STRUCTURAL STUDIES OF NATURAL AND SYNTHETIC
MACROMOLECULES STABILIZED BY METAL ION BINDING

by

ZHENG LI

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Dissertation Advisors: Dr. Alexander M. Jamieson and Dr. Stuart J. Rowan

Department of Macromolecular Science and Engineering
CASE WESTERN RESERVE UNIVERSITY

May, 2011
We hereby approve the thesis/dissertation of

ZHENG LI

candidate for the Doctor of Philosophy degree *.

(signed) Alexander M. Jamieson (chair of the committee)
Stuart J. Rowan
Lei Zhu
Sved Qutubuddin

(date) 1.18.2011

*We also certify that written approval has been obtained for any proprietary material contained therein.
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Structural Studies of Natural and Synthetic Macromolecules

Stabilized by Metal Ion Binding

Abstract

by

ZHENG LI

Metal ions can interact with both natural and synthetic macromolecules to produce changes in their structure and conformation. This thesis addresses structure-property relationships in several classes of macromolecular materials which fit this description. In Chapter II, the gelation mechanism of 50/50 w/w mixtures of guanosine (G) and 2',3',5'-tri-O-acetylguanosine (TAcG) in aqueous 0.354 M KCl is investigated using static light scattering (SLS), polarized and depolarized dynamic light scattering (VV and VH DLS), small-angle neutron and X-ray scattering (SANS and SAXS), and viscometry. The results indicate sol and microgel phases coexist up to the macroscopic gel point. An unusual transient contribution to the gel modulus is attributed to fibrillar species trapped within the gel network.

In Chapter III, a class of stimuli-responsive metallo-supramolecular gels self-assembled in good/poor solvent mixtures from metal ions and a ditopic macromonomer consisting of oligoethylene glycol end-capped with a 2,6-bis(1'-methylbenzimidazolyl)-4-pyridine ligand (MeBIP) is studied by SAXS and differential scanning calorimetry (DSC). An evolution from turbid to transparent gels with increase of good solvent content is shown to be due to the formation of a solvent-swollen lamellar structure. In Chapter IV,
with the goal of developing redox-active assembly of supramolecular polymers in solution, we describe structural studies using viscosity, SAXS, SLS and DLS of the supramolecular polymers formed via binding of Cu(II) and Cu(I) ions to a ditopic macromonomer consisting of polytetrahydrofuran end-capped with MeBIP ligands. The results demonstrate differences in the molecular weight of the metallo-supramolecular polymers formed at fixed stoichiometry via Cu(II)-MeBIP versus Cu(I)-MeBIP binding.

Chapter V describes structural characterization by SAXS of a cruciform base-paired ribonucleic acid (RNA) complex, which catalyzes an RNA splicing reaction. Comparison of the radii of gyration (\(R_g\)) of mutant complexes versus the wild type indicates a preference for one of four possible magnesium ion-driven folded conformers. Energy minimized atomistic structures are generated for the wild type and mutant complexes. Since the juxtaposition of catalytically-active groups in the selected conformer differs from that found in the native activated spliceosome, the results suggest a chaperoning role for spliceosomal proteins in ensuring the correct tertiary stacking of the base-paired helices.
CHAPTER I

Introduction

Preamble

Metal ions interact with both natural and synthetic macromolecules, and in doing so may produce changes in their structures and conformations. This thesis addresses structure-property relationships in several classes of macromolecular materials which fit this description. Supramolecular polymers, which employ non-covalent interactions, such as hydrogen bonding, $\pi - \pi$ stacking, hydrophobic interactions, and metal-ligand coordination bonding, to assemble smaller molecules into supramolecular structures have attracted much attention in recent years. We will investigate two examples which fall into the latter category, sometimes termed metallo-supramolecular polymers, which utilize interactions between metal ions and ligands covalently attached to synthetic macromolecules to build higher level structures. Each of these examples employs synthetic macromonomers end-functionalized by the same metal ion-binding ligand. One species has a linear oligomeric core and forms metallo-supramolecular gels with multi-stimuli-responsive properties, including optical responses derived from the metal ion and mechano-responsive behavior via the polymeric framework. The other has a linear polymeric core and forms viscous metallo-supramolecular polymer solutions in which we investigate the possibility that changes in the metallo-supramolecular polymer structure can be induced by changing the redox state of the metal ion. A second broad area relates to the interaction between metal ions and naturally-occurring molecules and more specifically the interaction between metal ions and nucleotides. We will investigate two systems in which such interactions play a role. One involves structural characterization of
gels formed via metal ion-induced association of guanosine derivatives into quartets, which further assemble into fibrillar aggregates. These gels have potential for application as biofunctional materials. The other investigation involves structural characterization of the magnesium ion-driven conformation of a small ribonucleic acid (RNA) complex which catalyzes a splicing reaction. This RNA complex, which we term a model spliceosome, serves as an in vitro model for the active site of the spliceosome, an enormous ribonucleoprotein complex which performs splicing reaction on pre-messenger RNA in the mammalian cell.

