential to cause NSF for patients with renal dysfunction.[183-185] Therefore, the determination of Gd complex stability and the development of stable, biocompatible complexes of Gd$^{3+}$ have increasingly become a primary focus from both a research and a clinical point of view.

\[
\text{GdL}^n \rightleftharpoons \text{Gd}^{3+} + \text{L}^{n-3} \quad (5.1)
\]

\[
\text{GdL}^n + \text{M}^{m+} \rightleftharpoons \text{Gd}^{3+} + \text{ML}^{n-3+m} \quad (5.2)
\]

\[
\text{GdL}^n + \text{L}_e^{m-3} \rightleftharpoons \text{GdL}_e^m + \text{L}^{n-3} \quad (5.3)
\]

There are three mechanisms reported in the literature to describe the dissociation of Gd and its ligands.[184, 186, 187] The first mechanism corresponds to the spontaneous or proton-assisted dissociation of the Gd$^{3+}$ complex, possibly catalyzed by endogenous metals (Equation 5.1).[188, 189] It has been demonstrated by Brucher that the rates of dissociation of Gd chelates are much faster in acidic solutions compared to pH 7.4.[190] However, it has also
been suggested that the proton-assisted dissociation plays a negligible role under typical physiological conditions.[188]

The second one is a transmetallation mechanism of the Gd chelate by endogenous metals, including Fe$^{3+}$, Ca$^{2+}$, Zn$^{2+}$ and Cu$^{2+}$ (Equation 5.3).[191] The in vitro studies performed by Sarka and coworkers suggested that the dissociation of Gd chelates occurred mostly by reactions with the exchangeable Cu$^{2+}$ and Zn$^{2+}$ in body fluids by a transmetallation mechanism.[188] Laurent and coworkers have pointed out that Zn$^{2+}$ is the most likely competing metal ion in endogenous systems for Gd chelates and proposed a relaxometric protocol for the study of transmetallation of a series of linear and macrocyclic Gd complexes by Zn$^{2+}$.[192] However, Idee and coworkers believe that transmetallation between Gd$^{3+}$ and Zn$^{2+}$ is unlikely due to the higher thermodynamical stability constant of Gd/L (L=Ligand) compared to that of Zn/L.[193] They hypothesized that the clinically reported zinc deficiency caused by administration of CAs is due to the excess free ligands added in the Gd chelate solutions to ensure the absence of free Gd$^{3+}$. Firstly, the zinc present in the body may have chelated with the additional free ligands. Secondly, for the patients with renal dysfunction, CAs will cause a transmetallation between Zn$^{2+}$ and Ca$^{2+}$ in the excipient calcium based chelates due to a higher thermodynamic constant for Zn/L than that of Ca/L. Rabiet and coworkers demonstrated that Fe$^{3+}$ and Cu$^{2+}$ have a strong affinity with the diethylenetriaminepentaacetic acid (DTPA) ligand commonly used in Gd chelates, and are able to dissociate Gd-DTPA, resulting in the release of free Gd$^{3+}$ in water, while Zn has a lower influence on the dissociation of Gd-DTPA.[191] They also showed that the level of
displacement largely depended on the concentration of competing ions. The release of free Gd\(^{3+}\) was caused when Cu\(^{2+}\) was present in a fivefold excess or Fe\(^{3+}\) in an equimolar or excess to Gd\(^{3+}\). However, Cu\(^{2+}\) is present in a very small concentration in the blood (1-10 \(\mu\)M) and the majority of Fe\(^{3+}\) is stably bound in ferritin and hemosiderin. Therefore the transmetallation is less likely to occur in the body for these two metal ions due to their low concentrations.[183, 192]

The third mechanism involves a ligand exchange with the competing anions such as PO\(_4\)\(^{3-}\) or CO\(_3\)\(^{2-}\) (Equation 5.3).[182, 194] Caroline and coworkers demonstrated that phosphate could result in Gd\(^{3+}\) release by ligand exchange when a Gd/phosphate ratio of 10\(^{-2}\) was reached in the buffer solution of Omniscan® (Gd-DTPA-BMA, BMA=bismethylamide).[194] They also found that the presence of endogenous cations such as Ca\(^{2+}\), Zn\(^{2+}\) or Cu\(^{2+}\) accelerated the kinetics of Gd release, which could result from catalyzing ligand exchange or inducing a transmetallation mechanism. Tweedle et al. found that Zn\(^{2+}\) and Cu\(^{2+}\) reacted with Gd(EDTA)\(^{-}\) (EDTA=ethylenediaminetetraacetic acid) and Gd(DTPA)\(^{2-}\) in the presence of phosphate to yield precipitated GdPO\(_4\) and chelated Cu/L.[195] They also concluded that the high thermodynamic stability constants of the chelates determined in water are neither necessary nor sufficient to indicate \textit{in vivo} stability, while kinetic inertia is a sufficient criteria.

These three mechanisms are described based on the small molecule Gd chelates. However, stability studies on Gd-based nanoparticles (GdNPs) are limited due to the fact that nanoparticle-based CAs are still in the early stage of development. Therefore, it is important that the stability of these nanoparticles systems is investigated if they are ever to reach
clinical use. In this research, the GdNPs used have a metal organic frame (MOF) structure with the general composition of Gd(1,4-BDC)\(_{1.5}(H_2O)_2\), which has chemical similarities to the Gd-chelates. In addition, the GdNPs are designed to be used as pH-responsive CAs, exposing them to different physiological pH’s. Therefore, it is critical to conduct stability studies of the GdNPs in pH solutions. However, the stability study of any system is a complex topic. As such, the primary focus for this chapter is on finding different pH solutions that are suitable to use for the in vitro evaluation of the performance of the GdNPs. In another word, the unmodified and polymer modified GdNPs will maintain in a good shape and size under the desired pH conditions. In this chapter, GdNPs were synthesized following the same procedure discussed in Chapter 4 and dispersed in solutions with different pH. The structure changes of the GdNPs after being treated with these solutions were then studied by transmission electron microscopy (TEM), fourier transform infrared (FTIR) spectroscopy and X-ray diffraction (XRD).

