Synthesis And Applications Of Ring Opening Metathesis Polymerization Based Functional Block Copolymers

Sanchita Biswas

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SYNTHESIS AND APPLICATIONS OF RING OPENING METATHESIS POLYMERIZATION BASED FUNCTIONAL BLOCK COPOLYMERS

by

SANCHITA BISWAS
B.Tech.University of Calcutta, India 2003

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Chemistry in the College of Sciences at the University of Central Florida Orlando, Florida

Spring Term 2010
Major Professor: Kevin D. Belfield
ABSTRACT

Ring opening metathesis polymerization (ROMP) is established as one of the efficient controlled living polymerization methods which have various applications in polymer science and technology fields. The research presented in this dissertation addresses several applications of multifunctional well-defined norbornene-based block copolymers synthesized by ROMP using ruthenium-based Grubbs catalysts. These novel block copolymers were applied to stabilize maghemite nanoparticles, creating the superparamagnetic polymeric nanocomposites. The J-aggregation properties of the porphyrin dyes were improved via self-assembly with a customized norbornene polymer. Novel multimodal copolymer probes were synthesized for two-photon fluorescence integrin-targeted bioimaging.

In Chapter 1 a brief overview of ROMP along with ruthenium metal catalysts and selected applications of the polymers related to this research is presented. Superparamagnetic maghemite nanoparticles are important in biotechnology fields, such as enhanced magnetic resonance imaging (MRI), magnetically controlled drug delivery, and biomimetics. However, cluster formation and eventual loss of nano-dimensions is a major obstacle for these materials. Chapter 2 presents a solution to this problem through nanoparticles stabilized in a polymer matrix. The synthesis and characterization of novel diblock copolymers, consisting of epoxy pendant anchoring groups to chelate maghemite nanoparticles and steric stabilizing groups, as well as generation of nanocomposites and their characterization, including surface morphologies and
magnetic properties, is discussed in Chapter 2. In Chapter 3, further improvement of the nanocomposites by ligand modification and the synthesis of pyrazole-templated diblock copolymers and their impact to stabilize the maghemite nanocomposite are presented. Additionally, the organic soluble magnetic nanocomposites with high magnetizations were encapsulated in an amphiphilic copolymer and dispersed in water to assess their water stability by TEM. To gain a preliminary measure of biocompatibility of the micelle-encapsulated polymeric magnetic nanocomposites, cell-viability was determined.

In Chapter 4, aggregation behaviors of two porphyrin-based dyes were investigated. A new amphiphilic homopolymer containing secondary amine moieties was synthesized and characterized. In low pH, the polymer became water soluble and initiated the stable J-aggregation of the porphyrin. Spectroscopic data supported the aggregation behavior.

Two photon fluorescence microscopy (2PFM) has become a powerful technique in bioimaging for non-invasive imaging and potential diagnosis and treatment of a number of diseases via excitation in the near-infrared (NIR) region. The fluorescence emission upon two-photon absorption (2PA) is quadratically dependent with the intensity of excitation light (compared to the linear dependence in the case of one-photon absorption), offering several advantages for biological applications over the conventional one-photon absorption (1PA) due to the high 3D spatial resolution that is confined near the focal point along with less photodamage and interference from the biological tissues at longer wavelength (~700-900 nm). Hence, efficient 2PA absorbing fluorophores conjugated with specific targeting moieties provides an even better bioimaging probe to diagnose desired cellular processes or areas of interest. The αvβ3 integrin
adhesive protein plays a significant role in regulating angiogenesis and is over-expressed in uncontrolled neovascularization during tumor growth, invasion, and metastasis. Cyclic-RGD peptides are well-known antagonists of $\alpha_V\beta_3$ integrin which suppress the angiogenesis process, thus preventing tumor growth. In Chapter 5 the synthesis, photophysical studies and bioimaging is reported for a versatile norbornene-based block copolymer multifunctional scaffold containing biocompatible (PEG), two-photon fluorescent (fluorenyl), and targeting (cyclic RGD peptide) moieties. This water-soluble polymeric multi scaffold probe with negligible cytotoxicity exhibited much stronger fluorescence and high localization in U87MG cells (that overexpress integrin) compared to control MCF7 cells.

The norbornene-based polymers and copolymers have quite remarkable versatility for the creation of advanced functional magnetic, photonic, and biophotonic materials.
To my family and special love and respect to my father late Asit Kumar Biswas.
First of all I want to thank my advisor Prof Kevin D. Belfield for providing his support, constant encouragement and valuable guidance throughout my graduate studies at UCF. His passion and enthusiasms for diverse research from two-photon optics, photonics to material science, polymer chemistry, nanomaterials and biological phenomenon enriched me to learn and understand the inter-relations between the multidisciplinary field and motivated to explore several applications during my research work. I am grateful for having the opportunity to think and develop the research independently and for his regular based discussion and meaningful suggestions about it.

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I would like thank my thesis committee members Dr. Andres Campiglia, Dr. Elloy Hernandez,
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Finally most important, my family, without whose love, support and encouragement I may have
not accomplish this endeavor. My mother and brother are my biggest inspirations. I am blessed
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immeasurable love and always being on my side. Also, I am grateful to all my friends and well-
wishers who made the life colorful, meaningful with their love and support.
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LIST OF ABBREVIATIONS

$^{13}$C  Carbon 13 isotope

2PFM  Two-photon fluorescence microscopy

2PA  Two-photon absorption

A-D-A  Acceptor-donor-acceptor

A-π-A  Acceptor-π-acceptor

Anal.  Analysis

Ar  Argon

ATRP  Atom transfer radical polymerization

CDCl$_3$  Deuterated chloroform

cm$^{-1}$  Wavenumber

EDC  1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide

FC  Field cooled

fs  Femtosecond ($10^{-15}$s)

FTIR  Fourier transform infrared
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>GPC</td>
<td>Gel permeation chromatography</td>
</tr>
<tr>
<td>GM</td>
<td>Goppert-Mayer unit for the 2PA cross-section (1 x 10^{-50} cm^4 s photon^{-1} molecule^{-1})</td>
</tr>
<tr>
<td>H_c</td>
<td>Coercive field</td>
</tr>
<tr>
<td>HRMS</td>
<td>High resolution mass spectrometry</td>
</tr>
<tr>
<td>M_s</td>
<td>Saturation magnetization</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter (10^{-3} L)</td>
</tr>
<tr>
<td>mM</td>
<td>Millimoles (10^{-3} moles)</td>
</tr>
<tr>
<td>NMM</td>
<td>N-methyl morpholine</td>
</tr>
<tr>
<td>NMP</td>
<td>Nitroxide mediated polymerization</td>
</tr>
<tr>
<td>PDI</td>
<td>Poly dispersity index</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>ROMP</td>
<td>Ring opening metathesis polymerization</td>
</tr>
<tr>
<td>RES</td>
<td>Reticulum endothelial system</td>
</tr>
<tr>
<td>NC</td>
<td>Nanocomposite</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer (10^{-9} m)</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
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</table>
THF  Tetrahydrofuran

TLC  Thin layer chromatography

$T_B$  Blocking temperature

TEM  Transmission electron microscopy

UV-Vis  Ultraviolet -Visible

USPIO  Ultra-small superparamagnetic iron oxide

XRD  X-ray diffraction

ZFC  Zero-field cooled

$\varepsilon_0$  Molar absorptivity coefficient

$\lambda_{\text{max}}$  Wavelength of absorption maxima

$\phi_{FL}$  Fluorescence quantum yield

$\tau$  Fluorescence lifetime

$\delta$  2PA cross-section
CHAPTER 1 : INTRODUCTION

1.1. Abstract

In the introduction chapter, the role of ring opening metathesis polymerization (ROMP) in polymer science is presented, particularly emphasizing their potential applications in cutting edge technological fields related to this dissertation research. This chapter addresses a brief synthetic and mechanistic aspect of ROMP, followed by some specific frontier topics in the polymer science field, including formation of organic–inorganic nanocomposites in biotechnology. The first part of this work focuses on the self-assembling and stabilization of inorganic nanoparticles via metal core coordination through pendant ligands of the polymers. Exploiting another kind of interaction, \( \pi-\pi \), ionic type, enhancement of optical properties of dyes and the significance of supramolecular assembly to further improve and stable aggregates in solution are discussed in the second part. Bioimaging techniques, particularly optical imaging with NIR fluorescent probes, offers safe and sensitive tools to visualize complex biological phenomenon at the molecular level as well as for therapeutic and high throughput drug screening applications. The third part of this dissertation presents the development of multi-scaffold biocompatible polymeric probes containing both a fluorescent reporter as well as a specifically labeled targeting moiety to image and target the desired location of interest. Also, this methodology provides the motivation to design and synthesize tailor-made multifunctional block copolymers containing additional therapeutic motifs along with the imaging probe and targeting
vector. The following chapters will employ these motivations to expand on the synthesis, characterization, and development of several ROMP-based well-defined multifunctional polymers and corresponding applications.

1.2. Introduction

Over the few past decades, there have been substantial researches on functional polymers, particularly since the discovery of controlled living polymerization techniques. “The term functional polymers is used to describe polymers that carry reactive functional groups that can participate in chemical processes without degradation of the original polymer chains”.1 The applications of functional block copolymers (made of two or more different blocks of chemically different monomers) are broad, ranging from electronics, nanofabrication, self-assembly, artificial organs, biomimetics, imaging and contrast agents, drug delivery, gene therapy, and therapeutics. Successful synthesis of well-defined block copolymers requires controlled living polymerizations, where polymers with low polydispersity are formed. Several functional polymers with a variety of architectures, such as linear, branched, star, and dendrite, have been reported in the literature.2-5 Different polymerization techniques have been employed to synthesize block copolymers, such as ring-opening metathesis polymerization (ROMP),6-8 atom transfer radical polymerization (ATRP),9,10 nitroxide mediated polymerization (NMP),11 and reversible addition-fragmentation transfer polymerization (RAFT).12,13 Ring-opening metathesis polymerization, a variation of the olefin metathesis reaction,14 represents a powerful and broadly applicable method among the living polymerization techniques.15
There are several advantages of ROMP polymerizations in the synthesis of well-defined block copolymers, including 1) living nature, 2) functional group (heteroatom containing) tolerance, 3) relatively faster initiation than propagation, 4) mild reaction condition, 5) commercially available different catalyst system, 6) polymer chain length can be modulated by varying ratio of [M]/[I] 8) narrow polydispersity 9) well-defined block copolymers can be prepared by addition of slower reactive monomer block first, and after fully consumed, followed by addition of faster reactive one, and 10) use of versatile capping agents for end functionalization. This results in greater control of the polymer properties and quantitative polymer functionalization.

![Figure 1-1](image.png)

Figure 1-1 A general example of ROMP.

ROMP uses strained cyclic olefin monomers to produce stereoregular and monodisperse polymers (Figure 1-1)\(^{16}\). The polymerization of such cyclic olefins is catalyzed by carbene complexes. i.e., transition metal complexes with a metal–carbon double bond. Such complexes have been synthesized with an impressive number of different transition metals. Many research groups, that of especially Schrock\(^{17-19}\) and Grubbs,\(^{15}\) have synthesized a wide variation of transition metal-based catalysts suitable for ROMP which have selected reactivity towards different functional groups (Table 1).\(^{20,21}\) Today, tungsten,\(^{22,23}\) molybdenum,\(^{24,25}\) and ruthenium\(^{26-29}\) carbenes are most commonly used in metathesis polymerization, some of which have become commercially available (Figure 1-2).
Figure 1-2 Different commercially available catalysts used for ROMP.

Table 1-1 Reactivity of transition metal complexes towards selected functional groups

<table>
<thead>
<tr>
<th></th>
<th>Titanium</th>
<th>Tungsten</th>
<th>Molybdenum</th>
<th>Ruthenium</th>
</tr>
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<tbody>
<tr>
<td>Acids</td>
<td>Acids</td>
<td>Acids</td>
<td>Acids</td>
<td>Olefins</td>
</tr>
<tr>
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<td>Alcohols, Water</td>
<td>Alcohols, Water</td>
<td>Acids</td>
<td></td>
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<tr>
<td>Aldehydes</td>
<td>Aldehydes</td>
<td>Aldehydes</td>
<td>Acids</td>
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<td>Olefins</td>
<td>Esters, Amides</td>
<td>Esters, Amides</td>
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</table>

A general mechanism of the ROMP, first proposed by Chauvin, is shown in Figure 1-3. Briefly, initiation proceeds through formation of coordination complex between transition metal of the
catalyst and cyclic olefin, followed by a four-membered metal-cyclobutane complex through \([2+2]\) cycloaddition. Release of strain of the cyclic olefin monomer is thermodynamically favorable. Similarly, this complex reacts with other olefin monomers and the chain grows in propagation step. Finally, the reaction is quenched by selective chain transfer reagents.\(^\text{16}\) The polymerization process is also dependant on number of physical factors such as monomer concentration, temperature, pressure, and the chemical nature and position of substituent on the ring.\(^\text{14,30}\)

**Figure 1-3 A general Chauvin mechanism for typical ROMP.**\(^\text{16}\)

ROMP is extensively reviewed in literature with a broad range of applications.\(^\text{16,31-33}\) Here, this research will instead focus on recent advances of supramolecular polymer chemistry for self-assembly through non-covalent as well as covalent interactions, which find significant applications from electronics to biotechnology fields. Collections of well-defined supramolecular
constructs, using a toolbox to design, construct, and build a multitude of functional nanomaterials related to this dissertation research is briefly discussed in the following pages.

1.3. Selected Applications of Polymers

1.3.1. Superparamagnetic nanocomposites.

Over the past several decades, extensive research investigations on nanomaterials in numerous fields has led to significant progress in unique optical, electronic, magnetic, and structural properties that exploit nanosized dimensions compared to the bulk materials. Inspite of intense development, for practical application purposes, a major obstacle of nanoscale particles is aggregation and cluster formation, thus losing the nanoscopic behavior. Theory as well as experimental analysis showed that ligand-mediated interaction strongly influences the metal nanostructure morphology. Herein, our primary focus is to investigate the effect of organic ligands to chelate and stabilize superparamagnetic iron oxide nanoparticles, which is of high biological interest in MRI imaging and magnetically-guided drug delivery and therapeutics.

Metal-ligand binding offers non-covalent but substantially strong interaction. Metal coordination occurs when lone pair electrons from a ligand are donated to an empty orbital in a metal ion. With the increasing strength of ligand, binding to a metallic nanoparticle increases and the rate at which the ligand dissociates from the metal ion decreases. There are many naturally abundant iron core-organic ligands which takes a vital role in may biological processes, such as heme (Figure 1-4).
Figure 1-4. The cytochrome c protein (colored ribbons), holds in its embrace a heme group (white honeycomb) that in turn clasps an iron atom (orange ball). This molecule is essential to life and any chemical that interferes with its activity is lethal. (Resource: Biocomputing, Visualization, and Informatics at the University of California, San Francisco)

There are several natural iron-protein complexes that play an important role in a number of physiological processes, e.g., transferrin, ferritin, and hemeproteins (e.g., hemoglobin, myoglobin, neuroglobin), where stable bonding between iron (III or II) core and protoporphyrin IX and the imidazole group of the histidine protein residue forms. Many FDA approved drugs for treatment thalassemia are also known for chelating irons (Figure 1-5) through the uni-, bi-, tri- or multidentate ligands like deferoxamine, deferiprone, myo-inositol hexaphosphoric acid, and desferrioxamine.

Figure 1-5 Different iron binding ligands used in drugs for treatment of thalasamia.
Recently, research has been reported to recognize and stabilize the metal ions or nanoparticle through noncovalent ligand anchoring by side chain functionalization of polymers (Figure 1-6), possessing applications ranging from electro-optics, magnetic, liquid crystal, and bioimaging to therapeutic purposes.\textsuperscript{41} Several research groups investigated different combinations of ligands to demonstrate the chelating ability between organic ligands (uni-, bi-, multi-dentate) and a metal core based on ‘modular or intermolecular functionalization’ strategies. Among these carboxylate,\textsuperscript{42-44} amine,\textsuperscript{45} thiol, phosphate, etc., containing ligands have been used to stabilize metal ions like Fe,\textsuperscript{46} Au,\textsuperscript{47} Pt, Cu, Zn, and Ru.\textsuperscript{48,49} Also, several researchers exploited the bi- or tri- dentate pyridine classic compound to generate a stable metallocomplex. Marcus Weck et al. investigated several noncovalent-based interactions like H-bonding and metal coordination to facilitate ROMP-based self-assembled polymeric system (Figure 1-7).\textsuperscript{50} This group introduced a ‘universal polymer backbone’ concept where the copolymers with a Pd-pincer complex along with diaminopyridine receptor in the side chain works orthogonally with the respective receptors pyridine and thymine,\textsuperscript{51-53} and extended the concept to produce complex crosslinked polymer structures.\textsuperscript{54} They reported research on both side chain functionalized and telechelic polymeric systems.

Recently, for OLED applications they reported the ROMP-based multiple motif copolymers having two norbornene-functionalized tetradentate cyclometalated platinum(II) complexes copolymerized with a bis(carbazolyl)benzene-based comonomer.\textsuperscript{55} Shubert et al., also reported a number of metal (Ru)-polymer complexes with respect to bipyridine complexes at the side
Kiessling et al. employed a ROMP-based polymer with a pendant ligand chelated with Gd\textsuperscript{2+} for potential MRI contrast agents.\textsuperscript{58}

Figure 1-6 Schematic representation of chelation of metal with ligands grafted on the side chain of polymer backbone.

Figure 1-7 Fully functional tri-block (ABA’) and diblock copolymer shows the combination of metal binding, H-bonding with crosslinked structure.
1.3.2. Self –assembly

Self-assembly is defined as “the spontaneous association of molecules under equilibrium conditions into stable, structurally well-defined aggregates joined by noncovalent bonds”,\textsuperscript{59} i.e, spontaneous formation of architecture based on the interaction between two complementary (recognition) pairs under equilibrium conditions. Self assembly chemistry is a platform to build the supramolecular structure, which relies on the non-covalent interaction of the molecular assembly. These self-assembly processes are reversible, selective, and spontaneous. Among the noncovalent strategies, such as hydrogen bonding, metal-coordination, π-π stacking, and van der Waals forces, acid-base interactions are prevalent in nature. The best example of self-assembly in nature includes DNA, RNA, and proteins.

Inspired by the nature, synthetic chemists attempt to simplify the complexities of nature to develop concepts and research based on supramolecular chemistry, utilizing non-covalent interactions to generate diverse synthetic self-assemblies. Supramolecular assemblies based on organized assemblies of macrocyclic chromophores have attracted widespread interest as molecular devices in many technologies like molecular electronics, artificial light harvesting, and pharmacology.

The aggregation of certain dye molecules in solution or at a solid interface at higher concentration sometimes results in a narrow, sharp, red shift of absorption bands compared to monomer (J-aggregates) or a blue shift of the absorption bands (H-aggregates). In the late 1930s, Gunter Scheibe\textsuperscript{60} and Edwin E. Jelly\textsuperscript{61} independently first discovered the phenomenon of J and H aggregation for cyanine-based dyes (Figure 1-8). Due to strong light absorption, the optical
properties of the J-aggregates play an important role in optoelectronic and photovoltaic devices including artificial light harvesting systems, optical switches, and demultiplexer.\textsuperscript{62-65} Among the many dyes, like phthalocyanine, pseudoisocyanine, porphyrin, hexabenzocoronene, azo dye, squarylium, and perylene bisamide that have been extensively studied,\textsuperscript{66} self-assembled synthetic porphyrins received more attention due to the unique photophysical properties as well as resemblance with chlorophylls found in nature in photosynthesis. It is desirable for the aggregates to possess stability for a long period of time, particularly in solution. It is also desirable to control the size the aggregates. However, stability and control of aggregate size remain significant challenges. A number of strategies have been attempted to address these issues, and one of the most promising approaches is stabilization through self-assembled polymers, a brief review follows.

Figure 1-8 Schematic representation of possible arrangements of J-aggregation of a dye in solution and in solid surface.