In what follows, we first review prior work on metallosupramolecular polymers in the literature, specifically focusing on the use of ligands based on 2,2':6,2''-terpyridine, then we discuss prior work on metallo-supramolecular polymers at CWRU that laid the groundwork for this thesis. Next we present an overview of the role of metal ions in biology, and review prior work on the development of the model spliceosome. Finally we provide an outline of the contents of this thesis.

1.1 Supramolecular Polymers Utilizing Interactions between Metal Ions and Synthetic Macromolecules

2,2':6,2''-terpyridine (Figure 1.1) was first isolated by Morgan and Burstall,\(^1\) and the three nitrogen atoms make it an effective tridentate ligand for various metal ions.\(^3\)\(^-\)\(^6\) This ligand has been widely used as building blocks in various metallo-supramolecular polymers and several very good reviews have discussed these systems.\(^7\)\(^-\)\(^10\) The thermodynamics and kinetics of binding interaction between ligand and metal ion determine the properties of the resulting metallo-supramolecular polymers. Terpyridine forms kinetically inert bonds with ruthenium\(^{2+}\), nickel\(^{2+}\), osmium\(^{2+}\) and iron\(^{2+}\) whose
strong binding constants result in stable metallo-supramolecular polymers, which can be characterized by standard techniques, including gel permeation chromatography (GPC), viscosity and analytical ultracentrifugation.\textsuperscript{11-15} For example, in the presence of specific solvents and additives such as inert salt to reduce interactions between these charged species and the GPC column material, metallo-supramolecular polymers can survive during GPC chromatography. Besides GPC as a convenient way to determine the molecular weight, viscosity and analytical ultracentrifugation have also proven effective in characterizing the molecular weight of such polymers.\textsuperscript{12} Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) does not work since the assemblies tend to fragment during the experiment.\textsuperscript{11}

\begin{center}
\includegraphics[width=0.5\textwidth]{structure.png}
\end{center}

\textbf{Figure 1.1.} Structure of 2,2':6,2"-terpyridine.

On the other hand, terpyridine forms much weaker bonds with other metal ions such as Zn\textsuperscript{2+}, Cu\textsuperscript{2+} and lanthanides. Hence the resulting metallo-supramolecular polymers exist in equilibrium with free ligand and metal ions, which is influenced by parameters such as temperature, solvent, concentration and the nature of the counterions. We are especially interested in such labile metallo-supramolecular polymers since their special properties make them sensitive to external stimuli. These materials are not stable in GPC columns but can be characterized by NMR, UV-vis spectroscopy and viscosity. Based on their different structures, these metallo-supramolecular polymers can be divided into two groups: metal-ligands incorporated to form main chain polymers, and the formation of side-chain polymers or networks.
1.1.1 Main Chain Metallo-supramolecular Polymers