5.2 Experimental

The following sections introduced materials used in this Chapter and described the relevant experiments and characterization techniques used.

5.2.1 Materials.

Cetyltrimethylammonium bromide (CTAB), gadolinium chloride hexahydrate (GdCl\(_3\cdot6H_2O\)), terephthalic acid, 1-hexanol, methylamine (40 wt% in water), sodium salicylate (NaSal), hexylamine, phosphoric acid, citric acid, 4-morpholineethanesulfonic acid (MES), 4-morpholinepropanesulfonic acid (MOPS), tris(hydroxymethyl)aminomethane (TRIS),
1,4-piperazinediethanesulfonic acid (PIPES) were purchased from Aldrich and used without further purification. Heptane, hydrochloric acid, sodium hydroxide, sodium chloride, potassium chloride, and ethanol (EtOH) were purchased from Macron Fine Chemicals. Sulfuric acid was purchased from EMD Millipore. Potassium phosphate monobasic, sodium phosphate dibasic, sodium phosphate monobasic, and calcium chloride were purchased from Fisher. Zinc chloride and ammonium phosphate dibasic were purchased from Fluka. Ammonium phosphate monobasic was purchased from J.T. Baker.

Gd nanoparticles (GdNPs) were synthesized via a reverse microemulsion system developed by Reiter and coworkers and modified by our group.[13, 162] Synthesis conditions were chosen to provide GdNPs with an average length of 136±30 nm and width of 31±7 nm. Detailed synthesis and characterization information is included in Chapter 4.

5.2.2 Instrumentation and characterization.

Transmission electron microscopy (TEM) was performed on a Philips CM200 with an accelerating voltage of 120kV for GdNPs. A Keen View Soft Imaging System coupled to iTEM Universal TEM Imaging Platform Software was utilized to acquire images. Powder X-ray diffraction (XRD) intensity data was collected on a Bruker D2 phaser diffractometer using CuKα radiation. Fourier transform infrared (FTIR) spectra were collected using a Nicolet iS50 FT-IR spectrometer (Thermo Scientific) operating with a liquid-nitrogen-cooled mercury cadmium telluride detector. The concentration of GdNPs in dialysate was acquired on a Perkin-Elmer Optima 3000 inductively coupled plasma atomic emission spectroscopy (ICP-AES) instrument following the EPA 200.7 standardized method. The instrument was
calibrated with an internal scandium standard and recalibrated if there was greater than 20% drift from 50 ppm concentration. Samples were diluted in 2% nitric acid solution and run against an internal quality control Gd standard from High Purity Standards.

5.2.3 Preparation of buffer solutions

Each buffer solution was prepared following the recipe in Table 5.1. For the same buffer solutions with different concentrations, the less concentrated solutions were prepared by dilution.

<table>
<thead>
<tr>
<th>Name</th>
<th>pH</th>
<th>Conc.</th>
<th>Buffer Recipe</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>7.5</td>
<td>10 mM</td>
<td>NaCl (0.8 g), KCl (0.02 g), Na$_2$HPO$_4$ (0.142 g), KH$_2$PO$_4$ (0.024 g), DIUF water (100 mL)</td>
</tr>
<tr>
<td>PCB</td>
<td>5.6</td>
<td>0.2 M</td>
<td>0.2 M Na$_2$HPO$_4$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.1 M citric acid</td>
</tr>
<tr>
<td></td>
<td>7.6</td>
<td>0.2 M</td>
<td>58 mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>42 mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>90 mL</td>
<td>10 mL</td>
</tr>
<tr>
<td>POB</td>
<td>5.8</td>
<td>0.1 M</td>
<td>0.2 M Na$_2$HPO$_4$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.2 M NaH$_2$PO$_4$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 mL</td>
<td>46 mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50 mL</td>
</tr>
<tr>
<td></td>
<td>7.2</td>
<td>0.1 M</td>
<td>36 mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>14 mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 mL</td>
<td>50 mL</td>
</tr>
<tr>
<td>APB</td>
<td>6.4</td>
<td>0.1 M</td>
<td>0.2 M (NH$_4$)$_2$HPO$_4$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.2 M NH$_4$H$_2$PO$_4$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16 mL</td>
<td>34 mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50 mL</td>
</tr>
<tr>
<td></td>
<td>7.3</td>
<td>0.1 M</td>
<td>40 mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10 mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 mL</td>
<td>50 mL</td>
</tr>
<tr>
<td>TRIS</td>
<td>7.4</td>
<td>0.1 M</td>
<td>Dissolve TRIS (1.21 g) in 50 mL DIUF, adjust pH with 1 M HCl, dilute to 100 mL with DIUF</td>
</tr>
<tr>
<td>MES</td>
<td>6.5</td>
<td>0.1 M</td>
<td>Dissolve MES (1.95 g) in 50 mL DIUF, adjust pH with 1 M NaOH, dilute to 100 mL with DIUF</td>
</tr>
<tr>
<td>MOPS</td>
<td>7.1</td>
<td>0.1 M</td>
<td>Dissolve MOPS (2.12 g) in 50 mL DIUF, adjust pH with 1 M NaOH, dilute to 100 mL with DIUF</td>
</tr>
<tr>
<td>PIPES</td>
<td>7.1</td>
<td>0.05 M</td>
<td>Dissolve PIPES (1.50 g) in 50 mL DIUF, adjust pH with 1 M NaOH, dilute to 100 mL with DIUF</td>
</tr>
</tbody>
</table>

5.2.4 Sample preparation for stability studies

One 800 mL batch of GdNPs was dispersed in 5 mL DIUF water to form a stock solution of GdNPs. All samples were prepared using a similar procedure from the stock solution. Initially,
0.1 mL of the stock solution was dispersed in 1.5 mL buffer solution and mixed well. After sitting for 2 h, the particles were collected by decanting the buffer solution and washed with DIUF water (2 mL) twice. The washed particles were re-dispersed in EtOH for preparing TEM samples. Then, the particles were dried for further characterization with FTIR or XRD.