Santoro et al. have demonstrated that the tetraanionic meso-tetrakis(4-sulfonatophenyl)porphine (H\textsubscript{2}TPPS) in the pH range 5-12 exists in a monomeric form, and its fluorescence is not pH-dependent.\textsuperscript{54} However, in the presence of polylysine, absorption, circular dichroism, and resonant light scattering data indicate extensive polymer-induced self-aggregation of the
porphyrins. In particular, at low pH (<7), the protonated polylysine promotes porphyrin binding and self-aggregation with consequent strong quenching of their fluorescence.\textsuperscript{67} Periasamy et al. observed that poly-(L, D, or DL)-lysine, depending on optical chirality, induces J-aggregation of TPPS more efficiently than monomeric lysine.\textsuperscript{55} Only micromolar concentration of polylysine was required for complete conversion of the porphyrin monomer to its J-aggregate.\textsuperscript{68} Whitten et al. demonstrated ‘superquenching’ of polyelectrolytes containing cyanine pendant polylysines (repeat unit: 1-900) both in solution and after adsorption onto silica nanoparticles.\textsuperscript{56} The self-assembled polymer-initiated surface activated quenching leads to formation of J-aggregates due to enhanced binding with increasing number of repeating unit of the polymers.\textsuperscript{69} Also, laponite clay behaved very similarly with cyanine dyes to induce J-aggregation.\textsuperscript{70} Zhao et al. recently reported the micellization of poly(ethylene glycol)-block-poly(4-vinylpyridine) (PEG114-b-P4VP61) induced by TPPS in acidic solution where the core contained TPPS/PV4P and shell was structured with PEG.\textsuperscript{58} TPPS formed aggregates in the micellar core to form J-aggregates and H-aggregates, respectively, at pH 1.5-2.5 and 3.0-4.0.\textsuperscript{71} Kano et al. found that the TPPS-acid form was stabilized to induce J-aggregation by binding with ferric myoglobin (metMb) in water at neutral pH due to encapsulation and fixation by the relatively rigid protein molecules.\textsuperscript{59} The hydrophobic core of the J-aggregate caused the deformation of the secondary structure of the metMb, and, thus, denaturation of the protein.\textsuperscript{72} Chmelka et al. reported that mesostructured silica-block copolymer thin films provided orientationally-ordered host matrixes for stable alignment of co-assembled porphyrin J-aggregates with anisotropic optical properties.\textsuperscript{73} Smith et al. reported the induction of J- and H-aggregation of TPPS by the cationic polyelectrolyte, poly(diallyldimethylammonium chloride) (PDDA) on films deposited on Si. The films were
made by dipping in alternating aqueous solutions containing film components (layer-by-layer deposition).\textsuperscript{74}

1.3.3. Biomedical applications

Polymeric bioconjugates, with the recent explosion of nanotechnology and biotechnology, have become a central topic in the biomedical and pharmaceutical fields. Several synthetic polymers have been developed for potential applications in optical imaging, magnetic resonance imaging (MRI), and positron emission tomography (PET) as well as in bio-sensors, bioenzymes, biomimetics and in targeted therapeutics. There are several problems related to small, low molecular weight compounds due to non-specificity, instability, toxicity, and rapid clearance from the body for the biological applications. Furthermore, the lack of functional sites on the small molecules as biological probes limits their further modification, resulting in reduced biocompatibility and lower efficiency for clinical practice. In contrast, polymeric agents can have prolonged half-lives, reduced toxicity, enhanced stability, and improved targeting. The combined integration of imaging modality and/or therapeutic probe conjugated with biocompatible/biodegradable polymers with a variety of design and architecture (linear, branched, dendrimer, star, multivalent, and block copolymer) has now opened up a useful and promising tool for biotechnology and medicinal fields.

ROMP provides a very powerful means of polymerization in order to synthesize biocompatible polymers containing different bio-molecules for several cell-specific targeting, diagnostic, and therapeutic purposes.\textsuperscript{75} Grubbs et al. reported polynorbornene substituted with two different sequences of RGD-containing integrin, the cell-binding domain of fibronectin, are potent
inhibitors of human foreskin fibroblast cell adhesion to fibronectin coated surfaces.\textsuperscript{63} They also demonstrated significantly enhanced biological effect of the homopolymer as well as copolymer-containing oligopeptides compared to the free oligopeptides due to multivalent interactions provided by the polymer scaffold.\textsuperscript{76}

![Figure 1-9 Cell-internalization of block copolymers](image)

Kiessling et al. reported ROMP-based synthesis and biological application of polynorbornene-based synthetic polymers,\textsuperscript{77,78} e.g., multivalent polymers conjugated with a fluorescein derivative as a reporter to an end-capped neoglycopolymer that had been shown\textsuperscript{79} to inhibit L-selectin function.\textsuperscript{80} They attached the fluorescein fluorophore at the terminal of the telechelic polymer by post-polymerization modification technology. Recently,\textsuperscript{81,82} the same group synthesized and characterized guanidine-containing multifunctional block copolymers, used as ‘artificial translocation domain’, to facilitate cell-endocytosis within a very short time period which was confirmed by confocal microscopy (Figure 1-9) and they also employed synthetic polymer based
antigen to recognize CD22, an inhibitory coreceptor on the surface of B cells that attenuates B cell antigen receptor (BCR) signaling and, therefore, B cell activation.\textsuperscript{83}  

Samson et al. used a ROMP based polymer conjugated with fluorescently labeled sperm ADAM protein (fertillin $\beta$) to specifically target a protein receptor on the mouse egg plasma membrane.\textsuperscript{84} \textsuperscript{85}  

Sleiman et al. used biotin-terminated telechelic block copolymers containing a ruthenium bipyridine luminescent block and a hydrophobic block for streptavidine detection and luminescent detection and amplification of biomolecules,\textsuperscript{48} while Nguyen et al. synthesized a doxorubin-containing ROMP polymer for cancer treatment.\textsuperscript{86}  

\section*{1.4.Dissertation outline}

The remainder of the dissertation is organized as follows.  

Chapter 2 first describes the synthesis and characterization of well-defined novel epoxy ester-containing norbornene backbone block copolymers and homopolymers using the Grubbs 1\textsuperscript{st} generation catalyst by a ROMP technique. This is followed by the synthesis of a 1:1 block copolymer-maghemite nanocomposite \textit{in situ} process stabilized by the anchoring ligand (epoxy ester) at the side chain of the backbone and steric stabilizing norbornene group. This composite was characterized by several techniques to confirm the surface morphology and presence of the nanoparticles. The magnetic properties of the composite were characterized by SQUID magnetometry, demonstrating the superparamagnetic, well-dispersed, stable maghemite nanoparticle dispersions in block copolymer had high magnetization.
In Chapter 3, further improvement of the pendant ligand system of the block copolymer was made by exploiting the epoxide chemistry. Another series of well-defined block copolymer series were prepared by ROMP method containing bidentate ligands (hydroxyl and dimethyl pyrazoles) along with the carboxylate ester to make even stronger chelating system with maghemite nanoparticles. All the polymers and nanocomposites were comprehensively characterized. Then, the superparamagnetic nanocomposites were encapsulated in a Pluronic F127 copolymer in order to disperse it in aqueous media by core-shell technology with confirmation of particle size and dispersion by TEM. The biocompatibility of the polymeric magnetic composite was also evaluated for its biocapability through a cell viability assay, suggesting it potential biomedical application, specifically as a MRI contrast agent.

Supramolecular self-assembly of two porphyrin-based dyes in a norbornene-based homopolymer with amphiphilic nature was conducted and characterized in Chapter 4. The problem regarding the long-term stability in solution of J-aggregation of the dyes was controlled under mild conditions. The optical properties were also measured to confirm J-aggregation.

In Chapter 5 a new multimodal water-soluble block copolymer with one block containing hydrophilic containing PEG groups and the other block containing amine-reactive succinimidyl ester groups were synthesized and well characterized. A well-structured copolymer scaffold was prepared that can be tuned on the demand depending on the applications and interest. The polymer was post-modified with an amine-containing two-photon absorbing fluorene-based chromophore and an integrin-targeting cyclic-RGD peptides, followed by the cell incubation, and 2PFM. The photophysical properties, biophysical properties, and imaging experiments resulted
in a promising multi-motif polymeric probe for non-invasive bioimaging for angiogenesis and tumor detection.

1.5. Conclusion

In the last decades there has been remarkable progress in the field of functional polymer chemistry, creating significant interest among researchers from multidisciplinary fields. Currently, a major focus of polymer chemists is to develop supramolecular structures that mimick nature’s beauty and elegance. In spite of some technological progress in mimicking nature, these artificial materials suffer from several challenges, such as selection of suitable material and their fabrication, along with optimum energy sources required to steer the artificial devices. These challenges need to be overcome in order to find practical applications in the future. Thus, more intensive research and deeper understanding is required to expand the horizon of functional polymers and supramolecular materials. This dissertation aims to develop well-defined functional polymeric materials for several applications, including superparamagnetic nanocomposites, supramolecular assembly of dyes templated by a polymer matrix, and biocompatible imaging probes for possible disease diagnosis and therapeutic purposes. More specifically, it aims to develop methodical synthesis of well-defined, functional polymers using ROMP methodology, and according to the demands of a particular application, exploit its functional groups of the side chain on a single polymer backbone through several specific interactions (non-covalent as well as covalent), metal-bonding, self-assembly, and investigate their effects on the magnetic and optical properties compared to small molecules.
1.6. References


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CHAPTER 2 : SYNTHESIS AND CHARACTERIZATION OF ROMP-BASED BLOCK COPOLYMERS TO STABILIZE MAGHEMITE NANOPARTICLES


Abstract

A series of well-defined diblock copolymers of bicyclo[2.2.1]hept-5-ene-2-carboxylic acid oxiranyl methyl ester and bicyclo[2.2.1]hept-5-ene (norbornene), having both anchoring and steric stabilizing blocks, have been prepared by ring opening metathesis polymerization (ROMP). The epoxy ester group provided strong chelation between iron-oxide nanoparticles and the polymeric siderophores, producing stable magnetic nanocomposites. The polymers were characterized by $^1$H NMR, GPC, TGA, and DSC. The morphology and crystalline structure of the maghemite - block copolymer nanocomposites were evaluated with TEM and XRD, revealing highly crystalline, monodisperse $\gamma$-Fe$_2$O$_3$ nanoparticles with an average size of 4-6 nm. Interactions between the maghemite nanoparticles and the polymer were confirmed by FTIR. SQUID magnetometric analysis of the nanocomposites demonstrated superparamagnetism at room temperature with high saturation magnetization, narrow magnetization curve (consistent with narrow particle size dispersity), hysteresis below the blocking temperature (indicative of anisotropic arrangement), and no hysteresis above the blocking temperature.
2.1. Introduction

Nanoparticles embedded in self-assembled block copolymers have generated interest as a tool in a number of applications due to several advantageous properties obtained from the combination of organic polymers and inorganic metal/metal oxide nanoparticles. Among the various magnetic nanoparticles, magnetic metal oxide nanoparticles, particularly maghemite and magnetite, have attracted attention due to their large ratio of surface area to volume, high magnetization, low magnetic remanence and coercivity, and low toxicity. Maghemite nanoparticles with diameters ranging from 1-10 nm exhibit superparamagnetism at room temperature, and have applications in ferrofluids, and biomedical imaging. The most significant applications of magnetic nanoparticles in the biomedical imaging field are as negative contrast agents in magnetic resonance imaging (MRI), non-invasive local drug and gene delivery, clinical diagnosis, bioseparations of DNA, cell surface receptor targeting, and treatment of hyperthermia. Superparamagnetic nanoparticles are promising for a variety of biomimetic engineering applications, including magnetosomes, nanobots, and artificial muscles. The important criteria for biomimetic applications are high instantaneous magnetization in the presence of an external magnetic field, complete removal of magnetic properties in the absence of a magnetic field, small particle size, and strong interactions between magnetic nanoparticles and the dispersing media so that all move together under magnetic stimulation without sacrificing stability.

A major, fundamental problem of nanoscale maghemite particles is aggregation and cluster formation that eventually nullifies the benefits related to their nanoscopic dimensions.
There is strong theoretical as well as experimental support that the morphology and behavior of the nanocomposites can be modulated by tailoring the ligands of well-defined, functional polymers, depending on the size of the nanoparticles. The magnetic core with polymeric shell-type structures isolate and disperse magnetic nanoparticles by the interaction of nanoparticles and polymers, mediated through ligands attached to the surface of the polymers. Ligands attached to the polymer matrix not only prevent the agglomeration of the nanoparticles but also provide a tool to tune the magnetic properties of the system. Commonly used ligands for magnetic nanoparticle (NP) stabilization include carboxyl, hydroxy, amine or imine, phosphine oxides, and phosphonic acid. Ligands markedly influence the particle’s spatial behavior as well as ultimate macroscopic properties of the polymer-nanocomposites. However, more efforts are needed in the design and synthesis of more efficient stabilizers for monodispersed maghemite nanocomposites with sufficient intrinsic magnetization and versatile surface functionality. Currently, the synthesis of well-defined nanocomposites in self-assembled structures, such as polymers or surfactants, has become simpler and more efficient relative to other complex processes, such as biomineralization. Diblock copolymer templates containing both steric stabilizing groups and anchoring ligands, to prevent the aggregation of the NPs offers microphase separation of the copolymer, thus controlling the spatial distribution and inherent properties of the nanocomposites.

Ring-opening metathesis polymerization (ROMP) is a well established tool in order to synthesize well-defined, highly functionalized block copolymers. The energetics of strained bicyclic olefin monomers is thermodynamically favorable to yield stereoregular and monodispersed polymers. The polymerization process is also dependant on number of physical
factors such as monomer concentration, temperature, pressure, and the chemical nature and position of substituent on the ring. Over the past decade, Grubbs’ ruthenium-based catalysts have shown a broad range of functionalization due to their high tolerance of heteroatom-containing groups which had poisoned earlier catalysts.\textsuperscript{13}

Herein, we demonstrate a strategy for the synthesis of well-defined diblock copolymers with a norbornene-based backbone, using ROMP. The copolymers contain ‘iron-loving’ siderophores in one block to chelate and interact with iron oxide nanoparticle surface and a steric stabilizing group in other block to prevent metal nanoparticle aggregation. The siderophores are designed with the following versatility: (1) use of epoxide/oxirane anchoring group to stabilize the maghemite nanoparticles while retaining supermagnetic properties, and (2) further flexibility of design by reaction of the oxirane group to modify the ligand via, e.g., nucleophilic reaction or hydrolysis.\textsuperscript{29-33} This leads to straightforward formation of maghemite – diblock copolymer nanocomposites and construction of a broad range of functionalities on the periphery of the block copolymers to stabilize the nanoparticles. Norbornene-based polymers have a number of interesting properties such as high thermal stability, optical transparency, and low dielectric constant with a generally amorphous morphology. We report strategies to synthesize epoxy-containing diblock copolymers via ROMP that are well-characterized by NMR, elemental analysis, gel permeation chromatography (GPC), thermogravimetric analysis (TGA), and differential scanning calorimetry (DSC). The polymer-maghemite nanocomposites with different polymer nanoparticle formulations were prepared through a non-hydrolytic method in the polymer microdomains with nanoparticles sizes ranging between 2-6 nm. The polymer composites were characterized by transmission electron microscopy (TEM), Fourier transform
infra-red spectroscopy (FTIR), and X-ray diffraction (XRD) in order to elucidate the nanoparticle size, nanoparticle-ligand interaction, and nanoparticle crystalline morphology, respectively. Magnetic properties of the nanocomposites were determined using superconducting quantum interference device (SQUID) through measuring magnetization as a function of temperature or applied magnetic field, and establish the superparamagnetism properties at room temperature.

2.2. Result and Discussion

Synthesis of Monomer 2. Monomer 2 was prepared according to Scheme 1. Acid chloride 1 was prepared in accordance to previous reports.23 Norbornenyl oxiranemethyl ester 2 was prepared by the addition of a mixture of triethylamine and glycidol slowly with acid chloride 1. After column chromatographic purification, colorless oil was isolated in high yield.

Scheme 2-1. Epoxy Monomer Synthesis

\[
\begin{align*}
&\text{SOCl}_2 & \quad \text{CHCl}_3 & \quad \text{Reflux} 4-5 \text{ hr} \\
&\rightarrow & \quad \text{HO} & \quad \text{Cl} \\
&\text{1} & \quad \text{THF, Et}_3\text{N} & \quad 1. 0^\circ \text{C}, 2\text{hr} \\
& & & \quad 2. \text{rt}, 8\text{hr} \\
&\rightarrow & \quad \text{2} \\
\end{align*}
\]

\(^1\text{H} \text{NMR analysis confirmed product formation by the appearance of new peaks at 4.39-3.78 ppm belonging to the proton adjacent to the ester, along with the characteristic vinyl peaks at 6.14-5.87 ppm for endo and exo monomers. Three new \(^13\text{C} \text{NMR peaks appeared at 65.17, 47.01, and 44.98 ppm. In addition, absorbances at 1247 and 1734 cm}^{-1} \text{ in the FTIR spectrum corresponded}\)
to characteristic C-O stretching vibrations of the epoxy ring and C=O stretching vibrations of
the ester, respectively.

**Synthesis of Polymers 4 and 5.** The homopolymer of epoxy monomer 2 and a diblock
copolymer, containing anchoring and steric stabilizing blocks in ca. 1:1 molar ratio, were
synthesized under mild conditions by ROMP, according to the procedure described in the
Experimental Section (and shown in Scheme 2). Due to the air- and water sensitivity of the
Grubbs’ catalyst, the catalyst solution was prepared in an anaerobic glovebox. Polymerization
reactions were carried out using a Schlenk line under N₂. In order to prepare narrowly dispersed,
well-defined block copolymers, the sequential order and time interval between addition of the
different monomers is quite significant.²⁸ Extent of polymerization of monomer 2 was

![Figure 2-1](image-url)

Figure 2-1. ¹H NMR of (1) monomer 2, (2) homopolymer 4, and (3) 1:1 block copolymer 3.
determined by $^1$H NMR in CDCl$_3$. This study showed a new broad peak between 5.12-5.24 ppm, along with the gradual disappearance of the vinyl peaks of the monomer around 5.87-6.12 ppm, due to polymer formation. It took about 3.5 hours for completion of the homopolymerization, i.e., complete monomer consumption (see Supporting Information). In general, the propagation rate of the polymers depend on the polarity and stereochemistry (exo/endo) of the substituted ligand since the catalyst initiates polymerization from the exo side of the norbornene vinylic bond. The polymerization of unsubstituted norbornene is more reactive relative to the norbornene substituted at the 2-position.$^{39,40}$ Hence, monomer 2 was used as the first block, followed by the addition of norbornene as the second block in the synthesis of diblock copolymer.

**Scheme 2-2. ROMP Block Copolymer Synthesis**

![Reaction scheme]

The molecular weight of the polymers was controlled by the monomer/initiator ([M]/ [I]) feed ratio.$^{41}$ The monomer concentrations were kept at ca. 0.15 M in dry CH$_2$Cl$_2$, and polymerizations was performed at ca. 30°C. Progression of the polymerization was followed by TLC (1:1 hexane:EtOAc). For block copolymer synthesis, after all the first block was consumed, the second monomer was added to the system. In general, for the block copolymer required longer reaction time to yield blocks with narrow polydispersity compared to the homopolymer.
After all monomer was consumed, the polymerization was quenched by adding excess ethyl vinyl ether, with vigorous stirring, followed by precipitation in cold CH$_3$OH. The polymer was purified by redispersing in CH$_2$Cl$_2$ and reprecipitating in CH$_3$OH several times, followed by vacuum drying.

<table>
<thead>
<tr>
<th>Entries</th>
<th>Target Block ratio (m:n)</th>
<th>[M$_1$]/[I]</th>
<th>[M$_2$]/[I]</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:0</td>
<td>350</td>
<td>0</td>
<td>67%</td>
</tr>
<tr>
<td>2</td>
<td>1:0</td>
<td>300</td>
<td>0</td>
<td>72%</td>
</tr>
<tr>
<td>3</td>
<td>1:10</td>
<td>350</td>
<td>3500</td>
<td>70%</td>
</tr>
<tr>
<td>4</td>
<td>1:5</td>
<td>350</td>
<td>1750</td>
<td>72%</td>
</tr>
<tr>
<td>5</td>
<td>1:1</td>
<td>350</td>
<td>350</td>
<td>74%</td>
</tr>
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<td>6</td>
<td>1:1</td>
<td>200</td>
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<td>92%</td>
</tr>
<tr>
<td>7</td>
<td>5:1</td>
<td>350</td>
<td>70</td>
<td>67%</td>
</tr>
</tbody>
</table>

[M$_1$] = Concentration of monomer 2; [M$_2$] = Concentration of norbornene

A series of five block copolymers with different molar ratios of the anchoring group and steric blocks, using Grubbs first generation catalyst by ROMP, to ensure the living nature and control of the polymer synthesis. The $^1$H NMR experiments proved the presence of ratiometric presence of integral area in NMR due to alkene ‘H’ (5.12-5.24 ppm) in the polymer compares to the proton due to ‘CH’ proton (4.39 ppm) next to the ester group (Figure 2-1). A 1:1 block copolymer was chosen for further study based on our previous success that indicated, among the different
norbornene block copolymers, 1:1 (200:200) diblock copolymers resulted in the best stabilization of maghemite nanoparticles.\textsuperscript{23}

Figure 2-2 1H NMR spectra of (1) 0:1 block, (2) 1:0 block, (3) 1:10 block, (4) 1:1 block.
Synthesis of Monomer 6: Monomer 6 was prepared by nucleophilic addition reaction with the epoxy group of 1 and carbon disulfide in presence of LiBr. After column chromatographic purification, colorless yellow oil was isolated in high yield. $^1$H NMR analysis confirmed product formation by the shift of CH$_2$-CH-CH After column chromatographic purification, colorless oil was isolated in high yield. $^1$H NMR analysis confirmed product formation by the shift of the proton peak at at 5.33 ppm which flanked between the dithiocarbonate group and the ester. Also, a new $^{13}$C NMR peaks appeared at 211.10 ppm due to C=S bond. FTIR showed also distinct absorbance at 1200 and 1746 cm$^{-1}$ corresponded to characteristic C=S (thiocarbonyl) stretching vibrations of the ring and C=O stretching vibrations of the ester, respectively.

