Spring 2010

Ratiometric fluorescent metal ion indicators based on functionalized poly(N-isopropylacrylamide)

Jie Du
University of New Hampshire, Durham

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RATIOMETRIC FLUORESCENT METAL ION INDICATORS
BASED ON FUNCTIONALIZED POLY(N-ISOPROPYLACRYLAMIDE)

BY

JIE DU

B.S., Tianjin University, P.R. China, 2003

DISSERTATION

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Doctor of Philosophy

in

Chemistry

May, 2010
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DEDICATION

To my parents, Qingde Du and Hong Lù,

with deepest love and respect.

Their support and encouragement have constantly inspired me along the way.
ACKNOWLEDGMENTS

First I would like to express my sincere gratitude to my advisor Dr. W. Rudolf Seitz, for all his guidance and encouragement throughout the years at UNH. His academic enthusiasm and positive energy deeply influenced me. It is he who helped me make all the important decisions.

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<tr>
<td>PNIPAM</td>
<td>poly(N-isopropylacrylamide)</td>
</tr>
<tr>
<td>NIPAM</td>
<td>N-isopropylacrylamide</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>IDA</td>
<td>iminodiacetic acid</td>
</tr>
<tr>
<td>SL</td>
<td>singly labeled</td>
</tr>
<tr>
<td>DL</td>
<td>doubly labeled</td>
</tr>
<tr>
<td>PVA</td>
<td>poly(vinyl alcohol)</td>
</tr>
<tr>
<td>PET</td>
<td>photoinduced electron transfer</td>
</tr>
<tr>
<td>EET</td>
<td>electronic energy transfer</td>
</tr>
<tr>
<td>FS</td>
<td>fluorescence solvatochromism</td>
</tr>
<tr>
<td>SOS</td>
<td>second-order scattering</td>
</tr>
<tr>
<td>LCST</td>
<td>lower critical solution temperature</td>
</tr>
<tr>
<td>RAFT</td>
<td>reversible addition-fragmentation chain transfer</td>
</tr>
<tr>
<td>ATRP</td>
<td>atom transfer radical polymerization</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>DNS-Cl</td>
<td>dansyl chloride</td>
</tr>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>D</td>
<td>donor</td>
</tr>
<tr>
<td>A</td>
<td>acceptor</td>
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<td>Abbreviation</td>
<td>Full Name</td>
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<td>-----------</td>
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<tr>
<td>RET</td>
<td>resonance energy transfer</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
</tr>
<tr>
<td>AIBN</td>
<td>azobisisobutyronitrile</td>
</tr>
<tr>
<td>MBA</td>
<td>N,N'-methylenebisacrylamide</td>
</tr>
<tr>
<td>VI</td>
<td>1-vinylimidazole</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>MAA</td>
<td>methacrylic acid</td>
</tr>
<tr>
<td>AEMA</td>
<td>N-(2-aminoethyl) methacrylamide hydrochloride</td>
</tr>
<tr>
<td>APMA</td>
<td>N-(3-aminopropyl) methacrylamide hydrochloride</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>st-IDA</td>
<td>N-(m-styryl)-iminodiacetic acid</td>
</tr>
<tr>
<td>AP-IDA</td>
<td>N-(3-acrylamidophenyl)-iminodiacetic acid</td>
</tr>
<tr>
<td>NAIDA</td>
<td>N-acryloyl-iminodiacetic acid</td>
</tr>
<tr>
<td>MWCO</td>
<td>molecular weight cut-off</td>
</tr>
<tr>
<td>DI</td>
<td>deionized</td>
</tr>
<tr>
<td>FP</td>
<td>fluorescein labeled polymer</td>
</tr>
<tr>
<td>RP</td>
<td>rhodamine labeled polymer</td>
</tr>
<tr>
<td>F</td>
<td>fluorescein</td>
</tr>
<tr>
<td>R</td>
<td>rhodamine</td>
</tr>
<tr>
<td>NMP</td>
<td>nitroxide-mediated radical polymerization</td>
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The abbreviations are listed according to their appearance in the dissertation. The abbreviations of chemicals used in the experiments can also be found after their names in Chapter 3 Experimental. Some of the structures can be found in Figure 3-1.
ABSTRACT

RATIO METRIC FLUORESCENT METAL ION INDICATORS BASED ON
FUNCTIONALIZED POLY(N-ISOPROPYLACRYLAMIDE)

By
Jie Du

University of New Hampshire, May 2010

A novel type of ratiometric fluorescent indicator based on the phase transition of a
well-known stimuli-responsive polymer poly(N-isopropylacrylamide) (PNIPAM) is
presented. The sensing mechanism involves the polymer conformational change resulting
from charge neutralization by the analyte metal ions. The ultimate goal is to develop
ratiometric fluorescent indicators for free Cu(II) in environmental water analysis.

The indicators are copolymers of N-isopropylacrylamide (NIPAM) with small
percentages of fluorophores and ligand monomers. The charges on the ligands prevent
PNIPAM from collapsing unless neutralized upon metal ion chelation. The polymer
phase transition is transduced by either fluorescence solvatochromism or fluorescence
resonance energy transfer (FRET), which forms the basis of two slightly different designs.

The first design incorporates the dansyl moiety, a polarity-sensitive fluorophore
which emits more strongly at a shorter wavelength when trapped in collapsed polymer
chains. By separating an iminodiacetic acid (IDA)-based ligand units from the dansyl
moieties, an enhanced fluorescence response was achieved with quenching metal ion
Cu(II). A ratiometric readout was generated based on the fluorescence peak shift and intensity enhancement. The indicator showed a temperature-dependent response to Cu(II) but no Cu(II) selectivity against other metal ions. The log K of the indicator-Cu(II) complex at 35 °C was determined to be 4.3.

Several FRET donor/acceptor pairs were attempted in the second design, which measures the average distance between the two as the chain conformation changes. Alexa Fluor 555 (donor) and 647 (acceptor) were reacted with the amine sites on the polymer either separately or simultaneously, yielding singly labeled (SL) or doubly labeled (DL) strands respectively. Through formulation optimization, both types of indicators showed satisfactory FRET intensity ratio response to Zn(II) as confirmed by the second-order scattering. Fluorescent response to Cu(II) was limited by a quenching mechanism postulated to be a cascade FRET process. The DL system also produced reasonable responses to other metal ions such as Ni(II), Hg(II) and Pb(II). It was found that the DL indicators have better sensitivity and shorter response time. Finally, the charge effect on the phase transition was studied briefly in an attempt to understand the phase transition mechanism.
CHAPTER 1

INTRODUCTION

1.1 Overview of Chemical Sensors

In the development of modern analytical chemistry, there has been a trend towards increased sophistication and hybridization of various state-of-the-art instrumentation techniques. These new techniques, often costly, have revolutionized sample analysis in terms of efficiency, accuracy and the limit of detection. In the mean time, the need for portable, low-cost, in situ, online analysis in complex industrial systems has spurred a reverse trend of minimizing, simplifying and decentralizing analytical techniques. The direct product of this trend is the rapid expansion of sensor research.¹

A sensor is initially defined as “a device capable of continuously and reversibly recording a physical parameter or the concentration of a chemical or biochemical species”.² Three types of sensors can be drawn from this definition, namely (a) physical sensors for measuring physical parameters such as distance, mass, temperature, pressure, etc. (b) chemical sensors which measure chemical substances by chemical or physical responses, and (c) biosensors which measure chemical substances by using a biological sensing element. Among them, biosensors are in fact a sub-set of chemical sensors, but are often treated as a topic in their own right.³
The focus of this dissertation is chemical sensors but defined in a broader sense. Specifically, the restrictive terms “device”, “reversibly” and “continuously” in the definition given above are no longer required to be considered a chemical sensor. First of all, the man-made substance does not have to be a visible solid device. A molecule, which executes the chemical sensing function, is still considered a chemical sensor, even though it is usually given the name “chemosensor”. Second, certain chemical sensing substances, not fully reversible but suitable for single use, can also be called chemical sensors. In a stricter sense, they are usually named probes. Third, the concept of chemical sensors in this dissertation also covers chemical indicators or dosimeters, which do not perform continuous measurements and are not reversible. In sum, the definition of a chemical sensor used in this dissertation is any substance that performs the function of chemical sensing—part of an information acquisition process in which some insight is obtained about the chemical composition of the system.

Chemical sensor research is a truly interdisciplinary subject in that the sensing mechanism can be based on most physical or chemical interactions. However diverse the sensing mechanisms are, all chemical sensors share the same signal transduction function and thus similar components. The prototype of a chemical sensor, as shown in Figure 1-1, usually consists of three components: (a) a chemical receptor, which is able to interact with the analyte or catalyze a reaction involving the analyte. Ideally, the receptor unit should be selective to particular substances or to a group of substances. (b) a transducer, which transforms the non-electric signals from the receptor into an electric signal—voltage, current or resistance. There can be multiple steps of energy conversion. (c) a signal processor, which processes the analog signals and outputs in the form of computer
digital signals. Its function includes signal amplification, integration, derivation, calculation, display, etc. The signal processor is sometimes not considered an integral part of a chemical sensor. One type of commercialized chemical sensor, for example, is the semiconductor gas sensor. They are typically composed of heated SnO₂ microcrystals, which exhibit elevated electrical resistance due to the positive charges in the space charge layer caused by O₂ adsorption. The analyte gas, usually a deoxidizing gas such as CO, induces a resistance decrease as a result of the electron transfer process. In this system, the SnO₂ microcrystal surface recepts the CO and the SnO₂ microcrystal itself transduces the CO concentration information into the resistance signal, which can be processed to be digital by a computer.

![Diagram of chemical sensor components](image)

**Figure 1-1** General model for chemical sensors

### 1.2 Classification of Chemical Sensors

There are many ways to classify chemical sensors. The most common method is to categorize them based on the type of signal generated by analyte-receptor interactions. Common groups are listed below:

1. Optical sensors, which follow absorbance, reflectance, fluorescence or refractive index
2. Electrochemical sensors, including voltammetric and potentiometric devices, potentiometric solid electrolyte gas sensors
3. Electrical sensors, which involve metal oxide and organic semiconductors
4. Mass sensitive sensors based on piezoelectric effect or surface acoustic waves
5. Magnetic sensors based on paramagnetic gas properties
6. Thermometric sensors measuring the heat effect of a specific chemical reaction or adsorption which involves the analyte

Since new advances in materials science have constantly found applications in chemical sensors, it is more enlightening to list chemical sensors according to the base material used. For example, the *Encyclopedia of Sensors* reviewed chemical sensors based on zeolites, conjugated polymers, molecularly imprinted polymers, carbon nanotubes, magnetic nanoparticles, ionic polymer-metal composites, etc. Other latest research highlighted chemical sensors based on quantum dots, nanowires, graphene materials, cyclodextrin derivatives, metallic nanoparticles, crown ether derivatives, functionalized hydrogels, ceramics and sol-gel materials. It is evident that polymer science and nanotechnology play an important role in chemical sensor development. The work in this dissertation deals with optical chemical sensing based on functionalized polymers and hydrogels.

### 1.3 Applications of Polymers in Chemical Sensors

Polymer science offers immense possibilities for chemical sensor research as new functional and “smart” polymer materials emerge. Some of the most common uses of polymers include polymeric electrolytes, hydrogels, gas-permeable membranes, diffusion barriers, immobilization matrices and permselective membranes for interference removal.
1. Polymeric electrolytes

Polymeric electrolytes are mostly used in electrochemical sensors as the electrolyte to conduct electrical current. Based on the conduction mechanism, two types of polymeric electrolyte are currently in use. The first type is the polyelectrolyte in which the polymer itself contains an anionic or cationic group on a side chain. The counter-ions for these groups are typically small, inorganic ions that are mobile within the polymer matrix. For instance, Nafion, a perfluorinated sulfonated ionomer made by DuPont, has been used as the electrolyte in several amperometric gas sensors. The second type of polymer electrolyte acts as the solvent for electrolyte ions which are able to move through the polymer matrix freely. Thus the polymer serves as a solid ionic conductor. An example of this type of polymer is polyethylene oxide in which lithium and other small cations have high mobility.

2. Hydrogels

Hydrogels are crosslinked polymer networks which can contain up to 98% water by volume. In a sense, hydrogels can be considered a “solid” form of water, which are more easily processed for practical applications. One of the most widely used hydrogels is the poly(vinyl alcohol) (PVA) hydrogel. PVA can be crosslinked by a variety of reagents such as boric acid and glutaraldehyde. The consistency and rigidity of the hydrogels can vary widely depending on the molecular weight of the PVA and the degree of crosslinking. PVA membranes have been used as an immobilization matrix in the previous research by former group members.

3. Gas-permeable membranes
Polymers are often used as gas-permeable membranes for gas sensors. They help reduce contamination of the sensor by incompatible materials in the environment and prevent excess electrolyte loss in electrochemical sensors. A typical gas-permeable membrane, preferably microporous, is hydrophobic and has a high rate of transport for the gas of interest relative to other species. Polymers that are commonly used as gas-permeable membranes are Teflon, silicone rubber and Langmuir-Blodgett membranes.  

4. Diffusion barriers

When the analyte concentration is extremely high, polymer membranes can be used as diffusion barriers to bring the effective analyte concentration down into the linear range of the response curve. The addition of a diffusion barrier can reduce the flux of analyte to the sensor and thus increase the sensor's usable measurement range.

5. Immobilization matrices

To retain the properties of a biomolecule or water compatible polymer, aqueous environment with controllable pH and ionic strength is preferred. An immobilization matrix such as a lightly crosslinked hydrogel reflects these preferences. Besides PVA hydrogel membranes mentioned above, polyurethane membranes has also been used as immobilization matrices in our research.

6. Permselective membrane for interference removal

Polymer membranes can also be used as permselective electrode coating to prevent electroactive interferences from reaching the electrode and therefore reduce background currents. Polymers with anionic or cationic charges exclude ions of similar charge by electrostatic repulsion.
The research in this dissertation explores the direct application of polymer solutions, instead of hydrogels, for chemical sensing as chemical indicators. The inherent aqueous phase transition properties of the functionalized polymer in this study enable it to serve the function of receptor and transducer.

1.4 Overview of Fluorescent Chemosensors

A fluorescent chemosensor is defined as “a compound of abiotic origin that complexes to an analyte reversibly with concomitant fluorescence signal transduction”. In a broader sense, this research presents an unconventional type of fluorescent chemosensor in that the sensing element is a functionalized polymer instead of a small molecule compound. The following reviews the principles and development in the area of fluorescent chemosensors.

A chemosensor can be considered a miniaturized chemical sensor. All the components, *i.e.* receptor, transducer and signal processor, are built within a molecule instead of a physical device. In many cases, they are not necessarily independent and physically separated. The molecular engineering of fluorescent chemosensors covers the following aspects: 1) receptor binding mechanism 2) fluorescence transduction method 3) connection between binding and fluorescence. One early example of a Zn(II) fluorescent chemosensor is a molecule based on anthracene shown in Figure 1-2. The anthracene fluorescence is quenched by electron transfer from the lone pairs of the amine to the excited fluorophore. Zn(II) complexation brings back the fluorescence by preventing the electron transfer process. In this example, the amine sites are the receptor unit and the
anthracene is the fluorescing unit. Prevention of the photoinduced electron transfer is the connecting mechanism between the two.

![Figure 1-2 Anthracene-based fluorescent chemosensor for Zn(II)](image)

In terms of analyte-sensor binding, high selectivity is desired. Fabrication of selective receptors from scratch is sometimes a long and multistep process. Lessons learned from biotic host-guest interactions often provide guidance in receptor design. On the other hand, the reversibility affects the application of the sensing substance. Irreversibly strong binding generates chemodosimeters instead of chemosensors.

As for the fluorescence transduction, three readouts are generally monitored—fluorescence intensity, intensity ratio and lifetime. In special cases, fluorescence anisotropy and solvatochromism also yield good results. Intensity measurement is by far the most commonly measured parameter. Depending on the fluorescence signal increase or decrease, two effects—chelation-enhanced fluorescence or chelation-enhanced quenching may be seen. The inherent drawback with absolute intensity measurement is that fluorescence intensity can vary in complex samples for reasons other than analyte concentration such as pH changes, light scattering, photobleaching, temperature quenching, etc. Intensity ratio measurement overcomes this problem by ratiometrically calibrating at two wavelengths. The intensity ratios are not dependent on the absolute
concentration of the chemosensor. Common fluorescence modes that yield ratiometric measurements include fluorescence resonance energy transfer (FRET) and ligand-induced excimer formation/dissociation.\(^{22}\) FRET will be detailed in Chapter 2.

\[
\begin{align*}
&\text{(a) Displacement} \\
&\quad \begin{array}{c}
F \quad Q \\
\text{Non-fluorescent}
\end{array} + A \quad \rightarrow \quad \begin{array}{c}
F \quad Q \\
\text{Fluorescent}
\end{array}
\end{align*}
\]

\[
\begin{align*}
&\text{(b) Disruption} \\
&\quad \begin{array}{c}
F \quad Q \\
\text{Non-fluorescent}
\end{array} + A \quad \rightarrow \quad \begin{array}{c}
\text{Fluorescent}
\end{array}
\end{align*}
\]

\[
\begin{align*}
&\text{(c) Bending} \\
&\quad \begin{array}{c}
F(D) \\
\text{Donor fluorescent}
\end{array} + A \quad \rightarrow \quad \begin{array}{c}
F(D) \\
F(A) \\
\text{Acceptor fluorescent}
\end{array}
\end{align*}
\]

\[
\begin{align*}
&\text{(d) Engaging} \\
&\quad \begin{array}{c}
F(M) \\
\text{Monomer fluorescent}
\end{array} + A \quad \rightarrow \quad \begin{array}{c}
F(M) \\
F(M) \\
F(E) \\
\text{Excimer fluorescent}
\end{array}
\end{align*}
\]

**Figure 1-3 Examples of fluorescent chemosensor designs**

A wealth of studies has successfully connected binding and fluorescence transduction with clever designs. Examples are illustrated in Figure 1-3.\(^{22,24}\) (a) depicts the displacement design, in which the analyte displaces the complexed quencher hence recovered fluorescence from the fluorophore. (b) shows the disruption design, in which the analyte disrupts the quenching by separating the fluorophore and quencher units. (c) involves FRET between the donor fluorophore and acceptor fluorophore. Analyte binding bends the molecular conformation leading to proximity of the donor and the acceptor,
which results in an increase of the acceptor emission and decrease of the donor emission. One of the systems studied in this dissertation is similar to this design. (d) deals with the transition from monomer fluorescence to excimer fluorescence. Fitting of the analyte in the cavity brings together the monomers resulting in stronger excimer emission. All these designs offer inspirations in developing new polymer-based fluorescent chemosensors.