5.2.5 Dialysis experiment

One 200 mL batch GdNPs was dispersed in 10 mL DIUF water to form a stock solution of GdNPs. Then 1 mL stock solution was dialyzed (molecular weight cut off of 3500 Da) against PBS solution (pH = 7.5, 10 mM) for 72 h with the aqueous dialysate solution being changed at 12 h and 48 h. Samples (5 mL) were taken at several time interval across the dialysis experiment (2 h, 12 h, 24 h, 48 h and 72 h). Another 3 congruent experiments were set to dialyze against PCB solution (pH = 7.6, 0.2 M), POB solution (pH= 7.2, 0.1 M) and APB solution (pH=7.3, 0.1 M), respectively, and followed similar protocols. The aqueous dialysate samples was then acidified in 2% nitric acid solution and used to determine the concentration of Gd$^{3+}$ leached to the dialysate from stock solution via ICP-AES.

5.3 Results and Discussion

In order to study the stability of GdNPs in different pH solutions, GdNPs were firstly synthesized and then dispersed in solutions with different pH, including aqueous solutions with HCl and NaOH, phosphate buffered saline (PBS), phosphate-citrate buffer (PCB), phosphate only buffered (POB), ammonium phosphate buffered (APB), tris(hydroxymethyl) aminomethane buffer (TRIS), and three Good’s buffers, 4-morpholineethanesulfonic acid (MES), 4-morpholinepropanesulfonic acid (MOPS), 1,4-piperazinediethanesulfonic acid
(PIPEC). These buffer solutions were chosen due to the fact that they are commonly used as buffer solutions in biological and biochemical research to imitate *in vivo* physiological situations. The results were discussed in the following sections.

### 5.3.1 GdNPs in aqueous solution of different pH adjusted with HCl and NaOH

![TEM images of the GdNPs in aqueous solution with different pH adjusted with HCl (0.1 M) and NaOH (0.1 M): (a) pH = 1, (b) pH = 3, (c) pH = 4.3, (d) pH = 5.0, (e) pH = 8.1, (f) pH = 11.](image)

**FIGURE 5.1** TEM images of the GdNPs in aqueous solution with different pH adjusted with HCl (0.1 M) and NaOH (0.1 M): (a) pH = 1, (b) pH = 3, (c) pH = 4.3, (d) pH = 5.0, (e) pH = 8.1, (f) pH = 11.

In order to study the stability of the GdNPs in different pH solutions, initially GdNPs were treated with aqueous solution of different pH using HCl and/or NaOH only. The aqueous
solutions with different pH were prepared by adding different amounts of HCl (0.1 M) and/or NaOH (0.1 M) into DIUF water to reach a pH range of 1.0-11.0. As seen from TEM images (Figure 5.1), the structures of the GdNPs were well maintained between pH of 3.0 to 11.0, for example, GdNPs after being treated with solution of pH 3 has an average length of 138±33 nm and width of 36±10 nm and GdNPs after being treated with solution of pH 8.1 has an average length of 150±45 nm and width of 40±10 nm, both of which have similar dimensions to the untreated GdNPs (length of 136±30 nm and width of 31±7 nm). However, when the pH dropped to 1.0, the nanoparticle structure was destroyed (Figure 5.1a), which was attributed to the proton-assisted dissociation of Gd$^{3+}$ and 1,4-BDC. The 1,4-BDC ligand, used in preparation of the GdNPs, becomes protonated when the solution pH is lower than its pKa of 3.5 and 4.8.[196] Since it is coordinated with Gd$^{3+}$, it requires a higher concentration of protons to disrupt the cation/ligand coordination and undergo the protonation reaction. This is probably why the structure of the GdNPs kept is still maintained at pH 3, but destroyed at pH 1. The protonation of the 1,4-BDC ligand at lower pH was confirmed by FTIR (Figure 5.2), by observing the formation of carboxylic acid groups, with characteristic stretches at 1280 cm$^{-1}$, 1675 cm$^{-1}$ and 2500-3100 cm$^{-1}$, and the disappearance of characteristic carboxylate stretches of carboxylate at 1395 cm$^{-1}$ and 1538 cm$^{-1}$, whereas the spectra of GdNPs at pH 5.0 and 8.1 remained the same as the untreated GdNPs. Although the GdNPs were proved to be stable after being treated with aqueous solutions with pH range in 3-11, the aqueous solution system is not the best solution system to disperse the GdNPs for the evaluation of CAs’ performance and also does not mimic in vivo conditions. In addition, as the pH of the aqueous solution is
hard to keep constant with just HCL and NaOH in the system to balance the pH, buffer agents will be need to prepare the solutions with constant pH.