![Scheme 2-3. Dithiocabonate Monomer Synthesis](image)

Synthesis of homo- and block copolymer of monomer 6 did not work successfully through ROMP process, several endeavor results in insoluble cross-linked polymer with in a short period of reaction. Possibly the presence of several S heteroatom in the pendant group of the polymer induces the cross-link reaction between intermolecular chain of the different polymer fragments. So, this route was avoided for further perusal.

Characterization of Polymers 4 and 5. The structures of the polymers were confirmed by $^1$H NMR analysis. The vinyl protons of both norbornene and the epoxy ester monomer (5.87-6.12 ppm) gradually disappeared while new alkene resonances at 5.12-5.24 ppm appeared, ascribed to
CH=CH protons in the polymer backbone (Figure 1). Each block ratio (m: n) was determined through integration of the proton NMR spectra. The new peaks at 5.12-5.24 ppm integrated to four protons, and were contributed to from both blocks of the 1:1 copolymer backbone. The methylene peak from the epoxy block appeared at 4.30 (1H) and 3.82 (1H) ppm. The calculated and experimental block ratio (m:n) were in good agreement, substantiating the desired block lengths in the polymer backbone.

Molecular weights of the polymer were estimated via GPC analysis by using a universal calibration curve and polystyrene standards. The experimental molecular weight of the polymers was ca. 60K and polydispersity indexes (PDIs) were between 1.12-1.43 (Table 2-2). The narrow PDI, as well as the close agreement of the number average molecular weight of the polymers with the calculated molecular weight, is consistent with a well-controlled, living ROMP system.

<table>
<thead>
<tr>
<th>Block ratio</th>
<th>m:n</th>
<th>Mₐ (Theo)</th>
<th>m:n</th>
<th>Mₐ (1H NMR)</th>
<th>Mₘ (GPC)</th>
<th>Mₚ (GPC)</th>
<th>PDI</th>
<th>Block ratio (calculated)</th>
<th>TGA °C</th>
<th>Tₘ °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>300:0</td>
<td>1:0</td>
<td>58227</td>
<td>1:0</td>
<td>62391</td>
<td>69968</td>
<td>1.12</td>
<td>321:0</td>
<td></td>
<td>340</td>
<td>55</td>
</tr>
<tr>
<td>200:200</td>
<td>1:1</td>
<td>57650</td>
<td>1:1</td>
<td>61464</td>
<td>88446</td>
<td>1.43</td>
<td>213:213</td>
<td></td>
<td>368</td>
<td>40</td>
</tr>
</tbody>
</table>

a. Theoretical molecular weight calculated from [M]/[I] feed ratio.

b. Mₐ, Mₚ, and PDI were obtained from GPC in THF relative to polystyrene standards.

c. Actual polymer block ratio was calculated from the ¹H NMR and GPC results.

d. Temperature at 10% weight loss.
Thermal properties of the polymers were evaluated by TGA and DSC, with results listed in Table 2-2. Homopolymer 4 started decomposing at nearly ca. 340 °C, slightly lower than the polynorbornene itself (ca. 400 °C), while the 1:1 block copolymer (3) was stable up to 370 °C (thermograms are presented in the Supporting Information). The norbornene homopolymer and that with epoxy ester homopolymer 4 had a T_g of 31 °C and 55 °C, respectively. The 1:1 block copolymer (4) exhibited a T_g of 40 °C, in good agreement with the theoretically predicted T_g value (40 °C) from Fox equation.

**Synthesis of Maghemite-Block Copolymer Nanocomposites.** The synthesis of polymer stabilized maghemite nanoparticle dispersions provided uniform, monodisperse, highly crystalline nanocrystallites. Stabilized γ-Fe_2O_3 nanoparticle ferrofluids were prepared with the 1:1 norbornene diblock copolymer in cyclohexanone by an *in situ* process. Maghemite-block copolymer nanocomposites were prepared using the 1:1 block copolymer as surfactant in cyclohexanone, via thermal decomposition of Fe(CO)_5, followed by oxidation with trimethylamine oxide. The 1:1 block copolymer was used for the nanocomposite preparation and its study, as we previously established that 1:1 balance between the chelating block and the steric stabilizing block in a norbornene-based copolymer results in good magnetic nanoparticle stabilization. Two different formulations were prepared by varying the wt% of 1:1 diblock copolymer (**NC1-B1**: 3.3 wt% ; **NC1-B2**: 0.97 wt%) relative to a constant volume of FeCO_5 feed, to evaluate this effect on the formation of nanoparticles, its morphology, and the magnetic properties of the nanocomposite.
Characterization of Maghemite-Block Copolymer Nanocomposite. TEM, X-ray diffraction, and FTIR were used to obtain information about the maghemite nanoparticles dispersed in the polymer matrix.

Figure 2-3. Transmission electron microscopy (TEM) images and electron diffraction pattern of (A) NC1-B1 and (B) NC1-B2.

Morphology and Particle Size. TEM analysis confirmed the generation of well-controlled uniform and spherical iron oxide nanoparticles, encapsulated by the self-assembled block copolymers. Interestingly, depending on the wt% of polymer loading, and, thus, the relative amount of chelating and steric stabilizing groups present in the polymer, the size of the nanoparticles can be controlled. The nanocomposite NC1-B1, with high polymer loading, resulted in smaller nanoparticles with 2-3 nm average diameters, while the nanocomposite NC1-
**B2** generated nanoparticles with an average diameter of 5-6 nm (Figure 2). In both cases, the 1:1 block copolymer was able to stabilize maghemite nanoparticles quite efficiently without any noticeable aggregation. In addition, selected area electron diffraction patterns (see Supporting Information) were obtained for both of the nanocomposites in order to examine the crystalline structure present in the nanocomposite (Figures 2c and 2f). Electron diffraction patterns of the nanoparticles were consistent with the standard crystal structure and d spacing of γ-Fe₂O₃.

![Figure 2-4](image)

**Figure 2-4** FTIR analysis for (A) γ-Fe₂O₃ nanoparticles stabilized by block copolymer 3 and 1:1 block copolymer 3 alone. (B) XRD pattern for γ-Fe₂O₃ nanoparticles stabilized by block copolymer 3 (NC1-B2) in powder form.

**Crystalline Phase.** The X-ray diffraction pattern (Figure 3B) of **NC1-B2** was in good agreement with the standard γ-Fe₂O₃ reflection [PDF#00-039-1346; Maghemite-C, syn; cubic], confirming the nanoparticles formed were Fe₂O₃. The Fe₂O₃ nanoparticles displayed several strong reflection peaks in the 2θ region of 20-70°. The strong Bragg reflections of Fe₂O₃ are at the 2θ angles of 30.24° (d = 2.95°Å), 35.35° (d = 2.51°Å), 43.15° (d = 2.09°Å), 56.80° (d = 1.64°Å), and 62.70° (d = 1.48°Å). These corresponded to the indices (220), (311), (400), (511), and (440),
respectively. The average diameters (d) of the singular γ-Fe₂O₃ nanocrystallites were estimated using Scherrer equation. The estimated d value associated with the strongest (311) reflection of the Fe₂O₃ at 2θ 35.35° was about 6 ± 1 nm, which was in good agreement with the average size of singular nanoparticles observed from TEM. However, the XRD analysis of NC1-B1 was unable to extract sufficient distinct peaks due to the ultra small particle size of the maghemite nanocrystals. For very small nanocrystals, this fact is supported by previous reports, although from the electron diffraction pattern in the TEM experiment the presence of γ-Fe₂O₃ was confirmed.

**Maghemite-Polymer Chemical Binding.** FTIR spectroscopy was quite informative in characterization of the block copolymer as well as the nanocomposite, suggesting complexation of the iron oxide nanoparticles and the pendant epoxy of the block copolymer, thereby rendering the iron oxide particles noninteracting. There was a distinct difference in the FTIR spectrum (Figure 3A) of the maghemite-polymer nanocomposite with respect to the polymer alone. In the nanocomposite, a single, broad band due to the Fe-O stretch at ca. 580 nm was present and is characteristic of γ-Fe₂O₃ particle less than 8 nm. A broad peak was observed at ca. 3350 cm⁻¹ in the polymer-maghemite nanocomposite, possibly due to OH stretching as a result of opening of the epoxy ring. Furthermore, the C=O stretching vibration shifted from 1729 cm⁻¹ in the polymer to 1692 cm⁻¹ in the nanocomposite, indicative of interaction between the metal core and the chelating block of the polymer. The coordination assembly of polymer to the iron-oxide core is believed to contain stable ring structures which assist in stabilizing the nanoparticles. This chelation is consistent with opening of the strained epoxy ring to generate free OH groups.
Magnetic Properties: Magnetic measurements were performed on both of the γ-Fe₂O₃-polymer nanocomposites (NC1-B1 and NC1-B2) in powder form using a Quantum Design Superconducting Quantum Interference Device (SQUID). Both nanocomposites exhibited behavior typical of non-interacting ferromagnetic nanoparticles with uniform size. The temperature dependent magnetizations M(T) for both field-cooled (FC) and zero-field-cooled (ZFC) cases are shown for the samples NC1-B1 and NC1-B2 in Figs. 2-5A and B respectively.

Figure 2-5 Magnetization (M) as a function of temperature (T) at 500 Oe applied magnetic field, for (A) NC1-B1 and (B) NC1-B2. Inset in Fig. B shows the linear behavior of $T_B^{1/3}$ as a function of particle diameter ($d$) through the origin.

The peak in the ZFC magnetization corresponds to the blocking temperature ($T_B$), shown by vertical dotted red arrows. The values of $T_B$ for the samples NC1-B1 and NC1-B2 are 5 K and 26 K respectively. The values are also listed in Table 2-3. The higher value of $T_B$ for the NC1-B2 compared to NC1-B1 is consistent with the fact that NC1-B2 has larger particle size (5.5 nm diameter) than NC1-B1 (3.5 nm diameter). The cube root of the ratio of $T_B(B2)$ to $T_B(B1)$ is 1.7 and the ratio of the respective diameters is 1.6. The close agreement of these ratios suggests that $T_B$ grows linearly with the volume of the particle as expressed by the equation,\(^{46}\)
where $K$ is the crystalline anisotropy constant, $k_B$ the Boltzmann constant, $V$ the volume of the particle, $\tau_m$ the measurement time (typically 100 sec), and $\tau_0$ the inverse of the attempt frequency associated with magnetic moments overcoming field-dependent energy barriers. The inset of Fig. 4B shows the dependence of $T_B^{1/3}$ on the particle diameter $d$ for the two samples with the linear fit (solid line) forced through the origin. This good agreement with Eq. 1 allows us to calculate $K = 8 \times 10^5$ ergs/cm$^3$ where we have set $\ln(\tau_m/\tau_0)$ to be on the order of 25, as is commonly done in this type of calculation. This value for $K$ is in good agreement with the previous observations on the same material.

Magnetization loops are shown in Fig. 5A and 5B at the indicated temperatures for the samples NC1-B1 and NC1-B2 respectively. Fig. 5A (5B I) shows the magnetization loop at 4.2 K for NC1-B1 (NC1-B2). Both of the loops show hysteretic behavior as expected below $T_B$. The coercive field $H_c$ determined from the loop is 60 Oe (340 Oe) for the NC1-B1 (NC1-B2). Since $H_c$ increases with increasing particle size, the particles are likely to be single domain and coherently rotating. The temperature dependence of $H_c$ for coherently rotating single domain particles with random orientations of the easy axis of magnetization is given by the relation

$$H_c = \frac{2K}{M_s} \left[ 1 - \left( \frac{T}{T_B} \right)^{3/4} \right]$$  \hspace{1cm} (2)$$

where $M_s$ is the saturation magnetization. Both $K$ and $M_s$ are intrinsic quantities independent of particle size. Using Eq. 2 the ratio $H_{cB2} : H_{cB1} \sim 6$ is calculated at $T = 4.2$ K. The ratio of the $H_c$'s
determined from the respective magnetization loops is (340/60) 5.7. This good agreement suggests that the particles are in fact uniform in size, non-interacting, single domain, and coherently rotating with the easy axis of the magnetization randomly oriented.

Figure 2-6 Magnetization (M) versus applied magnetic field (H) at constant temperature for (A) NC1-B1 and (B) NC1-B2. In each case, (I) shows hysteresis below the blocking temperature and (II) no hysteresis above the blocking temperature with H/T scaling as expected for superparamagnetic particles.

As expected, the magnetization loops for \( T > T_B \) are shown in Fig. 5A II (5B II) for the sample NC1-B1 (NC1-B2) do not show hysteresis, i.e., \( H_c = 0 \). Note the H/T scale on the x axis. All the
loops at different temperatures fall on top of each other. This type of scaling is the signature of non-interacting superparamagnetic particles.\textsuperscript{52}

<table>
<thead>
<tr>
<th>Nanocomposite</th>
<th>wt%</th>
<th>Average</th>
<th>$T_b$ (K)</th>
<th>$M_s$ (emu g$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC1-B1</td>
<td>3.3</td>
<td>3.5</td>
<td>5</td>
<td>63 at 50K</td>
</tr>
<tr>
<td>NC1-B2</td>
<td>0.97</td>
<td>5.5</td>
<td>26</td>
<td>64 at 100K</td>
</tr>
</tbody>
</table>

Table 2-3 Summary of Magnetic Properties of the Maghemite-Block Copolymer Nanocomposites

Figure 2-7. Photographs of (A) $\gamma$-Fe$_2$O$_3$ nanoparticles stabilized by 1:1 block copolymer NC1-B2 in cyclohexanone, and (B) the same ferrofluid under the influence of external magnetic field. The results from the magnetic measurements are listed in Table 2-3. The magnetization loop above the blocking temperature is fit with the Langevin function. The saturation magnetization $M_s$ is approximately 60 emu/g as determined from the fit. This is consistent with the previous observation.\textsuperscript{53,54} Figure 6A shows the 1:1 block copolymer-stabilized (NC1-B2) maghemite nanoparticle dispersion in cyclohexanone at room temperature. Figure 6B shows the effect of an
external magnetic field on the same ferrofluid, further demonstrating the magnetic properties of the system.

2.3. Conclusions

In this work, we demonstrated the synthesis and characterization of novel norbornene backbone-based well-defined block copolymers, containing both a steric stabilizing segment and an epoxy ester chelating group via ROMP. The molar ratios between these two blocks were determined to be 1:1 by $^1$H NMR study while the molecular weight was estimated from GPC analysis. The thermal properties of the polymer demonstrated that the polymers possess good thermal stability up to 360 °C with glass transition temperature of 40 °C, in good agreement with that calculated with the Fox equation and the $T_g$ of the two corresponding homopolymers. Well-controlled, monodisperse iron-oxide nanoparticles were synthesized in the presence of the 1:1 diblock polymer matrix with two different polymer loadings, resulting in uniform spherical nanocrystallites with an average diameter between 2-6 nm, depending on the amount of block copolymer present.

The morphology, chemical nature, and the crystalline structures of the maghemite-polymer nanocomposites were evaluated with TEM, FTIR, and XRD analysis, respectively. The stabilized maghemite–polymer nanocomposites had a monodisperse nanoparticle morphology, with a lattice structure resembling the maghemite –C-syn structure. FTIR analysis supports coordination assembly of the polymer to the iron-oxide core, likely containing stable ring structures that assist in stabilizing the nanoparticles. This chelation is consistent with opening of
the strained epoxy ring to generate free OH groups. The coordination between epoxy ester groups with the iron oxide nanoparticles prevent maghemite nanoparticle agglomeration and generated stabilized magnetic nanocomposites.

The magnetic properties of the nanocomposites were measured in dry powder form using a SQUID magnetometer, demonstrating the superparamagnetic nature of the nanocomposites at room temperature. The dc magnetization versus temperature ZFC curve indicated a low blocking temperatures of ca. 5 K for one polymer nanocomposite containing smaller particles and 26 K for the composite consisting of slightly larger particles. Our measurements of magnetic properties indicate the presence of single domain maghemite nanoparticles in the nanocomposite in which the 1:1 block copolymers were able to mask the nanoparticles efficiently by chelation through epoxy and ester groups. Both of the nanocomposites exhibited very small hysteresis below the blocking temperature and no hysteresis above this temperature, characteristic of superparamagnetism. Finally, the magnetization vs. applied magnetic field showed very high saturation magnetization values of ca. 60 emu g⁻¹ for the magnetic polymer nanocomposites. Thus, the magnetization data strongly suggest that the particles synthesized and stabilized by well-defined block copolymers, as described in this paper, are stable, non-interacting, single domain, coherently rotating, and uniform in size nanocomposites.
2.4. Experimental

2.4.1. Materials.
Bicyclo[2.2.1]hept-5-ene 2-carboxylic acid (98% mixture of endo and exo), norbornene (99%), thionyl chloride (99.5%), glycidol (96%), triethylamine (99.5%), Carbon disulfide, LiBr, bis(tricyclohexylphosphine)- benzylideneruthenium dichloride (Grubbs’ first generation catalyst, 3), bis(tricyclohexylphosphine)-benzylideneruthenium dichloride Grubbs’ second generation catalyst, 3, 5-dimethylpyrazole, Fe(CO)5 (99.9%), and trimethylamine N-oxide (98%), were purchased from Aldrich and used as received. CH2Cl2 and CHCl3 were dried over CaCl2 and distilled. THF was distilled over sodium and benzophenone ketyl under N2 before use. All catalyst solutions were prepared in a glovebox.

2.4.2. Synthesis

Synthesis of Bicyclo[2.2.1]hept-5-ene-2-carboxylic Acid Oxiranylmethyl Ester (2). The synthetic procedure is illustrated in Scheme 1. The acid chloride was prepared by refluxing a mixture of endo- and exo-bicyclo[2.2.1]-hept-5-en-2-carboxylic acid (25.0 g, 0.204 mol) and thionyl chloride (30 mL, 0.408 mol) in dry CHCl3 for 4-5 h under N2.34 Solvent was removed under reduced pressure and the residue was purified by vacuum distillation at 0.5 Torr at 42 °C, producing 1, as colorless liquid in 70% yield. Then, a mixture of triethylamine (22 mL, 0.16 mol) and glycidol (6.4 mL, 0.096 mol) was added over 2 h to the solution of the acid chloride (12 g, 0.08 mol) in dry THF at 0 °C. The mixture was then stirred at room temperature for 8 h. Et2O was added and the resulting white salt was filtered off. The organic filtrate was washed with aqueous 5% NaOH solution, followed by washing with 5% HCl, saturated Na2CO3, and water.
The solvent was removed under reduced pressure, and the residue was subjected to column chromatography (7:3 hexane:EtOAc on silica), affording 2 as a clear colorless oil (14.79 g, 95% yield). $^1$H NMR (500 MHz, CDCl$_3$) δ: 6.06-6.14 (m, 1.5H, HC=CH), 5.87 (m, 0.5H, HC=CH), 4.10-4.39 (m, 1H, CH$_2$-CH), 3.78-3.89 (m, 1H, O-CH$_2$-CH), 3.16 (s, 1H), 2.78 – 2.99 (m, 3.5H), 2.58 (s, 1H, -CH- epoxy ring), 2.22 (m, 0.5H), 1.83-1.87 (m, 1H), 1.19-1.48 (m, 4H). $^{13}$C NMR (125 MHz, CDCl$_3$) δ: 176.08, 174.58 (C=O exo and endo), 138.28, 138.05 (C=C), 135.83, 132.46, 77.78, 77.35, 76.93, 65.17, 64.99, 64.90, 49.97, 49.96, 49.78, 47.01, 47.00, 46.66, 46.07, 44.98, 44.94, 43.52, 43.31, 42.86, 41.98, 30.77, 29.65, 29.61. IR (neat): 2974, 1734 (C=O stretch), 1447, 1333, 1271, 1247 (C-O epoxide ring stretch), 1232, 1171, 1064, 1031, 904, 847 cm$^{-1}$. Anal. Calcd for C$_{11}$H$_{14}$O$_3$: C, 68.02, H, 7.27. Found: C, 67.89, H, 7.41.

**Polymerization by ROMP of Bicyclo[2.2.1]hept-5-ene-2-carboxylic Acid Oxiranylmethyl Ester.**

ROMP of epoxy monomer 2 with Grubbs’ first generation catalyst 3 was done according to a literature method and shown in Scheme 2. The catalyst solution was prepared by dissolving in anhydrous CH$_2$Cl$_2$ under N$_2$ atmosphere in a glovebox. The glassware was dried and purged with vacuum and N$_2$ in a Schlenk line several times prior to conducting the polymerization reaction.

(a) Preparation of Homopolymer 4. The epoxy monomer 2 (1.00 g, 5.15x10$^{-3}$ M, 300 equiv) was dissolved in 35 mL dry CH$_2$Cl$_2$ and purged with N$_2$ gas. Then, an adequate volume of the catalyst solution (14 mg, 17.13x10$^{-6}$ M, in 2 mL CH$_2$Cl$_2$, 1 equiv) was added to the reaction mixture and stirred for 4 h at 30 °C. The polymerization reaction mixture was terminated with ethyl vinyl ether (500 eq. relative to the catalyst) and stirred for another 1 h. Then, the reaction mixture was poured into cold methanol and stirred, purified, and dried under vacuum to give a
flaky white solid (72% yield). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$: of 1:1 diblock copolymer: 5.12-5.24 (br, 2H, -HC=CH-), 4.30 (br, 1H), 3.82 (br, 1H), 3.09 (br, 1H), 2.34-2.90 (br, 2H), 0.85–1.70 (br).