On the molecular level, most fluorescent chemosensors are based on two processes: photoinduced electron transfer (PET) or electronic energy transfer (EET).\textsuperscript{25,26} PET and EET are essentially two quenching processes in which the excited fluorophore loses its energy in different non-radiative decay processes. PET-based fluorescent chemosensors can be designed to turn on or off depending on whether the PET process occurs before or after the analyte binding. The example shown in Figure 1-2 shows the PET-based chemosensor with turn-on properties upon binding.

The use of synthetic polymers as fluorescent chemosensors is a recent development. So far, the majority of studies have been focused on conjugated polymer molecular wires. The initial motivation for connecting small chemosensor units in conjugation was to improve the sensitivity by signal amplification through energy transfer along the conjugated polymer backbone. This concept is shown in Figure 1-4.\textsuperscript{27} Compared to small molecules, conjugated polymers offer advantages such as processability, ease of structural modification and enhancements associated with electronic communication between receptors along the polymer backbone.\textsuperscript{26}

One example of fluorescent chemosensors based on a conjugated polymer is given in Figure 1-5.\textsuperscript{28} The polyarylene ethynylene backbone is known to be highly emissive with quantum yields higher than 0.7, while the terpyridyl pendant is a tridentate
Lewis base coordinating well to a large number of transition metal ions. The polymer showed enhanced sensitivity towards Cr(VI), Cd(II), Ni(II) and Mn(II) by chelation-enhanced quenching. It was found that the quenching mechanism involved complexation (static quenching) rather than collisional deactivation (dynamic quenching).

**Figure 1-4** Signal amplification in a conjugated polymer chemosensor

**Figure 1-5** Example of fluorescent chemosensor based on a conjugated polymer
The disadvantage of this type of chemosensor is its poor water solubility as a result of high conjugation. Fluorescence measurements have to be carried out with organic solvents such as tetrahydrofuran. This limitation makes it unsuitable to be used in real-time for environmental water analysis.

![Chemical structures](image)

**Figure 1-6** Example of water-soluble fluorescent chemosensor based on polymers

Meanwhile, a few recent studies reported polymer fluorescent chemosensors with good water solubility. Water solubility was imparted in the chemosensors through different routes as shown in Figure 1-6. (a) is a water-soluble conjugated polymer bearing 2,2'-bipyridine ligand in the main chain and sodium alkylsulfonate groups on the side chains of another conjugated monomer. The ionic groups keep the polymer soluble in water. (b) utilizes acrylamide as a comonomer to improve the water solubility. It is based
on the copolymer of acrylamide and a small-molecule PET chemosensor bearing a polymerizable double bond. (c) incorporates the methacrylate as the connecting unit to soften the polymer chain segments. It is also based on a small molecule chemosensor.

In summary, polymer-based fluorescent chemosensors developed so far involve a fluorescence-related electron transfer process, which is based on the same principles as small-molecule fluorescent chemosensors. To achieve the desired photophysical properties, intricate molecular design and laborious synthesis are often required. A simpler system that is more cost-effective and less time-consuming is needed. This dissertation presents a new type of polymer-based fluorescent chemosensor that differs from its predecessors in the underlying principles. It utilizes a simpler approach and thus has greater potential for practical applications.

1.5 Summary of the Dissertation Research

The primary goal of this research is to design and synthesize fluorescent metal ion indicators based on the conformational change of the PNIPAM chains in the phase transition process. The ultimate goal is to develop a ratiometric fluorescent indicator for Cu(II) with good selectivity to be used in environmental water analysis. The dissertation research is primarily the proof-of-concept work since the ligands currently used are not selective to Cu(II). Therefore, this general approach would be applied to other metal ion sensing as well. Selectivity to the analyte metal ion can be improved with the incorporation of ligands designed to have high affinity towards the analyte relative to the possible interfering substances.
Cu(II) was selected as the analyte not only because of its environmental and biological importance but also because of the challenge to develop a turn-on indicator/sensor to a quenching metal ion. By design, the metal binding unit and the fluorescing unit are separated on the polymer. This should theoretically circumvent the quenching problem.

The major tasks in this research include understanding the phase transition mechanism, synthesizing polymers with desired structural and compositional features, functionalizing the polymers by post-synthesis derivatization, purifying the polymers and evaluating their fluorescent responses under different experimental conditions. All these procedures affect the response of the final indicators.

In this dissertation, Chapter 2 describes the theoretical background information involved in the experimental design and data analysis. A review of the solution properties of polymers is followed by examples of polymers with inverse thermal solubility in water and the mechanism of PNIPAM phase transition. Details of the optical readouts including fluorescence solvatochromism (FS), fluorescence resonance energy transfer (FRET) and second-order scattering (SOS) on the fluorometer are also discussed. The chapter ends with theories on fluorescence quenching, examples of fluorophore labeling and the basics of the indicators. Chapter 3 reviews the experimental details of the synthesis, purification, derivatization and characterization of the polymers. Chapter 4 describes the development of dansyl-based indicators. The fluorescence response has been optimized and analyzed. The response to Cu(II) at different temperatures has been evaluated and possible explanation proposed. Chapter 5 presents FRET-based indicators. Two labeling schemes have been implemented and compared. The ratiometric responses
to Cu(II) and other metal ions such as Zn(II), Pb(II), Hg(II) and Ni(II) have been studied. The phase transition mechanism in terms of the effect of ionization has been briefly investigated. Chapter 6 summarizes the work of the dissertation and suggests future directions.
CHAPTER 2

THEORETICAL BASIS

2.1 Solution Properties of Polymers

The polymers synthesized in this research are used in aqueous solutions to complex metal ions. The understanding of polymer behavior in solution is very important for this reason.

Even in good solvents, the polymer chains are not fully extended as one would imagine. Polymer chains have different arrangements in dilute, semidilute and concentrated solutions. (Figure 2-1) In dilute solution, polymer chains exist as random coils, which form isolated spheres. The average diameter of these spheres is measured by the radius of gyration \( R_g \). The greater the affinity of the solvent for polymer or the longer the polymer chain, the larger the sphere. At 25 °C, for example, the \( R_g \) for
PNIPAM with a weight-average molecular weight of $1.2 \times 10^6$ g/mol was determined to be 45 nm by light scattering method, while a sample with a higher molecular weight of $2.3 \times 10^7$ g/mol yielded an $R_g$ of 230 nm.\(^{33}\) As the polymer concentration is increased, a point is reached where coils start to overlap. This critical concentration is called the coil overlap concentration $c^*$, which is estimated using equation (2-1):\(^ {34}\)

$$c^* = \frac{3M}{4\pi N_A R_g^3}$$

(2-1)

where $M$ and $R_g$ are the weight-average molecular weight and the radius of gyration of the polymer chains and $N_A$ is the Avogadro’s number. For a PNIPAM sample with a weight-average molecular weight of $2.35 \times 10^6$ g/mol and an average radius of gyration of 60 nm at 25 °C, $c^*$ was estimated to be 4.3 g/L or $3.8 \times 10^{-2}$ moles NIPAM per liter.\(^ {35}\) Using another estimation method based on intrinsic viscosity, PNIPAM sample with a viscosity-average molecular weight of $2 \times 10^6$ has an estimated $c^*$ of 5000 ppm or $4.4 \times 10^{-2}$ moles NIPAM per liter.\(^ {36}\) Above $c^*$, the polymer solution enters the concentrated solution regime. Polymer coils are more stretched out because a polymer chain can be considered a good solvent for another. Entanglement takes place among the chains.

In an extremely dilute polymer solution whose concentration is much lower than $c^*$, there exists a critical coil shrinking concentration $C_s$ at which the chain segments of the polymer coil start to feel the repulsive force between the segments of neighboring polymer coils in solution. The $C_s$ values were measured by light scattering.\(^ {37}\)

### 2.2 Polymers with Inverse Solubility versus Temperature in Water

The balance between hydrophilic and hydrophobic interactions determines the solubility of a polymer. Water soluble polymers usually contain hydrophilic side groups
such as amide, ether, alcohol or hydrated ionic groups which preferentially interact with water molecules through polar interactions, hydrogen bonding or ionic interactions. The entire polymer is solubilized if the number of hydrophilic groups along the polymer chain is high enough. On the other hand, water soluble polymers also contain hydrophobic moieties such as vinyl backbone or alkyl segments. These groups exert attraction towards one another giving rise to hydrophobic interactions. Clearly, every polymer chain experiences both hydrophilic and hydrophobic interactions.

Upon temperature increase, the hydrophilic interactions such as the hydrogen bond are disrupted. This results in the dominance of the hydrophobic interactions hence the insolubility of the polymer. The critical temperature at which such a transition happens is known as the lower critical solution temperature (LCST). It naturally follows that the LCST is determined by a balance between hydrophilic segment—water interactions and hydrophobic—hydrophobic segment interactions. The addition to either side shifts the LCST accordingly. Factors that increase the LCST include adding surfactants, having hydrophilic comonomers, introducing charged groups. On the other hand, increasing ionic strength, copolymerizing with hydrophobic monomers, and neutralizing charges tend to decrease the LCST. Our sensing scheme involves the charge effect brought about by metal ions.

There are a number of water soluble polymers that have the inverse solubility thus exhibiting an LCST. Figure 2-2 shows the structures of some examples.\textsuperscript{38,39} The best known of the group, PNIPAM, has a LCST between \textit{ca.} 30 and 35°C depending on the detailed microstructure of the polymer.\textsuperscript{40} When linear PNIPAM chains are crosslinked, a more versatile material PNIPAM hydrogel is produced. Similar to the LCST of linear
PNIPAM, the hydrogels have a critical temperature called the volume phase transition temperature above which dramatic shrinking of the gel network occurs. Through intelligent design and manipulation of chemical components and structure of the polymer, the thermosensitivity of this material has transcended into sensitivity/recognition towards other external stimuli such as pH, ionic strength, metal ions and specific molecules. To achieve well-defined macromolecular structures, controlled free radical polymerization methods have been employed in the synthesis. Examples of such include reversible addition-fragmentation chain transfer polymerization (RAFT) and atom transfer radical polymerization (ATRP).

![Diagram of polymers](image)

**Figure 2-2** Structures of some polymers having inverse thermal solubility
2.3 PNIPAM Phase Transition Mechanism

The sensing principles used in this research are based on fluorescence signal change resulting from the configuration or microenvironment change accompanying PNIPAM phase transition. The understanding of the phase transition on a molecular level is of primal importance in designing and optimizing our system. Results from mechanistic studies on the phase transition are summarized in this section. The investigation tools include laser light scattering, fluorescence spectroscopic techniques, quartz crystal microbalance, Fourier transform infrared spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, and microcalorimetry. The polymer systems under study range from simple systems such as linear PNIPAM homopolymer chains synthesized under different conditions to more complex systems such as PNIPAM copolymers and charged PNIPAM.

It is suggested that the PNIPAM phase transition proceeds in two stages: the first stage being intramolecular coil collapse (coil-to-globule transition), followed by a second stage of intermolecular aggregation between collapsed coils (globule aggregation). These two stages are not distinctive unless the temperature is at the LCST or slightly higher. In most cases when the temperature is above the LCST, the two processes happen simultaneously. A more recent study shows a concentration-dependent mechanism. The phase transition only involves intrachain contraction in an extremely diluted solution. When the concentration c is lower than the coil overlap concentration c*, both intrachain contraction and interchain association take place. When c is larger than c*, the phase transition involves only interchain association. Figure 2-3 illustrates the phase transition in a dilute solution above the LCST.
a. Intrachain contraction and interchain association take place simultaneously; concentration and temperature determine which process dominates.
b. More compact structure forms and the contraction/association reach maximum.

**Figure 2-3** Schematic PNIPAM phase transition in a dilute solution

Different ways to prevent the globule aggregation have led to the study of the coil-to-globule transition step alone. Such methods include using an extremely dilute solution, adding surfactant, and introducing charges. Kinetically, the coil-to-globule transition of a single chain involves two distinct stages. The chain-length-independent first stage can be attributed to the nucleation and initial growth of some “pearls” (locally contracting segments) on the chain, while the relatively slower second stage is related to the merging and coarsening of the “pearls”. Thermodynamically, the coil-to-globule transition involves four distinct stable states, namely the random coil, the crumpled coil, the molten globule and the fully collapsed globule. (Figure 2-4)

**Figure 2-4** Four thermodynamically stable states in the PNIPAM coil-to-globule transition
More relevant to our study is the research on the phase transition of lightly charged PNIPAM. This type of material is termed an ionomer, \textit{i.e.} an ion-containing polymer with a maximum ionic group content of about 15 mol\%.\textsuperscript{57} Ionomers in solutions have been studied using laser light scattering.\textsuperscript{55,58,59} Just like uncharged PNIPAM, the polymer chains undergo intrachain contraction and interchain association at the same time upon temperature increase. However, the small amount of charge adds an extra constraint on the polymer conformational change. The ionomer tends to adopt a core-shell type of configuration with the charges preferentially distributed on the surface. With the growth of the aggregates from multiple polymer chains, the charge density on the surface increases. A critical point is reached when there are enough charges to prevent further aggregation among the aggregates. This charge stabilization mechanism is similar to the surfactant effect on colloid particles. (Figure 2-5)

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2-5.png}
\caption{Thermal phase transition of PNIPAM ionomers}
\end{figure}

In fact, the specifics of the phase transition, \textit{i.e.} the size and density of the final aggregates, are determined by a number of synthetic and experimental conditions, which are detailed below:
1. The amount of charge on the polymer chains. The more charge, the smaller the aggregates. This is because the critical charge density on the surface of the aggregates is reached without aggregating as many chains.

2. The length and length distribution of the polymer chains. As shown in Figure 2-6, short chains are less likely to associate with each other; therefore they undergo intrachain contraction to form globules first before the globule aggregation. However, long chains favor interchain association over the intrachain collapse until the charges stabilize the aggregates. After the aggregation stops, the intrachain contraction can still take place, which results in a more compact structure.

\[ T < LCST \quad T > LCST \]

**Figure 2-6** Phase transition of PNIPAM ionomers with different chain lengths

3. The final solution temperature and the rate of temperature change. The higher the final solution temperature, the smaller the aggregates. This is due to a higher degree of PNIPAM contraction at high temperature. On the other hand, the faster the temperature is
raised, the less chance the polymer chains will aggregate before self-contraction, which means smaller aggregates will be formed.

4. Polymer concentration. The lower the concentration, the smaller the aggregates due to the lower probability of aggregation.

5. Ionic strength. The higher the ionic strength, the larger the aggregates. It is easy to understand because high concentrations of ions “mask” the charge effect making this similar to the low charge condition as described in 1.

6. Other factors not studied in the cited references such as pH and vigorous stirring. For anionic PNIPAM, the higher the pH, the smaller the aggregates should be because of deprotonation. Supposedly, vigorous stirring can facilitate the interchain association resulting in larger aggregates.

The understanding of the phase transition on a microscopic level gives us guidance in selecting the optimum conditions in the polymer synthesis and fluorescence measurement.

2.4 Optical Readouts

The optical readouts employed in this research include fluorescence and light scattering. Both signals can be measured on a conventional fluorometer.

Two fluorescence detection mechanisms were applied—fluorescence solvatochromism (FS) and fluorescence resonance energy transfer (FRET). The former involves the polarity sensitivity of a fluorophore, while the latter deals with energy transfer between two fluorophores designated as the donor and the acceptor. Another difference is the type of change detected by each phenomenon. Fluorescence
solvatochromism detects the polarity change of the fluorophore microenvironment, while FRET measures the spatial dimension change of the polymer.

Fluorescence solvatochromism refers to the appearance of new spectral bands, shifts in emission wavelengths or changes in fluorescence intensities as a result of the polarity change of the fluorophore microenvironment. Some of the well-known fluorophores exhibiting this type of polarity sensitivity are presented in Figure 2-7.

![Structures of well-known polarity sensitive fluorophores](image)

**Figure 2-7** Structures of well-known polarity sensitive fluorophores

Dansyl chloride (DNS-Cl) is one of the most widely used fluorescent probes in labeling biomolecules such as proteins. It is also extensively used to study the properties of hydrogels. The sulfonyl chloride moiety readily reacts with amine groups to form a stable sulfonamide bond. In polar media, dansyl fluorophores undergo a twisted intramolecular charge transfer reaction and the fluorescence is dominated by emission from the charge transfer state. In non-polar environment, the emission is stronger and is
mostly from locally excited state \textit{i.e.} before charge separation.\textsuperscript{63} The charge transfer state is a lower energy state than the locally excited state, therefore the dansyl emission shifts to lower wavelength (blue shifts) when the microenvironment changes from polar to non-polar.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fret_diagram.png}
\caption{Schematic diagram showing FRET between D and A}
\end{figure}

Fluorescence resonance energy transfer, or Förster resonance energy transfer (FRET), illustrated in Figure 2-8, is a nonradiative process whereby an excited state donor fluorophore (D) transfers energy to a proximal ground state acceptor (A) through long-range dipole-dipole interactions.\textsuperscript{64} The efficiency of energy transfer $E$, strongly dependent on the distance between the donor and the acceptor $r$, is given in equation (2-2):

$$E = \frac{1}{1+(r / R_0)^6}$$

where $R_0$ is the Förster distance of the FRET D/A pair, \textit{i.e.} the distance at which the energy transfer efficiency is 50\%. It is a function of the spectral overlap of the donor emission and acceptor absorption, refractive index of the medium, quantum yield of the donor and a factor $\kappa^2$ that depends on the relative orientation in space between the transition dipoles for donor and acceptor.\textsuperscript{65} As the distance $r$ increases, the efficiency
decreases dramatically. Therefore, it is a useful tool to monitor macromolecular interactions and concomitant conformational changes provided the donor and acceptor are properly tagged on the macromolecules.

Myriads of fluorophore materials have been utilized in FRET. These materials include organic materials such as traditional dye fluorophores, dark quenchers, and polymers; inorganic materials such as metal chelates and metal/semiconductor nanocrystals; fluorophores of biological origin such as fluorescent proteins and amino acids; and biological compounds that exhibit bioluminescence upon enzymatic catalysis. In this research, the Alexa Fluor family of fluorescent dyes produced by Invitrogen was used. They are generally more stable, brighter and less pH-sensitive than common dyes (e.g. fluorescein, rhodamine) of comparable excitation and emission, and to some extent the newer cyanine series.