FIGURE 5.2 FTIR spectra of GdNPs and the GdNPs treated with aqueous solution of HCl and/or NaOH.
FIGURE 5.3 TEM images of the GdNPs after being treated with different aqueous buffer solutions: 0.2 M PCB solutions: pH = 5.6 (a) and pH = 7.6 (b); 0.1 M POB solutions: pH = 5.8 (c) and pH = 7.2 (d); 0.1 M APB solution: pH = 6.4 (e) and pH = 7.3 (f); pH = 7.5 PBS solutions: 10 mM (g) and 1 mM (h).
5.3.2 GdNPs in phosphate contained buffer solutions

Phosphate buffer solutions have been widely used in literature to balance the solution pH for studying the pH-responsive properties of many different systems. However, as discussed in literature, phosphate has a strong affinity to most polyvalent cations and, as such, can form GdPO$_4$ with small Gd-chelates by a ligand exchange mechanism.[194, 197] Therefore, the structure of the GdNPs might change in the phosphate based buffer solution. To investigate this, GdNPs were treated with four different phosphate buffers, including PCB, POB, APB and PBS with a pH ranging from 5.6-7.6 and a phosphate ion concentration ranging from 0.2 M to 1 mM. This pH range was chosen as it closely matches that of the extracellular pH (pHe) of tumors and also was shown to produce stable nanoparticles structure in the previous aqueous study. The structures of the GdNPs in different phosphate based buffers were characterized by TEM. As seen from Figures 5.3, the structures of the nanoparticles were all significantly changed after the GdNPs were treated with the different phosphate buffer solutions. While the GdNPs treated with PCB solutions appeared to form new nanoparticles with a cross-linked network composed of short rod-like shaped (Figure 5.3a&b), GdNPs treated with POB solutions favored the formation of long needle-like shaped particles (Figure 5.3c&d). The presence of APB solutions seemed to help the formed particles to aggregate together (Figure5.3e&f). When the concentration of phosphate ions decreased to 1 mM, TEM figures demonstrated that some of the original GdNPs were still present in the system, in combination with big rod-like nanoparticles (Figure 5.3h).
FIGURE 5.4 FTIR spectra of untreated GdNPs and GdNPs treated with PBS solutions at different concentrations.

FIGURE 5.5 FTIR spectra of the GdNPs treated with different phosphate based buffer solutions.
The FTIR spectra (Figure 5.4&5.5) of the treated GdNPs matched the TEM results. For PBS 1 mM, the characteristic stretches of intermolecular water, which is coordinated to Gd$^{3+}$ ion, at 3460 cm$^{-1}$, the strong characteristic stretches of carboxylate at 1395 cm$^{-1}$ and 1538 cm$^{-1}$, and the characteristic stretch of phosphate at 1020 cm$^{-1}$ were all observed in the FTIR spectrum. For all the other samples being treated with 10 mM PBS, PCB, POB and APB solutions, the FTIR spectra all showed a strong phosphate stretch and a weak carboxylate stretch (Figure 5.4&5.5). It should be noted that the samples were washed and dried before the characterization, thus the characteristic groups observed in FTIR spectra are attributed to the carboxylate or phosphate groups coordinated to Gd since the free phosphate or carboxylate in the solution will be washed away in the sample preparation.

FIGURE 5.6 XRD spectra of untreated GdNPs and GdNPs treated with 10 mM PBS solution.

XRD spectra also showed that the samples treated with 10 mM PBS solution lost the crystalline structure that was observed in the untreated GdNPs with a rod-like shape and
average length of 138±33 nm and width of 36±10 nm (Figure 5.6). These results suggested that
the original structure of GdNPs with a composition of Gd(1,4-BDC)_{1.5}(H_{2}O)_{2} is destroyed
when the concentration of the phosphate ion in solution is higher than 1 mM, possibly via a
ligand exchange mechanism.

FIGURE 5.7 (a) TEM image of nanoparticles collected by mixing 1 mM GdCl_{3} and 1 mM
Na_{2}HPO_{4} and TEM images of the GdNPs after being treated with different aqueous solution:
(b) 0.1 M NaH_{2}PO_{4}, pH = 4.5, (c) 0.1 M Na_{2}HPO_{4}, pH = 8.9, (d) 0.1 M citric acid, pH = 2.1,
(e) H_{3}PO_{4} solution, pH = 3.4, (f) H_{2}SO_{4} solution, pH = 3.1.

To further confirm that the phosphate ions were responsible for the observed changes in
TEM images, two more experiments were performed. The first one was to mix a 1 mM GdCl_{3}
solution and a 1 mM Na$_2$HPO$_4$ solution, which is documented as a generic method for fabrication Gd phosphate.[198] The solution turned from clear to cloudy once the two solutions were mixed and the formed precipitate in this system was gadolinium phosphate compound in a hydrated state. As seen from TEM images (Figure 5.7a), the Gd phosphate precipitate formed in this system has a long needle like shape which is similar to the observed shape of nanoparticles in TEM images of GdNPs treated with low concentration phosphate buffer solution (Figure 5.3 c&d).

After knowing the free Gd ions in solution will form precipitate with phosphate ions in solution, the second set of experiments was designed to investigate whether the coordinated Gd$^{3+}$ in the GdNPs will react with phosphate ions in solution and if it does react, are there differences in the shape of formed nanoparticles by the existence of different anions which are used in the preparation of phosphate buffers. Therefore, the GdNPs were treated with 0.1 M NaH$_2$PO$_4$ (pH = 4.5), 0.1 M Na$_2$HPO$_4$ (pH = 8.9), 0.1 M citric acid solution (pH = 2.1), respectively. TEM images of the treated GdNPs are included in Figure 5.7. Nanoparticles with a cross-linked net shape or a long needle shape were observed in all of the TEM images for particles treated with the phosphate solutions and were not observed for particles treated with citric acid solution. However, the structure of the GdNPs did change after being treated with 0.1 M citric acid solution (pH = 2.1). FTIR spectrum of the citric acid treated GdNPs showed the formation of carboxylic acid, the same as in the FTIR results of the GdNPs sample treated with 0.1 M HCl solution (Figure 5.8), in which the change was attributed to the proton-assisted dissociation of the Gd$^{3+}$ and 1,4-BDC due to a high concentration of protons existed in
solution. Further, when the GdNPs were treated with a less concentrated citric acid (pH = 3.0), the FTIR spectrum showed the presence of both carboxylate and carboxylic acid (Figure 5.8).