(b) Preparation of Diblock Copolymer 5. The epoxy monomer 2 (1.15 g, 5.93x10$^{-3}$ M, 200 equiv) was dissolved in 40 ml dry CH$_2$Cl$_2$ under N$_2$ gas. Then, the catalyst solution (25 mg, 30.38x10$^{-6}$ M, in 2 mL CH$_2$Cl$_2$, 1 equiv) was added to the reaction mixture and stirred for 4 h at 30 $^\circ$C. The pink color of the solution turned dark brown. The norbornene solution (0.55 g, 5.84x10$^{-3}$M in 40 mL CH$_2$Cl$_2$, 200 equiv.) was injected and stirred for another 5 h. The polymerization reaction mixture was terminated with excess ethyl vinyl ether (500 eq. relative to the catalyst) and stirred for another 1 h. Then, the reaction mixture was poured into cold methanol and stirred, purified, and dried under vacuum to give flaky white solid with 92% yield. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$: of 1:1 diblock copolymer: 5.19-5.44 (br, 4H), 4.35 (br, 1H), 3.82 (br, 1H), 3.18 (br), 2.98, 2.64, 2.43, 1.97 (br.), 0.85 – 1.70 (br). In a very similar fashion different block copolymers were prepared listed in Table 2-1.

**Synthesis of (2-thioxo-1, 3-oxathiolan-5-yl)methyl bicyclo[2.2.1]hept-5-ene-2-carboxylate 6.**

To a solution of epoxy monomer 1 (0.20g, 1.03 mmol) and catalytic amount of LiBr (0.005g, 0.05 mmol) in THF, carbondisulfide (0.09ml, 1.55 mmol) was added dropwise to the solution. The reaction mixture was stirred for about 3 hrs. The excess solvent was removed by rotary evaporator and then, yellow, oil was derived as pure product by using column chromatography (Hexane: ethyl acetate = 7:3) followed by drying under vacuum (0.26g, yield:92%). $^1$H NMR (300 MHz, CDCl$_3$) $\delta$: 6.20-6.12 (m, 1.5H, HC=CH), 5.94 (m, 0.5H, HC=CH), 5.33 (s, 1H), 4.48-
4.33 (b, 2H), 3.61 – 3.24 (m, 2H), 3.06-2.94 (d, 2H), 2.31-1.95 (m, 2H), 1.60 -1.31 (m, 4H). $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$: 211.10 (C=S), 176.05, 174.54, (C=O exo and endo) 138.38, 138.31, 138.24, 132. 40 (=C-CH=C), 87.87, 69.35, 63.03 (O-CH$_2$-CH), 50.06, 46.70, 43.54, 42.87, 42.01, 36.34, 36.23, 30.86, 29.67 IR (neat): 3500 (b), 3120, 2976, 1746 (vs, C=O), 1601, 1444, 1330, 1200, 1162, 1038, 716 cm$^{-1}$. Anal. calcd for C$_{12}$H$_{14}$O$_3$S$_2$: C, 53.31, H, 5.22. Found: C, 53.72, H, 5.26.

**Polymerization by ROMP of (2-thioxo-1, 3-oxathiolan-5-yl)methyl bicyclo[2.2.1]hept-5-ene-2-carboxylate 7**

ROMP was performed on monomer 6 in a similar process as described before. The dithiocarbonate monomer 6 was dissolved in dry CH$_2$Cl$_2$ and purged with N$_2$ gas. Then, an adequate volume of the catalyst was added to the reaction mixture and stirred at 30 °C. But with time the polymer formed from the reaction phase separated out from the solvent, although thereafter the reaction was terminated with diethyl ether. The white-grey polymer was insoluble in all commonly used solvents. So, further characterization could not be performed with that. Also, copolymers of monomer 6 and norbornene did not improve the solubility.

**Preparation of Stabilized Magnetic Nanoparticle Dispersion.** Preparation of monodisperse maghemite nanoparticles within copolymer matrices was accomplished by modification of known methods$^{36,37}$ as follows: the diblock copolymer was dissolved in cyclohexanone and heated to 100°C, followed by addition of 0.2 mL of Fe(CO)$_5$ (1.52mmol) under Ar. The mixture was refluxed for about 2 h until the yellow color of the solution turned black. It was then cooled to ambient temperature, and 0.34 g of trimethylamine N-oxide (4.56 mmol) was added to oxidize
the iron nanoparticles. The mixture was refluxed for another 4 h, and the black dispersion of diblock copolymer-stabilized nanoparticles was observed. The dispersion was centrifuged, and the supernatant was collected. Ethanol was added to the supernatant and black-brown precipitate was collected after further centrifugation. The black-brown precipitate was then redispersed in cyclohexanone or hexane. Two different formulations of maghemite-polymer nanocomposites were synthesized by varying the polymer weight (NC1-B1 - 3.3 wt% polymer; NC1-B2 – 0.97 wt% polymer) relative to a fixed amount of Fe(CO)₅ (0.2 mL) loading according to the procedure described above.

2.4.3. Characterization.

¹H NMR and ¹³C NMR spectra were acquired on a Varian Mercury Gemini spectrometer at 500 and 125 MHz, respectively, using CDCl₃ as the solvent for all monomers and polymers. Elemental analysis was performed at Atlantic Microlab, Inc., Norcross, GA. All FTIR studies were done using a Perkin-Elmer Spectrum One FTIR spectrometer from 4000 -500 cm⁻¹. Thermogravimetric analysis (TGA) was performed with a TA Instruments model Q5000 TGA, from room temperature to 750 °C at 20 °C /min. All samples were dried under vacuum for 2 days before measurement. Differential scanning calorimetry (DSC) was conducted with a TA Instruments Q1000 DSC, from -10 to 250 °C at a rate of 10°C/min. Transmission electron microscopy (TEM) was accomplished using a JEOL 1011 TEM, operated at 100 kV. A FEI Tecnai F30 TEM was used for ultra small nanocomposites. The samples were prepared by evaporation of a dispersion of nanoparticles in the polymer on carbon-coated copper TEM grids. Selected area electron diffraction patterns were obtained in both cases. X-ray diffraction (XRD) (Geigerflex Rigaku2, 2θ = 10 – 80°, step = 0.05, dwell (s) = 3) was used to obtain powder X-ray
diffraction pattern spectra using Cu-Kα radiation (λ = 0.154 nm), and noise corrections were made by using MDI Jade 7 software. Gel permeation chromatography (GPC) was conducted with a Waters 2414 refractive index detector, Waters 2996 photodiode array, and Waters 1525 binary HPLC pump (THF as the mobile phase, flow rate of 1 mL/min) using Waters styragel HR2, HR5E columns and polystyrene as the standards.

Magnetic properties of the nanocomposites were measured using a superconducting quantum interference device (SQUID) magnetometer from Quantum Design. All the measurements were done in powder form of the sample after vacuum drying. The temperature dependence of the magnetization was determined by zero field-cooled (ZFC) and field-cooled (FC) measurements. The ZFC curve was obtained by cooling down to 5 K at zero magnetic fields and then measuring the magnetization under a 500 Oe applied magnetic field. The magnetization was measured during heating from 5 K to room temperature at 10 K intervals. The ZFC curve was obtained by cooling down to 2 K at zero fields and then measuring the magnetization under a 500 Oe applied magnetic field (for NC1-B1) up to 50 K. The corresponding FC curves were similarly obtained except that this time the sample was cooled while applying a 500 Oe magnetic field. The magnetizations as a function of applied magnetic field were also studied under constant temperature (below and above the blocking temperature).

2.5. References


(13) Thompson, R. B.; Ginzburg, V. V.; Matsen, M. W.; Balazs, A. C. Science 2001, 292, 2469-2472.


3.1. Abstract.

Maghemite nanoparticles were templated by a novel, well-defined pyrazole-containing norbornene block copolymer to improve the chelation between iron-oxide nanoparticles with the polymer. This copolymer-maghemite nanocomposite provided superparamagnetism with high saturation magnetization at room temperature under an applied magnetic field. The synthesis, physical, morphological, and magnetic characterization of the nanocomposites are reported. Then, exploiting a core-shell (hydrophobic core, hydrophilic shell) strategy, the organic solvent dispersible nanocomposites were dispersed in water using Pluronic F127 copolymer, followed by TEM study and cell-viability (MTS) assay with Hela cell-lines, suggesting the potential of the polymeric magnetic nanocomposite in biological applications such as a contrast agent for MRI.

3.2. Introduction.

There has been great interest in magnetic nanoparticles for potential biomedical applications like medical diagnosis and imaging, magnetic separation, magnetic hyperthermia and controlled drug delivery\textsuperscript{1-6} and many biomimetic applications as magnetically controlled locomotion of artificial bacterial flagellar motors,\textsuperscript{7} artificial muscles.\textsuperscript{8} Magnetic resonance imaging (MRI) is one of the powerful and useful 3D techniques for non-invasively diagnosis of anatomic and physicochemical details of blood flow as well as many diseases. To improve the MRI resolution
and image quality several paramagnetic iron oxide nanoparticles, mostly Fe$_2$O$_3$ and Fe$_3$O$_4$ based, as negative contrast agent have been already effectively applied in many clinical trials.$^{1,9-12}$ Among these, newly developed ultrasmall superparamagnetic iron oxide (USPIO) are the most promising contrast agent because of its no magnetic hysteresis at ambient temperature, high magnetization change under applied magnetic field, tunability of sizes of the nanoparticles, non-toxicity, rapid clearance from the organs.$^{13-16}$

The USPIO enhances the MRI image by its superparamagnetic iron oxide core by decreasing the $T_1$- and $T_2$- relaxation behavior of the water molecules, inducing signal increase on $T_1$-weighted images ($T_1$-w) and signal reduction on $T_2$-weighted-gradient-echo ($T^*_2$-w) images.$^{17-19}$ Although control of uniform and stable magnetic nanoparticles are often challenging because of their inherent nature of forming clusters once they form nano dimensions to reduce the surface energy. Hence, considering the stable dispersion of the magnetic nanoparticles, surface coating with the ligands to anchor the iron oxides are of great importance. Ligands grafted on the polymer backbone give better control to handle the stability as well as the spatial behavior.$^{20}$ Our previous success in formation of stable and well-dispersed maghemite ($\gamma$-Fe$_2$O$_3$) –polymer nanocomposite led us to investigate to explore more flexibility and designs of even better ligand system by exploiting the chemistry of oxirane group.$^{21,22}$ Also, considering further biochemical applications, biocompatibility and aqueous dispersion of the nanocomposites is a very significant issue.

Herein, we present a strategy for the synthesis of a novel norbornene-based self-assembled diblock copolymer via ROMP of a functional monomer, and subsequent stabilization of
maghemite nanoparticles to form superparamagnetic nanocomposites. An iron-binding, anchoring ligand (siderophore) is present in one block of the diblock copolymer to chelate iron-oxide nanoparticles while the other block contains a steric stabilizing group which helps isolate and disperse the magnetic nanoparticles from each other. The anchoring group was synthesized by taking advantage of the versatility of the oxirane ring-opening by the heterocyclic pyrazole, creating a ligand to stabilize maghemite nanoparticles through strong coordination. The polynorbornene backbone was selected for its high thermal stability, optical transparency, low dielectric constant, and good mechanical properties. In addition, functionalized norbornene-based monomers are readily polymerized by highly tolerant Grubbs catalysts into low polydispersity copolymers with readily tailored block ratios. Well-defined novel diblock copolymers, containing 1:1 anchoring and steric stabilizing blocks, were characterized by NMR, gel permeation chromatography (GPC), thermogravimetric analysis (TGA), and differential scanning calorimetry (DSC). The ligand-stabilized organic-inorganic hybrid nanocomposites were prepared into self-assembled 1:1 block copolymer microdomains in situ by a non-hydrolytic procedure using a previously reported method. Transmission electron microscopy (TEM) studies on the nanocomposite revealed that the particles are highly monodisperse and spherical in shape, with an average diameter of 5 nm. X-ray diffraction (XRD) and selected area electron diffraction (SAED) patterns suggested the presence of crystalline maghemite-C type nanoparticles. FTIR analysis on the polymer nanocomposite supported the assembly of the polymer and iron oxide core through chelation, most likely forming stable ring structures. Surface topography of the nanocomposite and the block copolymer itself were studied by scanning electron microscope (SEM). Magnetic properties of the nanocomposite were
characterized by superconducting quantum interference device (SQUID) magnetometer, showing excellent superparamagnetic properties at room temperature.

![Pictorial representation of Core-shell micelles structure of pluronic coated nanocomposite](image)

**Figure 3-1** Pictorial representation of Core-shell micelles structure of pluronic coated nanocomposite

For biological application of the superparamagnetic nanocomposite, solubility in the aqueous medium is an important criterion. Pluronic copolymers are well known in pharmaceutical industry to improve the solubility of the hydrophobic drugs\(^{24}\) and to increase the biocompatibility and blood circulation time.\(^{25,26}\) Pluronic F127 is a triblock copolymer of poly(ethylene oxide)-poly(propylene-oxide)-poly(ethylene oxide) (PEO-PPO-PEO) consisting of 70 wt% PEO. Also, steric stabilization of the several nanoparticles by Pluronic F127 maintains stable suspensions in a high ionic strength environment.\(^{27}\) Its already been in literature that that oleic acid coated iron-oxide nanoparticles were stabilized in water by phase transfer from organic medium almost
keeping its inherent magnetic properties. Besides, Pluronic F127 also provides antifouling properties to prevent aggregation, protein adsorption along with recognition by RES. So, Pluronic F127 was used to encapsulate the pyrazole block copolymer stabilized nanocomposites to make it water soluble and biocompatible (Figure 3-1). The hydrophobic inner core (PPO) encapsulated the ligand stabilized maghemite nanocomposite whereas hydrophilic outer shell (PEO) helped to disperse it in water. Aqueous dispersions of the maghemite-polymer nanocomposites were further characterized with TEM to study the morphology and the cytotoxicity was studied by the cell-viability assay (MTS) with Hela cells, demonstrating good biocompatibility of the nanocomposites in cellular media.

3.3. Results and Discussion

Scheme 3-1 Synthesis of monomer

![Synthesis of monomer and polymer](image)

**Synthesis of monomer and polymer.** Scheme 3-1 illustrates the approach for the synthesis of the functional monomer and well-defined block copolymer. The monomer and polymers were designed and prepared by exploiting the versatility of the epoxy precursor in order to generate maghemite-polymer nanocomposites that were responsive to an external applied magnetic field. In the first step, a novel monomer 2 was synthesized from 2, by opening the epoxide ring with 3,5-dimethyl pyrazole in presence of base (see Experimental section for details). This provides
an efficient metal ligating framework to anchor the iron oxide core. Next, a 1:1 diblock copolymer was synthesized according to Scheme 3-2 via ROMP using Grubbs second generation catalyst by sequential addition of monomer 2 and nobornene. The molecular weight and the polydispersity of the polymer were controlled on the basis of a time-dependent study, monitoring monomer consumption by $^1$H NMR and controlling the [M]/[I] ratio, where M is monomer and I is initiator.

Scheme 3-2 Synthesis of block copolymer

The self-assembled diblock copolymer template contained the ligand system in one block, which can chelate through both N- (from pyrazole) and O-donors (from hydroxy and/or carboxylate groups) with the metal oxide core, while the second block contained the steric stabilizing group to disperse the nanoparticle composite and reduce interaction between nanoparticles. These ligands play a vital role in stabilizing the maghemite nanoparticles through coordination, and can adopt different bridging modes to coordinate the metal nanoparticles, affording a supramolecular metal-organic framework. The formations of desired polymers were characterized by $^1$H NMR (see Appendix B) and FTIR. The molecular weights of the polymers were determined by GPC and thermal analysis from TGA and DSC analyses (see Table 3-1 for results).
Table 3-1 Properties of the polymers

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Block ratio (m:n)</th>
<th>M_n (Theo)</th>
<th>m:n H NMR</th>
<th>M_n</th>
<th>PDI</th>
<th>Block Ratio</th>
<th>T_g (°C)</th>
<th>T_d (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>200:0</td>
<td>58072</td>
<td>1:0</td>
<td>65085</td>
<td>1.42</td>
<td>224:0</td>
<td>46</td>
<td>170</td>
</tr>
<tr>
<td>9</td>
<td>175:175</td>
<td>67291</td>
<td>1.08:1</td>
<td>66331</td>
<td>1.33</td>
<td>176:163</td>
<td>39</td>
<td>217</td>
</tr>
</tbody>
</table>

a Theoretical molecular weight calculated from [M]/[I] feed
b Mn and PDI were obtained from GPC in THF relative to polystyrene standards
c. Actual polymer block ratio was calculated from the 1H NMR and GPC results.
d. Temperature at 10% weight loss.

The second step was to generate monodisperse, uniform, highly crystalline maghemite nanoparticles \textit{in situ} from an iron pentacarbonyl precursor in the presence of the diblock copolymer (0.68 wt%) in cyclohexanone. Trimethylamine N-oxide was used as an oxidizer to prepare the maghemite nanocomposite.

\textbf{Characterization of Polymer-Nanocomposite.} The block copolymer-stabilized nanocomposites were characterized by TEM, SEM, XRD, and FTIR. TEM (Figure 3-2 (a, b)), confirming the generation of well-controlled, uniform and spherical iron oxide nanoparticles, encapsulated by the self-assembled block copolymer throughout the sample. The size distribution (Figure 1 (b)) was measured using ImageJ software, and the average size of the nanoparticles were 4.3nm with the range 3-6 nm. Evidence of formation of a crystalline structure of the Fe_2O_3 nanoparticles was obtained by XRD and SAED (Figure. 3-2 (c) and (d)), revealing cubic maghemite-C type structures of the nanoparticles, corresponding well with standard data (PDF#00-039-1346). The average diameter (d) of the singular $\gamma$-Fe_2O_3 nanocrystallites, estimated using the Scherrer equation, was 4 nm, corresponding to the strongest reflection (311) of Fe_2O_3 nanoparticles at 2$\theta$ value of 35.72°. This correlates well with TEM analysis of the
nanoparticles. SEM data revealed the bulk morphology of the 1:1 block copolymer and nanocomposite (Figure 3-3, (e) and (f)). The self-assembled nature of maghemite with the 1:1 block copolymer is prominent from SEM observation.

Figure 3-2 (a) TEM images of magnetic nanocomposite; (b) size distribution analysis of iron-oxide nanoparticles from TEM studies; (c) electron diffraction pattern of nanocomposite; (d) XRD analysis showing the presence of crystalline maghemite-C;
FTIR spectroscopic analysis provided compelling evidence of ligand-mediated binding of iron oxide with the pendant chelating group of the diblock copolymer (Figure 3-4(a)). The presence of a single broad band at about 574 cm\(^{-1}\) in the FTIR spectrum of the nanocomposite is

Figure 3-4  (a) FTIR analysis of 1:1 block copolymer (labeled 6) and magnetic nanocomposite (labeled NC); (b) magnetization (M) versus temperature (T) of the nanocomposite at 500 Oe applied magnetic field (H)
characteristic of Fe-O stretching for less than 8 nm nanoparticles.\textsuperscript{33} Also, the shift from 1732 cm\textsuperscript{-1} to 1690 cm\textsuperscript{-1} corresponding to C=O stretch of the polymer to nanocomposite respectively establish the binding between iron-oxide core with the diblock copolymer.

Figure 3-5. (a) M-H loop below $T_B$ shows coercive field ($H_c$) of 100 Oe. (b) M-H loop above $T_B$, coercive field ($H_c$) is zero.

Magnetization ($M$) as a function of temperature ($T$) was performed on $\gamma$-Fe$_2$O$_3$ – diblock copolymer nanocomposites in powder form using SQUID. Figure 3-4.(b) shows the zero field cooled (ZFC) and field cooled (FC) versus temperature profile, where the blocking temperature ($T_B$) of 9 K is denoted by the peak of the ZFC curve. The experimentation was similar to that described in Chapter 2. The low and sharp blocking temperature of the nanocomposite is consistent with the small size of the maghemite nanoparticles. Uniform distribution of the magnetic nanoparticles within the polymer matrix is further strengthened by very close superimposition of the ZFC and FC curves after passing the $T_B$. The magnetic hysteresis loop at 4.2 K, temperature below $T_B$, is shown in Figure. 3-5 (a). The coercive field was of the order of 100 Oe. Figure.3-5(b) shows the magnetization loops at three different temperatures ($T$)
For potential biomedical applications, solubility and biocompatibility of pyrazole-containing block copolymer stabilized maghemite nanocomposites is very significant. Thus, organic soluble maghemite-polymer nanocomposites were dispersed in water using an amphiphilic Pluronic F127 triblock copolymer (PEO-PPO-PEO), according to the standard solvent evaporation method discussed in Experimental Section. The water dispersible nanocomposite, encapsulated in the Pluronic triblock copolymer, was generated by mixing equal volume of organic soluble nanocomposite in methylene chloride and an aqueous solution of Pluronic F127, followed by vigorous stirring to evaporate the volatile organic solvent. The outer hydrophilic (EO) core of Pluronic F127 helps the dispersion in water and the inner hydrophobic core helps to stabilize the polymer-coated maghemite nanocomposite. The morphology of the Pluronic F127-pyrazole-maghemite nanocomposites in water were studied by TEM, showing (Figure 3-6) the size of the core maghemite nanoparticles before and after dispersion in water were similar and well-dispersed.