![Figure 2-9 Spectra overlap of the Alexa 555 excitation and Alexa 647 emission shown in shaded area](image)

The excitation and emission spectra of the FRET pair, *i.e.* Alexa Fluor 555 and Alexa Fluor 647, were obtained from Invitrogen Fluorescence SpectraViewer (Figure 2-9). The shaded area is the spectral overlap of the donor emission and acceptor excitation. Alexa Fluor 555 and Alexa Fluor 647 are superior alternatives to Cyanine 3 (Cy3) and
Cyanine (Cy5) respectively. The Cy3/Cy5 pair, a commonly used FRET pair in single-molecule experiments, has a Förster distance larger than 50 nm.\textsuperscript{64,68,69}

Several processes in fluorescence experiments complicate the measurements and sometimes lead to spurious results. Such processes include fluorescence quenching, photobleaching, excess light scattering background \textit{etc.}

Fluorescence quenching refers to any process which decreases the fluorescence of a sample. It usually occurs without any permanent change in the molecules, that is, without a photochemical reaction. A variety of molecular interactions and processes can result in quenching. Four pathways are discussed below:\textsuperscript{61,70,71}

a). Dynamic quenching, also known as collisional quenching, results from collisional encounters between the fluorophore and the quencher. Energy is lost as heat instead of emitted light. In solution samples, collisional quenching is always present to some extent. Elevating the temperature of the solution accelerates molecular movement and therefore generally increases collisional quenching.

b). Static quenching occurs as a result of the formation of a non-fluorescent complex between the fluorophore and the quencher. When the complex absorbs light, it immediately returns to the ground state without emission of a photon. This complex typically has a different absorption spectrum from the fluorophore; therefore an absorption change often accompanies this type of quenching. Both dynamic and static quenching require molecular contact between the fluorophore and quencher.

c). The third type of quenching process is resonance energy transfer (RET), which does not require molecular contact between the fluorophore and the quencher. It is a similar process as FRET except that the acceptor does not have to be a fluorophore. Resonance
energy transfer is an excited-state interaction, in which an excited donor transfers energy to ground state donor by electronic coupling. The efficiency of RET quenching is strongly dependent upon the distance between the donor and the quencher (acceptor). Self-quenching is a special case of static quenching in that the fluorophore and the quencher are the same species. It is often a measurement artifact as a result of high absorbance of the fluorophore. It is generally advisable to keep the absorbance of the fluorescent analyte to be under 0.1.

In addition to fluorescence quenching, photobleaching is another cause for reduced fluorescence signal. Photobleaching, also known as fading, is the photochemical destruction of a fluorophore. The fluorophore permanently loses the ability to fluoresce due to photon-induced chemical damage and covalent modification. It usually occurs when the fluorophore is repeatedly excited with high energy beam for a long period of time. To reduce the effect of photobleaching, it is advisable to reduce the intensity and time-span of light exposure or increase the concentration of the fluorophores. Photobleaching is characteristic of individual type of fluorophores. Robust fluorophores such as Alexa Fluors used in this research are less prone to bleaching.\textsuperscript{66, 70}

In contrast to quenching and photobleaching, excess light scattering signal usually leads to falsely high readings in the fluorescence signal when the wavelength of the scattered light falls in the wavelength range of the fluorescence emission. Time-resolved detection, proper use of filters and synchronous luminescence spectroscopy can provide different approaches to reduce the interference of the scattered light.\textsuperscript{61, 72} Second-order scattering (SOS), usually treated as an instrumental artifact, is utilized in this study. It can be conveniently measured on a conventional fluorescence spectrophotometer. SOS is the
strong scattering of light that commonly appears at double the wavelength of the incident light ($\lambda_{em} = 2\lambda_{ex}$). As it interferes with fluorescence measurements, SOS is usually minimized off or eliminated as a harmful phenomenon in the fluorometric analysis. Even though there are no thorough studies discussing the properties of SOS, it is generally considered a type of nonlinear Rayleigh scattering resulted from the light transmission of the second-order diffraction in monochromator. SOS has been successfully applied to the study of nanoparticles, biological macromolecules, and determination of inorganic ions. It was found that the SOS intensity was dependent on the shape, diameter, rigidity, refractive index and absorption characteristics of the supramolecules or nanoparticles. In this research, the SOS signal serves as a complementary confirmative technique to study polymer phase transition. As the polymer chains collapse, the SOS intensity increases as a result of higher refractive index and possibly larger aggregates.

2.5 Fluorescence Labeling of PNIPAM

Various fluorescence techniques have been used on PNIPAM for both mechanistic research and application studies. All these systems can be classified in the following three categories:

a) Free fluorophores in solution

It is possible to dissolve a polarity-sensitive fluorophore probe in PNIPAM solution. When the PNIPAM phase transition takes place, the fluorophores will be trapped in the polymer phase leading to a local polarity change of the fluorophores. Pyrene is one of the fluorophores used in the study of the conformational switch of
PNIPAM. It was found that the vibrational fine structures of the emission changed upon phase transition and the intensity ratio of two bands on the profile increased from 0.58 to 0.7. This method is not suitable for sensing applications because of possible interference from the free probes.

b) One type of covalently attached fluorophore

This is the most commonly used labeling method. The fluorophore to be used should be sensitive to the change of the physical or chemical properties during the PNIPAM phase transition. As an alternative to category a), pyrene was covalently attached to PNIPAM through an activated ester. A higher pyrene excimer band was observed when PNIPAM chains collapse. Another example is the application of PNIPAM tagged with a viscosity-sensitive fluorophore boradiazaindacene (BODIPY) as a fluorescent thermometer. Enhanced BODIPY fluorescence was observed when higher viscosity was induced due to PNIPAM chains collapsing. Other polarity-sensitive fluorophores employed in transducing PNIPAM phase transition include rhodamine, benzofurazan, hemicyanine, carbazole, dicyanomethylene-4H-pyran derivatives and dansyl. A more facile dansyl system was introduced in this research.

c) Two types of covalently attached fluorophores

This system is usually based on FRET. Examples of the FRET pairs used in signaling PNIPAM phase transition are listed in Table 2-1. Two-fluorophore (donor and acceptor) system yields more significant intensity ratio change and is less prone to environment perturbation. In this study, an Alexa Fluor-based FRET system was studied.
Table 2-1 FRET pairs used in PNIPAM phase transition studies

<table>
<thead>
<tr>
<th>Donor</th>
<th>Acceptor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenanthryl derivative</td>
<td>Anthryl derivative</td>
<td>83</td>
</tr>
<tr>
<td>Cy5</td>
<td>Cy5.5</td>
<td>84</td>
</tr>
<tr>
<td>NBD derivative</td>
<td>Spiropyran derivative</td>
<td>85</td>
</tr>
<tr>
<td>Naphthalene derivative</td>
<td>Pyrene derivative</td>
<td>86</td>
</tr>
<tr>
<td>2.5 nm CdTe nanocrystals</td>
<td>3.5 nm CdTe nanocrystals</td>
<td>87</td>
</tr>
</tbody>
</table>

2.6 Basics of Metal Ion Indicators

Metal ion indicators are defined as “compounds whose color changes when they bind to a metal ion”. They are typically used to detect the endpoints of complexometric titrations. The equilibrium constant $K_{eq}$ of the metal ion (M) and indicator (In) reaction is given in equation (2-3). The concentration of the metal ion is determined from the ratio of the complexed and uncomplexed indicator and the equilibrium constant for the reaction.

$$M + In \leftrightarrow MIn \quad K_{eq} = [MIn]/[M][In] \quad (2-3)$$

When $[MIn] = [In]$, $[M] = 1/K_{eq}$. The range of concentrations sensed by an indicator is thus centered around this point and is generally considered to go from $[M] = 10/K_{eq}$ to $[M] = 1/10K_{eq}$. In terms of pM, the useful metal ion sensing range is from $pK_{eq} + 1$ to $pK_{eq} - 1$. For specific concentration ranges, the $K_{eq}$ has to be varied accordingly so that the $1/K_{eq}$ value is close to the center of the range.

Metal ion indicators directly measure the activity of the free metal ion because the detection mechanism is based on the aforementioned reaction. When added to the solution, the indicator should have a concentration much lower than the total amount of metal ions. Otherwise, the metal ion-indicator reaction will lead to a shift in the equilibrium causing false reading of the free metal ion. The dissertation work presents a type of fluorescent ratiometric indicators for direct sensing of traces amount of free metal ions.
ions. The fluorescent readout is supposed to be more sensitive than colorimetric readout. The concentration of the indicator was kept at least twenty times lower than that of the total metal ion concentration.
CHAPTER 3

EXPERIMENTAL

3.1 Reagents

Reagents are listed by their sources. The abbreviations are included in the parenthesis in bold. The structures of the major reagents are illustrated in Figure 3-1.

**Sigma-Aldrich**

N-isopropylacrylamide (NIPAM), 97%

Dansyl chloride (DNS-Cl), 99% HPLC grade

Acetonitrile (ACN), anhydrous, 99.8%, HPLC grade

Ammonium persulfate (APS), 98+%, ACS reagent

Azobisisobutyronitrile (AIBN), 98%

N,N'-Methylenebisacrylamide (MBA), 99%

1-Vinylimidazole (VI), 99+% 

Sodium iminodiacetate dibasic hydrate, 98%

Ethlyenediaminetetraacetic acid (EDTA) tetrasodium salt hydrate, 99.0+% 

N,N,N',N'-Tetramethylethlenediamine (TEMED), 99% ReagentPlus 

Methacrylic acid (MAA), 99%

9-Vinylanthracene, 97%

MES hydrate, 99.5+% 

Fluorescein o-acrylate, 97%

Triethylamine, 99.5+%
Copper(II) nitrate hemipentahydrate, 98%, ACS reagent
Zinc nitrate hexahydrate, 98% reagent grade
Mercury(II) nitrate monohydrate, 98%
Fluorescein o-acrylate, 97%

**J.T. Baker**
Nickel(II) nitrate hexahydrate

**Fisher Scientific**
Lead(II) nitrate, 99%

**Invitrogen**
Alexa Fluor 555 carboxylic acid, succinimidyl ester
Alexa Fluor 647 carboxylic acid, succinimidyl ester

**Polysciences**
N-(2-aminoethyl) methacrylamide hydrochloride (**AEMA**), 98+%  
N-(3-aminopropyl) methacrylamide hydrochloride (**APMA**), 98+%  
PolyFluor 570: Methacryloxyethyl thiocarbamoyl rhodamine B

**Pharmco-AAPER**
Ethyl ether, anhydrous, 99+% , ACS reagent

**EMD Chemicals Inc.**
Tetrahydrofuran (**THF**), 99.99+%  
Dimethylformamide (**DMF**), 99.99+%  

**Alfa Aesar**
Trifluoroacetic acid (**TFA**), 98%

**Synthesized by Nick Bencivenga of the Planalp group**
N-(m-styryl)-iminodiacetic acid (St-IDA) disodium salt
N-(3-acrylamidophenyl)-iminodiacetic acid (AP-IDA) disodium salt
N-(3-acrylamidophenyl)-iminodiacetic acid diethyl ester
N-acryloyl-iminodiacetic acid (NAIDA) disodium salt
N-acryloyl-iminodiacetic acid diethyl ester
N-acryloyl-iminodiacetic acid di-tert-butyl ester

All buffers were prepared at 0.1 M concentration and adjusted to 0.1 M ionic strength with sodium nitrate unless otherwise noted. Aqueous solutions were prepared from doubly distilled water prepared from a Corning Mega-Pure distillation apparatus.