Ditopic ligands react with metal ions and result in formation of main-chain linear metallo-homopolymers as shown in Figure 1.2. The core connecting the two ligands can be either a flexible or a rodlike spacer. Schubert and coworkers reported the synthesis of such ditopic ligands based on diethylene glycol, poly or oligo(ethylene oxide), poly(tetrahydrofuran) spacers (Figure 1.3) and their self-assembly behavior in solution in the presence of different metal ions (Cd$^{2+}$, Cu$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Fe$^{2+}$).\textsuperscript{16-21} Complex formation between the terpyridine ligand and metal ions could be easily monitored by the red shift of the $\pi-\pi^*$ band and the appearance of a metal-to-ligand charge transfer band in UV-Vis spectroscopy.\textsuperscript{16-19} NMR measurement of proton chemical shifts was also able to detect the existence of complexes.\textsuperscript{16-19} Atomic force spectroscopy solution of cast films revealed phase separation and the presence of a lamellar structure.\textsuperscript{18} Solution viscosity measurement was the primary technique used to prove the existence of higher molecular weight of metallo-supramolecular polymers.\textsuperscript{19-20} Gucht et al.\textsuperscript{22} reported detection of the increase of degree of polymerization of metallo-supramolecular polymers constructed from terpyridine end-capped poly(ethylene oxide) (Figure 1.3 (ii)) in the presence of Cd$^{2+}$, by monitoring not only the increase of solution viscosity but also a decrease of the diffusion limited voltammetric current. The results were found to be in good quantitative agreement with a theoretical model for self-association based on the theory of Jacobson and Stockmayer for polycondensation polymers.\textsuperscript{23} In addition to the formation of linear supramolecular polymers, it was suggested that rings can also be formed, indicated by the occurrence of a viscosity dip at low concentration and the appropriate chemical stoichiometry, where all ligands and Cd$^{2+}$ form 2:1 complexes.\textsuperscript{22,24}
Figure 1.2. Schematic representation of the main-chain linear metallo-homopolymers.
Figure 1.3. Structure of 2,2':6,2"-terpyridine-bisfunctionalized diethylene glycol (i), poly or oligo(ethylene oxide) (ii), poly(tetrahydrofuran) (iii).

Terpyridine-Ru$^{2+}$ and –Fe$^{2+}$ macrocyclic complexes were synthesized by both stepwise and self-assembly procedures, and the structures were supported by NMR, UV/Vis spectroscopy, mass spectroscopy and TEM. Interaction of Fe$^{2+}$ with a short chain ditopic ligand based on terpyridine resulted in formation of two different metallo-macroyclic assemblies (Figure 1.4) which could be separated using column chromatography. These were identified as ring structures by both mass spectroscopy
and NMR spectroscopy. Newkome, Wesdemiotis and their coworkers applied traveling wave ion mobility mass spectrometry to analyze hexacadmium-terpyridine macrocycles, and demonstrated the capability to identify isomeric structures in supramolecular assemblies: distinguishing rings from linear chains.\textsuperscript{27}

\textbf{Figure 1.4.} Metallo-macrocyclic assemblies formed from terpyridine ligand terminated short chains and ferrous ions.\textsuperscript{26}
Metallo-supramolecular polymers based on ditopic ligand with rodlike spacers, usually π-conjugated oligomers, has also attracted much attention because of their special electro-optical properties. Kelch and Rehahn\textsuperscript{28} reported the synthesis of rodlike coordination polymers via the interaction of 4,4''-bis(2,2':6',2''-terpyridine)-2',5'-dihexyl-p-terphenyl (Figure 1.5 (i)) with Ru\textsuperscript{2+} salts. \textsuperscript{1}H and \textsuperscript{13}C NMR spectroscopy confirmed the formation of high molecular weight (Pn \( \geq 30 \)) supramolecular polymer, which was further supported by viscosimetry experiments. Since the coordination polymers are rigid rods with limited conformational degree of freedom in solution, it is presumed they should exhibit less complex polyelectrolyte behavior and thus could perform as a model system. By a combination of continuous wave (CW) and pulsed EPR spectroscopy with double electron-electron resonance (DEER) spectroscopy, it was deduced that the divalent nitrosodisulfonate counterions locate near and interact preferentially with the metal ions of the coordinated polymers.\textsuperscript{29}