Figure 3-6 (a) TEM image of Pluronic F127 coated polymer-maghemite nanocomposites, (b) Size distribution of the nanoparticles from TEM micrograph
Infact, the Pluronic encapsulation even decreased the aggregation and enhanced the stability in water, consistent with reports in the literature.\textsuperscript{30,34}

Figure 3-7 (a) Photograph of pluronic 127 stabilized polymer-Fe$_2$O$_3$ nanocomposites(I) and the same under external applied magnetic field (II)

A colloidal solution (core-shell method) is formed with the nanocomposites in the presence of water in Pluronic F127 by the solvent evaporation method which was very stable without precipitation for weeks.

Cytotoxicity studies (Figure 3-8) of Pluronic and pyrazole block copolymer-coated superparamagnetic nanoparticles were carried out in Hela cells out in order to examine the stability and toxicity cell-viability assay (MTS). Hela cells were incubated for 24 h with varying concentrations of the nanocomposites (0-50 µM), exhibiting minimal toxicity of the nanocomposites as the cells were viable (90-100%) depending on the concentration. This infers that the novel pyrazole-containing ligand block copolymer was capable of stabilizing maghemite nanoparticles. The nanocomposite dispersion in water, via Pluronic F127 micelle encapsulation,
makes it a potential candidate as a contrast agent for MRI imaging, or as carrier for drug delivery, subjects of possible future investigation.

![Figure 3-8 Cell viability of MNCs in Hela cells at the various concentrations.](image)

**3.4. Conclusion**

In summary, it is clear that well-defined N,N-dimethyl pyrazole-functionalized norbornene-based diblock copolymers are effective in stabilizing maghemite nanoparticles. The resulting nanocomposite exhibited strong superparamagnetism in the presence of an external magnetic field. Nanoparticle agglomeration was prevented through specific binding between the pendant ligands in the anchoring block and the iron oxide metal core. This well structured organic block copolymer-inorganic maghemite nanocomposite underwent dynamic changes in magnetic properties and directional motion in response to an external magnetic field without destroying the integrity of the system. The pyrazole-stabilized magnetic
nanocomposites exhibited good aqueous stability and low cytotoxicity. These results hold immense promise for the design of a magnetically-actuated systems capable of mimicking biological systems, smart organic-inorganic hybrids, regulated drug delivery, and nano-bioengineering applications, aspects that are subjects of future investigation.

3.5. Experimental

3.5.1. Materials.

Monomer 1, bis(tricyclohexylphosphine)-benzylideneruthenium dichloride Grubbs’ second generation catalyst, 3, 5-dimethylpyrazole, Fe(CO)5 (99.9%), trimethylamine N-oxide (98%), and DMF were purchased from Aldrich and used as received. CH2Cl2 and CHCl3 were dried over CaCl2 and distilled. THF was distilled over sodium and benzophenone ketyl under N2 before use. All catalyst solutions were prepared in a glovebox. Pluoronic F127 (Mw~ 12600) was purchased from BASF. Promega CellTiter 96® AQeuous One Solution Reagent was purchased from Fisher. Hela cells were purchased from ATCC (America Type Culture Collection, Manssas, VA, USA). All cells were incubated in Minimum Essential Media (MEM, Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA, USA), 100 units/mL penicillin-streptomycin (Atlanta Biologicals, Lawrenceville, GA, USA), and incubated at 37 °C in a 95% humidified atmosphere containing 5% CO2.
3.5.2. Synthesis

Synthesis of 3-(3, 5-dimethyl-1H-pyrazol-1-yl)-2-hydroxypropyl bicyclo[2.2.1]hept-5-ene-2-carboxylate (7). Monomer 2 was prepared according to our previous published procedure.\textsuperscript{31} Epoxy monomer 1 (0.23g, 1.2 mmol) in DMF was added dropwise to a suspension of 3, 5-dimethylpyrazole (0.107g, 1.11 mmol) and anhydrous potassium carbonate (0.166g, 1.2 mmol) in 10 mL of anhydrous DMF. The reaction was stirred at 100 °C for 9 h until the disappearance of starting materials (followed by TLC). It was then cooled to room temperature, the salt was filtered off, and the solvent was removed under reduced pressure. A white creamy solid (0.15 g, 55% yield) was obtained after column chromatography (1:1 hexane:ethyl acetate on silica) followed by vacuum drying overnight. \textsuperscript{1}H NMR (500 MHz, CDCl$_3$) $\delta$: 6.14-6.03 (m, 1.5H, HC=CH), 5.87 (m, 0.5H, HC=CH), 5.74 (s, 1H-Py ring), 4.52 (b, 1H, -OH), 4.17 – 3.85 (m, 6H), 3.15 (s, 0.5H), 2.98-2.90 ((m, 1H), 2.89-2.86 (m, 1H), 2.20 – 2.13 (m, 6H, 2 -CH$_3$ in Py ring), 1.88 - 1.84 (s, 1H), 1.69 -1.19 (m, 3H). \textsuperscript{13}C NMR (125 MHz, CDCl$_3$) $\delta$: 176.05, 174.54, (C=O exo and endo) 148.24, 139.80, 138.14, 137.96, 137.92, 135.64, 132. 26, 105.12 (=C -CH=C), 69.24, 65.19, 65.17, 65.04, 64.97, 49.72, 46.67, 46.36, 45.80, 45.77, 43.26, 43.04, 42.53, 41.64, 30.45, 29.31, 13.43 (Py-CH$_3$) , 10.95 (Py-CH$_3$) IR (neat): 3349.48 (b, -OH), 3025.81, 2925.08, 1732.36 (vs, C=O), 1601.25,1553.78, 1492.89, 1452.06, 1333.81, 1272.80, 1232.39, 1180.06, 1029.02, 906.84, 756.10, 620.99, 540.10 cm$^{-1}$. Anal. calcd for C$_{16}$H$_{22}$N$_2$O$_3$: C, 66.18, H, 7.64. Found: C, 65.96, H, 7.65.

Preparation of diblock copolymer 5 by ROMP of 3-(3, 5-dimethyl-1H-pyrazol-1-yl)-2-hydroxypropyl bicyclo[2.2.1]hept-5-ene-2-carboxylate (9) ROMP of monomer 2 with Grubbs second generation catalyst 3 was performed as shown in Scheme 1. The glassware was dried and
purged with vacuum and N₂ in a Schlenk line several times prior to conducting the polymerization reaction. A solution (0.2 M) of monomer 2 (120 mg, 4.13 x 10⁻⁴ M, 175 eq) was prepared in dry CH₂Cl₂ under N₂. The catalyst solution was prepared by dissolving the catalyst in anhydrous CH₂Cl₂ under N₂ in a glovebox. The catalyst solution (2 mg, 2.36 x 10⁻⁶ M in 0.5 mL CH₂Cl₂, 1 eq) was added to the reaction mixture and stirred for 8 h at 30 °C. A norbornene solution (38 mg, 4.13 x 10⁻⁴M, 175 eq) was injected and stirred for another 9 h. The polymerization reaction mixture was terminated with excess ethyl vinyl ether (300 eq relative to catalyst) and stirred for another 1 h. The reaction mixture was then poured into cold methanol and stirred, purified, and dried under vacuum, yielding a flaky white solid in 72% yield. ¹H NMR (500 MHz, CDCl₃) δ: 5.82 (b, CH, Py ring), 5.35 -5.22 (b, -HC=CH-), 4.17- 3.85, 3.18, 2.96, 2.44, 2.23-2.19, 1.87-1.27(b).

Preparation of Stabilized Magnetic Nanoparticle Dispersions. Preparation of monodisperse maghemite nanoparticles within copolymer matrices was accomplished by our previously reported method in Chapter 2 using cyclohexanone as solvent or others as required.²²

Preparation of Micelles Stabilized Magnetic Nanoparticle Dispersions in water. 120 mg of F127 was dissolved in 20 ml (to confirm the concentration is higher than CMC; Critical micelle concentrations (CMCs) at room temperature of Pluronic F127 is 0.007 g/cm³)³⁵,³⁶ of water by vigorously stirring for 3 hrs. Maghemite-polymer nanocomposites (7mg) were dispersed in CH₂Cl₂. Equal volume of these two mixtures were added together and stirred under N₂ at 40 °C (waterbath) for until all CH₂Cl₂ evaporated in a fume hude and nanocomposite covered by the F127 coating was prepared in water. Then, it was subjected to centrifuge and finally, the light
brown colored supernatant was collected which contains blockcopolymer-maghemite naocomposites entrapped in pluronic micelles.

3.5.3. Characterization

$^1$H NMR and $^{13}$C NMR spectra were acquired on a Varian spectrometer at 500 and 125 MHz, respectively, using CDCl$_3$ as the solvent for all monomers and polymers. Elemental analysis was performed at Atlantic Microlab, Inc., Norcross, GA. All FTIR studies were done using a Perkin-Elmer Spectrum One FTIR spectrometer from 4000-500 cm$^{-1}$. Gel permeation chromatography (GPC) was conducted with a Waters 2414 refractive index detector, Waters 2996 photodiode array, and Waters 1525 binary HPLC pump (THF as the mobile phase, flow rate of 1 mL/min) using Waters styragel HR2 and HR5E columns, and polystyrene standards. Thermogravimetric analysis (TGA) was performed with a TA Instruments model Q5000 TGA, from room temperature to 600 °C at 20 °C /min. All samples were dried under vacuum for 2 days before measurement. Differential scanning calorimetry (DSC) was conducted with a TA Instruments Q1000 DSC, from -10 to 120 °C at a rate of 10 °C/min. Transmission electron microscopy (TEM) was accomplished using a JEOL 1011 TEM, operated at 100 kV. The samples were prepared by evaporation of a dispersion of nanoparticles in the polymer on carbon-coated copper TEM grids. Particles size distribution, on the base of the profile of randomly selected quasi-spherically shaped particles, has been obtained using the ImageJ program. Selected area electron diffraction patterns were also obtained. XRD (Geigerflex Rigaku2, 20 = 0 – 80°, step = 0.05, dwell (s) = 3) was used to obtain powder X-ray diffraction pattern spectra using Cu-Kα radiation ($\lambda = 0.154$ nm). Noise corrections were made by using MDI Jade 7 software. The morphology of the block copolymers and the nanocomposite in powder form (sputter coated on sample holder)
were examined by SEM (Zeiss ultra 55). Magnetic properties of the nanocomposites were measured using a superconducting quantum interference device (SQUID) magnetometer from Quantum Design. All the measurements were done in powder form of the sample after vacuum drying. The temperature dependence of the magnetization was determined by zero field-cooled (ZFC) and field-cooled (FC) measurements. The ZFC curve was obtained by cooling down to 4 K at zero magnetic fields and then measuring the magnetization under a 500 Oe applied magnetic field up to 300 K. The corresponding FC curves were similarly obtained, except that this time the sample was cooled while applying a 500 Oe magnetic field. The magnetizations as a function of applied magnetic field were also studied at 100 K above the blocking temperature.

Cytotoxicity (MTS) assay. To test the cytotoxicity of the nanoparticles, $4 \times 10^3$ per well of Hela cells in 96-well plates were incubated in 90 µL of RPMI medium without phenol red, supplemented with 10% FBS and 100 units/mL penicillin-streptomycin for 24h. Then the cells were incubated with various amounts of nanoparticles (1µg/mL, 10 µg/mL, 20 µg/mL, 50 µg/mL) for additional 20 hours. Subsequently, 20 µL of CellTiter 96® AQueous One Solution reagent was added into each well, followed by further incubation for 4h at 37 °C. The relative viability of the cells incubated with nanoparticles to untreated cells was determined by measuring the MTS-formazan absorbance on a Kinetic microplate reader (Spectra Max M5, Molecular Devices, Sunnyvale, CA, USA) at 490 nm with a subtraction of the absorbance of cell-free blank volume at 490 nm. The results from three individual experiments were averaged.
3.6. References


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CHAPTER 4: ENHANCEMENT OF J-AGGREGATION OF PORPHYRIN-BASED DYES IN SELF-ASSEMBLED POLYMER MATRIX

4.1. Abstract

Supramolecular structures based on organized assemblies of macrocyclic chromophores, particularly porphyrin-based dyes, have attracted widespread interest as molecular devices with potential applications in molecular electronics, artificial light harvesting, and pharmacology. We report the formation of J-aggregates of two porphyrin-based dyes (TPPS, 12) and another synthesized analog of porphyrin (13) in water using functionalized norbornene-based homopolymers, synthesized by the ring opening metathesis polymerization technique. The J-aggregation of the dyes is remarkably enhanced, exploiting the ionic interaction of the cationic siderophores (tertiary amino group) of the polymer under acidic condition with the negatively charged sulfonate groups of the dyes.

4.2. Introduction

Supramolecular structures based on organized assemblies/aggregation of macrocyclic chromophores have attracted widespread interest as molecular devices with potential applications in molecular electronics, artificial light harvesting and pharmacology. Among the aggregations, J-aggregation (side-by-side) and H-aggregation (face-to-face) are well-studied which are characterized by strong, narrow, red-shifted absorption wavelengths and blue shifted absorption respectively with respect to its monomeric form. Aggregation, driven by non-covalent interactions mostly, causes remarkable change the optical and electronic properties in the
molecular aggregates. Strong intermolecular interactions between the dyes and delocalized excitonic energy over the whole assembly of aggregation are the characteristics of this phenomenon. Kasha et al.\textsuperscript{1} proposed the following equation to explain the dipole–dipole coupling interaction energy (V) in aggregations:

\[ V = -\frac{M^2}{r^3}(1 - 3\cos^2\theta) \]

where M is the transition dipole moment, r is the distance from center-center between dipoles, \( \theta \) represents the geometrical factor related to mutual inclination of the aligned monomers.

Also, molecules with strong 2PA properties are of rapidly growing interest because of their potential applications in photodynamic therapy (PDT), optical power limiting, 3D fluorescence microscopy, high density data storage, and micro- and nano-fabrication. The 2PA cross section of an organic molecule can be enhanced, in general, by two methods: (a) design of chromophores with long conjugation and push-pull system by appropriate donor, acceptor system (intramolecular), and (b) molecular assembly formation by the aggregation of the organic dyes (intermolecular). The first approach was investigated by the several research groups and still is an area of interest. More recently however, the second approach attracted interest as some theoretical (one dimensional Frenkel-exciton model) and experimental results revealed the immense potential of the net increase of 2PA of the self-assembled aggregation of the dyes forming J-aggregates. A strong enhancement of the 2PA cross section (\( \delta \)) of porphyrin systems (symmetric or asymmetric) forming J-aggregates was reported by Bozio,\textsuperscript{2-4} Sainudeen,\textsuperscript{5} and an enhancement of the 2PA cross section in the J-aggregation of PIC dyes was also observed in
water, first by Belfield and later by others. An electronic cooperative effect was responsible for the enhancement in both cases. Recently, Prasad reported aggregation-enhanced fluorescence and 2PA in nanoaggregates due to the hindering of molecular internal rotation.

Among the known molecular building blocks, porphyrins constitute a highly attractive class of “synthons” for functional nanomaterials due to their unique photonic and electronic properties, specifically for their potential applications in PDT, nonlinear optics, and for investigation of artificial light harvesting system mimicking natural photosynthesis. Tetrakis(4-sulfonatophenyl)porphyrin (TPPS) is probably one of the most studied synthetic porphyrins. Under acidic aqueous conditions (usually pH <1), the diacid species of the TPPS porphyrin, forms J-aggregates due to hydrophobic π-π stacking and electrostatic interaction between the anionic sulfonated phenyl group and the cationic core. The tuning factors of porphyrin aggregation in aqueous solution vary depending on the porphyrin structure and concentration, as well as the pH, ionic strength, and counterions of inorganic salts in the media. Also, the medium, such as copolymer micelles, polymers ionic liquids, nucleic acids, polypeptides, proteins, and carbon nanotubes, is capable of influencing porphyrin aggregation behavior. Several research progressions with polymer based self-assembled J-aggregation of the dyes were discussed earlier in the Chapter 1. Examples of well-defined, molecularly assembled porphyrinic aggregation in solution, that can be systematically built up from the nano- to microscale in a hierarchical and controlled fashion, are still rare.

Herein, our main focus is to determine whether a functional copolymer can serve as the foundation to build a supramolecular structure containing two porphyrin-based dyes (separately),
facilitating J-aggregation of the porphyrin dyes. We report the synthesis and characterization of a norbornene-based monomer, containing dimethylamine with a linker at the sideophore, and corresponding homopolymers by ROMP. Then, the J-aggregation of two porphyrin-based dyes, commercial TPPS (12) and a synthetic analog (13), in acidic aqueous solution was studied with and without presence of the homopolymer templates. The aggregation properties were evaluated by UV-vis absorption, fluorescence spectroscopy, and fluorescence lifetime decay studies, clearly demonstrating J-aggregation was more pronounced induced in dyes by the polymer, possibly due to enhanced stabilization of the anionic dye’s periphery by the cationic nature of the sideophore (-NHMe₂⁺) of the polymers at low pH.

4.3. Results and Discussion

Scheme 4-1. Synthesis of monomers and homopolymer

Synthesis. The synthesis of monomer 10 is shown in Scheme 4-1. Compound 1 was reacted with 6-dimethylaminohexanol under basic condition to give 10 as a product by esterification. The NMR spectra and GCMS results confirmed product formation. The homopolymer of 10 was prepared ROMP, using Grubbs first generation catalyst, and characterized by NMR, (see Experimental Section). The polymer was soluble in organic solvents such as chloroform and
methylene chloride. However, the solubility of the homopolymer in THF was poor, which restricted measurement of the molecular weight via GPC with polystyrene as standard in THF. The polymer became water soluble after lowering the pH with HCl due to protonation at the amino group. As the porphyrin derivatives generally undergo aggregation at low pH (<1) in aqueous medium, polymer 11 should be suitable to be used as a template.

**Scheme 4-2. Synthesis of dye 13**

The analog of commercially available TPPS dye, 13 was synthesized by a three-step process (Scheme 4-2). Briefly, first tetraphenylporphyrin was selectively nitrated (mono) with fuming nitric acid, then the nitro group was reduced with SnCl2/conc. HCl, transform it into an amino group, followed by exhaustive sulfonation to produce water soluble porphyrin derivative 13, an analog of 12.

The solutions of two porphyrin dyes (12 and 13) were prepared in aqueous solution at low pH by acidification with different buffer (HCl/KCl – pH range 2.2, 2.0, 1.5, 1.0). The solutions of dyes were prepared in neutral condition by dissolving them in ultra-pure water (pH ~ 7.0) and another in acidic condition by adding 0.2 M HCl (without adding any salt, pH 2.0). All the solutions were prepared at the same concentration of the dyes in them ([C] = 4 x 10⁻⁶ M). In another set, first the homopolymer 11 was first dissolved in the same buffer as mentioned and a certain
amount (0.4g/L) of the polymer was gradually added (Scheme 4-3). All solution preparation and photophysical studies were done in the dark. The aggregation properties were studied by time-dependent UV-vis absorption, fluorescence, and fluorescence lifetime decay for both the dye solutions themselves as well as polymer-templated dye solutions.

**Scheme 4-3. Scheme for dye aggregations on polymeric template**

Figure 4-1. (a) UV-vis absorption spectra of (12) solution; (b) formation of J-aggregates of (12) at different pH (HCl/KCl aqueous buffer).

Figure 4-1. (a) UV-vis absorption spectra of (12) solution; (b) formation of J-aggregates of (12) at different pH (HCl/KCl aqueous buffer).
UV-vis Absorption Spectroscopy: The absorption bands of TPPS (12) and its analog (13) in the acidified water with or without polymer templates at $10^{-5}$M concentration of the dyes were measured. Commercially available TSPP (12) is known to form J-aggregates under strong acidic condition. In neutral aqueous solution, TPPS remains as a monomeric free base form considering the electrostatic anionic repulsion from the sulfonate group. The absorption bands correspond to its non-protonated form (Figure 4-1), having an intense Soret band at 414 nm and weak Q bands at 516, 550, 581, and 635 nm.