The structures of major reagents used in the experiments are illustrated in Figure 3-1. The structures of the Alexa Fluor are proprietary and cannot be obtained.

```
NIPAM

APMA

NAIDA

St-IDA

AP-IDA

DNS-Cl
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3.2 Apparatus

Fluorescence measurements were carried out on a Cary Eclipse fluorescence spectrophotometer equipped with a Peltier thermostatted single cell holder. Both the Scan mode and Kinetic mode of the program were used. UV-Vis absorption spectra were collected on a Varian Cary 50 UV-Vis spectrophotometer thermostatted with a Peltier
temperature control. Proton NMR spectra were obtained on a Varian Mercury 400 MHz NMR spectrometer. An Orion 901 digital analyzer with an Orion 91/55 combination pH meter was used to measure the pH. The molecular weight information was obtained on an Agilent 1100 GPC at 25 °C. The mobile phase was phosphate buffer (pH 7.2). A series of polyacrylic acid standards were used as the calibration standards.

A Branson model 1210 sonicator was used for reagent dissolution and sonication. A Buchi RE111 Rotavapor was used to evaporate the solvents. Separation of precipitated polymer from the solution was performed on a Fisher laboratory centrifuge (3400 rpm). A home-made freeze drying apparatus, built from parts bought from Ace Glass Inc. (Catalogue No: 6696), was used to freeze dry small amount of polymer aqueous solution. Dialysis tubing with a molecular weight cut-off (MWCO) of 12,000-14,000 was purchased from Fisher scientific.

3.3 General Procedures

The following describes the general experimental procedures or a typical example of the synthesis, purification, derivatization and characterization of the polymer indicators. Minor adjustments were made to specific samples. The formulations of samples whose spectra are analyzed and discussed in Chapter 4 and 5 are listed in 3.5.
3.3.1 Dansylated PNIPAM (without ligand monomer)

Free radical polymerization was carried out with varied percentages of APMA in the feed. The polymer was either synthesized in acetonitrile or deionized (DI) water. The following procedure is for PNIPAM with 0.1% amine sites synthesized in acetonitrile.

**Polymer synthesis**

APMA stock solution ($10^{-4}$ mol/mL) was prepared by dissolving 0.0894 g APMA in 5 mL DI water. In a 100 mL round bottom flask, 100 μL APMA stock solution and 10 mmol (1.1316 g) NIPAM were added to 40 mL acetonitrile and sonicated to facilitate dissolution. The reaction mixture was capped and purged under N$_2$ for 20 min under vigorous stirring. After the mixture was brought to 65 °C in a thermostat water/oil bath, 0.0236 g AIBN dissolved in 0.5 mL acetonitrile was injected into the flask to start the reaction. The reaction proceeded for 16 h.

**Polymer purification**

After the polymerization, the crude solution was concentrated in a Rotavapor to almost dryness. 100 mL diethyl ether was added to precipitate the polymer. The white polymer suspension was stirred and sonicated for 10 min. Excess diethyl ether was decanted 10 min after stopping agitation. The remaining solvent was vaporized in a Rotavapor to yield white powdery polymer.

**Dansylation**

Dried polymer was dissolved in 20 mL dichloromethane. 0.005 g dansyl chloride and 50 μL triethylamine were added to the solution. Dansylation reaction was carried out at room temperature for 6 h.
Polymer purification

The polymer purification procedure is the same as before except that ether wash was performed three times until the decanted solvent had no yellow color. The dried polymer was dissolved in 35 mL DI water afterwards. 7 mL aqueous polymer solution was added to dialysis tubing with MWCO of 12,000-14,000 and dialyzed against DI water for 2 days.

The following describes the procedure for PNIPAM with 0.1% amine sites synthesized in DI water.

Polymer synthesis

APMA stock solution (10^-4 mol/mL) was prepared by dissolving 0.0894 g APMA in 5 mL DI water. 100 µL APMA stock solution, 10 mmol (1.1316 g) NIPAM and 0.0113 g ammonium persulfate were added to 40 mL water in a 100 mL round bottom flask and sonicated for 1 min. The reaction mixture was capped and purged under N₂ for 20 min under vigorous stirring. 75 µL TEMED was then injected into the flask to start the reaction. The reaction proceeded at room temperature for 16 h.

Polymer purification

The crude polymer solution was heated to 60 °C and white precipitate formed and settled at the bottom of the flask, which was separated by decantation. The polymer was redissolved in DI water and heated again followed by decantation. Solid polymer separated from the solution was in a clear gel-like form. It was left to dry in the air overnight.
Dansylation

Dansyl chloride (0.001 g) was dissolved in 5 mL acetone to form dansyl chloride stock solution. 0.05 g dried polymer was dissolved in 1 mL 0.1 M bicarbonate buffer (pH=9.0). Sonication was used to facilitate dissolution. Upon the addition of 500 μL dansyl chloride stock solution to the polymer buffer solution, a yellow suspension formed. Dansylation reaction was carried out at room temperature until the yellow color disappeared. A clear homogenous solution was produced at the end of the reaction.

Polymer purification

All the dansylated polymer solution was transferred to dialysis tubing with MWCO of 12,000-14,000 and dialyzed against acetone and then DI water until no significant dansyl emission was detected in the solution outside the tubing.

3.3.2 Fluorescent indicator (dansylated PNIPAM with ligand monomer)

The dansyl-based indicators were synthesized in the same fashion as the dansylated PNIPAM without ligand monomer except that the ligand monomer was added to the formulation. The percentages of APMA and the ligand monomer NAIDA were varied for optimization. When NAIDA was in its sodium salt form, the polymer synthesis was carried out in DI water. When NAIDA was in its ethyl or tert-butyl ester form, the polymerization was done in acetonitrile. The following procedure shows an example of the synthesis in DI water using the sodium salt form of NAIDA.
Polymer synthesis

APMA stock solution ($10^{-4}$ mol/mL) was prepared by dissolving 0.0894 g APMA in 5 mL DI water. 50 μL APMA stock solution, 5 mmol (0.5658 g) NIPAM, 0.0187 g NAIDA sodium salt and 0.0100 g ammonium persulfate were added to 50 mL water in a 100 mL round-bottom flask and sonicated for 1 min. The reaction mixture was capped and purged under N₂ for 20 min under vigorous stirring. 80 μL TEMED was then injected into the flask to start the reaction. The reaction proceeded at room temperature for 16 h.

Polymer purification

The crude polymer solution was heated to 70 °C and a few drops of diluted hydrochloric acid were added. White precipitate formed and settled at the bottom of the flask, which was separated by decantation. The polymer was redissolved in DI water, acidified and heated again followed by decantation. Solid polymer separated from the solution was in a clear gel-like form. It was left to dry in the air overnight.

Dansylation

Dansyl chloride (0.022 g) was dissolved in 2 mL acetone to form dansyl chloride stock solution. 0.0343 g dried polymer was dissolved in 3 mL 0.1 M bicarbonate buffer (pH=9.0). Sonication was used to facilitate dissolution. Upon the addition of 120 μL dansyl chloride stock solution to the polymer buffer solution, a yellow suspension formed. Dansylation reaction was carried out at room temperature until the yellow color disappeared. A clear homogenous solution was produced at the end of the reaction.
Polymer purification

All the dansylated polymer solution was transferred to dialysis tubing with MWCO of 12,000-14,000 and dialyzed against acetone followed by DI water until no significant dansyl emission was detected in the solution outside the tubing.

The following procedure is for polymer synthesized in acetonitrile using NAIDA di-ethyl ester.

Polymer synthesis

In a 100 mL round bottom flask, 0.0486 g NAIDA ethyl ester and 10 mmol (1.1316 g) NIPAM were added to 40 mL acetonitrile and sonicated to facilitate dissolution. 0.0089g APMA dissolved in 1 mL water was added to the flask. The reaction mixture was capped and purged under N₂ for 20 min under vigorous stirring. After the mixture was brought to 65 °C in a thermostat water/oil bath, 0.0236 g AIBN dissolved in 0.5 mL acetonitrile was injected into the flask to start the reaction. The reaction proceeded for 16 h.

Polymer purification

After the polymerization, the crude solution was concentrated in a Rotavapor to almost dryness. 100 mL diethyl ether was added to precipitate the polymer. The white polymer suspension was stirred and sonicated for 10 min. Excess diethyl ether was decanted 10 min after stopping agitation. The remaining solvent was vaporized in a Rotavapor to yield white powdery polymer.
Dansylation

Dried polymer was dissolved in 20 mL dichloromethane. 0.005 g dansyl chloride and 50 \( \mu \)L triethylamine were added to the solution. Dansylation reaction was carried out at room temperature for 6 h.

Polymer purification

The polymer purification procedure is the same as before except that ether wash was performed three times until the decanted solvent had no yellow color.

Ester hydrolysis

The dried polymer was dissolved in a mixture of 30 mL ethanol and 15 mL DI water. 1 pellet (~0.06 g) sodium hydroxide was added and dissolved by sonication. In the case of \textit{tert}-butyl esters, trifluoroacetic acid was used. The reaction solution was refluxed at 85 °C for 1 h and let react at 45 °C overnight. After the reaction, the solvent was evaporated in a Rotavapor to yield sticky glue-like polymer. The polymer was dissolved in 35 mL DI water by sonication. 7 mL of the solution was added to dialysis tubing with MWCO of 12,000-14,000 and dialyzed against DI water for 2 days.

**Indicators Based on FRET (Chapter 5)**

3.3.3 Fluorescein-Rhodamine system

Polymer synthesis

Fluorescein labeled polymer (FP) was synthesized in the following fashion. In 50 mL acetonitrile, dissolve 10 mmol (1.132 g) NIPAM, 5 \( \mu \)L VI, 4 mg fluorescein o-acrylate to form homogeneous solution. The reaction mixture was capped and purged under \( \text{N}_2 \) for 20 min under vigorous stirring. After the mixture was brought to 65 °C in a
thermostat water/oil bath, 0.057 g AIBN dissolved in 0.5 mL acetonitrile was injected into the flask to start the reaction. The reaction proceeded for 16 h.

**Polymer purification**

After the polymerization, the crude solution was concentrated in a Rotavapor to almost dryness. 100 mL diethyl ether was added to precipitate the polymer. The polymer precipitate was collected by centrifugation. All the precipitate was dissolved in DI water and dialyzed in dialysis tubing with MWCO of 12,000-14,000 against DI water until no significant fluorescein emission was detected in the solution outside the tubing.

Rhodamine labeled polymer (RP) was synthesized in the same manner except that 7 mg PolyFluor 570 replaced the fluorescein o-acrylate. The purification process is the same as the FP.

### 3.3.4 Alexa Fluor system

The Alexa fluorophores were dissolved in 100 μL DMF to form the stock solutions. Based on the molecular weight information obtained from Invitrogen, the concentrations of the Alexa 555 and 647 stock solutions were calculated to be approximately $8 \times 10^{-9}$ mol/μL.

The following description shows an example of the Alexa Fluor-based metal ion indicator synthesized in DI water at room temperature.

**Polymer synthesis**

In a 50 mL round bottom flask, dissolve 0.0321 g AP-IDA disodium salt, 5 mmol (0.5658 g) NIPAM, 0.0179 g APMA and 0.0113 g APS in 30 mL DI water. The reaction
mixture was capped and purged under N₂ for 20 min under vigorous stirring. 60 μL TEMED was injected to start the reaction. The reaction proceeded for 16 h.

**Polymer purification**

The crude polymer solution was heated to 70 °C and a few drops of diluted hydrochloric acid were added. White precipitate formed and settled at the bottom of the flask, which was separated by decantation. The polymer was redissolved in DI water, acidified and heated again followed by decantation. Solid polymer was dissolved in DI water followed by extensive dialysis.

**Alexa Fluor labeling**

In a small vial, combine 1 mL 0.1 M pH 8.7 bicarbonate buffer, 5 μL Alexa 555 stock solution and 12 μL polymer solution. The exact amount of polymer solution depends on the amount of amine in relation to the amount of Alexa 555. The Alexa 555 is at least 1:1 with respect to the amines on the polymer. The reaction was carried out at room temperature overnight. The Alexa 647 labeling was carried out in the same fashion except that 5 μL Alexa 647 stock solution was added in place of the Alexa 555. In the case of DL polymers, the Alexa 555 and 647 were both added to the same vial. The solution after labeling was dialyzed extensively in DI water.

The following procedure shows an example of the Alexa Fluor-based metal ion indicator synthesized in acetonitrile.

**Polymer synthesis**

In a 50 mL round bottom flask, 8.4 mg AP-IDA ethyl ester and 5 mmol (0.5658 g) NIPAM were added to 35 mL acetonitrile and sonicated to facilitate dissolution. 0.0089 g
APMA dissolved in 1 mL water was added to the flask. The reaction mixture was capped
and purged under N₂ for 20 min under vigorous stirring. After the mixture was brought to
65 °C in a thermostat water/oil bath, 0.0113 g AIBN dissolved in 0.5 mL acetonitrile was
injected into the flask to start the reaction. The reaction proceeded for 16 h.

**Polymer purification**

After the polymerization, the crude solution was concentrated in a Rotavapor to
almost dryness. 100 mL diethyl ether was added to precipitate the polymer. The white
polymer suspension was stirred and sonicated for 10 min. Excess diethyl ether was
decanted 10 min after stopping agitation. The remaining solvent was vaporized in a
Rotavapor to yield white powdery polymer.

**Ester hydrolysis**

The dried polymer was dissolved in a mixture of 30 mL ethanol and 15 mL DI
water. 1 pellet (~0.06 g) sodium hydroxide was added and dissolved by sonication. In the
case of tert-butyl esters, trifluoroacetic acid was used. The reaction solution was refluxed
at 85 °C for 1 h and let react at 45 °C overnight. After the reaction, the solvent was
evaporated in a Rotavapor to yield sticky glue-like polymer. The polymer was dissolved
in 35 mL DI water by sonication. 7 mL of the solution was added to dialysis tubing with
MWCO of 12,000-14,000 and dialyzed against DI water for 2 days.

**Alexa Fluor labeling**

In a small vial, combine 1 mL 0.1 M pH 8.7 bicarbonate buffer, 5 μL Alexa 555
stock solution and 12 μL polymer solution. The exact amount of polymer solution
depends on the amount of amine in relation to the amount of Alexa 555. The Alexa 555 is
at least 1:1 with respect to the amines on the polymer. The reaction was carried out at
room temperature overnight. The Alexa 647 labeling was carried out in the same fashion except that 5 µL Alexa 647 stock solution was added in place of the Alexa 555. In the case of DL polymers, the Alexa 555 and 647 were both added to the same vial. The solution after labeling was dialyzed extensively in DI water.

3.4 Polymer Characterization

3.4.1 Fluorescence measurement

20 µL aqueous polymer solution after dialysis was added to 3 mL DI water in the cuvette. The excitation wavelength used in dansyl system (Chapter 4) is 330 nm and that for Alex 555 and 647 pair (Chapter 5) is 525 nm unless otherwise noted. The SOS signal was shown at 660 nm in the dansyl system. In the FRET system, the SOS was collected at 800 nm with the excitation wavelength set at 400 nm. The slit widths were set so that the absolute fluorescence intensity is optimal. The volume of DI water or buffer in the cuvette is 3 mL before the addition of dialyzed aqueous polymer solution in the order of tens of µLs. Temperature was increased stepwise on the thermostat. Approximately ten minutes were allowed for the signal to stabilize.

All the measurements on the dansyl system were made using the scan mode of the fluorescence spectrophotometer, which scans the fluorescence intensity over a preset wavelength range. In addition to the scan mode, the kinetic mode was used on the FRET system. The kinetic mode monitors the fluorescence intensities at one or more specified wavelengths as a function of time. The two wavelengths corresponding to the donor and acceptor emission were selected based on the fluorescence spectra obtained in the scan mode. For polymers labeled with Alexa 555 and 647, the two wavelengths were usually
set at 565 nm and 647 nm respectively. An additional wavelength at 800 nm when excited at 400 nm was also monitored for the SOS signal readout. During the kinetic scan, conditions such as temperature, pH and metal ion concentration were varied. The resulting fluorescence response was recorded after the intensity signals stabilized. Using the math function of the software program, a new trace of the intensity ratio can be generated directly by dividing the two traces of absolute intensities.

Metal ion titration was performed by adding proper amounts of 0.001M, 0.01M or 0.1M Cu(II) stock solutions to the cuvette containing the indicator solution. The kinetic signal shows the time it requires to equilibrate.

**Fluorometric titration of the indicators**

The spectrofluorometric titration of the dansyl-based indicators with the metal ions was performed in the quartz cuvette. 10 to 30 µL polymer indicator solution was added to 3 mL DI water or buffer solutions. The concentration of the polymer in terms of the ligand groups was estimated from the absorption of dansyl fluorophores at 330 nm and the relative amounts of the fluorophore and ligand in the feed. The extinction coefficient used in the estimation was 3300 M\(^{-1}\)·cm\(^{-1}\). The indicator solution was brought to desired temperature and equilibrated. For a specific concentration of Cu(II), proper amounts of 0.001M, 0.01M or 0.1M Cu(II) stock solutions were added to the cuvette followed by stirring. The volume added each time was lower than 100 µL. The volume increase was minimal and omitted in the calculation. Fluorescence spectra were collected after at least 5-min equilibration time. Kinetic scan of the FRET-based indicators was conducted in the similar fashion. The solution was not stirred and additional metal ions were added when the signal stabilized. Fluorometric titration of the indicator by other
metal ions including Zn(II), Pb(II), Ni(II) and Hg(II) was carried out in the same way as Cu(II).

3.4.2 UV-Vis absorption measurement

To assess the concentration of the Alexa fluorophores in the cuvette, the UV-Vis absorption of the polymer indicator was measured before the fluorescence measurement. The scan speed was set at medium for good resolution. The intensities at the Alexa 555 and 647 absorption maximum were recorded for further calculation. The concentrations of Alexa 555 and 647 can be estimated using the provided extinction coefficients of 150,000 and 239,000 M$^{-1} \cdot$cm$^{-1}$ respectively.

To study the effect of the ionic groups on the PNIPAM phase transition, UV-Vis turbidity measurements were carried out kinetically by monitoring the UV-Vis absorption at 500 nm. The absorbance signal was recorded after it stabilized when temperature was increased. The absorbance was then plotted against the temperature in the figures in Chapter 5.

3.5 Critical Formulation Information and Experimental Conditions

The polymer samples used in the discussion are named according to their appearance in the corresponding chapters. The symbols in the parentheses provide simplified codes for the formulations. The names of the fluorophores or ligands are abbreviated, the percentages of which are shown by the numbers preceding the abbreviation. The fluorophores are listed before the ligands followed by the synthetic medium, all of which are connected by the dash sign “-”. In the case of FRET pair using
Alexa Fluor 555 and 647, the labeling method (SL or DL) is first noticed before the percentage of total amine sites. For polymers not containing fluorophore or ligand, the corresponding abbreviations in the codes are omitted as well.

**Sample 4-1 (0.1DNS-DI):** 0.1 mole% APMA, synthesized in DI water at room temperature with redox initiation. Spectra were collected in DI water with 10 min intervals allowing thermal equilibration. Slit widths were set at 20-20 nm. Concentration of the polymer in the cuvette was kept low but not monitored.

**Sample 4-2 (0.5DNS-2NAIDA-ACN):** 0.5 mole% APMA, 2 mole% NAIDA di-tert-butyl ester, synthesized in acetonitrile. Spectra were collected on the hydrolyzed sample in DI water with 10 min intervals allowing thermal equilibration. Slit widths were set at 10-10 nm. Concentration of the polymer in the cuvette was kept low but not monitored.

**Sample 4-3 (0.1DNS-2NAIDA-ACN):** 0.1 mole% APMA, 2 mole% NAIDA di-tert-butyl ester, synthesized in acetonitrile at 65 °C. Spectra were collected on the hydrolyzed sample in DI water with 10 min intervals allowing thermal equilibration. Slit widths were set at 10-10 nm. Concentration of the polymer in the cuvette was kept low but not monitored.

**Sample 4-4 (0.1DNS-2NAIDA-DI):** 0.1 mole% APMA, 2 mole% NAIDA disodium salt, synthesized in DI water at room temperature with redox initiation. Spectra were collected in DI water with 10 min intervals allowing thermal equilibration. Slit widths were set at 20-10 nm. Concentration of the polymer in terms of the IDA ligand was estimated to be $4 \times 10^{-6}$ M using the IDA/dansyl molar ratio.
Sample 5-1 (0.2FLU/0.08RHO-ACN): 0.2 mole% fluorescein o-acrylate, 0.08 mole% PolyFluor 570, 5 mole% MBA, synthesized in acetonitrile at 65 °C. Concentration of the polymer microspheres in the cuvette was kept low but not monitored.