![FTIR spectra of the GdNPs treated with HCl solution (0.1 M) and citric acid solutions with different concentration.](image)

**FIGURE 5.8** FTIR spectra of the GdNPs treated with HCl solution (0.1 M) and citric acid solutions with different concentration.

To compare the effect of acid structure on the stability of the GdNPs, the nanoparticles were treated with a citric acid solution (pH = 3.0), HSO₄ solution (pH = 3.1), H₃PO₄ solution (pH = 3.4), and HCl solution (pH = 3.0), respectively. While the structure of GdNPs treated with HSO₄ solution (pH = 3.1) (Figure 5.7f) and HCl solution (pH = 3.0) (Figure 5.1b) did not change, two structures were observed in the TEM images of the GdNPs treated with H₃PO₄ solution (pH = 3.4) (Figure 5.7e), including the rod-like shape from the original GdNPs and long needle-like shape from the Gd phosphate compound. A comparison of the FTIR spectra of the GdNPs treated with different acid solutions (Figure 5.9) suggests two possible statements. Firstly, the presented phosphate ions in H₃PO₄ solution will react with
coordinated Gd ions, and the more phosphate ions present the more the 1,4-BDC ligand will be exchanged from the Gd$^{3+}$ ions. Secondly, the presence of citric acid promotes the proton-assisted dissociation of the Gd$^{3+}$ and 1,4-BDC because the citric acid can provide more protons once the free protons in the solution have reacted with the 1,4-BDC ligand.

![FTIR spectra](image)

**FIGURE 5.9** FTIR spectra of the GdNPs treated with different acid solutions with a similar pH.

To further investigate the presence of this promoted dissociation, the dialysis experiments of the GdNPs against the different phosphate based buffer solutions were conducted. In these experiments, three main reactions were present, including the spontaneous or proton-assisted dissociation between Gd$^{3+}$ and 1,4-BDC ligands, the ligand exchange reaction between 1,4-BDC and phosphate, the dissociation of the formed Gd phosphate compound. As seen
from Figure 5.10, the amount of Gd\textsuperscript{3+} leached to the dialysate of PCB solution was about 10 times higher than the amounts in the other three dialysates, which suggests that the presence of citric acid in PCB solution promotes the proton-assisted dissociation between Gd and its ligands, resulting in a higher leach of Gd\textsuperscript{3+}. Interestingly, the accumulated amount of Gd\textsuperscript{3+} leached to the dialysate at 12 h was less than the amount at 2 h, which happened to all four dialysis experiments. This phenomena was explained by the time-dependence of the ligand exchange reaction between phosphate and 1,4-BDC. During the first two hours of the dialysis, ‘free’ Gd\textsuperscript{3+} was leached to the dialysate when the ligand exchange reaction was ongoing in the dialysis tube. The leached Gd\textsuperscript{3+} can then react with the PO\textsubscript{4}\textsuperscript{3-} in the dialysate to form Gd phosphate compound resulting in a low amount of Gd\textsuperscript{3+} being detected at 12 h. After 24 h, while the amount of leached Gd\textsuperscript{3+} remained constant in the PBS, POB and APB solutions, the amount of leached Gd\textsuperscript{3+} increased slightly in the PCB solution, which again was attributed to the presence of citric acid. It was also observed that the dialysis against the APB solution gave the lowest amount of Gd\textsuperscript{3+} leached to the dialysate. This result is consistent with the TEM observations discussed earlier about the presence of APB solutions apparently causing accumulation of the newly formed particles (Figure5.3e&f), which might hinder the dissociation of the Gd complex. However, the mechanisms behind these systems are complex, and will require significantly more work to be performed to fully understand them. As mentioned in the introduction, the focus of this chapter was to find the suitable pH buffer solutions to keep GdNPs stable in solutions for the \textit{in vitro} studies. As such, while these
results are interesting, a complete understanding of these systems would be extremely time consuming, so the next step was to examine other buffer systems.

![Graph showing Gd³⁺ leached to different dialysates](image)

**FIGURE 5.10** The Gd³⁺ leached to the different dialysates during the dialysis experiments as a function of time.

### 5.3.3 GdNPs in TRIS and Good’s buffer solutions

After the phosphate based buffer solutions were demonstrated to have a strong affect on the structure and stability of the GdNPs, TRIS and Good’s buffer solutions were investigated. TRIS and Good’s buffer solutions have been widely used in biochemical and biological research because their pKa’s are close to physiological pH and they have good stability and compatibility with biological fluids.[169-171, 199-201] MES, MOPS and PIPES, as members of Good’s buffer family, were specifically selected due to their low affinities for metal ions.[197, 202] Therefore, unlike phosphate base buffers, TRIS, MES, MOPS and PIPES
buffers are not expected to form significant complexes with most metals by the ligand exchange mechanism.

FIGURE 5.11 TEM images of the GdNPs in different aqueous buffer solution: (a) 0.01 M TRIS, pH = 7.4, (b) 0.1 M TRIS, pH=7.4. (c) 0.01 M MES, pH = 6.6, (d) 0.1 M MES, pH = 6.5, (e) 0.01 M MOPS, pH = 7.1, (f) 0.005 M PIPES, pH=7.1.

The GdNPs were treated with TRIS, MES, MOPS and PIPES solutions of different concentrations. As seen from the TEM images (Figure 5.11), no major changes in the structure of the GdNPs were observed, not only for a solution concentration of 0.01 M but also for 0.1 M, for example, GdNPs after being treated with 0.1 M TRIS solution with pH of 7.4 has an
average length of 131±32 nm and width of 37±10 nm and GdNPs after being treated with 0.01 M MOPS solution of pH 7.1 has an average length of 128±40 nm and width of 41±13 nm. FTIR spectra (Figure 5.12) also clearly demonstrated that the characteristic stretches of carboxylate at 1395 cm⁻¹ and 1538 cm⁻¹ and other stretches all matched well with the untreated GdNPs. The TEM and FTIR results suggested that the GdNPs have a good stability in TRIS, MES, MOPS and PIPES buffer solutions.