In the presence of acid, protonation takes place at the 2 N of the porphyrin core. Thus, the absorption maxima of Soret band shifts to 435 nm and the two Q bands shift to 590 and 645 nm. The presence of J-type aggregation is usually signaled by an intense and narrow absorbance at 490 nm (J-band), which is bathochromically shifted relative to the monomer absorption band at about 435 nm (the Soret band), and a weaker broad band at 705 nm. The 490 nm J-band of the aggregates has been assigned to a Frenkel exciton transition.\textsuperscript{15,16} Porphyrins tend to self-assemble through balancing π-π oblique stacking interactions of their hydrophobic porphyrin rings, and charged substituent groups, present at inner (cationic N) core and outer surface (anionic sulfonate groups) by electrostatic forces. The induction of J-aggregation of 12 has been studied using HCl/KCl buffers (pH range 2.2 -1.0) at room temperature in the dark. Dye 12 formed stronger J-bands with decreasing pH (Figure 4-2 (a)). At pH 1.0, with increasing time, more molecules of 12 participates in the J-aggregation, as shown in Figure 4-2 (b). This behavior is consistent with other reports.\textsuperscript{17}
In the presence of polymer 11, dye 12 produced a well defined strong and sharp J-band at 491 nm along with a broad band at 706 nm, while an H band at 422 nm appeared at slightly higher pH (>1.0). Formation of the H-aggregates is generally attributed to π-conjugation of the porphyrin rings with the combined effect of attractive σ-π and repulsive π-π interactions. Also, with increasing time periods (0-30 min), it has been noticed that more molecules were transformed from H-aggregation to J-aggregation with a very sharp and narrow J-band at 490 nm, possibly due to a stabilization effect of cationic polymer 11. Also, the spectral width (full-width at half-maximum, FWHM) of the absorption peak of the J-aggregate varies with the coherence length as $N^{-1/2}$, where $N$ is the spectroscopic aggregation number and effect of external ammonium ions were evaluated in literature.

The J-aggregation study of dye 13 has not been reported to date. Dye 13, in its non-protonated form, exhibited the soret band at 416 nm along with a shoulder at 444 nm and a weak band at
654 nm. In presence of 0.2 M HCl, the soret band of dye 13 bathochromically shifted to 433 nm and the Q band blue shifted to 647 nm.

Dye 13 behaved differently with respect to dye 12 in the pH range 1.0-2.2 due to the presence of the amine group instead of the fourth sulfonate group at the para position of one of the phenyl substituents. Dye 13 showed increasing J-band with increasing pH (Figure 4-3 (a)) at sharp J-band at 484 nm and another red shifted band at 696 nm. With the addition of polymer 11, stronger J-aggregation of 13 occurred at the same concentration of the dye at pH 1.0, which is clear from the higher ratio and sharper nature of the peak corresponding to J band at 484 nm to the peak at 432 nm. In addition to π-π hydrophobic interactions and the electrostatic attraction force between imidazolium ring and sulfonated phenyl group, the interaction of the cationic
dimethylammonium in the J-aggregated species reduced the repulsive forces among porphyrin rings, thus, making the J-aggregation solution more stable in aqueous solution.

**Fluorescence Spectroscopy:** Steady state fluorescence emission spectra were recorded for TPPS in its neutral condition, acidic condition (10⁻⁶ M) and after formation of J-aggregates with or without polymer (10⁻⁵ M) at room temperature in 1 cm cuvettes using a PTI Quantamaster spectrofluorimeter.

![Fluorescence Spectra](image)

**Figure 4-4.** Steady state fluorescence spectra.

Figure 4-4 shows the fluorescence spectra resulting from the excitation of Soret band (B-band) of the species at 413, 434, and 490 nm for free base, dianion monomer, and J-aggregated species, respectively. The emission spectra did not alter whether excited at the B or Q bands, as reported in literature.²²,²³ Emission of the aggregated species (with or without polymer) was considerably weaker, with almost negligible Stokes shifts, compared to the monomer and dianion species, likely due to the predominant effect of radiative quenching pathways.²³
**Lifetime studies:** To shed light on the photodynamics of the porphyrin J-aggregates, fluorescence lifetime decays were studied using a time-correlated single photon counting system (PicoHarp 300) under linear polarized femtosecond excitation.

| Table 4-1. Photophysical properties of the TPPS (12, free base and dianion) and related J-aggregations with or without polymer templates |
|-----------------|-----------------|-----------------|----------------|-----------------|-----------------|-----------------|-----------------|
|                 | $\lambda_{\text{abs}}$ (nm) | $\lambda_{\text{em}}$ (nm) | $\lambda_{\text{exc}}$ (nm) | $t_1$ | Lifetime (ns) $t_2$ | $R^2$ |
| TPPS (12)       | 413, 515, 635   | 472             | 413(826)       | 6.87 |                 | 0.99246 |
| TPPS (pH 2.0)   | 434, 644        | 466             | 434 (868)      | 3.74 |                 | 0.99788 |
| 12 (J)          | 434, 490, 708   | 709             | 710            | 0.32 | 0.06935         | 0.99711 |
| 11 + 12 (J)     | 421, 491, 711   | 712             | 710            | 0.25 |                 | 0.99728 |

The free base and dianion monomer had a single exponential decay with excited state lifetimes of 6.87 and 3.74 ns for nonlinear excitation at 826 and 868 nm, respectively. As anticipated, the lifetime of the J-aggregated species were very short when excited at Q band (710 nm). Specifically, with the polymer template a shorter (250 ps) lifetime was observed, as shown in Table x. Fluorescence quenching and shortening of the excited state lifetime can be explained by different mechanisms, e.g., efficient internal conversion between different energy states and fast quenching of fluorescence intermolecular phonon modes that controls motion in the aggregate formation direction.$^{23,24}$
We have successfully examined the effect of a ROMP-based water soluble polymeric template on the J-aggregation properties of two porphyrin-based dyes (TPPS, 12, and its amine-containing analog 13). The photophysical properties of the J-aggregated species showed facilitation of J-aggregation by the amphiphilic polymer, with the polymer inducing J0-aggregation faster than the dye alone under similar conditions. These results support the use of cationic polymer

4.4. Conclusion
templates to enhance the J-aggregation in solution through possible attractive interaction between anionic porphyrin periphery, establishing the foundation for the design of polymer-templated supramolecular aggregates.

**4.5. Experimental**

### 4.5.1. Materials

5,10,15,20-Tetrakis(4-sulfonatophenyl)porphyrin (TPPS) and TPP were purchased from Strem and Aldrich, respectively, and used without further purification. Dimethylaminohexanol was obtained from TCI America. Grubbs first generation catalyst and norbornene carboxylic acid were purchased from Aldrich. Compound 1 was prepared according to our previously reported procedure. All solvents were purified and drieds according to standard procedures.

### 4.5.2. Synthesis

*Synthesis of the 6-(dimethylamino)hexyl bicyclo[2.2.1]hept-5-ene-2-carboxylate, 10.* Compound 1 (10.8 g, 0.069 M) was dissolved in freshly prepared THF. Then, a mixture of 6-dimethylaminohexanol (14.22 mL, 0.086 M) and NaHCO₃ (11.6 g, 0.14 M) were added to the solution at room temperature under N₂ atmosphere and refluxed overnight. After the reaction was complete, the mixture was filtered to remove the salt, and THF was removed under reduced pressure. This was followed by washing with water and extraction with CH₂Cl₂, then drying over anhyd. Na₂SO₄. Colorless oil was obtained after column chromatography with 3:1 CH₂Cl₂:MeOH, solvent removal, and vacuum drying. $^1$H NMR (500 MHz, CDCl₃) δ: 8.98 (NH, 1H), 6.18-5.79 (m, 2H, HC=CH), 4.09 – 3.82 (m, 2H), 3.13 (s, 0.5H), 3.04-2.68 (m, 2H), 2.42-2.06
(m, 8H), 1.92 - 1.73 (s, 1H), 1.64 -1.06 (m, 11H). $^{13}$C NMR (125 MHz, CDCl$_3$) δ: 176.24, 174.69, (C=O exo and endo) 138.02, 137.55, 135.74, 132. 36, 64.29, 63.38, 59.40, 46.74, 45.76, 43.07, 43.31, 30.40, 30.22, 29.27,28.88, 28.34, 26.90, 25.79. HR-MS-ESI theoretical m/z [M+H]$^+$ = 266.21, found 266.21.

Synthesis of polymer II. ROMP of monomer 10 with Grubbs first generation catalyst was performed as shown in Scheme 4-1. The glassware was dried and purged with vacuum and N$_2$ in a Schlenk line several times prior to conducting the polymerization reaction. A solution (0.2 M) of monomer 10 (265 mg, 1 x 10$^{-3}$ M, 175 eq) was prepared in dry CH$_2$Cl$_2$ under N$_2$. The catalyst solution was prepared by dissolving the catalyst in anhydrous CH$_2$Cl$_2$ under N$_2$ in a glovebox. The catalyst solution (8.5 mg, 1 x 10$^{-6}$ M in 0.5 mL CH$_2$Cl$_2$, 1 eq) was added to the reaction mixture and stirred for 1 h at 30 °C. The polymerization reaction mixture was terminated with excess ethyl vinyl ether (300 eq relative to catalyst) and stirred for another 1 h. The reaction mixture was then poured into cold methanol, stirred, collected by filtration, and dried under vacuum, yielding flaky white solid in 82% yield. $^1$H NMR (500 MHz, CDCl$_3$) δ: 5.54 -5.09 (b, -HC=CH-), 4.22- 3.84, 3.26- 2.61, 2.58-2.22, 2.15-1.85, 1.80-1.05(b). M$_w$ (GPC) =26590 (cald.), n = 100.

4.5.3. Characterization

$^1$H NMR and $^{13}$C NMR spectra were acquired on a Varian Mercury Gemini spectrometer at 500 and 125 MHz, respectively, using CDCl$_3$ as the solvent for all monomers and polymers. High resolution mass spectrometry (HR-MS) analysis was performed in the Department of Chemistry, University of Florida, Gainesville, FL. Samples for the spectroscopy measurements were
prepared by dissolving the dyes in ultrapure water and acidified with 0.2M HCl solution. Different buffer solutions (pH: 2.2-1.0) were prepared according to CRC handbook\textsuperscript{25} procedure.

Linear photophysical properties were investigated in spectroscopic-grade solvents (DMSO and ultrapure water) at room temperature. The steady state absorption spectra were obtained with an Agilent 8453 UV-visible spectrophotometer using 1 cm path length quartz cuvettes with dye concentrations of $1 \times 10^{-5}$ M. The steady state fluorescence spectra and excitation anisotropy spectra were obtained with a Photon Technologies, Inc. (PTI) QuantaMaster spectrofluorimeter, using 10 mm spectrofluorometric quartz cuvettes and low concentration solutions $C \leq 10^{-6}$ M. All fluorescence spectra were corrected for the spectral sensitivity of the PTI emission monochromator and photomultiplier tube (PMT) detector. The values of the fluorescence lifetime of was measured with a time-correlated single photon counting system (PicoQuant PicoHarp 300) under linear polarized femtosecond excitation oriented by the magic angle, with time resolution $\sim 80$ ps.

### 4.6. References


CHAPTER 5: SYNTHESIS OF MULTI-SCAFFOLD BLOCK COPOLYMERS FOR TARGETED TWO-PHOTON MEDIATED BIOIMAGING

5.1. Abstract

The synthesis and characterization of multi-scaffold block copolymers for targeted bioimaging is detailed. Targeted molecular imaging with two-photon fluorescence microscopy (2PFM) is a powerful technique for chemical biology and, potentially, for non-invasive diagnosis and treatment of a number of diseases. The synthesis, photophysical studies, and bioimaging is reported for a versatile norbornene-based block copolymer multifunctional scaffold containing biocompatible (PEG), two-photon fluorescent (fluorenyl), and targeting (cyclic-RGD peptide) moieties. Cell-viability and 2PFM imaging of the new probes with human epithelial U87MG cell lines that over express αvβ3 integrin, along with control studies using MCF-7 cells and blocking experiments, are reported.

5.2. Introduction

Two photon fluorescence microscopy (2PFM) is fast becoming an important tool for immunological research and three-dimensional (3D) optical imaging of biological samples, ranging from cellular membranes to millimeter-thick brain slices. Several excellent reviews have appeared that describe the theory, experimentation, and applications of 2PFM.1-7 Briefly, a fluorophore is first excited to a singlet state by the simultaneous absorption of two low energy photons, bridging the energy gap between the excited state and the ground state, followed by the
fluorescence emission. Effective quasi-simultaneous absorption of two photons requires a high temporal and spatial photon density, facilitated by mode-locked, high peak-power laser pulses. Two-photon absorption (2PA) processes offers several advantages over one-photon absorption (1PA). For 2PA, fluorescence emission is quadratically dependent on the excitation irradiance (intensity of excitation light), and the volume of fluorescence emission is largely restricted to the region of the focal point (least out of focus) of interest. In addition, the longer wavelength, typically in the near-infrared (NIR) spectral range, used in 2PA process is much more favorable for the biological applications for its minimal photodamage and deeper penetration depth for imaging and therapeutic applications.

For efficient two-photon assisted imaging, a fluorescent probe should be highly fluorescent (high fluorescence quantum yield) with high 2PA cross section at wavelengths suitable for biological imaging (typically 690-1000 nm to avoid scattering effect of tissues and absorption of hemoglobin), and have high photostability. These parameters helps for improved penetration depth and a low laser power for fluorescence excitement helps in maintaining biocompatibility without causing photo cytotoxicity. Recently, remarkable progress in the design and synthesis of organic chromophores with very high 2PA cross sections (>1000 GM) has been reported. However, most of the efficient organic 2PA chromophores are hydrophobic, posing a serious limitation for their use in biological applications.

To overcome the problem of poor water solubility, a few 2PA hydrophilic dyes have been recently reported, although their syntheses and purifications are tedious. Some other approaches, such as dye-doped silica nanoparticles and the use of micelles from block copolymers has
been demonstrated to make organic dyes soluble for several biological applications, although the stability of the micelles or nanoparticles incorporated with noncovalently attached dyes for long times in the bloodstream is matter of concern. Thus, covalent attachment of 2PA organic probes, without affecting their photophysical properties, in a hydrophilic block copolymer is a promising strategy.

Another important dimension is for the probe is to specifically target particular moieties of a cell for imaging purposes. Angiogenesis is an important physiological process that involves in growth of new blood vessels from the pre-existing vessels. It has significant role in embryonic development, tissue growth and wound healing in normal cell and also in cancer and malignancies. This ‘new blood vessels’ accelerates tumor growth by providing the oxygen and nutrients. Integrin receptors play an important role to activate and initiate the angiogenesis process. Identification of the $\alpha_v\beta_3$ integrin that regulates angiogenesis, to understand the process of angiogenic cascade role in tumor growth, and anti-integrin treatment efficiency are major concerns facing researchers in the cancer field. Certain integrin proteins are significantly up-regulated in growing tumor cells and their expression levels correlate well with the aggressiveness of the disease.$^{15-18}$ Endothelial tumor cells that overexpress $\alpha_v\beta_3$ integrin selectively bind the short peptide sequence Arginine-Glycine-Aspertic (RGD).$^{19-21}$ Preclinical studies as well as phase I/II clinical trials showed that RGD-containing peptides inhibit metastasis and tumor growth, while a number of studies demonstrated the effectiveness of RGD as a target to specifically bind $\alpha_v\beta_3$ integrin in PET, SPECT, and NIR imaging.$^{22-27}$ It has been demonstrated that cyclic (RGD) is more stable and selective with respect to the linear analog.$^{28,29}$ Cyclic RGD acts as an antagonist of $\alpha_v\beta_3$ integrin which suppress the angiogenesis process, thus
preventing tumor growth. Grubbs et al. reported that ROMP-based copolymers, substituted with GRGDS peptides, enhanced the inhibitory effect for cell-adhesion to the ECM fibronectin protein significantly (3300%) compared to the GRGDS peptide itself. Also, recent reports claim that synthetic polymer bioconjugates based on N-(2-hydroxypropyl) methacrylamide (HPMA-RGDfK) selectively delivers the diagnostic agent/therapeutic agent efficiently due to its multivalency, combination of active targeting and passive tumor localization, and extravascularization.

Figure 5-1. Design of multi-scaffold copolymer.

Multimeric RGD has even superior activity in targeting the integrins with respect to monomeric analogs. Many researchers demonstrated that polymer-RGD bioconjugates significantly enhance tumor localization, tumor-to-background ratio in comparison to either the polymer or the peptide alone, and also applied these to targeted radiotherapy in cancer treatment.

Here, we report the synthesis of novel norbornene-based block copolymers, comprised of PEG groups in one block, to impart hydrophilicity and biocompatibility, and succinimidy ester groups in the other block, to facilitate covalent conjugation with amine-terminated 2PA fluorenyl probes.
and an amine-terminated cyclic-RGD (c-RGDfK) peptide. PEG groups are known to impart aqueous solubility as well as prevent non-specific adsorption to general substances in a living body while reducing toxicity and immunogenicity. Synthesis of the norbonene-based monomers containing PEG and succinimidyl groups were accomplished via ROMP. Two different series of block copolymers were synthesized by varying the ratio of the PEG. These were characterized by GPC and $^1$H NMR. A two-photon absorbing fluorenyl dye were synthesized by modification of our previously reported method. A primary amine-terminated linker was attached to the 2PA fluorescent probe to facilitate the reaction with the succinimidyl ester.

In order to investigate the photophysical behavior of the polymeric probe, a model adduct was synthesized and characterized. Postpolymerization modification was the performed on the succinimidyl-containing block copolymers by reacting with a calculated amount of the amine-containing 2PA dye and cRGDFk, yielding the polymeric multi-scaffold bioimaging probe. Both one-photon and two-photon photophysical studies of the model compound and polymers are reported. Cell viability of the polymers were evaluated with an MTS assay. 2PFM imaging with human glioblastomal U87MG cell lines that over express $\alpha_v\beta_3$ integrin, along with control studies using human breast cancer MCF-7 cells ($\alpha_v\beta_3$ integrin negative) and blocking experiments, showed high targeting efficiency of the polymeric probe at the integrin region of the U87MG cell, useful for further investigation of the angiogenesis and early detection of cancer metastasis by 2PFM.
5.3. Results and Discussion

Synthesis and characterization of monomers and block copolymers: The two monomers, one containing a succinimidyl ester and another containing PEG, were synthesized to according to Schemes 5-1 and 5-2. The succinimidyl ester is a well-known amine reactive group, forming an amide upon reaction at very mild condition, and is widely used in biological chemistry. Hence, a norbornene derivative with the succinimidyl ester group was prepared for further derivatization to react with amine-containing two-photon fluorescent dyes as well as lysine terminated cyclic-RGD. Compound 14 was prepared according to literature with little modification. Briefly, first the carboxyl group was protected with trimethylsilylchloride and then amidation with 1, followed by the deprotection under basic condition was conducted. Then, succinimidyl ester group was introduced to 14 via esterification to obtain 15. PEG-functionalized monomer 16 was also prepared from intermediate 1 to provide hydrophilicity. The detailed synthesis is described in the Experimental Section.

Scheme 5-1. Synthesis of Monomers
The homopolymers of each monomer (15 and 16) were synthesized using ROMP in order to establish the reactivity and time for polymerization. This also directs the order of

Figure 5-2. $^1$H NMR study in (A) and (B) for the monomers 15 and 16 to monitor the homopolymerization respectively

Scheme 5-2. Synthesis of block copolymer
monomers addition during synthesis of the block copolymers with narrow PDI; in general, slower reactive monomers added first. To accomplish this, each monomer was dissolved in deuterated THF and $^1$H NMR spectra were collected at different time intervals until it was observed that all the monomer was transformed to the corresponding polymer (with the progress of polymerization, the signal for the monomer alkene CH at ~6 ppm decreased and a new polymer olefinic CH signal at ~5.3 ppm was observed). $^1$H NMR studies showed that PEG containing monomer 16 reacted more slowly to yield the corresponding homopolymer relative to 15 (Figure 5-2). Two different series of block copolymers were then synthesized by ROMP, using Grubbs second generation catalyst in THF, by varying the ratio of the monomers 15 and 16, according to Scheme 5-2. For bioimaging purposes, the solubility of the polymer in water was essential. Both of the block copolymers that were prepared were water soluble.

**Scheme 5-3. Synthesis of the amine terminated 2PA dye.**
Synthesis of 2PA dye 22 and model compound 23: The amine-terminated 2PA probe was synthesized according to Scheme 5-3. Key intermediate 19 was prepared following our previously published procedure.43 Considering the efficiency of the reaction of a primary alkyl amine with the succinimidyl ester group, a linker on the 2PA probe was introduced to provide a primary amine for conjugation. Hence, intermediate 20 was prepared by protecting the amine group, followed by coupling with 19 to give 21. The protecting group was then removed under acidic conditions, affording primary amine-containing 2PA probe 22.

Scheme 5-4. Synthesis of model compound 23

To investigate the linear and two-photon photophysical behavior of the amine reactive probe 22 after conjugation, a model compound was prepared by simple reaction with the norbornene derivative of succinimidyl compound 15 with 22, as shown in Scheme 5-4. The reaction was carried out in DMSO at room temperature using N-methylmorpholine. Structures of all new compounds were confirmed by $^1$H and $^{13}$C NMR spectra along with HRMS, with exception of the oxidatively labile amine 22, which was used immediately after isolation.

Postmodification on block copolymer. Post-modification on polymers provides an efficient tool to prepare functionalized polymers, depending on the desired applications. Complex, bulky substituents with multiheteroatoms often interfere with the catalyst reactivity, initiation, and
propagation reactions, often resulting in poorly controlled polymerization reactions with broad polydispersity index (PDI). Also, the solubility and purification of polymers with complex functional groups are challenging issues.\textsuperscript{45,46} Thus, post-modification is an attractive strategy for the synthesis of highly functionalized polymers. Post-modifications on block copolymers were performed by reaction of the succinimidyl ester-containing block and amine reactive 2PA probe 22 as well as the integrin-targeting small cyclic peptide cRGDfK at room temperature under mild conditions. In order to monitor the post-modification process, both different block copolymers were reacted with excess 22, followed by purification (confirmed by TLC that no excess – unreacted - dye was present), according to Scheme 5-5.