Sample 5-2 (0.1FLU-0.5VI-ACN & 0.1RHO-0.5VI-ACN): Fluorescein labeled polymer (FP) and rhodamine labeled polymer (RP) were both synthesized in acetonitrile at 65 °C with 0.5 mole% 1-vinylimidazole in the feed. FR has 0.1 mole% fluorescein o-acrylate while RP has 0.1 mole% PolyFluor 570. Equal volume of FP and RP were combined the dialyzed in DI water. Spectra were collected DI water using both the scan mode and kinetic mode. Slit widths were set at 10-10 nm. Concentration of the polymer in the cuvette was kept low but not monitored.

Sample 5-3 (SL-1ALX-DI): 1 mole% APMA, synthesized in DI water at room temperature with redox initiation. Spectra were collected in DI water. Slit widths were set at 10-10 nm. Concentrations of the Alexa 555 and 647 were estimated to be $2.4 \times 10^{-7} M$ and $2.0 \times 10^{-7} M$ respectively.

Sample 5-4 (SL-2ALX-2StIDA-DI): 2 mole% APMA, 2 mole% St-IDA disodium salt, synthesized in DI water at room temperature with redox initiation. Spectra were collected in pH 6 MES buffer. Slit widths were set at 10-10 nm. Concentrations of the Alexa 555 and 647 were estimated to be $1.7 \times 10^{-7} M$ and $9.6 \times 10^{-8} M$ respectively.

Sample 5-5 (SL-2ALX-2APIDA-DI): 2 mole% APMA, 2 mole% AP-IDA disodium salt, synthesized in DI water at room temperature with redox initiation. Spectra were collected in pH 6 MES buffer. Slit widths were set at 10-10 nm. Concentrations of the Alexa 555 and 647 were estimated to be $1.7 \times 10^{-7} M$ and $1.8 \times 10^{-7} M$ respectively.
Sample 5-6 (SL-1ALX-2APIDA-ACN): 1 mole% APMA, 2 mole% AP-IDA di-ethyl ester, synthesized in acetonitrile at 65 °C. Spectra were collected in pH 5.5 and 6.0 MES buffers. Slit widths were set at 10-10 nm. Concentrations of the Alexa 555 and 647 were estimated to be $1.3 \times 10^{-7} \text{ M}$ and $1.1 \times 10^{-7} \text{ M}$ respectively.

Sample 5-7 (SL-1ALX-6APIDA-ACN): 1 mole% APMA, 6 mole% AP-IDA di-ethyl ester, synthesized in acetonitrile at 65 °C. Spectra were collected in pH 6.0 MES buffers. Slit widths were set at 10-10 nm. Concentrations of the Alexa 555 and 647 were estimated to be $9.2 \times 10^{-8} \text{ M}$ and $9.1 \times 10^{-8} \text{ M}$ respectively.

Sample 5-8 (SL-1ALX-4APIDA-ACN): 1 mole% APMA, 4 mole% AP-IDA di-ethyl ester, synthesized in acetonitrile at 65 °C. Spectra are not shown.

Sample 5-9 (SL-1ALX-0.5APIDA-ACN): 1 mole% APMA, 0.5 mole% AP-IDA di-ethyl ester, synthesized in acetonitrile at 65 °C. Spectra were collected in pH 6.0 MES buffers. Slit widths were set at 10-10 nm. Concentrations of the Alexa 555 and 647 were estimated to be $1.6 \times 10^{-7} \text{ M}$ and $1.4 \times 10^{-7} \text{ M}$ respectively.

Sample 5-10 (SL/DL-2ALX-DI): 2 mole% APMA, synthesized in DI water at room temperature with redox initiation. Spectra were collected in DI water. Slit widths were set at 10-10 nm. Concentrations of the Alexa 555 and 647 were varied in the experiment. Alexa 555 and Alexa 647 were added to the polymer separately or together, which produces SL polymer or DL polymer respectively.

Sample 5-11 (DL-4ALX-2-APIDA-ACN): 4 mole% APMA, 2 mole% AP-IDA di-ethyl ester, synthesized in acetonitrile at 65 °C. Equal amount of Alexa 555 and 647 corresponding to the equivalent of 2 mole% APMA was added to the polymer. Spectra
were collected in DI water. Slit widths were set at 10-10 nm. Concentrations of the Alexa 555 and 647 were estimated to be $1.8 \times 10^{-7} \text{M}$ and $1.9 \times 10^{-7} \text{M}$ respectively.

**Sample 5-12 (0.5VI-ACN):** 0.5 mole% VI, synthesized in acetonitrile at 65 °C. UV-Vis Spectra were collected in a series of pH citrate-phosphate buffers.

**Sample 5-13(5VI-ACN):** 5 mole% VI, synthesized in acetonitrile at 65 °C. Spectra were collected in a series of pH citrate-phosphate buffers.

**Sample 5-14(5MAA-ACN):** 5 mole% MAA, synthesized in acetonitrile at 65 °C. Spectra were collected in a series of pH citrate-phosphate buffers.
CHAPTER 4

A RATIOMETRIC FLUORESCENT INDICATOR FOR METAL IONS BASED ON DANSYLATED PNIPAM

4.1 Introduction

Metal ion contamination in the environment is a potential health hazard for living organisms. The bioaccumulation of toxic metal ions often leads to chronic poisoning especially in human beings and other organisms at the top of the ecological food chain. The toxicity and bioavailability of the concerning metal ions have been shown to be related to the activity of free metal ions under most circumstances. It is therefore of great significance to measure traces amount of free metal ions unbound to the natural organic ligands.

Several conditions render free metal ion analysis a challenging task. First, the complexed and free forms of a metal ion cannot be distinguished by spectroscopic techniques such as atomic absorption spectroscopy. Second, multiple sample manipulations, required for many instrumental techniques, may disturb the complexation equilibria resulting in false readings. Third, the amount of metal ions existing in the free form is extremely low. For instance, copper is thought to be >99% complexed in seawater. This presents a detection limit challenge.
A few analytical methods have been developed for directly measuring free metal ion concentration, each having its advantages and limitations. Ion selective electrodes are commonly used to measure free metal ions. However, selective electrodes are not available for many metal ions. Moreover, they are prone to interference from other ions and usually have a relatively high detection limit. Low detection limits can be achieved by voltammetry but the application of current disturbs the complexation equilibrium when the amount of metal ion in the redox reaction is large compared to the bulk. More recent techniques include using a cation-exchange resin, a diffusion gradient in thin-films technique and a Donnan membrane technique. One of the oldest and most convenient methods of free metal ion analysis is using metal ion indicators. The fluorescent metal ion indicator is simply added to an aqueous sample and the free metal ion concentration is then calculated from the fluorescence calibration curve. The measurement can be continuous or intermittent depending on the application. This method is completely analogous to the use of pH indicators to sense pH. The range of metal ion activity sensed centers around \( pM = \log K \), where \( K \) is the formation constant for the indicator-metal ion complex.

A variety of fluorescent metal ion indicators have been developed for the detection of biologically relevant metal ions such as Ca(II) and Zn(II). It is more difficult to develop fluorescent indicators for quenching metal ions such as Cu(II). For most fluorescent Cu(II) indicators developed so far, the sensing mechanisms are based on fluorescence quenching due to the paramagnetic nature of Cu(II). However, fluorescence “turn-on” sensing can distinguish weak signal from the “dark” background, thus offering better sensitivity in general. Moreover, a “turn-off” or quenching indicator
is more prone to false positive signals arising from the quenchers in the matrix or photobleaching.

There has been increasing interest in fluorescent "turn-on" sensing for quenching metal ions thanks to its inherent advantages. Some of the sensing mechanisms involved include spiro ring-opening reaction,\textsuperscript{102} ion-catalyzed reaction,\textsuperscript{103} internal charge transfer,\textsuperscript{104} photoinduced electron transfer\textsuperscript{105} and metal ion displacement.\textsuperscript{106} However, these indicators are unsuitable for quantitative measurements in environments where it is not feasible to control the amount of indicator in the optical path, \textit{e.g.} single cell measurements.

The metal ion indicator developed in this study is designed to address the quenching problem by spatially separating the metal chelator from the fluorescent signaling moiety on a macromolecule. Furthermore, the dansyl peak shift provides a ratiometric sensing method, which is independent of the indicator concentration and less susceptible to the environmental effect. It is extremely important in applications under conditions where the concentration of the indicator is not known, \textit{i.e.} in the cell.

4.2 Overall Sensing Mechanism

As described in Chapter 2, PNIPAM has an interesting solution phase transition behavior in response to external stimuli, which allows it to be used for sensing applications. The metal ion indicators developed in this chapter as well as in Chapter 5 both take advantage of the concomitant physical property change during the phase transition. Figure 4-1 further summarizes the sensing mechanism which involves two steps of signal transduction occurring simultaneously.
The first step is from metal ion concentration signal to polymer (indicator) conformational change. This is achieved by labeling the polymer with ligands that chelate metal ions. Ideally, the ligand should have good selectivity towards the analyte metal ion and appropriate affinity for the concentration range of the analyte. When metal ions bind to the ligand sites on the polymer, positive charges are introduced on the polymer causing the polymer chains to expand or contract depending on the initial charge state of the ligands. For example, polymers attached with carboxylate ligands are negatively charged at pH 6 or higher. The positive charges on the metal ions neutralize the negative charges on the polymer and cause the polymer chains to contract, \textit{i.e.} to undergo a phase transition. In contrast, the polymer phase transition would be reversed if the ligand used is neutral at the sensing pH, as in the case of bipyridine ligands.

The second step is from the polymer conformational change to a fluorescence signal. This is achieved by tagging the polymer with fluorophores. As reviewed in Chapter 2, various tagging methods have been applied. This chapter deals with polarity-sensitive dansyl fluorophore and Chapter 5 deals with the FRET system.

In sum, the indicator consists of three major components covalently attached together—the polymer backbone, the fluorophores and the ligand. When the metal ions bind to the ligand sites, the polymer backbones (chains) expand or contract leading to a phase transition from a solubilized state to an insoluble state or vice versa. With proper
fluorescence tagging, the phase transition can cause a change in the fluorescence signal. Through the two-step signal transduction, metal ion concentration is consequently correlated with the fluorescence readout.

In this chapter, a polarity-sensitive fluorophore (dansyl) system was first studied on thermally induced PNIPAM phase transition to validate the fluorescence transduction. Then a series of experiments were carried out to develop a PNIPAM-based metal ion indicator. Response to the quenching metal ion Cu(II) was used to demonstrate the enhanced fluorescence response. Finally, the responses to other metal ions were compared briefly and the effects of synthetic conditions were discussed.

4.3 Validation of Dansyl Fluorescence Transduction

The attachment of dansyl groups to PNIPAM is through APMA, a commercially available amine-containing monomer from Polysciences Inc. The structure of APMA is shown in Figure 3-1. APMA is expected to have similar reactivity as the principal monomer NIPAM because it is a methacrylamide. This structure similarity insures even distribution of amine sites on the polymer for dansyl fluorophore attachment. APMA can only be used in aqueous medium or highly polar organic solvent because the primary amine is in its hydrochloride salt form. It is worth mentioning that other amine-containing monomers are also available from Polysciences Inc, which include the t-BOC protected APMA for synthesis in organic media and AEMA, whose side chain is one methylene shorter than that of APMA.

Figure 4-2 shows the fluorescence spectra of PNIPAM with 0.1% dansyl labeling (Sample 4-1) as the temperature was increased. The detailed formulation information and
experimental conditions can be found in Chapter 3 for all the samples whose spectra are analyzed here. Polymers synthesized in DI water and acetonitrile showed no significant difference in fluorescence spectra.

![Thermal response of Sample 4-1](image)

**Figure 4-2** Thermal response of Sample 4-1

As the temperature increased from 25 °C to 40 °C, the dansyl emission shifted from 545 nm to 503 nm with increased intensity. This agrees with our expectation—PNIPAM collapses with increasing temperature leading to a more hydrophobic microenvironment around the dansyl fluorophores. As described in the Chapter 2, the stronger emission at the shorter wavelength is from the locally excited state before charge separation when dansyl groups are in a non-polar environment.

The LCST, as demonstrated by the peak shift, was found to be around 32.2 °C in the dansylated PNIPAM, which is in good agreement with the literature value. This result suggested that 0.1% fluorescent tagging did not affect the PNIPAM phase
transition. Our result also agrees well with laser light scattering results on the PNIPAM coil-to-globule transition. The θ temperature for PNIPAM was shown to be around 30.5 °C, slightly below the temperature at which the PNIPAM coil started to contract towards its center. No apparent change in fluorescence was seen. When the PNIPAM solution entered the two-phase region at around 32 °C, the chain density increased to the point at which collapsed globule was formed. It is at this point (32.2 °C) that significant fluorescence peak shift and intensity increase took place. The globule aggregates started to form at 32.4 °C as shown by increased scattering background at ca. 420 nm. As the aggregates got larger and larger, the fluorescence peak shift and intensity increase were not as significant, which suggested an increasing number of dansyl groups “merged” into the non-polar environment but there was only a minor change in polarity for those that were already in the non-polar environment.

This result shows that the coil-to-globule transition and the initial stage of the globule aggregation result in quite significant fluorescence change, which can be used in developing the fluorescent indicators. The sharp phase transition may be disadvantageous for developing an on-line sensing device for measuring a concentration span. Wider molecular weight distribution, often undesirable in polymer synthesis, would otherwise be suitable in this application because PNIPAM with different molecular weight undergoes phase transition at slightly different temperatures making the phase transition less sharp. Alternatively, crosslinked PNIPAM hydrogel particles would have a continuous phase transition thus making them responsive to wider concentration range.

From Figure 4-2, a calibration curve can be generated based on the intensity enhancement (Figure 4-3). However, this response is dependent on the polymer
concentration. Figure 4-4 shows a calibration curve based on the peak shift and a ratiometric calibration combining the two effects. They are less susceptible to environmental perturbation and independent of the polymer concentration and are therefore preferred. The intensity ratio was plotted at 503 nm (non-polar) over 545 nm (polar) versus temperature.

![Figure 4-3 Calibration of Sample 4-1 thermal response based on wavelength max](image)

**Figure 4-3** Calibration of Sample 4-1 thermal response based on wavelength max

In conclusion, fluorescence transduction on PNIPAM phase transition through dansyl labeling is validated using a low percentage of tagging. A ratiometric calibration as a function of temperature has been established. Good agreement with the literature studies has been found. The next step is to incorporate NAIDA for metal ion sensing.
4.4 IDA-based Metal Ion Indicator

IDA is a common tridentate ligand that form a complex with two fused five membered rings. Two carboxylate groups and the secondary amine are the metal binding sites. (Figure 3-1) In order to attach IDA to the polymer, a polymerizable double bond has to be introduced at the secondary amine site to form NAIDA. The derivatization greatly weakens the binding of the metal ions by IDA because the nitrogen in the amide is a very weak chelating site. The derivatized IDA basically becomes a bidentate diacetate. Take the formation constants for Cu(II) for instance. The log $K$ for IDA is 10.57 and that for glutaric acid is 2.4. The log $K$ for NAIDA should be between these two values. The deprotonated NAIDA carries negative two charges. Once it complexes divalent metal ions such as Cu(II), the negative charges are neutralized. This -2 to neutral

Figure 4-4 Calibration of Sample 4-1 thermal response based on intensity ratio and peak wavelength
transition is expected to cause the dansylated PNIPAM to change from the expanded state to a collapsed state, *i.e.* a phase transition.

The NAIDA monomer was synthesized by Nick Bencivenga from Dr. Roy Planalp’s lab either in the ester or acid form for synthesis in organic or aqueous media. The monomer synthesis involved a facile reaction of acryloyl chloride with IDA ester. The polarity of the monomers and solvents may affect the relative positioning of the IDA groups and the APMA, on which the dansyl groups attach. It would also determine the percentage incorporation in the final polymer for both since the reactivity ratios are different. For example, APMA may form ion pairs with the NAIDA in its acid form during the synthesis but not the ester form. The average distance between the two also depends on the amount of each in the feed.

4.4.1 Signal optimization

The percentage of dansyl labeling was compared at several levels while the NAIDA ligand was kept constant at 2%. The samples involved were all synthesized in acetonitrile. Polymers with low percentage of dansyl tagging (0.01%) had a weak fluorescence signal, therefore required high polymer concentration or wider slit width. In both cases, background scattering at high temperature was so intense that it affected the fluorescence reading. On the other hand, 0.5% dansyl labeling (Sample 4-2) increased the hydrophobicity of the polymer chains resulting in insignificant peak shift from 522 nm to 508 nm as shown in Figure 4-5. In Figure 4-6, polymers with 0.1% dansyl labeling (Sample 4-3) showed larger peak shift from 542 nm to 513 nm. The slight hydrophobicity increase in this case is optimal for a large intensity ratio response.
Figure 4-5 Thermal response of Sample 4-2

Figure 4-6 Thermal response of Sample 4-3
Sample 4-4 has the same percentages of dansyl labeling and the NAIDA ligand as Sample 4-2 but it was synthesized in DI water using the NAIDA disodium salt. Figure 4-7 shows its thermal response. Compared to the peak shift in Figure 4-6, a larger shift from 546 nm to 508 nm suggests the dansyl group underwent the largest change in the polarity of its microenvironment. Therefore, for the synthesis of the indicators, DI water and disodium salt form of the NAIDA were used and the percentage of dansyl labeling was kept at 0.1%.

![Figure 4-7 Thermal response of Sample 4-4]

**Figure 4-7** Thermal response of Sample 4-4

### 4.4.2 Signal calibration

Extracted from the spectra in Figure 4-7, calibration curves based on the intensity ratio at 508 nm over 546 nm and the peak wavelength are plotted in the same graph in Figure 4-8. Both curves show a gradual phase transition for the indicator from...
approximately 32 °C to 41 °C. The LCST of the indicator is determined to be approximately 37 °C at the midpoint of the transition. The intensity ratio increases by almost a factor of 2 as the dansyl environment shifts from hydrophilic to hydrophobic.

![Figure 4-8 Calibration curves of Sample 4-4 thermal response based on intensity ratio and peak wavelength](image)

Since Sample 4-1 and Sample 4-4 only differ in the absence or presence of 2% NAIDA ligand, a careful comparison of the two reveals the effect of ligand on the fluorescence behavior. First, the phase transition taking place between 31 °C and 33 °C in Figure 4-4 is much sharper than that between 32 °C to 41 °C in Figure 4-8. The scattering background accompanying the polymer phase transition is also higher in Figure 4-2. Second, the LCST value in Figure 4-4 is about 5 °C lower than that in Figure 4-8. The differences can be attributed to the core-shell type structure the charged PNIPAM assumes at elevated temperatures as shown in Figure 2-5. The NAIDA ligand is thought to be partially deprotonated in DI water as the pK$_{a1}$ and pK$_{a2}$ of a similar ligand monomer.
were determined to be 3.4 and 5.6 respectively. Negative charges on the ligands, preferentially distributed on the surface of the polymer globule, exert charge repulsion forces deferring the phase transition. This leads to a more gradual phase transition and a higher LCST. The charges also stabilize the globules, preventing further coagulation hence lowering scattering background in Figure 4-7.