FIGURE 5.12 FTIR spectra of the GdNPs after being treated with different aqueous Good buffer solutions and TRIS buffer solution.
5.3.4 GdNPs in aqueous solutions containing different cations

A transmetallation mechanism has been widely discussed and used to explain the dissociation of the small molecule Gd chelate-based CAs in literature.[175, 176, 183, 188, 191, 193, 203-205] Transmetallation discussed in this area usually refers to the capacity of the endogenously available cations (e.g. zinc, copper, iron and calcium) to displace Gd in the chelates.

FIGURE 5.13 TEM images of the GdNPs in aqueous solution with different cations: (a) 0.15 M NaCl, (b) 2 mM CaCl\(_2\), and (c) 1 mM ZnCl\(_2\).

In this work, the effect of sodium, calcium and zinc were investigated to find their effect on the stability of the GdNPs. Copper was not investigated due to its low concentration in the blood and iron was not investigated because the majority of iron in the body is tightly bonded in ferritin and hemosiderin resulting in a low concentration of free Fe\(^{3+}\).[176, 193] Although sodium has a low affinity to most of the ligands, it was investigated here because it has the
highest concentration in the blood compared to all other metal ions and it is the main cation in the phosphate based buffer solutions.[206]

FIGURE 5.14 FTIR spectra of the GdNPs treated with aqueous solutions containing different cations

The GdNPs were treated with NaCl, CaCl$_2$ and ZnCl$_2$ solutions with comparable concentrations in the blood, 0.15 M for NaCl compared to 0.137 M in blood, 2 mM for CaCl$_2$ compared to 1-1.3 mM in blood and 1 mM for ZnCl$_2$ compared to 0.1 mM in blood. The TEM images for the treated GdNPs are shown in Figure 5.11. As expected, no major changes in the structure of the GdNPs were observed from the TEM images for NaCl and CaCl$_2$ due to their low affinities to the ligands,[176, 206] for example, GdNPs after being treated with NaCl solution has an average length of 126±31 nm and width of 36±9 nm. Further, the results also demonstrate that zinc, with a moderate affinity to the ligand, did not cause a significant change.
in the structure of GdNPs, probably due to its relatively low concentration. The FTIR spectra (Figure 5.14) of these three samples all matched well with the untreated GdNPs with the general composition of Gd(1,4-BDC)\(_{1.5}(\text{H}_2\text{O})_2\).

5.4 Conclusions

Due to the free Gd\(^{3+}\) ion is toxic in body and the Gd complex has the potential to cause NSF for patients with renal dysfunction, the stability study of Gd based nanoparticles is important if they are ever to reach clinical use. However, the stability study for any system is a complex study. In this chapter, we mostly focused on finding suitable pH buffer solutions to use for the \textit{in vitro} evaluation of the performance of the pH-responsive MRI CAs. Interesting observations during this finding process were presented and discussed based on our understanding of the system. The stability of the GdNPs with a general composition of Gd(1,4-BDC)\(_{1.5}(\text{H}_2\text{O})_2\) in different pH solutions was studied because the GdNPs synthesized in this research were used for the preparation of pH-responsive CAs which will be evaluated and eventually exposure to different physiological pH conditions. The results demonstrated that the stability of the GdNPs in pH solutions were mainly affected by two mechanisms: a spontaneous or proton-assisted dissociation of the Gd\(^{3+}\) and the 1,4-BDC ligand, and a ligand exchange with phosphate ions which tend to precipitate most polyvalent cations. Although the structure of GdNPs was remained in aqueous solutions with pH range of 3-11 adjusted with just HCl and NaOH, the structure of GdNPs had significant changes if they were dispersed in solutions with a high concentration of protons, such as 0.1 M HCl solution and citric acid solution. Further, the structure of the GdNPs was destroyed after they were treated with
different phosphate contained solutions, including PCB, POB, APB, H$_3$PO$_4$ solutions. However, the stability of the GdNPs in TRIS, MES, MOPS and PIPES solution was sufficient for the *in vitro* study of the GdNPs in different pH solutions, such as their possibility to be used as a pH-responsive CAs. The transmetallation, as another main mechanism to explain Gd$^{3+}$ release from Gd chelates, does not affect the stability of the GdNPs while the cations are sodium, calcium and zinc. Therefore, TRIS, MES, MOPS and PIPES buffer solutions are strongly recommended for the *in vitro* test related to use of GdNPs with MOF structures, if pH buffer solution is ever needed in the system.
6.1 Conclusions

Due to the lack of clinical methods for the *in vivo* measurement of extracellular pH (pHe) in solid tumors, new contrast agent (CA) platforms containing the required chemistry and material properties need to be developed. One of the most promising new approaches to better imaging the pHe of tumors involves the combined use of polymer-based pH-responsive systems, due to the specific pH profile of solid tumors, and gadolinium (Gd) nanoparticles, due to drawbacks with Gd chelates. Current pH-responsive CAs with low molecular weight materials based on Gd chelates are limited in their performance, by low changes in relaxivity limiting their sensitivity, poor specificity due to lack of molecular targeting, solubility issues due to changes in the ligand structure, and difficulty in tuning the pH response to match tumors.