**Scheme 5-5. Synthesis of polymer-dye adduct**

![Scheme 5-5](image)

After conjugation, the $^1$H NMR spectrum showed the disappearance of succinimidyl peaks (2.74-2.78 ppm) and the appearance of new aromatic peaks (7-8.2 ppm) from probe 22. $^1$H NMR spectral analysis also demonstrated the desired block ratio formation from the integration of the
proton adjacent to the benzothiazole group (8.15 ppm-1H) to the alkene proton at (5.35-5.20 ppm). The solubility of the block copolymer containing a 2:1 ratio of PEG and dye-containing blocks (25) was more favorable in water compared to its 1:1 analog (24), as expected due to the greater amount of PEG. The result led to selection of block copolymer 18 for further modification as a better candidate for polymeric 2PA bioimaging probes. The bioconjugate polymeric probe was prepared from 18 by the post-modification of the succinimidyl block with a calculated amount of 2PA dye 22 and cRGDfK (3:1), determined by 1H NMR spectroscopic analysis.

Scheme 5-6. Synthesis of polymer-dye-cRGDfK adduct

The block copolymer 18 was post-modified with a specific ratio (1:1) of dye 22 and cyclic-RGD in a similar manner as stated above (Scheme 5-6). The polymer was purified by passing through a SEC column, using water as eluent, to remove the traces of excess of unreacted dye and cRGD. The formation of the target polymer was confirmed by the 1H NMR as well as GPC studies. The appearance of a new peak at about 8.59 ppm, in addition to the dye’s proton resonances in the aromatic region, due to the –COOH presence in cRGD was observed.
Single and two-photon photophysical studies: Linear photophysical properties of model compound 23 were first thoroughly characterized by UV-vis absorption, steady-state fluorescence, quantum yield, lifetime, and anisotropy in DMSO.

![Graph showing photophysical properties of model compound 23.](image)

Table 5-1. Photophysical properties of model compound, 23

<table>
<thead>
<tr>
<th>λ_{Abs}^{max} (nm)</th>
<th>λ_{Em}^{max} (nm)</th>
<th>Stokes Shift (nm)</th>
<th>ε_{max.} (10^{-3}M^{-1}cm^{-1})</th>
<th>φ_{Fl}</th>
<th>τ (ns)</th>
<th>R²</th>
<th>δ (GM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>360</td>
<td>439</td>
<td>79</td>
<td>51</td>
<td>0.95</td>
<td>1.36</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>30 at 740nm</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All photophysical studies were done in DMSO. Maxima of absorption, λ_{Abs}^{max}, fluorescence, λ_{Em}^{max} and extinction coefficients, ε_{max}; fluorescence quantum yields, φ_{Fl}; and lifetimes, τ.

The linear absorption and Compound 23 exhibited a single exponential fluorescence decay process with a lifetime of 1.36 ns. 2PA cross sections were determined by the upconversion fluorescence method, using a femtosecond Ti:sapphire laser as the excitation source. The 2PA cross section of 23 was ~30 GM at 740 nm, while 23 exhibited a high fluorescence quantum
yield (0.95). Thus, the two-photon action cross section was ~ 29 GM, reasonable enough for 2PFM bioimaging purposes. Results are shown in Figure 5-3 and Table 5-1; emission maxima of 23 were 360 nm and 439 nm, respectively, with considerably high fluorescence quantum yield of 0.95 using DPA as standard. The photophysical characterization of the model compound 23, helped us understand the behavior of the bioconjugated polymer 26 because the same chromophore is responsible for the photophysical properties of the polymer, as the polymer does not contain any other chromophores to contribute to the photophysical behavior. The linear photophysical properties of the two polymer conjugates with 26 or without RGD (25) were evaluated because of their favorable aqueous solubility in both DMSO and in water and compared with the model adduct, shown in Figure 5-4 and Table 5-2.

![Figure 5-4. Normalized UV-Vis and Fluorescence studies of the conjugated polymer 25 and 26.](image)

The result of the dye conjugated polymer 25 showed good agreement with the model compound, although in the more polar solvent water, a slight blue shift of the absorption peak (355 nm) and red shift of the emission peak (447 nm) were observed along with a reduction in the fluorescence
quantum yield (0.47). Also, it was noticed, 26 had slightly lower fluorescence compared to 25 because of the lower number of chromophores present in that block (cRGDfK does not contribute in the photophysical properties) of the polymer.

### Table 5-2. Linear photophysical properties of Conjugated polymer

<table>
<thead>
<tr>
<th></th>
<th>$\lambda_{\text{Abs}}^\text{max}$ (nm)</th>
<th>$\lambda_{\text{Em}}^\text{max}$ (nm)</th>
<th>Stokes Shift (nm)</th>
<th>$\phi_{\text{FL}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 in water</td>
<td>355</td>
<td>447</td>
<td>91</td>
<td>0.47</td>
</tr>
<tr>
<td>26 in water</td>
<td>355</td>
<td>447</td>
<td>91</td>
<td>0.40</td>
</tr>
<tr>
<td>26 in DMSO</td>
<td>360</td>
<td>439</td>
<td>79</td>
<td>0.78</td>
</tr>
</tbody>
</table>

**Cell-viability Study.** Cytotoxicity of the polymers (25 and 26) were investigated using an MTS assay prior to biological applications, shown below in Figure 5-5.

Figure 5-5. Nonradioactive MTS based cell proliferation assay was applied with U87MG cell lines. The cell was incubated with various amounts of fluorene dye conjugated polymer derivatives without RGD, 25 (A) and with RGD, 26 (B) for 24 hours at 37 °C. The relative viability of the cells incubated with the derivatives to untreated cells was determined by measuring the MTS-formazan absorbance on a Kinetic microplate reader (Spectra Max M5, Molecular Devices, Sunnyvale, CA, USA) at 490 nm.
Both of the conjugated copolymers showed excellent cell viability for the U87MG cell lines after incubation for 24 h. Fluorenyl dye conjugated polymer 25 was incubated with 1-30 µM dye concentration, resulting in more than 85% viable cells, demonstrating the minimal cytotoxicity of the functionalized block copolymer. Also, greater than 90% of the U87MG cells were viable after incubation with the cRGDfK/dye-containing polymer 26 at 0.1-5 µM concentration of the dye. These results confirmed the biocompatibility of the both dye-conjugated polymers.

![Image](image_url)

**26 + U87MG**  **Block c(RGDfK) + 26 + U87MG**  **26 + MCF7**

Figure 5-6. Comparison of 3D reconstruction from overlaid 2PFM of different cells incubated with fluorescence conjugate 26 (1 µM, 2h) all taken with 60x, oil immersion objective at the same experimental condition. (Ex: 740 nm; Power: 20mW; Em. long-pass filter 690 nm; 10 µm grid)

**One- and two-photon targeted fluorescence bioimaging.** 2PFM imaging is emerging as a powerful tool for non-invasive targeted *in vitro* and *in vivo* bioimaging. This process offers a degree of biological compatibility, with deep penetration and 3D focused imaging at the point of biological interest in the NIR (700-1000 nm). 2PFM overcomes the general problem related with one-photon fluorescence optical imaging; significant limitations due to auto-fluorescence, absorption and light scattering by the tissues below NIR range and above that range water, lipids are the primary absorbers of IR.\(^\text{39}\)
Receptor affinity of the polymeric bioconjugate containing a 2PA dye and cRGD (26) for integrin αvβ3 positive human glioblastoma U87MG tumor cells was investigated. To demonstrate and compare the integrin specificity and localization, polymer 26 was incubated for 2 h with a U87MG cell line and a αvβ3 negative control breast cancer cell line MCF7 under the same experimental conditions. Images were taken by both one-photon and two-photon fluorescence microscopy. The imaging results demonstrated the specificity of the cRGDs at the peri-nuclear area of the cell, after receptor mediated endocytosis, by strong fluorescence in the U87MG cells, even at very low concentration of the dye (1 µM) by both techniques. Meanwhile, in the negative control MCF7 cells there was only dim/no fluorescence without any specificity (largely autofluorescence). 2PFM afforded better resolution and sharper images with respect to conventional (one-photon) fluorescence microscopy imaging. Figure 5-6 shows the 3D reconstruction of the 2PFM images, comparing the effectiveness of the polymeric probe in U87MG cells with respect to a blocking experiment and integrin negative MCF7 cells. Integrin receptor specificity was further validated by a blocking experiment. The U87MG cells were blocked (incubated) with the unlabeled cRGDfK peptide (2 mg/mL) prior to the incubation with the polymeric conjugate 26 (1 µM), followed by fluorescence microscopy, resulting in reduced tumor contrast.

One-photon fluorescence images were taken using a mercury lamp excitation source with a modified Olympus Fluoveiw FV300 microscope system and two-photon images were taken with the same system coupled with a tunable Coherent Mira 900F Ti:sapphire laser as excitation source. Differential interference contrast (DIC), one-photon fluorescence, and two-photon fluorescence images are shown in Figures 5-7, 5-8, and 5-9.
Figure 5-7. Images of U87MG cells incubated with fluorescence conjugate \textbf{26} (1 \(\mu\)M, 2 h) all taken with 60x, oil immersion objective. a) DIC, 40 ms. b) One-photon fluorescence image, 100 ms (filter cube Ex: 377/50 DM: 409 Em: 525/40). c) one layer of 2PFM image (Ex: 740 nm; power: 40mW; Em. long-pass filter 690 nm).

Figure 5-8. Images of U87MG cells incubated first with 2 mg/mL RGD for 2 h and then with fluorescence conjugate \textbf{26} (1 \(\mu\)M, 2 h) all taken with 60x, oil immersion objective. a) DIC, 40 ms. b) One-photon fluorescence image, 100 ms (filter cube Ex: 377/50 DM: 409 Em: 525/40). c) one layer of 2PFM image (Ex: 740 nm; power: 40 mW; Em. long-pass filter 690 nm).
Figure 5-9. Images of MCF-7 cells incubated with fluorescent conjugate 26 (1 µM, 2 h) all taken with 60x, oil immersion objective. a) DIC, 40 ms. b) One-photon fluorescence image, 100 ms (filter cube Ex: 377/50 DM: 409 Em: 525/40). c) one layer of 2PFM image (Ex: 740 nm; power: 40mW; Em. long-pass filter 690 nm).

Another interesting finding of the polymer mediated integrin targeted bioimaging with the 2PA dye-containing copolymer 26 was that it exhibited better localization, higher affinity, and brighter fluorescence in the cycloplasmic region of the U87MG cells at much lower probe concentration and low power with respect to free dye (published elsewhere) possibly due to multivalency of the polymeric probe.47 This multi-scaffold polymeric probe could be a very effective carrier of both fluorescent probe for 2PFM and cell-specific targeting along. In addition, the PEG group attached to the polymer imparts biocompatibility and should provide longer circulatory retention time for in vivo use. In the future, this approach can be further broadened by incorporating a therapeutic moiety (e.g., photosensitizer for photodynamic therapy) in the construct so that it will lead to an integrated probe having all desired theranostic utilities.
5.4. Conclusion

In this study, we report a multi-scaffold copolymer conjugated with a 2PA fluorenyl dye and cRGDfK peptide to target human glioblastoma cancer cell U87MG, a α₃β₃ positive cell, and 2PFM bioimaging. This biocompatible PEG-containing block copolymer was synthesized by ROMP polymerization using Grubbs second generation catalyst and further post-modified with an amine-reactive fluorene dye and cRGDfK, and characterized by NMR and GPC. The biocompatibilities of the system were evaluated by an MTS assay, revealing minimal cytotoxicity of the system in U87MG cells. The efficiency of the targeting ability of the polymeric probe was studied by both conventional (one-photon) and two-photon fluorescence microscopy of the α₃β₃ positive U87MG cells with respect to a negative control (MCF7 cells) and a blocking experiment with U87MG cells incubated first with unlabeled cRGDfK. The results reported open a new dimension to use of multi-scaffold copolymers to prepare target-specific 2PF probes for 2PFM with potential applications in angiogenesis imaging and cancer detection.

5.5. Experimental

5.5.1. Materials

Bicyclo[2.2.1]hept-5-ene 2-carboxylic acid (98% mixture of endo and exo), norbornene (99%), thionyl chloride (99.5%), trimethylchloro silane, triethylamine (99.5%), Polyethyleneglycol monomethyl ether (Mₙ = 550), 6-aminocaproic acid, N-hydroxy succinimide, EDC, NMM, and Grubb’s second generation catalyst were purchased from Aldrich or ACROS and used as
received. CH$_2$Cl$_2$ and CHCl$_3$ were dried over CaCl$_2$ and distilled. THF was distilled over sodium and benzophenone ketyl under N$_2$ before use. All catalyst solutions were prepared in a glovebox. N,N’-Dicyclohexylcarbodiimide was distilled before use. CH$_2$Cl$_2$ was dried over CaH$_2$. 7-(benzothiazol-2-yl)-9,9-di(2-(2-methoxyethoxy)-ethyl)fluoren-2-amine was prepared as described previously.$^{43}$ Promega CellTiter 96® AQueous One Solution Reagent was purchased from Fisher.

Cell lines. The U87MG cells and MCF7 cells were purchased from ATCC (America Type Culture Collection, Manassas, VA, USA). All cells were incubated in Minimum Essential Media (MEM, Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA, USA), 100 units/mL penicillin-streptomycin (Atlanta Biologicals, Lawrenceville, GA, USA), and incubated at 37 °C in a 95% humidified atmosphere containing 5% CO$_2$.

5.5.2. Characterization
The $^1$H and $^{13}$C NMR spectroscopic measurements were performed using a Varian 500 NMR spectrometer at 500 MHz with tetramethysilane (TMS) as internal reference; $^1$H (referenced to TMS at $\delta = 0.0$ ppm) and $^{13}$C (125 MHz, referenced to CDCl$_3$ at $\delta = 77.0$ ppm). Chemical shifts of $^1$H and $^{13}$C spectra were interpreted with the support of CS ChemDraw Ultra version 11.0 and NMR spectra were analyzed with Mestrec software. High resolution mass spectrometry (HR-MS) analysis was performed in the Department of Chemistry, University of Florida, Gainesville, FL. Gel permeation chromatography (GPC) was conducted with a Waters 2414 refractive index detector, Waters 2996 photodiode array, and Waters 1525 binary HPLC pump (THF as the
mobile phase, flow rate of 1.5 mL/min) using Waters styragel HR1, HR4, and HR5E columns, with reference to polystyrene standards.

**Synthesis of 2, 5-dioxopyrrolidin-1-yl 6 (bicyclo[2.2.1]hept-5-enecarboxamido)hexanoate 15.** 6-Norborn-2-ene 5-carbonylaminoheptane acid was prepared by literature method with a slight modification. Briefly, 6-aminocaproic acid (7.95 g, 0.061 mol) was dissolved in CH₂Cl₂, then trimethylchlorosilane (15.6 mL, 0.122 mol) was added and refluxed for 4 h. The reaction mixture was cooled to 0 °C and 1 (9.49 g, 0.061 mol) was added slowly. The temperature was increased to room temperature and stirred overnight. CH₂Cl₂ was removed under reduced pressure, then the residue was cooled to 0 °C and treated with saturated NaHCO₃ solution and stirred for 7 h. The solution was then acidified with HCl, extracted with CH₂Cl₂, and dried with Na₂SO₄. After solvent removal, viscous oily product was obtained by column chromatography using Et₂O as the mobile phase (yield: 51%).

6-Norborn-2-ene 5-carbonylaminoheptane acid (0.60 g, 2.4 mmol), N-hydroxysuccinimide (0.54 g, 4.69 mmol), and EDC (0.72 g, 3.75 mmol) were mixed together in CH₂Cl₂ at room temperature under N₂ atmosphere and stirred overnight. After CH₂Cl₂ removal under reduced pressure, column chromatography followed (1:9 hexanes:CH₂Cl₂ on silica) to obtain white solid (0.71 g, 85% yield). ¹H NMR (500 MHz, CDCl₃) δ: 6.09-6.01 (m, 2H, HC=CH), 3.63 (s, 1H), 3.18 (s, 2H, N-CH₂), 2.83-2.74 (b, 5H, Succ -CH₂), 2.55 (s, 2H, CO-CH₂), 1.83 (s, 1H), 1.70 (m, 1H), 1.69-1.63 (m, 3H), 1.49 – 1.39 (m, 4H), 1.26 - 1.19 (s, 3H). ¹³C NMR (125 MHz, CDCl₃) δ: 175.73, (C=O exo and endo) 169.41, 168.42, 138.19, 138.00, 136.15, 135.96, 135.64, 47.44, 47.02, 44.45, 44.42, 41.72, 41.30, 39.03, 31.05, 30.56, 30.38, 28.95, 25.98, 25.80, 25.58,
Synthesis of Bicyclo[2.2.1]hept-5-ene-2-carboxylic Acid polyethyleneglycol monomethyl ether Ester 16. A mixture of triethylamine (22 mL, 0.16 mol) and polyethyleneglycol monomethyl ether (6.4 mL, 0.096 mol) was added over 2 h to the solution of the acid chloride (12 g, 0.08 mol) in dry THF at 0 °C. The mixture was then stirred at room temperature for 8 h. Et₂O was added and the resulting white salt was filtered off. The organic filtrate was washed with aqueous 5% NaOH solution, followed by washing with 5% HCl, saturated Na₂CO₃, and water. The solvent was removed under reduced pressure, and the residue was subjected to column chromatography (9:1 methanol: CH₂Cl₂ on silica), affording 2 as clear light yellow oil (14.79 g, 95% yield). ¹H NMR (500 MHz, CDCl₃) δ: 6.19-6.11 (m, 1.5H, HC=C), 5.94 (m, 0.5H, HC=C), 4.26-4.15 (m, 2H, COO- CH₂ ), 3.77 (m, 1H), 3.67 – 3.51 (m, 66 H, -CH₂ Peg), 3.38 (s, 3H, -CH₃), 2.78 – 2.99 (m, 3H), 1.93 (m, 0.5H), 1.88-1.26 (m, 4H). ¹³C NMR (125 MHz, CDCl₃) δ: 175.76, 174.23, 137.94, 137.30 (C=C), 135.62, 135.42, 132.30, 132.09, 77.78, 77.52, 73.01, 72.34, 71.46-67.64 (multiple peak due to PEG), 63.46 -61.29, 58.92 -57.91, 49.49, 49.37, 49.23, 46.55, 46.30-45.36, 43.16-41.28, 36.81, 30.24, 29.99, 29.01, 28.07. HRMS-ESI was verified (see appendix for details).

Synthesis of tert-butyl 7-(7-(benzo[d]thiazol-2-yl)-9,9-bis(2-(2-methoxyethoxy)ethyl)-9H-fluoren-2-ylamino)-7-oxoheptylcarbamate 21. 7-(benzothiazol-2-yl)-9,9-di(2-(2-methoxyethoxy)-ethyl)fluoren-2-amine 19 was prepared according to literature published procedure. To a stirred solution of 19 (0.1 g, 0.19 mmol) dissolved in CH₂Cl₂ (3 mL) and pyridine (0.15 mL) under N₂,
6-(tert-butoxycarbonylamino)hexanoic acid 20 (0.080 g, 0.29 mmol) was added to the mixture at room temperature. \(N,N'\)-Dicyclohexylcarbodiimide (DCC) (0.080 g, 0.38 mmol) was added and the mixture was stirred at room temperature for 18 h. The mixture was then filtered and concentrated under reduce pressure. Purification was carried out by silica gel column chromatography using hexanes/ethyl acetate (2:3) providing 0.13 g of yellow oil (90 % yields).

\(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\): 8.15(d, \(J = \), 1H), 8.09 (t, \(J=8\) Hz, 2H), 7.93 (d, \(J= 8\) Hz, 1H), 7.75-7.67 (m, 4H), 7.56 (s, 1H), 7.56 (t, \(J=7\) Hz, 1H), 7.41 ( t, \(J= 7\) Hz, 1H), 4.65 (s, 1H), 4.13-3.13 (m, 16H), 2.84-2.79 (m, 4H), 2.54-2.48 (m, 2H), 2.45-2.39 (m, 4H), 1.82-1.76 (1:4:6:4:1 q, \(J= 7\) Hz, 4H), 1.57-1.53 (1:4:6:4:1 q, \(J= 6.5\) Hz, 2H), 1.45 (s, 9H). \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) \(\delta\): 171.25, 168.28, 156.12, 154.18, 150.73, 149.71, 143.09, 138.51, 135.39, 134.96, 132.10, 127.50, 127.34, 123.13, 122.96, 122.09, 121.90, 121.53, 120.94, 119.92, 79.15, 71.71, 69.85, 66.96, 60.42, 58.84, 51.58, 39.61, 37.59, 29.74, 28.52, 28.20, 26.30, 25.06. HR-MS-ESI theoretical m/z [M+H]\(^+\) = 732.36, found 732.36, theoretical m/z [M+Na]\(^+\) = 754.35, found 754.34.