Another minor difference can also be seen that the intensity ratio and the peak wavelength in the hydrophilic state, i.e. at low temperature, are slightly lower for the polymer without ligands. This suggests that the 2% NAIDA ligand on the polymer increases the hydrophobicity of the PNIPAM slightly. It can also be attributed to the core-shell structure shown in Figure 2-5 since the hydrophobic functional groups are forced closer together in the conformational rearrangement. The gradual phase transition as mentioned before is advantageous in sensing metal ions over a large range of concentrations.

4.4.3 Response to Cu(II)

Sample 4-4 was spectrofluometrically titrated with Cu(II) at different temperatures. Temperature points were selected below, at and above the LCST as shown by the two curves in Figure 4-8. The fluorescence titration spectra at 35 °C are shown in Figure 4-9 with the titration curves extracted in Figure 4-10. The dansyl peak shifted to lower wavelength with enhanced intensity as Cu(II) was added. This is due to the neutralization of charges on the IDA ligand sites leading to the collapse of the polymer coils. However when the Cu(II) concentration reaches $5 \times 10^{-4}$ M, significant scattering background and additional peaks appear on the spectrum. This scattering background
may be due to the globule coagulation caused by excess Cu(II) crosslinking among the globules. The fluorescence peak splitting is thought to be caused by the scattering from the aggregated globules. On the other hand, the spectra in Figure 4-7 show no significant scattering background up to 50 °C. This once again confirms the charge stabilization on the globule surface. Cu(II) binds to the ligand sites and changes the conformation of the charge stabilized coils at concentrations lower than $5 \times 10^{-4}$ M. Above this threshold, Cu(II) is assumed to act as crosslinks among the coils similar to the role of Ca(II) in another light scattering study.\textsuperscript{114}

\textbf{Figure 4-9} Cu(II) titration of Sample 4-4 at 35 °C
Figure 4-10 Cu(II) titration curves of Sample 4-4 based on intensity ratio and peak wavelength at 35 °C.

The apparent formation constant $K$ can be obtained from the ratiometric titration curve by using the intensity ratio at zero Cu(II) concentration (not shown in Figure 4-10) and the final intensity ratio. The log $K$ of the indicator-Cu(II) complex at 35 °C is determined to be 4.3 corresponding to the intensity ratio value close to the midpoint of the slope in Figure 4-10. This value lies in between the log $K$ of IDA-Cu(II) complex and that of glutaric acid-Cu(II) complex confirming the weakened chelating ability of NAIDA. The indicator is useful in the pCu range between 3.3 and 5.3.

The same titration was done at 34 °C and the fluorescence spectra and titration curves are shown in Figure 4-11 and Figure 4-12 respectively. However, the intensity ratio curve shifted to the right yielding a log $K$ of 3.2. The lower log $K$ at 34 °C is not unexpected.
Figure 4-11 Cu(II) titration of Sample 4-4 at 34 °C

Figure 4-12 Cu(II) titration curves of Sample 4-4 based on intensity ratio and peak wavelength at 34 °C
Figure 4-13 (a) Polymer segment under microscopic force balance (b) Mechanistic view of the dansyl system in response to metal ions

As shown in Figure 4-13 (a), a polymer segment is expected to experience two opposing forces—the collapsing force, \( f_o \), and the expanding force including the hydrophilic force \( f_i \) and the electrostatic repulsion force \( f_r \) originating from the charged ligand sites. Without any disturbance, the polymer chains are considered to be in a metastable "stressed" coil state when the three forces are balanced as expressed in equation (4-1). Any change in the delicate force balance results in a different polymer conformation where a new metastable state will be reached. The addition of metal ions weakens the electrostatic repulsion force by neutralizing the negative charges and therefore the metastable coils undergo the coil-to-globule transition followed by globule aggregation. This metal ion sensing mechanism is illustrated in Figure 4-13 (b) where negatively charged ligands are used.

\[
f_o = f_i + f_r
\]  

(4-1)
Based on this concept, the polymer coils at 35 °C are more “stressed” and therefore more compact than those at 34 °C. At 35 °C, the $f_i$ in equation (4-1) is weakened as a result of two effects. First, the more compact coils contain less water. Second, the hydrogen bonding is slightly weaker at 35 °C. Meanwhile, the $f_o$ and $f_r$ are strengthened due to the compactness. Consequently, the $f_r$ becomes the major expanding force that counterbalances the increasing collapsing force $f_o$. It therefore takes smaller amount of Cu(II) to generate force imbalance and chain conformational change at 35 °C.

This explanation holds at lower and higher temperatures. At 33 °C and below, the titration spectra showed almost no change up to $5 \times 10^{-4}$ M Cu(II). Only 1 °C lower, the polymer coils are larger and the $f_i$ is the major expanding force. A significant decrease in $f_r$ as a result of high Cu(II) concentration is still not enough to generate force imbalance. Conversely, the log K's at 36 °C and 37 °C were determined to be higher—4.6 and 5.0 respectively. (Figure 4-14 and Figure 4-15) However, the polymer coils were already very compact without Cu(II) at these temperatures leaving little space for more compression. The insignificant peak shift and small intensity ratio change make the measurement susceptible to instrument noise and scattering background artifact.

Therefore, the indicator functions best near the temperature at which the intensity ratio slope begins to form in Figure 4-8. The polymer coils are at a critical condition and ready to undergo phase transition.
Figure 4-14 Cu(II) titration curves of Sample 4-4 based on intensity ratio and peak wavelength at 36 °C

Figure 4-15 Cu(II) titration curves of Sample 4-4 based on intensity ratio and peak wavelength at 37 °C
4.4.4 Selectivity, stability, response time and reversibility

By using an IDA ligand which does not selectively bind Cu(II), it is expected that the indicator will respond to other metal ions as well. For instance, the indicator shows a similar response to Zn(II) and Pb(II) as shown in Figure 4-16 and Figure 4-17 respectively. The affinity for these metal ions is slightly different from that for Cu(II).

The fluorescence signal from the indicator is stable before and after the phase transition, which is partly due to the low concentration of indicator used in the cuvette. Measurements are reproducible after storing the indicator solution for several months. The response time is usually less than 5 min after the addition of the metal ions. This response time is acceptable for practical applications.

To ensure no irreversible Cu(II) catalyzed reaction takes place during the measurements, the Cu(II)-indicator complex was pulled apart by adding excess EDTA, which is a much stronger chelating reagent. Figure 4-18 shows this effect on the spectra. At 35 °C, the addition of $3 \times 10^{-4} M$ Cu(II) induced a peak shift and intensity increase. Then excess EDTA solution was added and the emission peak shifted back to the starting position. The SOS confirms that the polymer chains became extended again when Cu(II) was extracted from the polymer by EDTA. Therefore, the observed effect with Cu(II) was due to Cu(II) complexation instead of Cu(II) catalyzed hydrolysis.
Figure 4-16 Zn(II) titration curves of Sample 4-4 based on intensity ratio and peak wavelength at 35 °C

Figure 4-17 Pb(II) titration curves of Sample 4-4 based on intensity ratio and peak wavelength at 35 °C
4.4.5 Considerations of polymer synthetic conditions

All polymers in this dissertation research were synthesized by random free radical polymerization, which generally has poor control over the molecular weight and polymer composition. Table 4-1 summarizes the effect of formulation and reaction conditions on the rate of polymerization and the molecular weight of the final polymer. The + sign shows a positive correlation and the – sign means a negative correlation. During the synthesis, the effects of these conditions were considered in order to produce polymers with high molecular weight. High molecular weight was thought to be conducive to the effective separation of the ligands and fluorophores on the same chain.

Figure 4-18 Reversibility of the Sample 4-4 response proven by the addition of excess EDTA at 35 °C
Table 4-1 Effect of formulations and reaction conditions on the rate of polymerization and the molecular weight

<table>
<thead>
<tr>
<th>Correlation</th>
<th>Monomer concentration</th>
<th>Initiator concentration</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate of polymerization</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Since the polymer indicators are copolymers of NIPAM with APMA and ligand monomer, the composition and sequence distribution of the copolymer are of great importance. When the comonomers have very different reactivity ratios, the one with higher reactivity ratio is preferentially incorporated in the final polymer. Take the copolymerization between NIPAM (monomer 1 or $M_1$) and MAA (monomer 2 or $M_2$) for example. The reactivity ratios for $M_1$ and $M_2$ were reported to be 10.2±1.4 and 0.01±0.03 respectively. For a feed ratio of MAA to NIPAM=5:100, the mole fraction of NIPAM ($F_1$) in the final copolymer is calculated to be 0.9951 using equation (4-2).

$$F_1 = \frac{r_1 f_1^2 + f_1 f_2}{r_1 f_1^2 + 2 f_1 f_2 + r_2 f_2^2} \quad (4-2)$$

where $f_1$ and $f_2$ are the mole fractions of monomer $M_1$ and $M_2$ in the feed while $r_1$ and $r_2$ are the reactivity ratios.

In the current study, the comonomers used in the synthesis were either acrylamide or methacrylamide. By using monomers with similar structures, similar reactivity ratios are expected and consequently the copolymer composition is expected to be close to the monomer composition in the feed.

Another important factor, the sequence-length distribution, can be calculated by using equation (4-3) and (4-4). Using the same example, the mole fraction ($N_{MAA}$)$_x$ of forming MAA sequence of length $x$ is calculated as follows: \(^{116}\)
\[
(N_2)_x = (p_{22})^{(x-1)} p_{21}
\]  
(4-3)

\[
P_{21} = \frac{[M_1]}{r_2[M_2] + [M_1]}
\]  
(4-4)

where \(p_{21}\) and \(p_{22}\) are the probabilities of forming a M2M1 and a M2M2 dyad respectively.

The mole fraction of segregated MAA (single M2) is equal to \(p_{21}\) because \(x\) equals 1 in equation (4-3). The \((N_2)_i\) is calculated to be 0.9995, which means the possibility of forming MAA aggregates is almost zero. Similarly, in our system, it is very unlikely to form APMA aggregates or ligand monomer aggregates because their mole fractions in the feed are very low.

Generally, the reactivity ratios are independent of the reaction medium in the radical copolymerization. However, for an acidic or basic monomer, the reactivity ratio is dependent on the pH since the identity of the monomer changes with pH. Take for example the copolymerization of acrylic acid (M1) and acrylamide (M2). At low pH, \(r_1\) is larger than \(r_2\); however the reverse is true at high pH. Acrylic acid shows a decreased tendency to homopropagate and add to propagating centers with electron-rich substituents such as the amide group when the acid groups are deprotonated.\(^{116}\)

The dependence of reactivity ratio on the charge state of the monomer could explain the difference between indicators synthesized using the ester and acid forms of NAIDA. Polymer indicators synthesized in acetonitrile used NAIDA esters, which need to be hydrolyzed. The hydrolysis of the NAIDA ester on the polymer was monitored by \(^1\)H NMR. The disappearance of the methyl peak signal from the tert-butyl group confirmed the effectiveness of the hydrolysis. The number-average molecular weight obtained from the GPC was \(1.7 \times 10^5\) and the dispersity index was 2.2 for the purified and dialyzed polymer sample.

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Contrary to the polymers synthesized using the acid form of NAIDA, this type of polymers showed a completely different behavior towards Cu(II). The fluorescence intensity showed a decrease with the addition of Cu(II). Cu(II) titration spectra of Sample 4-2 are presented in Figure 4-19. This suggests that the effective separation of the NAIDA and dansyl groups was not achieved in this system. However, the dansyl peak still shifted to lower wavelength as expected. Even though Cu(II) quenches the fluorescence, the dansyl fluorophores still experienced an environment change. The intensity ratio change and peak shift are very small in this case as shown in Figure 4-20.

**Figure 4-19** Cu(II) titration of Sample 4-2 at 33 °C
Figure 4-20 Cu(II) titration curves of Sample 4-2 based on intensity ratio and peak wavelength at 33 °C

4.5 Conclusion

Based on the understanding of the phase transition mechanism of PNIPAM, a dansyl-based metal ion indicator has been developed. The thermal phase transition did cause a change in the polarity of the dansyl microenvironment as proven by the emission enhancement and peak shift. Two calibration methods based on the intensity ratio and the peak wavelength were generated. Both methods are independent on the indicator concentration and thus suitable for use under conditions where the indicator concentration cannot be obtained. Formulation optimization took the following factors into consideration—polarity of the synthetic medium, hydrophobicity of the monomers and the percentage of dansyl labeling. A combination of all these factors affect the relative positioning of the ligand and fluorophore, the percentage of incorporation into the polymer as well as their conformation on the polymer chains. It was found that
polymers synthesized using the disodium salt form of the ligand in DI water with 0.1% dansyl labeling realized effective ligand-fluorophore separation and thus showed a enhanced fluorescence response towards Cu(II). The response was proven to be temperature-dependent. The optimum temperature was determined to be 35 °C, at which the log K of the indicator-Cu(II) complex was found to be 4.3.

In conclusion, we developed a ratiometric fluorescent indicator for Cu(II) whose affinity is temperature-dependent. It circumvented the quenching effect of Cu(II) and achieved the goal of a ratiometric response.
CHAPTER 5

A RATIOMETRIC FLUORESCENT INDICATOR FOR METAL IONS BASED ON FLUORESCENCE RESONANCE ENERGY TRANSFER

5.1 Introduction

FRET is a widely used fluorescence technique in the biological sciences. For example, FRET has been used to study protein folding on the single-molecule level.\(^{117}\) It is generally applied as a sensitive tool to study the dimension alteration in a dynamic process. In addition to fundamental research, sensing applications of FRET keep emerging in the literature. For instance, a highly sensitive and specific graphene FRET aptasensor for thrombin has been developed recently.\(^{118}\) In the area of ion sensing using FRET, which is more related to this research, the analyte ions range from hydrogen ion,\(^{119}\) Fe(III),\(^{120}\) Cu(II),\(^{121}\) to Cr(III)\(^{122}\) and Cu(I)\(^{123}\) in a living cell. Most of these studies involve a conformational change in response to the analyte.

The most cited advantage of using FRET for sensing is its ratiometric character. Increased energy transfer from the donor (D) to the acceptor (A) results in a higher intensity ratio A/D. This ratio change should be much larger than that stemming from a single peak shift as in the case of dansyl system in Chapter 4.
The current study was inspired by one of the early studies discussing the sensing application of the PNIPAM conformational change.\textsuperscript{124} By varying the identity and quantity of the functional groups, the PNIPAM was demonstrated to response to H\textsuperscript{+}, K\textsuperscript{+} and SO\textsubscript{4}\textsuperscript{2-}. The fluorescence transduction used in this study was based on a polarity-sensitive fluorophore benzofurazan, which is similar to the dansyl system in Chapter 4. However, the signal readout was based on absolute intensity, which is susceptible to artifacts such as concentration changes and matrix effects. It was also not aimed at sensing quenching metal ion. Our goal is to develop a ratiometric FRET indicator that shows an enhanced response to Cu(II).

In this chapter, several donor-acceptor pairs have been evaluated for labeling PNIPAM to follow its conformational change. The Alexa Fluor pair was chosen for their distinct advantages over other pairs to develop a FRET-based indicator for Cu(II). Conditions such as the type and percentage of the IDA ligand, the labeling ratio of A/D and labeling approaches were optimized. Finally, a brief mechanistic study of ionization on the phase transition of PNIPAM was conducted.

5.2 Early FRET Systems

Several different approaches to label PNIPAM with two fluorophores have been attempted. The initial plan was to use two fluorophore monomers which are commercially available. The two fluorophore monomers would then be incorporated into the polymer in a batch random copolymerization. Two FRET donor-acceptor pairs in their monomer form were studied so far. The first pair—2-naphthyl methacrylate (D) and 9-vinylanthracene (A) pair is based on naphthalene-anthracene FRET pair, which has a
 Förster distance of ~16 Å.\textsuperscript{125} This short Förster distance poses several limitations. For example, the polymer has to collapse to an extremely tightly packed structure for the donor-to-acceptor distance to be close enough for FRET. Alternatively, the polymer has to be labeled with high percentage of fluorophores, which may induce self-quenching. Another disadvantage with this pair is the low excitation wavelength (~285 nm). \textit{In vivo} application of the indicator would not be possible because of background fluorescence at this excitation wavelength. A FRET system using this pair has been studied by our group member John Osambo.\textsuperscript{126}

The second pair—fluorescein o-acrylate (D) and PolyFluor 570 (A)—overcomes some of the limitations with the first pair. PolyFluor 570, also known as methacryloxyethyl thiocarbonyl rhodamine B, acts as an efficient acceptor for fluorescein emission. The Förster distance of fluorescein-rhodamine B pair was found to be 65.5 Å,\textsuperscript{127} which is much larger than that of naphthalene-anthracene pair. As a result, a high degree of polymer chain contraction or high percentage of labeling is not required for FRET to occur. Background fluorescence can be minimized as well because of the longer excitation wavelength.

The initial focus of the research was on crosslinked polymer systems. A simple experiment (Sample 5-1) was carried out to prove that FRET takes place on the crosslinked polymer labeled with the fluorescein-rhodamine pair, designated as F and R respectively. After copolymerizing NIPAM with fluorescein o-acrylate and PolyFluor 570, the crude product was dialyzed in DI water. Fluorescence spectra were collected on both the solutions inside and outside the dialysis tubing. The solutes in the outside solution are supposed to be mostly free fluorophores and small polymers with molecular
weight less than the MWCO 12,000. Under this condition, no close contact between the F and R results in almost no FRET. As shown in Figure 5-1, when excited at the donor wavelength 450 nm, only F emission and very weak R emission were observed as shown by the tailing of the profile. The weak R emission is not due to the absence of R but the lack of FRET. The presence of the R was verified by exciting at the acceptor wavelength 550 nm. (Figure 5-2)

![Fluorescence spectrum of free D and A dialyzed from Sample 5-1 solution when excited at the D wavelength](image)

**Figure 5-1** Fluorescence spectrum of free D and A dialyzed from Sample 5-1 solution when excited at the D wavelength

On the contrary, the fluorescence spectrum of the inside solution, supposedly the PNIPAM tagged with F and R, showed a significant R emission peak when excited at the donor wavelength 450 nm in DI water at room temperature. (Figure 5-3) This proves that FRET only takes place on the polymer when the F and R are closely positioned. In their free form, there is no energy transfer.
Figure 5-2 Fluorescence spectrum of free D and A dialyzed from Sample 5-1 solution when excited at the A wavelength

Figure 5-3 Fluorescence spectrum of Sample 5-1 when excited at D wavelength
As the temperature was increased, the intensity ratio of the emission at 576 nm to 525 nm was monitored kinetically using the kinetic mode at pH 6. The intensity ratio vs. temperature was plotted in Figure 5-4.

![Figure 5-4 Intensity ratio as a function of temperature at pH 6 for Sample 5-1](image)

The increasing ratio of R emission to F emission indicates the presence of FRET as the temperature-induced shrinking of the polymer takes place. However, a decrease in the ratio was seen when the temperature was raised to 45 °C from 40 °C. This may be due to preferential thermal quenching on the R. It could also be the result of precipitation of the shrunken polymer since the polymer was crosslinked. The results pointed to a disadvantage of crosslinking in this system. With uncrosslinked polymer, precipitation would not be very significant.