For the pH-responsive CAs based on Gd nanoparticles (GdNPs), the effects of pH on the performance of GdNPs in magnetic resonance imaging (MRI) are not well understood. In this research, we have designed pH-responsive CAs for MRI by modifying GdNPs with pH-responsive block copolymers and investigated the effect of surface attachment of pH-responsive polymers on the performance of the polymer modified Gd nanoparticles as CAs for MRI.

Firstly, pH-responsive block copolymers with pKa’s at relevant physiological pH, consisting of poly(ethylene glycol) (PEG) as the biocompatible block and tertiary amine-based
methacrylates as the pH-responsive block, were successfully synthesized via reversible addition-fragmentation chain transfer (RAFT) polymerization. 4-cyanopentanoic acid dithiobenzoate (CPAD) was found to be a better RAFT agent for mediating the polymerizations of 2-(dibutylamino)ethyl methacrylate (DBAEMA), 2-(diisopropylamino)ethyl methacrylate (DPAEMA) and 2-(piperidino)ethyl methacrylate (PPDEMA) by a RAFT process when compared to the s-1-dodecyl s’-(α,α-dimethylacetic acid) trithio-carbonate (DATC). 1,4-Dioxane, DMF/2-propanol (1/1 volume ratio) and acidic buffer were demonstrated to be good solvent systems for the synthesis of homopolymers of the three tertiary amine-based monomers. Kinetics studies and gel permeation chromatography (GPC) results confirmed the controlled character of the polymerizations via a RAFT process by using CPAD as a RAFT agent for the homopolymers and PEG-CPAD as a macro-RAFT agent for the block copolymers in the 1,4-dioxane solvent system. The semilogarithmic plots of conversion versus time for the preparation of the different homopolymers and block copolymers indicated that the polymerization of PPDEMA has the highest rate of polymerization compared to the polymerization of DBAEMA and DPAEMA. However, the semilogarithmic plots of conversion versus time also suggested both a lower RAFT agent initiation efficiency and an increased amount of termination at higher conversions in the polymerization of DBAEMA occurred, which may be due to the steric bulk of the two n-butyl groups. The experimental pKa values for PDBAEMA and PEG-b-PDBAEMA were approximately 5.1, for PDPAEMA and PEG-b-PDPAEMA were approximately 6.4, and for PPPDEMA and PEG-b-PPPDEMA were approximately 6.8. These results suggested that pKa,
as a critical value to characterize the dissociation ability of the different ionizable functional groups, remains constant for the polymers prepared from the same tertiary amine monomers within a certain range of molecular weight. The dynamic light scattering (DLS) results demonstrated that the pH-responsive block copolymers undergo a transition from unimers to micelles in aqueous media in response to changes in solution pH. The hydrodynamic diameter of the formed micelles was not only dependent on the molecular weight of the block copolymers but also on the composition of the block copolymers. The polymer solution of PEG-b-PPPDEMA formed the largest micelle, compared to the PEG-b-PDPAEMA and PEG-b-PDBAEMA with a similar molecular weight, due to the decreased flexibility of the piperidino groups in PPDEMA.

Secondly, the well-defined block copolymers were successfully attached to the surface of GdNPs after aminolysis of the trithiocarbonate end groups in basic conditions. The PEG-b-PPPDEMA with a pKa of 6.8 and PEG-b-PDPAEMA with a pKa of 6.4 were chosen as their pKa values are close to the pHe of solid tumors which is 6.2-6.8. The successful modification of the GdNPs was verified by several different characterization techniques, including TEM, FTIR, TGA and DLS. While a thin layer of polymers was observed on the surface of the GdNPs in TEM images, the characteristic stretches of polymers were clearly shown in the FTIR spectra of the polymer modified GdNPs. The grafting densities were determined by using the polymer weight loss observed by TGA and the surface area of GdNPs which was calculated based on the rod-like shape and dimensions observed in TEM. The calculated grafting densities, 0.071 chains nm⁻² for the PEG-b-PDPAEMA modified GdNPs,
0.19 chains nm\(^{-2}\) for the PEG-b-PPPDEMA modified GdNPs and 0.30 chains nm\(^{-2}\) for the three two polymer modified GdNPs, showed the modification of the GdNPs with RAFT polymerized pH-responsive polymer was effective. Zeta potential, measured by DLS, confirmed that the PEG-b-PPPDEMA was attached on the surface of the GdNPs by showing a dramatic decrease when pH increased from 6.9 to 7.3. The potential of pH-responsive polymer modified GdNPs as smart contrast agents for MRI has also been demonstrated by NMR. The longitudinal relaxivity (r\(_1\)) of the pH-responsive polymer modified GdNPs was increased when the solution pH was lower than the pKa of the pH-responsive polymers and decreased when the solution pH was higher than the pKa of the pH-responsive polymers. Increasing the grating density increased the differences of the relaxivity created by the changing pH. While the PEG-b-PDPAEMA or PEG-b-PPPDEMA modified GdNPs had one dramatic decreasing in r\(_1\), the combined polymer modified GdNPs showed continuous decreasing in r\(_1\) from pH 6.6 until 7.3.

Finally, stability studies of the GdNPs, with a general composition of Gd(1,4-BDC)\(_{1.5}\)(H\(_2\)O)\(_2\), in different pH solutions were conducted. The results suggested that the phosphate ions in solution reacted with the GdNPs to form a Gd phosphate compound by a ligand exchange mechanism and destroyed the crystalline structure of the GdNPs. Excess acid in the solution also affected the structure of the GdNPs by a proton-assisted dissociation mechanism. However, the GdNPs were stable in TRIS, MES, MOPS and PIPES buffer solutions. The Gd\(^{3+}\) ions in the metal organic frame structure also were not displaced by other
metal ions in solution with a comparable concentration to physiological concentration, including sodium, calcium and zinc.

In summary, the use of pH-responsive polymers and gadolinium nanoparticles for the preparation of pH-responsive imaging agents offers tremendous potential in the area of pHe imaging. However, further work is required, which will be discussed in the following section.