Synthesis of 7-amino-N-(7-(benzo[d]thiazol-2-yl)-9,9-bis(2-(2-methoxyethoxy)ethyl)-9H-fluoren-2-yl)heptanamide 22. \(N\)-Protected aminofluorene 22. 21 (0.1 g, 0.13 mmol) was placed into a sealed tube under N\(_2\) atmosphere and dissolved in CH\(_2\)Cl\(_2\) (2 mL) at room temperature. To this, TFA (0.05 mL, 0.67 mmol) was added and the reaction was heated at 80 °C for 40 min. Solvent was removed under vacuum and the brownish oil residue was neutralized with a 10% solution of NH\(_4\)OH. The residue was purified by silica gel column chromatography, eluting with 2:3 EtOAc/MeOH, affording 0.082 g of yellow oil (95 % yield). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\): 8.15 (s, 1H), 8.09 (t, \(J= 16.5\) Hz, 2H), 7.93 (d, \(J= 8\) Hz, 1H), 7.82 (s, 1H), 7.75-7.66 (m, 3H), 7.55 (s,
1H), 7.52 (t, J=15.5 Hz, 1H), 7.41(t, J= 15 Hz, 1H), 3.65-3.18 (m, 14 H), 2.85-2.73 (m, 6H),
2.54-2.40 (m, 6H), 2.04 (s, 2H), 1.82 (t, J= 14 Hz, 2H), 1.55-1.46 (m, 4H). This intermediate was
not further characterized and used directly in the next step due to oxidative lability.

*Synthesis of model compound 23.* Compound 15 (85 mg, 0.24 mmol) was dissolved in DMSO
under N₂ followed by the addition of 22 (70 mg, 0.10 mmol) and a catalytic amount of NMM (23
mg, 0.23 mmol) at room temperature. The reaction was completed within 7 h. After which time
DMSO was removed under reduced pressure, and the crude product was purified by column
chromatography with 9:1 EtOAc:MeOH elutant, followed by drying under vacuum to yield
yellowish oily product (61 mg, 70% yield). ¹H NMR (500 MHz, CDCl₃) δ: 8.67 (s, 1H), 8.15 (s,
1H), 8.07 (d, 2H), 7.93 (s, 1H), 7.78-7.61 (m, 4H), 7.38(t, 1H), 6.24-5.96 (3H), 3.65-3.15 (m, 20
H), 2.92-2.79 (m, 6H), 2.54-2.19 (m, 7H), 2.05 (s, 2H), 1.80-1.25 (t, 15H). ¹³C NMR (125 MHz,
CDCl₃) δ: 176.06, 174.66, 173.28,171.82, 168.36, 154.15, 150.62, 149.63, 143.25, 139.12,
137.99, 137.82, 135.14, 134.95, 132.31, 132.15, 132.0, 127.48, 126.49,
126.34,125.25,125.08,123.03,122, 121.69,121, 119.93, 119.31, 114.58, 114.45,
71.75,69.88,67.11, 66.97,66.83, 58.96, 51.51, 50.11,46.37, 46.30, 44.95,44.81, 44.69,
42.84,42.69,39.67 39.55, 39.44, 37.39,36.52, 30.53, 29.22, 26.40,26.33, 25.33,25.03. HR-MS-
ESI theoretical m/z [M+H]⁺ = 865.45, found 865.45, theoretical m/z [M+Na]⁺ = 887.43, found
887.43. theoretical m/z [M+K]⁺ = 903.41, found 903.41.

**Polymerization by ROMP.** General polymerization method for homopolymers was as follows.
A solution of monomer (n equiv) in THF was prepared in a very dry and clean flask at room
temperature under N₂. The ruthenium-based Grubbs second generation catalyst (1 equiv) in THF
was added to the monomer solution, and the reaction mixture was stirred. After a specific time (determined by the kinetics through the NMR study of each monomer), an excess of ethyl vinyl ether was added to terminate the polymerization. The resulting brown solution was stirred for 12 h. The reaction was precipitated into cold Et₂O/MeOH as required. The mixture was centrifuged, and the solvent was decanted. The resulting solid was dried and purified by passing through a polymeric resin based column (Polymer labs, Stratosphere-PL-Thiourea MP SPE 003) in MeOH, followed by vacuum drying to yield white to light brown solid as the product.

**Homopolymer of 2,5-dioxopyrrolidin-1-yl 6 (bicyclo[2.2.1]hept-5-enecarboxamido)hexanoate.**

^1H NMR (500 MHz, CDCl₃) δ: 5.47 -5.10 (b, -HC=CH-), 3.38- 3.10, 3.01, 2.92-2.76, 2.61, 2.23-1.66, 1.63-1.22(b).

**Homopolymer of bicyclo[2.2.1]hept-5-ene-2-carboxylic acid polyethylene glycol monomethyl ether ester.** ^1H NMR (500 MHz, CDCl₃) δ: 5.58 -5.14 (b, -HC=CH-), 4.30- 3.97, 3.82-3.37, 3.34-3.10, 3.07-2.78, 2.65-2.38, 2.82-1.58, 1.54-1.15(b).

**Block copolymer synthesis.** A solution of monomer 16 (m equiv) in THF was prepared in a very dry and clean flask at room temperature under N₂. The ruthenium-based Grubbs second generation catalyst (1 equiv) in THF was added to the monomer solution, and the reaction mixture was stirred. After a specific time (determined by the kinetics through the NMR study of each monomers), the second monomer 15 (n equiv) was added and stirred. The reaction was monitored by TLC and, when there was no trace of monomer, the reaction was terminated by adding excess of ethyl vinyl ether. The polymer was then precipitated in cold Et₂O and passed through a polymeric resin based column (Polymer labs, Stratosphere-PL-Thiourea MP SPE 003)
in MeOH to get rid of any traces of Ru-catalyst, followed by vacuum drying to yield white to light brown solid as the product.

Two different (2:1 and 1:1) block copolymers with respect to PEG:succinimidyl group of the norbornene monomer (15 and 16) were prepared. $^1$H NMR chemical shifts were similar for both of them except the integration ratio of the alkene protons $\sim 5.3$ ppm to that of the succinimidyl protons $\sim 2.78-2.74$ ppm.

17: Yield: 75%. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$: 5.47 - 5.10 (b, -HC=CH-), 4.16-3.89, 3.67-3.28, 3.27- 3.19, 3.08-3.83, 2.89-2.72, 2.69-2.40, 2.07-1.66, 1.69-1.52(b), 1.47-1.18. $M_n$ (Theoretical) = 36000 (it was an approximate value because of polydispersity of the PEG group, m:n = 35:35); $M_n$ (Experimental-GPC) = 36931, PDI = 1.39 [m:n= 1:1(theoretical); m:n =1: 1.26 from $^1$H].

18: Yield:81% $^1$H NMR (500 MHz, CDCl$_3$) $\delta$: 5.53 - 5.02 (b, -HC=CH-), 4.25-3.87, 3.78-3.28, 3.27- 3.19, 3.08-3.83, 2.89-2.72, 2.69-2.40, 2.07-1.66, 1.69-1.52(b), 1.47-1.18. $M_n$ (Theoretical) = 34240 (m:n = 40:20); $M_n$ (Experimental-GPC) = 37006 PDI =1.75 [m:n= 2:1(theoretical); m:n =2: 0.9 from $^1$H].

**Post-modification on block copolymer.**

*Polymer PEG–2PA dye adducts 24 and 25.* Polymer 17 (60 mg) was dissolved in 1 mL DMSO under $N_2$ followed by the addition of dye 22 (19 mg) and a catalytic amount of NMM (3 mg) at room temperature. The reaction was completed within 24 h (monitored with TLC). The crude product was then precipitated with cold Et$_2$O to precipitate the polymer, followed by washing several times to remove the excess dye and DMSO. Then the polymer was dried under vacuum.
(61 mg, 70% yield). In a similar manner polymer 18 was also modified with 22 to obtain polymer 25.

24: Yield: 88%. \( ^1H \) NMR (500 MHz, CDCl\(_3\)) \( \delta: 8.15, 8.09-8.04, 7.92-7.90, 7.78-7.68, 7.50, 7.39, 5.35-5.20 (b, -HC=CH-), 4.25- 4.08, 3.79-3.50, 3.28-3.19, 2.96, 2.81, 2.60-2.42, 2.17-1.78, 1.58-1.21. \( M_n \) (Theoretical) =62016 (approx.m:n = 35:44); \( M_n \) (Experimental-GPC) = 73925, PDI = 1.13 [m:n= 1:1(theoretical); m:n =1:1.29 from \(^1H\) NMR]

25: Yield: 91%. \( ^1H \) NMR (500 MHz, CDCl\(_3\)) \( \delta: 8.15, 8.09-8.04, 7.92-7.90, 7.78-7.60, 7.50, 7.39, 5.35-5.20 (b, -HC=CH-), 4.23-4.09, 3.78-3.43, 3.28-3.19, 2.99 (some trace of DMSO ~ 2.6 present), 2.81, 2.60-2.38, 2.17-1.21. \( M_n \) (Theoretical) = 42834(approx.m:n = 40:18); \( M_n \) (Experimental-GPC) = 45765, PDI = 1.56(m:n = 2.00:0.83 from \(^1H\) NMR)

Polymer PEG–2PA dye-RGD adducts 26. Polymer 26 (52 mg) was dissolved in 1 mL DMSO at room temperature under N\(_2\). Then, cRGDfK (8 mg) was added to the solution and reacted for 4.30 h. The solution of dye 22 (15 mg in 0.2 mL DMSO) was added and the reaction stirred overnight. The crude product was precipitated in cold Et\(_2\)O and washed several times to remove unreacted dye and RGD, followed by drying under vacuum. The product was then passed through a size exclusion column (12 cm length, Bio-Rad Econo-Pac 10DG, MWCO) in pure water to allow collection of pure bioconjugate fractions, yielding 57 mg. \( ^1H \) NMR (500 MHz, CDCl\(_3\)) \( \delta: 8.61, 8.15, 8.09-8.04, 7.92-7.90, 7.78-7.68, 7.50, 7.39, 5.35-5.20 (b, -HC=CH-), 4.25-4.08, 3.79-3.50, 3.28-3.19, 2.96, 2.81, 2.60-2.42, 2.17-1.78, 1.58-1.21. \( M_n \) (Theoretical) = 42574; \( M_n \) (Experimental-GPC) = 45387, PDI = 1.42.
**Uptake and localization of probe in cancer cells.** U87MG cells or MCF-7 cells were placed onto poly-D-lysine coated glass coverslips in 24-well plates (40,000 cells per well), and the cells were incubated for 48 h before incubating with the fluorescent polymer conjugate. Stock solution of fluorescent conjugate 26 dissolved in water was prepared as $6 \times 10^{-4}$ M solution. The solution was diluted to a 1 µM by complete growth medium, Minimum Essential Media (MEM), and then freshly placed over the cells for a 2 h period. After incubation, the cells were washed with PBS (3–5x) and fixed using 3.7% formaldehyde solution for 15 min at 37 °C. Then 0.5 mL/well NaBH$_4$ (1 mg/mL) solution in PBS (pH=8.0) was added, which was prepared by adding few drops of 6N NaOH solution into PBS (pH=7.2), for 15 min. Then the plates were washed twice with PBS and once with water. Finally, the glass coverslips were mounted using Prolong Gold mounting media for microscopy.

**Cytotoxicity Assay.** To assess the cytotoxicity of conjugates 25 and 26, $5 \times 10^3$ per well of U87MG cells in 96-well plates were incubated in 90 µL of MEM medium without phenol red, supplemented with 10% FBS and 100 units/mL penicillin-streptomycin for 24 h. Then the cells were incubated with various amounts of polymer probes 25 and 26 (30 µM, 20 µM, 10 µM, 5 µM, 1 µM) and (5 µM, 1 µM, 0.5 µM, 0.1 µM), respectively, for an additional 20 h. Subsequently, 20 µL of CellTiter 96® AQueous One Solution reagent was added into each well, followed by further incubation for 4 h at 37 °C. The relative viability of the cells incubated with the polymer probe to untreated cells was determined by measuring the MTS-formazan absorbance on a microplate reader (Spectra Max M5, Molecular Devices, Sunnyvale, CA, USA) at 490 nm with subtraction of the absorbance of cell-free blank volume at 490 nm. The results from three individual experiments were averaged.
**Blocking Experiment.** The blocking experiment was performed to verify the integrin targeting ability of the RGD containing polymeric probe 26. U87MG cells were placed onto poly-D-lysine coated glasses in 24-well plates (40,000 cells per well), and the cells were incubated for 48 h. Then, the cells were incubated with unlabeled cRGDiK (2 mg/mL of MEM) for 1 h. After that, 1 µM solution of 26 in MEM was added over the cells and incubated for a 2 h period. After incubation, cells were washed with PBS (3–5x) and fixed using 3.7% formaldehyde solution for 15 min at 37 °C. Then 0.5 mL/well NaBH₄ (1 mg/mL) solution in PBS (pH=8.0), which was prepared by adding few drops of 6N NaOH solution into PBS (pH=7.2), was added and left for 15 min. The plates were the washed twice with PBS and once with water. Finally, the glass coverslips were mounted with Prolong Gold mounting media for microscopy.

**One-Photon Spectral Measurements.** Linear photophysical properties of new fluorescent probes were investigated in spectroscopic-grade solvents (DMSO and ultrapure water) at room temperature. The steadystate absorption spectra were obtained with Agilent 8453 UV-visible spectrophotometer using 1 cm path length quartz cuvettes with dye concentrations of $1 \times 10^{-5}$ M. The steadystate fluorescence spectra and excitation anisotropy spectra were obtained with a Photon Technologies, Inc. (PTI) QuantaMaster spectrofluorimeter, using 10 mm spectrofluorometric quartz cuvettes and low concentration solutions $C \leq 10^{-6}$ M. All fluorescence spectra were corrected for the spectral sensitivity of the PTI emission monochromator and photomultiplier tube (PMT) detector. Excitation anisotropy spectra were measured using an L-format configuration geometry, with extraction of the scattered light and solvent emission. Fluorescence quantum yields of the compounds were determined by a relative method with 9,10 diphenylanthracene in cyclohexane as a standard. Fluorescence lifetimes were measured with a
time-correlated single photon counting system PicoQuant PicoHarp 300) under linear polarized femtosecond excitation oriented by the magic angle, with time resolution ~80 ps.

Conventional single-photon fluorescence images were obtained using an inverted microscope (Olympus IX70) equipped with a QImaging cooled CCD (Model Retiga EXi) and mercury lamp 100 W. In order to improve the fluorescence background-to-image ratios, a customized filter cube (Ex 377/50, DM 409, Em 460/50) was used for the one-photon fluorescence images. The specifications of the filter cube were tailored to match the excitation wavelength of the probe, and to capture most of the probe’s emission profile

**Two-Photon Absorption and Imaging Measurements.** 2PA spectra of 23 were obtained over a broad spectral region via a typical two-photon induced fluorescence (2PF) method relative to Rhodamine B in methanol and Fluorescein in water (pH =11) as a standards.50 A PTI QuantaMaster spectrofluorimeter and femtosecond Clark-MXR CPA-2010 laser that pumped an optical parametric generator/amplifier (TOPAS, Light Conversion), with pulse duration, ~140 fs, (FWHM), tuning range 600-900 nm, pulse energies 0.15 μJ, and 1 kHz repetition rate. Two-photon fluorescence measurements were performed in 10 mm fluorometric quartz cuvettes with dye concentrations of 3 × 10^{-5} M in DMSO. The quadratic dependence of two-photon induced fluorescence intensity on the excitation power was confirmed for each excitation wavelength.

Two-photon fluorescence microscopic images were obtained with a modified Olympus Fluoview FV300 microscope system combined with a tunable Coherent Mira 900F Ti:sapphire laser. The femtosecond NIR laser beam (with 220 fs pulse width and 76 MHz repetition rate) was tuned to 730 nm and used as the two-photon excitation source. The two-photon induced fluorescence was
collected by a 60× microscopic objective (UPlanSApo 60×, NA 1.35, Olympus). A high-transmittance (>95%) short-pass filter (cutoff 685 nm, Semrock) was placed in front of the PMT detector of the FV300 scanhead in order to filter off background radiation from the laser source (730 nm).

5.6. References


(37) Fischbach, C.; Mooney, D. J. Biomaterials 2007, 28, 2069-2076.


APPENDIX A: RESULTS RELATED TO CHAPTER 2

NMR
$^1$H NMR of 2
$^{13}\text{C NMR}$ of 2
$^1$H NMR of 6
$^{13}$C NMR of 6
$^1$H NMR spectra to study the progress of the homopolymerization of 2 as a function of time, showing the disappearance of vinyl peaks of monomer 2 and formation of the homopolymer.
Thermal Properties of Polymer:

Comparison of thermal stability of the polymers. (A) TGA analysis and (B) DSC analysis.

GPC Analysis of Polymer (4,5):
Electron diffraction pattern of NC1-B1 from TEM.

Table. Comparison between crystal structure of NC1-B1 and standard maghemite-C-syn nanocrystal

<table>
<thead>
<tr>
<th>Ring #</th>
<th>( r_2 ) (nm)</th>
<th>( d_2 ) (nm)</th>
<th>d(spacing) of ( \gamma\text{-Fe}_2\text{O}_3 ) (nm) std.</th>
<th>( hkl )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.950</td>
<td>0.2514</td>
<td>0.2518</td>
<td>311</td>
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<tr>
<td>2</td>
<td>2.339</td>
<td>0.2096</td>
<td>0.2089</td>
<td>400</td>
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<tr>
<td>3</td>
<td>2.695</td>
<td>0.1818</td>
<td>0.1822</td>
<td>421</td>
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<tr>
<td>4</td>
<td>3.232</td>
<td>0.1517</td>
<td>0.1525</td>
<td>521</td>
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<tr>
<td>5</td>
<td>3.827</td>
<td>0.1280</td>
<td>0.1273</td>
<td>533</td>
</tr>
<tr>
<td>6</td>
<td>4.476</td>
<td>0.1095</td>
<td>0.1096</td>
<td>730</td>
</tr>
</tbody>
</table>

where the camera length was 20 cm, \( r_1 = 2.097 \), \( d_1 = 0.2338 \) nm were used from the calibration file.\(^{127}\)
Electron diffraction pattern of NC1-B2 from TEM.

Table Comparison between crystal structure of NC1-B2 and standard maghemite-C-syn nanocrystal

<table>
<thead>
<tr>
<th>Ring #</th>
<th>$r_2$</th>
<th>$d_2$(nm)</th>
<th>d(spacing) of $\gamma$-Fe$_2$O$_3$ (nm)</th>
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</tr>
</thead>
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<td>5</td>
<td>4.636</td>
<td>0.1440</td>
<td>0.1432</td>
<td>530</td>
</tr>
</tbody>
</table>

where the camera length was 30 cm, $r_1 = 2.857$, $d_1 = 0.2338$ nm were used from the calibration file.

References:

APPENDIX B: RESULTS RELATED TO CHAPTER 3
$^1$H NMR analysis of 7.
\textsuperscript{13}C NMR analysis of 7
$^1$H NMR analysis of 1:0 homopolymer, 8
$^1$H NMR analysis of 1:1 diblock copolymer; (a) monomer 7, (b) homopolymer 8, and (c) 1:1 diblock copolymer 9.
TGA and DSC of the polymers.

GPC analysis of homo and block copolymer
Electron diffraction pattern:

Comparison between crystal structure of block copolymer-maghemite nanocomposite and standard maghemite-C-syn (std):

<table>
<thead>
<tr>
<th>Ring #</th>
<th>$r_2$</th>
<th>$d_2$(nm)</th>
<th>d(spacing) of $\gamma$-Fe$_2$O$_3$ (nm)</th>
<th>hkl</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.2412</td>
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<tr>
<td>3</td>
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<tr>
<td>4</td>
<td>4.509</td>
<td>0.1481</td>
<td>0.1476</td>
<td>440</td>
</tr>
</tbody>
</table>

where camera length was 30cm, $r_1 = 2.857$, $d_1 = 0.2338$ nm were used from calibration file.

References:

APPENDIX C: RESULTS RELATED TO CHAPTER 4
$^{13}$C NMR of 10
$^1$H NMR of 11
HRMS Spectra of 10
APPENDIX D: RESULTS RELATED TO CHAPTER 5
$^1$H NMR of 14
Chemical formula: $C_{18}H_{24}N_2O_5$

$^1H$ NMR of 15
$^{13}$C NMR of 15
$^1$H NMR 16
$^{13}$C NMR of 16
$^1$H NMR of 23
$^{13}$C NMR of 23
Homopolypeg
$^1\text{H NMR 17 (m:n =2:1)}$
$^1$H NMR 18 (m:n =1:1)
$^1$H NMR 24 (m:n = 1:1)
$^1$H NMR of 25 (m:n = 2:1)
$^1$H NMR 26 (R$_1$=dye, R$_2$ = cRGDfK)
HRMS Spectra

Theoretical [M+H]+ = 349.1680
[M+Na]+ = 371.1577
[M+K]+ = 387.1317
MALDI-TOF spectra of 16
HRMS Spectra
Experimental setup for two-photon up-converted fluorescence measurements.