The drawback of this FRET pair used on a crosslinked polymer is its instability when exposed to basic pH buffers. Figure 5-5 shows an example of the observation in pH
8 phosphate buffer. With no external disturbance, the fluorescein peak increased with time. Fluorescein is known to be highly sensitive to pH. This behavior may be related to the slow enclosure of fluorescein into the polymer as the polymer shrinks or possibly slow hydrolysis of the acrylate bond.

![Fluorescence spectra of Sample 5-1 obtained at 10-min intervals in pH 8 buffer](image)

**Figure 5-5** Fluorescence spectra of Sample 5-1 obtained at 10-min intervals in pH 8 buffer

To overcome the aforementioned disadvantages, an uncrosslinked system was employed. An additional modification to the previous system is that the fluorescein containing strands and the rhodamine containing strands were synthesized separately before mixing them together. In order to introduce Cu(II) binding sites, 1-vinylimidazole, known to form stable complex with Cu(II), was added in the formulation of Sample 5-2. Figure 5-6 shows the fluorescence spectra of Sample 5-2 at low and high temperatures. Significant R emission can be seen at 45 °C as a result of FRET when PNIPAM collapses.
It can also be observed that the F emission is slightly influenced by the environment since the maximum wavelength shifts 2 nm to lower wavelength when the polymer collapses.

![Fluorescence spectra for Sample 5-2 at 25 °C and 42 °C](image)

**Figure 5-6** Fluorescence spectra for Sample 5-2 at 25 °C and 42 °C

Figure 5-7 illustrates the kinetic scan of the F and R emissions when the polymer was added to DI water and the temperature was ramped up followed by the addition of Cu(II). Using the math function of the program, the intensity ratio of the R/F was calculated and plotted in Figure 5-8. The y-axis is the intensity ratio, however, the program shows it as Intensity (a.u.).

Before the polymer coil aggregation takes place, *i.e.* from 25 °C to 29 °C, both the F and R emissions decreased as a result of thermal quenching. The intensity ratio decreases indicating that the thermal quenching coefficient for the R is larger. Once the PNIPAM reaches the phase transition temperature, the polymer collapses resulting in
FRET. At 45 °C, the decrease in the intensity ratio upon adding Cu(II) may be due to its quenching effect.

**Figure 5-7** Kinetic scan of the D & A intensities for Sample 5-2 upon temperature increase

**Figure 5-8** Intensity ratio change of Sample 5-2 with temperature and Cu(II)
Further experiment was performed to test the stability and reversibility of this system. Temperature was oscillated between 25 °C and 42 °C twice. The F and R emissions were kinetically monitored as shown in Figure 5-9. The R signal is quite reversible but the F response is slow and acting unpredictably. However, the final intensity ratio showed good reversibility as shown in Figure 5-10. The equilibration time was shown to be as long as 40 min. This behavior may be related to slow conformational rearrangements with regard to the fluorescein on the polymer. Also by synthesizing two batches of polymers, the reproducibility is an issue. As a result, the F-R pair was not separately labeled on different polymer strands in the following study.

Figure 5-9 Kinetic scan of the D & A intensities for Sample 5-2 upon temperature oscillation
5.3 Alexa Fluor FRET System

Pre-synthesis fluorophore tagging was used for the previous FRET pairs—the donor and acceptor fluorophores were derivatized into monomers and copolymerized with NIPAM. However, the derivatization requires extensive synthesis and it is impractical to derivatize the expensive fluorescent dyes. There are only a few commercially available fluorophore monomers. On the contrary, post-synthesis tagging can be easily done using the commercial protein/nucleic acid labeling packages. Polymer synthesis introduces functionalities such as amines or thiols to which the fluorescent dyes can be attached. By avoiding synthesizing two batches of polymer, better reproducibility can be achieved. Most of the work was concentrated on singly labeled (SL) system—polymer strands from the same batch of polymer labeled separately with the donor fluorophore and the acceptor fluorophore. This situation is similar to Sample 5-2 except that the labeling was done after polymer synthesis. Experiments with the doubly labeled...
(DL) system—polymer strands labeled with the donor and acceptor at the same time—were carried out briefly and compared. This system is analogous to Sample 5-1 except that it uses post-synthesis labeling.

As discussed in the introduction, the Alexa 555 and 647 were selected as the donor and acceptor. PNIPAM with amine sites introduced by APMA was separately labeled with Alexa 555 and Alexa 647. Figure 5-11 shows the thermal response of SL equally mixed strands. (Sample 5-3) There was a very weak Alexa 647 peak when PNIPAM chains are extended at 25 °C. As the temperature was increased to 45 °C, PNIPAM chains undergo interchain association leading to shorten distance between Alexa 555 and 647. A prominent Alexa 647 peak was seen due to FRET. The results validate that this FRET pair is suitable for labeling PNIPAM.

![Figure 5-11 Fluorescence spectra for Sample 5-3 at 25 °C and 40 °C](image-url)
5.4 Metal Ion Indicator Based on Alexa Fluor FRET System

We studied two IDA derivatives, St-IDA and AP-IDA, as the chelator groups on the PNIPAM chains. They were synthesized by Nick Bencivenga and their structures are shown in Figure 3-1. In both structures, the amine site of the IDA was connected to a benzene ring, which exerts a mesomeric effect on the lone pair of the nitrogen. Consequently, the log stability constant (log K) of the N-phenyliminodiacetic acid (phenyl-IDA)-Cu(II) complex is 6.62, lower than that of the IDA-Cu(II) complex (10.57). The difference between the two ligand monomers lies in the linking group. The benzene ring in St-IDA is connected to the double bond directly while that in AP-IDA is connected through an amide linker to double bond. Since the reactivity ratios of the monomers determine the final composition of the copolymer and the sequence length distribution, AP-IDA is supposed to yield more homogeneous chains with predictable compositions. However, both monomers were used in the study.

The concentration of the indicator used in the fluorescence study was in the regime of extremely dilute solution, where the concentration is much smaller than the coil overlap concentration c*. Therefore, each polymer chain experiences intrachain interaction before interchain interference.

Figure 5-12 and Figure 5-13 show the A/D intensity ratio response of Sample 5-4 with and without the analyte metal ion. Sample 5-4 uses 2% St-IDA disodium salt. The intensity ratio was monitored as the temperature was increased stepwise from 25 °C to 45 °C. Two conditions were analyzed—without Cu(II) and with 1.0×10⁻⁴ M Cu(II) as in Figure 5-12, without Zn(II) and with 1.7×10⁻⁴ M Zn(II) as shown in Figure 5-13.
Figure 5-12 Intensity ratio as a function of temperature for Sample 5-4 with & without Cu(II)

Figure 5-13 Intensity ratio as a function of temperature for Sample 5-4 with & without Zn(II)
The first observation is the slight increase of the LCST of the polymer to 35 °C as compared to pure PNIPAM. This suggests that under the experimental conditions, the partially deprotonated St-IDA introduces negative charges on the polymer deferring the phase transition to higher temperature. Supposedly, the presence of metal ions such as Cu(II) or Zn(II) should neutralize the negative charges on St-IDA and therefore the polymer can collapse to a greater extent. Consequently, the intensity ratio should be higher at high temperature. This is the case with Zn(II). However, the opposite was observed with Cu(II). In both cases, the SOS signal increased in the presence of the metal ion (not shown) suggesting that the metal ions can bind to the St-IDA causing the polymer to collapse. The addition of Cu(II) at high temperatures decreased the absolute intensities of both Alexa 555 and Alexa 647. Surprisingly, minimal quenching was seen when Cu(II) was added to the polymer solution at low temperature.

The quenching mechanism is proposed as follows. First, the Cu(II) quenching is through energy transfer to the Cu-IDA complex, not by Cu(II) itself. This explains the lack of Cu(II) quenching at low temperature when the Alexa fluorophores and the ligands are far apart from each other. Second, the quenching mechanism follows a cascade FRET process as suggested in a paper.\(^{121}\) Basically, the resonance energy transfers from Alexa 555 to Alexa 647 and finally to Cu(II)-IDA complex.
Figure 5-14 UV-Vis absorption of 0.1 M Cu(II) and Cu(II)-IDA complex

Figure 5-14 shows the UV-Vis absorption spectra of Cu(II) and Cu(II)-IDA complex at 0.1 M. When the Cu(II) complex is formed, the maximum absorption undergoes a blue shift, which mainly covers the Alexa 647 emission profile centered around 670 nm. This suggests the possibility of the energy transfer from Alexa 647 to the Cu(II) complex. Another observation is the weak Cu(II) quenching to the Alexa 647 strands alone. In other words, the quenching only occurs in the cascade FRET process. In almost all the spectra showing FRET at low and high temperatures, the Alexa 647 emission peak shifted to higher wavelength, e.g. in Figure 5-11. It was thought to be due to the environmental sensitivity of the Alexa 647. However, no peak shift was observed with the temperature increase when Alexa 647 strands were tested alone whether excited at 525 nm or 600 nm. (Figure 5-15 and Figure 5-16) The reason for this phenomenon is not clear. One possible explanation might be that the resonance energy transfer from Alexa 555 excites Alexa 647 to a slight different vibrational energy level than that associated with direct excitation. This explanation also validates the weak Cu(II)
quenching to the Alexa 647 strands alone since the energy level associated with direct excitation does not match the energy gap for the Cu(II) complex.

As discussed before, the positioning of the AP-IDA with regard to the fluorophores may be important in that the quenching depends on the distance between the two. In this sense, it would be very difficult to circumvent the problem of Cu(II) quenching in the IDA system because the polymer responds to Cu(II) through the conformational change from expanded state to collapsed state, which inevitably leads to close positioning of the Cu(II) complex and the fluorophores. Another key point is that the distance between the AP-IDA and the fluorophores may influence the extent of quenching the fluorophores experience as a result of the binding-induced conformational change.

Due to the reactivity difference of St-IDA to NIPAM, the following study uses AP-IDA instead of St-IDA. Sample 5-5 has similar formulation to Sample 5-4 except that the ligand is AP-IDA. The intensity ratio response of Sample 5-5 is shown in Figure 5-17. Again, the presence of $6.7 \times 10^{-5}$ M Cu(II) lead to a decreased intensity ratio, which is the opposite of our expectation. Meanwhile, the SOS signal was not affected by Cu(II) as shown in Figure 5-18. This again suggests a Cu(II) quenching behavior taking place at high temperatures.
Figure 5-15 Fluorescence spectra of the Alexa 647 strands alone at 25 °C and 45 °C when excited at 525 nm

Figure 5-16 Fluorescence spectra of the Alexa 647 strands alone at 25 °C and 45 °C when excited at 600 nm
Figure 5-17 Intensity ratio as a function of temperature for Sample 5-5 with & without Cu(II)

Figure 5-18 SOS as a function of temperature for Sample 5-5 with & without Cu(II)
In Sample 5-6, AP-IDA ethyl ester was used instead of the sodium salt form as a means to vary the polarity of the monomers and synthetic medium. The intensity ratio and SOS responses are shown in Figure 5-19 and Figure 5-20. Measurements were made at two pH buffers for comparison.

![Graph showing intensity ratio as a function of temperature for Sample 5-6 with and without Cu(II) at pH 5.5 and pH 6.0.](image)

**Figure 5-19** Intensity ratio as a function of temperature for Sample 5-6 with & without Cu(II) at pH 5.5 and pH 6.0

Similar response was obtained under two pH buffer conditions—pH 5.5 and pH 6.0 MES buffers. The general trend is that the presence of Cu(II) increases the SOS signal at high temperatures but decreases the intensity ratio. The increase in SOS is more significant in Sample 5-6 than those in Sample 5-4 and Sample 5-5 suggesting the Cu(II) binding leads to a larger degree of polymer chain aggregation in this batch of polymer. As a result, the cascade FRET quenching effect is even more significant as shown by the low intensity ratio at high temperatures when Cu(II) is present. Comparing the intensity ratio and second order scattering values at pH 5.5 and 6.0 when Cu(II) was absent, both
the values at pH 5.5 are slightly higher. This is because slightly more IDA groups are
protonated at pH 5.5 resulting in less charge repulsion. The pKa of N-phenyliminodiacetic acid (phenyl IDA) was found to be 5.1. Polymers at pH 5.5 can therefore collapse to a greater extent.

![Figure 5-20 SOS as a function of temperature for Sample 5-6 with & without Cu(II) at pH 5.5 and pH 6.0](image)

The SOS curves show an interesting behavior. The globule aggregation takes
place at a lower temperature when Cu(II) is present. This indicates the charge
stabilization effect of the globules by the charges on the IDA ligands is lost when Cu(II)
is present. This once again confirms the chelating ability of the AP-IDA ligand to Cu(II).

The indicator response to Zn(II) is within our expectation. Figure 5-21 and Figure 5-22 show the intensity ratio and SOS signals upon the addition of Zn(II) at pH 6. The increase in both the intensity ratio and second order scattering suggests the effectiveness
of the indicator for Zn(II). However, the increase in the intensity ratio is weak. When Zn(II) concentration is half of the Cu(II) concentration used in Figure 5-20, the increase in the SOS was not very significant. It is when the Zn(II) concentration reaches 5 times the concentration of Cu(II) that the SOS signal increases substantially. This may be due to a much lower stability constant of Zn(II)-IDA complex as compared to that of Cu(II)-IDA complex. The log $K$ of phenyl IDA-Cu(II) is 6.62 while that of phenyl IDA-Zn(II) is 3.36\textsuperscript{110}.

![Figure 5-21 Kinetic scan of intensity ratio with the addition of Zn(II) for Sample 5-6](image-url)
5.5 Optimization of FRET Intensity Ratio Response

In order to optimize the intensity ratio response, the percentage of AP-IDA was varied in the polymer. Based on the response from 2% AP-IDA in Sample 5-6, the percentage was increased in Sample 5-7 and Sample 5-8 to 6% and 4% respectively while the percentage was decreased to 0.5% in Sample 5-9. Sample 5-7 and Sample 5-8 have similar responses to increasing temperature and Cu(II). Figure 5-23 and Figure 5-24 show the intensity ratio and SOS signals of Sample 5-23 at pH 6 respectively. The increase in the SOS upon addition of Cu(II) confirms binding. However, the intensity ratio shows no significant change. With 6% or 4% charges on the polymer coils, charge stabilized individual globules form at high temperatures. This prevents interchain association, hence the very weak thermal response when the temperature was increased from 25 °C to 45 °C. The addition of Cu(II) neutralizes the surface charges and the collapsed donor or
acceptor globules can aggregate again. This explains the increased scattering signal. However, since the collapsed globules are more rigid than polymer coils, the distance between the donor and acceptor globules are still too large. This may be the reason why the intensity ratio is minimally affected.

The indicator response is greatly improved when there is only a small percentage of AP-IDA on the polymer as in Sample 5-9. Figure 5-25 show the kinetic data of the donor emission, acceptor emission and the SOS during temperature ramps without and with Cu(II) for Sample 5-9 at pH 6. The intensity ratio and SOS were plotted as a function of temperature in Figure 5-26 and Figure 5-27 respectively.

![Figure 5-23 Kinetic scan of intensity ratio with the addition of Cu(II) for Sample 5-7](image)
Figure 5-24 Kinetic scan of SOS, D and A with the addition of Cu(II) for Sample 5-7

Figure 5-25 Kinetic scan of SOS, D and A with temperature and the addition of Cu(II) for Sample 5-9
Figure 5-26 Intensity ratio as a function of temperature for Sample 5-9 with & without Cu(II) at pH 6

Figure 5-27 SOS as a function of temperature for Sample 5-9 with & without Cu(II) at pH 6

The intensity ratio increased by 6 times when the temperature was raised from 25 °C to 42 °C, which is much larger than the previous samples. The beginning of the phase
transition is slightly above 32 °C suggesting that a small percentage of charges does not significantly inhibit the phase transition. For comparison, the incipient phase transition temperature for Sample 5-6 (2% AP-IDA) is about 34 °C and that for Sample 5-7 (6% AP-IDA) is above 45 °C. Therefore the aggregates formed at high temperatures contain both donor and acceptor strands and significant energy transfer can take place. As seen in Figure 5-25, the scattering signal undergoes a sharp increase followed by a gradual decrease at the point of phase transition. This is due to the transition from “liquid-like” mesoglobules to “solid-like” mesoglobules as suggested in a similar FRET study of PNIPAM phase transition.86

The indicator response to Cu(II) is consistent with the results in the previous samples. A decreased intensity ratio and increased scattering are seen when Cu(II) is present. Even though AP-IDA binds Cu(II) causing polymer chain aggregation, the postulated cascade FRET process results in a lower intensity ratio.

Using a non-quenching metal ion such as Zn(II), the validity of the intensity ratio readout can be tested. The intensity ratio and SOS signal as a function of temperature without and with Zn(II) are shown in Figure 5-28 and Figure 5-29 respectively. Because the stability constant for Cu(II) is 1000 times higher than that for Zn(II), Zn(II) concentration used in the study is 10 times higher than that of Cu(II). The intensity ratio increased slightly when Zn(II) is present, which agrees with our expectation. Meanwhile, the second order scattering does not show much difference. The slight decrease may be due to minor sampling error or slight precipitation of the aggregated polymer chains at high temperature. Generally, the response to Zn(II) is weak as a result of the small stability constant.
Figure 5-28 Intensity ratio as a function of temperature for Sample 5-9 with & without Zn(II) at pH 6

Figure 5-29 SOS as a function of temperature for Sample 5-9 with & without Zn(II) at pH 6
The Cu(II) quenching effect on Sample 5-9 donor strands and acceptor strands was tested separately by comparing the intensities with and without Cu(II). The results are summarized in Table 5-1.

Table 5-1 Cu(II) quenching effect on separate donor or acceptor strands for Sample 5-9

<table>
<thead>
<tr>
<th>Conditions</th>
<th>25°C no Cu(II)</th>
<th>25 °C 3.3E-5 M Cu(II)</th>
<th>45 °C no Cu(II)</th>
<th>45 °C 3.3E-5 M Cu(II)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Donor strand</strong></td>
<td>413.99</td>
<td>399.80</td>
<td>350.04</td>
<td>273.51</td>
</tr>
<tr>
<td><strong>Acceptor strand</strong></td>
<td>287.04</td>
<td>267.46</td>
<td>137.89</td>
<td>107.96</td>
</tr>
<tr>
<td><strong>A/D ratio</strong></td>
<td>0.69</td>
<td>0.67</td>
<td>0.39</td>
<td>0.40</td>
</tr>
</tbody>
</table>

* Numbers are in absolute units (a.u.) given by the fluorescence spectrophotometer

If measured separately, Cu(II) quenches the fluorescence of the two strands to the same extent as shown by the close intensity ratio values. As suggested before, the quenching mechanism is a cascade FRET process and possibly excites the donor to a different energy state as shown by the acceptor peak shift.

5.6 Comparative Study of the SL and DL Systems

A new fluorophore labeling method of reacting the polymer with both Alexa 555 and 647 at the same time was suggested towards the end of the study. This method produces polymers with donor and acceptor labeled on the same strand, doubly labeled (DL) polymer. The fluorescence spectra of the SL and DL systems are compared and the intensity ratio readout is optimized. Figure 5-30 shows the thermal response of a mixture of 10 μL Alexa 555 labeled strands and 10 μL Alexa 647 labeled strands (10+10 μL)
from Sample 5-10. The molar absorptivity values for Alexa 555 and 647 are $1.5 \times 10^5$ and $2.39 \times 10^5$ M$^{-1}$·cm$^{-1}$ respectively as provided by Invitrogen. Based on the UV-Vis absorption, the concentration is estimated to be approximately $1 \times 10^{-7}$ M for both fluorophores. Concentrations of other volumes can be calculated accordingly. Figure 5-31 shows the spectra of 20 µL DL in 3 mL DI water at 25 °C and 45 °C.

![Graph showing spectra at 25°C and 45°C]