6.2 Future work

Despite the remarkable work that has already been conducted in the development of pH-responsive imaging agents for the next generation of diagnostics in cancer, there is much work to be conducted before these systems reach the clinic. Firstly, the vast majority of current reports on these responsive imaging agents as OI or MRS/MRI CAs have focused on in vitro studies. Secondly, while there has been a significant amount of research into the measurement of pHe in vivo using low molecular weight materials, to date all of the techniques developed suffer from significant limitations that have precluded their use clinically. The work presented in this thesis has demonstrated that the use of pH-responsive polymers and GdNPs offer tremendous potential for the preparation of pH-responsive MRI contrast agents for use in the area of pHe imaging. However, before the translation of these materials from the lab to the clinic, comprehensive in vivo studies investigating the pharmacological and toxicological properties of the imaging agents are particularly required. Asides from the in vivo studies, the following work and problems should also be considered.

Firstly, in order to effectively use the pH-responsive MRI CAs in the area of tumor pHe imaging, a relationship between the CA relaxivity and the solution pH need to be developed.
According to the results from this work, the surface modification of GdNPs with pH-responsive polymers creates changes in the relaxivity of the GdNPs upon the environmental pH changing. However, the detailed mechanisms behind this phenomenon need to be studied to answer the following questions: (1) Since the system does not exhibit dramatic changes in the relaxivity of the pH-responsive CAs right after the pKa of the attached pH-responsive polymer, what pH does it happen at and how far does it deviate from the pKa? (2) Is there a time-dependent relationship exist in the system for the conformational change of the pH-responsive polymer chains on the surface of the nanoparticles? (3) Do the conformational changes of the pH-responsive polymer chains upon pH change perform differently after they have been attached to the nanoparticles’ surface, compared to as free polymer chains in the solution? The mechanisms behind the relaxivity change and pH change need to be justified by both experimental results and theoretical modeling studies of the effect of environmental pH on the conformation changes on the pH-responsive polymer chains.[207-209] Furthermore, the following questions come after the mechanism questions: (1) Is it possible to develop a linear relationship between the relaxivity exhibited by the GdNPs and the solution pH by attaching different pH-responsive polymers on the surface of the nanoparticles? (2) If it is possible, how do the compositions of the different polymer being attached can be determined?

Secondly, the pKa results from this work suggested that pKa remains constant for the polymers prepared from the same tertiary amine monomers within a certain range of molecular weight. Therefore, more pH-responsive block copolymers with pKa’s ranging from 6.2 to 7.4
need to be developed. Synthesis of pH-responsive copolymers with combinations of different monomers could be an option as some preliminary results suggest that the pH-responsive random block copolymers can exhibit a new pKa between the two pKa’s of the two individual block copolymers.

FIGURE 6.1 Titration curves of pH-responsive copolymers and mixed block copolymer samples.

As seen from the titration curves in Figure 6.1, the copolymer of PEG-b-P(DBAEMA-co-DPAEMA) has a different transition in the pH titration experiment, which sits between the two transitions of PEG-b-PDBAEMA and PEG-b-PDPAEMA. Similar results were observed for the copolymer of PEG-b-P(DBAEMA-co-PPDEMA). Therefore, by synthesis copolymers with different pH-responsive monomers or different monomer ratios, it is possible to develop pH-responsive polymers with a series of different pKa’s to meet the
specific, desired pH for use in preparation of pH-responsive imaging agents or drug delivery system.

Lastly, to demonstrate the potential of pH-responsive polymers for use in preparation of smart contrast agents, GdNPs with a composition of Gd(1,4-BDC)$_{1.5}$(H$_2$O)$_2$ were used. However, in order to investigate the relationship between the relaxivity of CA and the pH of the targeted area \textit{in vivo}, the \textit{in vivo} concentrations of CA in the target area need to be determined first. Therefore, the synthesis of hybrid nanoparticles, which have the ability to determine the concentrations of CAs, represents another goal for researchers in this field. One of such candidates is Gd and gold (Au) hybrid nanoparticles that have potential to be used as a multi-modal image probe for MRI and computed tomography (CT). With the \textit{in vivo} concentration of Au in the targeted area can be determined via CT, the \textit{in vivo} concentration of Gd will be able to be determined at the same targeted area. However, in order to realize this, the well-defined Gd/Au hybrid nanoparticles with an accurate ratio of Gd and Au need to be developed first.

To summarize, there is a clinical need for the development of new techniques that enable the non-invasive \textit{in vivo} determination of tumor pH due to the correlation between low tumor pH and increased cancer morbidity and mortality. While many new imaging agents have been developed to image pH using low molecular weight materials or associating with polymer systems, more future work in the area is required to accomplish the system before these new imaging agents can reach the clinic. Further, other responsive contrast agents whose signal
varies as a function of the presence of biomarkers such as small molecules, metal ions, proteins, or enzymes, are also of significant interest to researchers in this field.
REFERENCES


APPENDIX A

FIGURE A.1 $^1$H NMR of DBAEMA

FIGURE A.2 $^1$H NMR of PPDEMA
FIGURE AA.3 $^1$H NMR of PEG-CPAD
FIGURE AA.4 Titration curves of PDBAEMA and PEG-b-PDBAEMA.

FIGURE AA.5 Titration curves of PDPAEMA and PEG-b-PDPAEMA.
FIGURE AB.1 TGA weight lost curve of GdNPs with the general composition of Gd(1,4-BDC)$_{1.5}$(H$_2$O)$_2$.

FIGURE AB.2 XRD spectrum of GdNPs with the general composition of Gd(1,4-BDC)$_{1.5}$(H$_2$O)$_2$. 
FIGURE AB.3 TGA curve of the block copolymers