A set of rigid ligands employing different spacers (Figure 1.5 (ii)), reported by Schubert and coworkers,\textsuperscript{30} has poly(ε-caprolactone) side chains, and thus their Zn\textsuperscript{2+} based supramolecular polymers exhibit enhanced processability and film-forming properties. Coupled with their useful photophysical properties, they have good potential in OLED applications. Kurth and coworkers\textsuperscript{31} investigated the assembly in solution of a shorter rigid ligand 1,4-bis(2,2':6',2''-terpyridine-4'-yl) benzene (Figure 1.5 (iii)) with different metal ions including Fe\textsuperscript{2+}, Co\textsuperscript{2+}, Ni\textsuperscript{2+} and Zn\textsuperscript{2+}, by conductivity, viscosity and small angle neutron scattering (SANS) as well as on surfaces by atomic force microscopy (AFM). The AFM and SANS results proved that rigid rod supramolecular polymers were formed from these rigid ligands and metal ions. The same group also investigated the optical,
electrochemical and electrochromic properties of a series of supramolecular polymers composed of metal ions (Fe$^{2+}$, Ru$^{2+}$ and Co$^{2+}$) and ditopic ligands based on terpyridine derivatives with mono-(or- poly)phenylene as the core (Figure 1.5 (iv)). Structure-property relationship studies revealed that switching reversibility between oxidation state of metal ions (e.g. Fe$^{2+}$/Fe$^{3+}$) and stability were influenced by the electronic properties of the substituents at the peripheral pyridine rings. With an electron-rich group R (see Figure 1.5 (iv)) the supramolecular polymers exhibit faster and more prolonged switching ability, and a lower switching potential than with an electron-deficient R-group.$^{32-33}$
Figure 1.5. Structures of bis(terpyridine) rigid ligands: (i) 4,4''-bis(2,2':6',2''-terpyridine)-2',5'-dihexyl-p-terphenyl; (ii) bisterpyridine based macroligands bearing poly(ε-caprolactone) chains; (iii) 1,4-bis(2,2':6',2''-terpyridine-4'-yl) benzene. (iv) functionalized bisterpyridine based ligands having mono-(or- poly)phenylene as the core.

1.1.2 Metallo-supramolecular Polymers with Side Chain, Crosslinks and Network Architectures.

Schubert and coworkers\textsuperscript{34} prepared metallo-supramolecular graft copolymers by grafting different organic and polymeric species having terpyridine end groups to a copolymer of methyl methacrylate with terpyridine-terminated methacrylate esters through metal ion-ligand interactions. (Figure 1.6) Ru\textsuperscript{2+} was used as the metal ion so that
the resultant metallo-supramolecular polymers could be characterized like normal polymers. Dendronized polymer brushes with metallo-supramolecular polymer side chains were also reported using the same ligands and metal ions. The synthetic strategy, as shown in Figure 1.6, was to attach different organic and polymeric species having ligand-Ru$^{2+}$ end groups as side-chains to the free pendant ligands on the polymer backbones through formation of coordinate bonds. Another strategy was to first synthesize an amine-functionalized side chain containing ligand-Ru$^{2+}$-ligand complexes and then anchor the side chains to a poly(methymethacrylic acid) backbones via coupling through the amine group (Figure 1.7).
Figure 1.6. The synthesis of metallo-supramolecular graft copolymers incorporating Ru$^{2+}$-ligand binding in the side chains. Metal ion-ligand coordination and attaching the side chain are at the same time.$^{29}$
Figure 1.7. The synthesis of metallo-supramolecular graft copolymers incorporating Ru$^{2+}$-ligand binding in the side chains. Metal ion-ligand coordination is before attaching the side chain.\textsuperscript{31}

One step beyond incorporating metal ion coordination in polymer side chains is to carry out supramolecular crosslinking through metal ion-ligand binding. If Fe$^{2+}$ or Zn$^{2+}$ is added to the poly(methyl methacrylate) copolymers shown in Figure 1.6, the pendant terpyridine groups on two different copolymer backbones can complex with one of these metal ions to create a ligand - metal ion - ligand complex crosslink between the copolymer chains.\textsuperscript{37} Specifically, in fact, one expects that intermolecular crosslinking will occur at high concentration while intramolecular complexation will become predominant at low concentrations (Figure 1.8). This synthetic approach could be extended to other polymers, as long as they have pendant ligands.\textsuperscript{38-39}
1.1.3 Prior Work on Metallo-Supramolecular Polymers at CWRU.