**Figure 5-30** Thermal response of 10+10 µL SL Sample 5-10

A noticeable difference is the presence of moderately strong Alexa 647 emission at 25 °C in the case of DL sample. (Figure 5-31) This suggests FRET can occur when the polymer is in its expanded state at current labeling level. It therefore can be concluded that the donor and acceptor fluorophores labeled on the same polymer chains are more closely positioned than those labeled on separate chains. Even when the SL system is completely collapsed at high temperature, the average distance between the donor and
acceptor fluorophores is still much larger than that in the DL system. The DL system, requiring less fluorophores, should also result in better sensitivity.

![Graph showing thermal response of 20 μL DL Sample 5-10](image)

**Figure 5-31** Thermal response of 20 μL DL Sample 5-10

Another advantage of the DL system is the quicker response time as shown in the kinetic scans. Figure 5-32 and 5-33 compare the kinetic data as the temperature is raised from 25 °C to 45 °C. It takes less time for the intensity ratio of the DL polymer to reach a plateau. This is easily explainable since the SL system requires interchain association, which occurs extremely slowly in dilute solution.
Figure 5-32 Kinetic scan of 10 +10 μL SL Sample 5-10 with increasing temperature

Figure 5-33 Kinetic scan of 20 μL DL Sample 5-10 with increasing temperature
The third advantage of DL system is the weak dependence of the intensity ratio change on the indicator concentration. Table 5-2 shows the relationship between the intensity ratio and the concentration. The signal becomes noisy as the concentration approaches the limit of detection for the spectrofluorometer. This may explain the decrease in the percent change at the lowest concentration in the table. By comparison, the concentration dependence of the intensity ratio change is much stronger in the case of SL system as shown in Table 5-3. Again, this is due to the requirement of interchain association in the SL system.

The intensity ratio of the SL system can be adjusted by varying the relative amount of donor strands and acceptor strands. The optimization table is shown in Table 5-4. Based on the limited formulations, larger intensity ratio changes are observed when the donor is in excess. As stated before, the average distance in the SL system at high temperature is still quite large as compared to the DL system. The more donors there are, the more chances that a donor is close to an acceptor.

<table>
<thead>
<tr>
<th>Concentration presented by volume (µL)</th>
<th>Intensity ratio at 25 °C</th>
<th>Intensity ratio at 45 °C</th>
<th>Percent change</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.31</td>
<td>0.71</td>
<td>129%</td>
</tr>
<tr>
<td>1</td>
<td>0.30</td>
<td>0.79</td>
<td>163%</td>
</tr>
<tr>
<td>20</td>
<td>0.31</td>
<td>0.84</td>
<td>170%</td>
</tr>
<tr>
<td>200</td>
<td>0.32</td>
<td>0.85</td>
<td>166%</td>
</tr>
</tbody>
</table>
Table 5-3 Intensity ratio change with concentration at equal D&A for SL Sample 5-10

<table>
<thead>
<tr>
<th>Concentration presented by volume* (µL)</th>
<th>Intensity ratio at 25°C</th>
<th>Intensity ratio at 45°C</th>
<th>Percent change</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1+0.1</td>
<td>0.10</td>
<td>0.20</td>
<td>100%</td>
</tr>
<tr>
<td>1+1</td>
<td>0.091</td>
<td>0.22</td>
<td>142%</td>
</tr>
<tr>
<td>10+10</td>
<td>0.092</td>
<td>0.25</td>
<td>172%</td>
</tr>
<tr>
<td>100+100</td>
<td>0.093</td>
<td>0.29</td>
<td>212%</td>
</tr>
</tbody>
</table>

* For 10+10, the concentration for each fluorophore is approximately $1\times10^{-7}$ M

Table 5-4 Optimization of intensity ratio change by varying the D/A for SL Sample 5-10

<table>
<thead>
<tr>
<th>Concentration presented by volume* (µL)</th>
<th>Intensity ratio at 25°C</th>
<th>Intensity ratio at 45°C</th>
<th>Percent change</th>
</tr>
</thead>
<tbody>
<tr>
<td>10+10</td>
<td>0.092</td>
<td>0.25</td>
<td>172%</td>
</tr>
<tr>
<td>10+30</td>
<td>0.200</td>
<td>0.39</td>
<td>95%</td>
</tr>
<tr>
<td>30+10</td>
<td>0.053</td>
<td>0.18</td>
<td>240%</td>
</tr>
<tr>
<td>30+5</td>
<td>0.045</td>
<td>0.13</td>
<td>189%</td>
</tr>
<tr>
<td>30+15</td>
<td>0.062</td>
<td>0.20</td>
<td>223%</td>
</tr>
</tbody>
</table>

* For 10+30, the D is 10 µL and the A is 30 µL.

5.7 Metal Ion Indicator Based on DL FRET System

Sample 5-11 has 4% APMA in the feed plus 2% AP-IDA. Since the Alexa 555 and 647 were added to the polymer solution at the same time, the percent labeling for each depends on the relative reactivity. It is assumed the Alexa fluorophores have similar reactivity towards the amine sites. In the case of 4% APMA, the percent labeling of each is assumed to be 2%. The relative amount of each calculated from the UV-Vis absorption shows a similar value for both, which confirms the assumption.

Since the indicator contains carboxylic acid groups, protonation difference by external acid or base should also result a FRET response. For instance, the addition of
acid should protonate the carboxylic groups leading to a phase transition and increased FRET ratio. The intensity ratio response of Sample 5-11 to acid at 25 °C is shown in Figure 5-34. For comparison, the intensity ratio and SOS responses of Sample 5-11 to acid at 45 °C are shown in Figure 5-35 and Figure 5-36 respectively.

**Figure 5-34** Intensity ratio change of Sample 5-11 upon addition of acid 25 °C
**Figure 5-35** Intensity ratio change of Sample 5-11 upon addition of acid 45 °C

**Figure 5-36** SOS change of Sample 5-11 upon addition of acid 45 °C
At 25 °C, the intensity ratio only increased very slightly with the addition of 50 μL 0.1 M HCl as shown in Figure 5-34. The SOS does not show much change either. However, in Figure 4-36, the intensity ratio increased dramatically at 45 °C when HCl was added. So did the SOS in Figure 4-37. At 45 °C, the polymer globules, stabilized by negative charges, cannot undergo further aggregation as shown by only slight intensity ratio and scattering increase from 25 °C to 45 °C. The addition of HCl cancels out the charge expulsion effect leading to substantial increase of both signals. On the other hand, when the globules are not in a “stressed” state, the protonation of carboxylic groups does not affect the polymer aggregation much. That is the case at 25 °C. This behavior agrees well with the temperature-dependent response of the dansyl system in Chapter 4. The polymer responds to H⁺ ions as expected.

Figure 5-37 and Figure 5-38 show the kinetic data for the intensity ratio and scattering in response to Cu(II). A significant increase in intensity ratio and scattering occurred when temperature was raised from 40 °C to 45 °C proving that the charges caused deferred phase transition by stabilizing the globules. The addition of Cu(II) results in lowered intensity ratio and increased scattering, which is the same as the previous samples. Further addition of Cu(II) lead to increased intensity and scattering. One possible explanation is that excess Cu(II) may cause interchain crosslinking, which was shown in the irreversible scattering signal when the temperature was lowered to 25 °C.
**Figure 5-37** Kinetic scan of intensity ratio upon addition of Cu(II) at 45 °C for Sample 5-11

**Figure 5-38** Kinetic scan of SOS, A & D upon addition of Cu(II) at 45 °C for Sample 5-11
The indicator response was evaluated to other metal ions including Zn(II), Ni(II), Hg(II) and Pb(II). Even though Cu(II) does not show the results as expected possibly due to quenching, other metal ions show expected response. Figure 5-39 and Figure 5-40 plot the intensity ratio and SOS versus metal ion concentration during a fluorescence titration. The intensity ratio and SOS both increase as more and more metal ions were added indicating a binding induced phase transition. All of the intensity ratio curves show a drop at high metal ion concentration. This may be due to interchain crosslinking as a result of large amount of charges. Based on the highest value and the value before the addition of metal ions, the stability constants of the complexes can be estimated from the curves. Table 5-5 compares the log K values of the indicator obtained from the titration curves and the log K values of the metal-phenyl IDA complexes given in the reference.

![Figure 5-39](image)

**Figure 5-39** Intensity ratio change as a function of metal ion concentration for Sample 5-11
Figure 5-40 SOS change as a function of metal ion concentration for Sample 5-11

The values are reasonable as compared to the database values. It is expected that the chelating ability of phenyl-IDA ligand, when connected to PNIPAM, should change slightly. Among the metal ions, Hg(II) has the highest intensity ratio increase while Pb(II) has the highest scattering increase. Also the response to Pb(II) was found to be slow compared to other ions.

Table 5-5 Log stability constants of the metal ion-indicator and metal ion-phenyl IDA

<table>
<thead>
<tr>
<th></th>
<th>Log K (M^{2+}-indicator)</th>
<th>Log K (M^{2+}-phenyl IDA)\textsuperscript{110}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn(II)</td>
<td>3.7</td>
<td>3.36</td>
</tr>
<tr>
<td>Ni(II)</td>
<td>4.0</td>
<td>3.60</td>
</tr>
<tr>
<td>Hg(II)</td>
<td>3.2</td>
<td>N/A</td>
</tr>
<tr>
<td>Pb(II)</td>
<td>3.1</td>
<td>3.49</td>
</tr>
</tbody>
</table>
5.8 Effect of the Ionic Groups on the PNIPAM Phase Transition

As shown in the previous experiments, understanding how the charge stabilizes the polymer globules is the key to interpret the fluorescence intensity ratio behavior and possibly solve the quenching problem. This part of research aims to elucidate the effect of the ionic groups on the PNIPAM phase transition. PNIPAM copolymers containing 0.5% VI (Sample 5-12), 5% VI (Sample 5-13) and 5% MAA (Sample 5-14) were synthesized under similar conditions. The temperature of the copolymers in different pH buffers was raised and the UV-Vis absorbance at equilibrium was monitored.

The copolymers were tested in pH buffers higher and lower than the pKa value. It was reported that the pKa's of the PNIPAM-VI and PNIPAM-MAA are 5.2 and 5.6 respectively. Therefore in pH 4.0 buffer, the PNIPAM-VI carries positive charges due to protonation at the imidazole sites. At pH 9.2, the polymer should carry no charge. The scenario is reversed for PNIPAM-MAA. At pH 9.2, the MAA moieties are deprotonated resulting in negative charges on the polymer while no charges are present at pH 4.0. The absorbance or indirectly the turbidity is a measurement of the size of the aggregates in solution, which is a direct result of interchain association.

Figure 5-41 shows the results of Sample 5-12 when positively charged (pH 4.0) and uncharged (pH 9.2). Without charges, larger aggregates form at high temperature due to the lack of charge repulsion. When the imidazoles are protonated, the interchain association only proceeds to the point where the surface charge density is high enough to stabilize the aggregates. Therefore, smaller aggregates with protons on the surface are formed at pH 4.0. The scenario is similar to that shown in Figure 2-5 except that the positive charges are from external protons. However, with ten times the amount of
imidazoles in Sample 5-13, the amount of charges is sufficient to stabilize a single coil thus completely prevents interchain association as suggested by the pH 4.0 trace in Figure 5-42.

![Figure 5-41 Thermal phase transition of Sample 5-12 in two pH buffers measured by UV-Vis](image)

The situation with Sample 5-14 is different. At pH 4.0, the carboxylic groups are protonated. Interchain association can take place without charges on the polymer. When the polymer is negatively charged at pH 9.2, the inception of the phase transition is deferred by about 3 °C and the absorbance at high temperatures is lower than that at pH 4.0. Figure 5-43 suggests 5% MAA is not sufficient to stabilize a single polymer chain. It should be noted that the percentage is the monomer ratio in the feed. The actual percentage in the polymer may differ. Also, the distribution of MAA and VI on the polymer chain may also contribute to the difference in Figure 5-43 and Figure 5-42. A
possible explanation for the deferred phase transition is proposed as follows. From 32 °C to 34 °C, the amount of negative charges on the deprotonated carboxylic groups is enough to stabilize a single polymer chain. As the temperature is raised to 36 °C, the stabilization is compromised by the increased contraction force. The negative charges innate to the polymer chains, as opposed to the positive charges resulted from the external protons in the case of VI, are able to rearrange so as to incorporate more polymer chains. Clearly, the phase transition behaviors of positively and negatively charged polymer chains are different.

Figure 5-42 Thermal phase transition of Sample 5-13 in two pH buffers measured by UV-Vis
Figure 5-43 Thermal phase transition of Sample 5-14 in two pH buffers measured by UV-Vis

The phase transition of the 5%VI polymer was studied at more pH buffers as shown in Figure 5-44. The higher the pH is, the fewer charges there are on the polymer. Consequently, the larger the aggregates can grow at high temperatures. Note that most of the effect occurs at only a small percentage of ionization. At pH 6.0, the absorbance value is about 35% of that at pH 9.2. By using the pKa value of 5.2, the percent ionization can be calculated to be 14%, which corresponds to 0.7 mole% on the polymer. This suggests that a large response for less than 1 mole% metal ion bound to the polymer can be realized. This is important for high sensitivity applications, e.g. the measurement of free Cu(II) in wastewater, because it minimizes the amount of Cu(II) that needs to be bound to get a signal, thereby minimizing perturbations to the free Cu(II) at equilibrium that arise due to Cu(II) binding by the indicator.
Figure 5-44 Thermal phase transition of Sample 5-13 in 4 pH buffers measured by UV-Vis

5.9 Conclusion

A FRET pair Alexa 555 (D) and Alexa 647 (A) was used to label PNIPAM, which successfully transduced the thermal phase transition. Metal ion indicator based on the same pair showed increased A/D ratio in response to metal ions such as Zn(II), Ni(II), Pb(II) and Hg(II). However, the intensity ratio decreased with the addition of Cu(II), which is postulated to be due to cascade FRET quenching by the Cu(II) complex. The goal of developing a ratiometric indicator that shows a enhanced response to Cu(II) was not met.

Synthetic parameters that were optimized include the reactivity and percentage of the ligand monomer, the polarity of the environment and the fluorophore labeling methods. It was found that 0.5% AP-IDA synthesized in acetonitrile resulted in a large
response in the SL system. In the DL system, the Alexa fluorophores are closer to each other, which makes it more sensitive. Meanwhile, the effects of positive and negative charges on the PNIPAM phase transition were found to differ. Further study of the mechanism is needed.
CHAPTER 6

CONCLUSION AND FUTURE WORK

Inspired by the high sensitivity of the PNIPAM conformation in response to a small percentage of charges on a metastable polymer chain, we have successfully utilized this special phenomenon for fluorescent metal ion sensing. The underlying principles are related to the transfer of positive charges carried by the metal ions to ligand sites on the PNIPAM. By labeling the PNIPAM with either an environment-sensitive fluorophore or a FRET pair, two types of ratiometric fluorescent metal ion indicators have been developed, which differ slightly in design.

The first system based on the solvatochromism of dansyl fluorophores served its purpose as a “proof-of-concept” study. Through formulation optimization, a ratiometric indicator showing a fairly large fluorescence intensity ratio response to Cu(II) was constructed. The goal of effective separation of chelating units from the fluorescing units was achieved as shown by an enhanced fluorescence response to Cu(II). In the research, the percentage of the ligand NAIDA was kept constant at 2%. This number can be varied for further optimization.

The second system based on the FRET pair Alexa 555 and Alexa 647 yielded a larger intensity ratio response toward a number of metal ions. The apparent stability constants obtained closely match the literature values. However, a Cu(II) indicator showing enhanced fluorescence response could not be fabricated possibly due to an
unknown Cu(II) quenching process. It is found the DL polymer has more closely positioned fluorophores and produces better sensitivity and larger response. The future work should focus on the DL system thanks to its inherent advantages over the SL system.

The ligand used in this dissertation work is based on IDA, therefore a negative to neutral transition takes place upon metal ion binding. It was also found that the positive and negative charges influenced the PNIPAM phase transition differently. It is advisable to use a neutral ligand at the sensing pH in both types of indicators so as to evaluate the opposite process of PNIPAM expanding. This design may circumvent the problem of quenching if the Cu(II)-ligand complex quenches fluorescence.

In-depth mechanistic study should also be conducted on the phase transition of charged PNIPAM since the fluorescence response depends on this process. The phase transition process of PNIPAM without charges has been studied extensively by fluorescence and light scattering. However, few investigations were aimed at charged PNIPAM. Similar studies using FRET or light scattering can be conducted to illuminate the phase transition mechanism. Equally important is the characterization of the polymers in terms of molecular weight and distribution, percent charges and copolymer compositions.

In designing both systems, the complexation selectivity towards a specific metal ion was not taken into consideration as the chelator groups are common ligands that bind to many metal ions with similar affinities. In practical sensing applications such as environmental analysis or biochemical analysis, the selectivity is vital in that the matrices tend to be very complex. While total selectivity towards one analyte ion is not achievable at present, the selectivity can be improved by using ligands that have a high affinity for
the analyte metal ions and low affinity for other ions. For example, acetylacetone, ethylacetoacetate and salicyldehyde form chelates with Cu(II) of high stability as compared to other metals. These chelators can be derivatized to be incorporated into the polymer. A number of specific host-guest interactions and natural biomolecular interactions can also be used in our system if the analyte extends beyond the metal ions. Additionally, the molecular imprinting/ion imprinting would help improve the selectivity. This is another research direction in process—using uncrosslinked PNIPAM to do molecular imprinting. However, the marriage of ion imprinting and conformation-based sensing could be problematic.

Even though the formulation optimization in random free radical polymerization was to some extent effective in this research, a precise control of the polymer synthesis can save time wasted in trial and error. Controlled free radical polymerization or living polymerization can make polymers with well defined structures and regulated molecular weight. Such polymerization techniques include reversible addition fragmentation chain transfer (RAFT) polymerization, nitroxide-mediated radical polymerization (NMP) and atom transfer free radical polymerization (ATRP).

In conclusion, our systems demonstrated a new sensing approach and opened the door to other sensing applications. New improvements can be made based on the success of the current systems.
REFERENCE


42. Szczubialka, K.; Nowakowska, M., Response of micelles formed by smart terpolymers to stimuli studied by dynamic light scattering. Polymer 2003, 44 (18), 5269-5274.


90. Buckley, P. J. M.; van den Berg, C. M. G., Copper complexation profiles in the
Atlantic Ocean: A comparative study using electrochemical and ion exchange techniques.

91. Holm, P. E.; Christensen, T. H.; Tjell, J. C.; McGrath, S. P., Speciation of
Cadmium and Zinc with Application to Soil Solutions. J. Environ. Qual. 1995, 24 (1),
183-190.

92. Davison, W.; Zhang, H., In situ speciation measurements of trace components in

93. Temminghoff, E. J. M.; Plette, A. C. C.; Van Eck, R.; Van Riemsdijk, W. H.,
Determination of the chemical speciation of trace metals in aqueous systems by the

94. Grynkiewicz, G.; Poenie, M.; Tsien, R. Y., A new generation of Ca\(^{2+}\) indicators

Tsien, R. Y., Calcium Green FlAsH as a genetically targeted small-molecule calcium


97. Chang, C. J.; Lippard, S. J., Zinc Metalloneurochemistry: Physiology, Pathology,
and Probes. In Neurodegenerative Diseases and Metal Ions, Astrid Sigel, H. S., Roland K.


100. Domaille, D. W.; Que, E. L.; Chang, C. J., Synthetic fluorescent sensors for

101. Que, E. L.; Domaille, D. W.; Chang, C. J., Metals in Neurobiology: Probing Their

Photochromism of Spirobenzopyran via Selective Metal Ion Coordination: An Efficient
(9), 3466-3475.


129. Maeda, Y.; Yamamoto, H.; Ikeda, I., Effects of Ionization of Incorporated Imidazole Groups on the Phase Transitions of Poly(N-isopropylacrylamide), Poly(N,N-