With a view to obtain metallo-supramolecular materials with superior thermal and photoelectric properties, Rowan and coworkers developed ditopic macromonomers based on different macromonomer cores end-capped with 2,6-bis(1'-methylbenzimidazolyl)-4-pyridine (MeBIP) ligands. The MeBIP ligand, which as seen in the schematic in Figure 1.9, may be viewed as a derivative of terpyridine, has the properties of binding transition metal (II) ions in the ratio of 2:1, and lanthanide ions in the ratio of 3:1. This offers the possibility to create gel networks from metallo-supramolecular polymers, in which the divalent transition metal ion binds two MeBIP...
ligands and functions as a chain extender, while the trivalent lanthanide ions binds three MeBIP ligands and functions as a chain stoppers or a crosslinker (Figure 1.9). Beck et al.\textsuperscript{40-41} demonstrated that, indeed, metallo-supramolecular polymers, derived from self-assembly of a macromonomer, composed of penta(ethylene glycol) end-capped by the MeBIP ligand, dissolved in organic solvents containing metal ion combinations (Co\textsuperscript{2+}/La\textsuperscript{3+}, Co\textsuperscript{2+}/Eu\textsuperscript{3+}, Zn\textsuperscript{2+}/La\textsuperscript{3+}, and Zn\textsuperscript{2+}/Eu\textsuperscript{3+}), formed turbid gels which have multi-stimuli responsive properties (photo-, chemo-, thermo- and mechano-responses). Subsequently, this gel system was studied in great detail, including measurement of rheological properties and structural analysis by wide angle X-ray scattering and optical microscopy, and it was established that the gelation mechanism is highly unusual, involving phase separation to form semi-crystalline, colloidal particles, which self organize into a gel network at sufficiently high concentrations (e.g. 50 g/L).\textsuperscript{42-44} It was further established that, in good-poor solvent mixtures, when the mixture is sufficiently rich in the good solvent, the gels become transparent, while maintaining their mechanical strength. In this thesis (Chapter III), these gels will be investigated further, utilizing differential scanning calorimetry (DSC) to gain insight into how the length of the oligoethylene oxide core influences the gel properties, and applying small angle X-ray scattering to probe the structural transformation that accompanies the onset of transparency in the gels.
**Figure 1.9.** Schematic representation of the construction of a metallo-supramolecular gel using a combination of lanthanide$^{3+}$ and transition metal ion$^{2+}$ mixed with penta(ethylene glycol) end-capped by MeBIP ligands.

Besides oligo(ethylene glycol), a flexible polymeric core, poly(tetrahydrofuran) (PTHF), was also utilized to make a ditopic macromonomer (Figure 1.10 (i))$^{45-46}$. Metal ions including Fe$^{2+}$, Co$^{2+}$, Zn$^{2+}$, Cd$^{2+}$ or Zn$^{2+}$/Eu$^{3+}$ were mixed with these macromonomers in solution and the formation of self-assembling aggregates confirmed, as reflected from solution viscosity measurements. Furthermore, mechanically stable films were obtained by casting from solution, and Zn$^{2+}$/Eu$^{3+}$ containing films shown to exhibit stimuli-responsive properties.$^{45-46}$ In this thesis (Chapter II) the self-assembly behavior of a MeBIP end-capped flexible macromonomer (Figure 1.10 (i)) with metal ions in different oxidation states ($Cu^{+}$ and $Cu^{2+}$) will be investigated in solution by scattering and viscosity techniques.
To explore the possibility of improved photoelectric properties, poly(2,5-dialkoxy-p-phenylene ethynylene) (PPE) and poly(2,5-dioctyloxy-p-xylylene) (PPX) were introduced as the core of ditopic macromonomers based on the MeBip ligand by Rowan and Weder (Figure 1.10 (ii), (iii)).\textsuperscript{47-50} The resulting materials are readily solution-processable and exhibit good mechanical and interesting optoelectric properties.
1.2 Metallo-Supramolecular Gels, Hydrogels and Supramolecular Hydrogels.

Based on the attempts to describe and define gels starting from about 150 years ago, one can define a substance as a gel if “it has a continuous microscopic structure with macroscopic dimensions that is permanent on the time scale of an analytical experiment and is solid-like in its rheological behavior despite being mostly liquid.” Kruyt, Hermans and Flory discuss several different types of gel: gels with well-ordered lamellar structures; gels with cross-linked polymeric networks swollen with solvent; gels with polymer networks involving physical chain-chain interactions and gels with a disordered particulate structure. Cross-linked polymeric networks may be viewed as essentially a single macromolecule having infinite molecular weight. They are usually obtained from high molecular weight linear chains by crosslinking via functional groups attached to the chains, or from step-addition polymerization of oligomeric multifunctional precursors. Instead of chemical crosslinks, noncovalent interactions between
polymer chains could also lead to the formation of gels. These weaker interactions which function as crosslink junctions include the van der Waals force, hydrogen bonding, $\pi-\pi$ stacking, metal-ligand coordination, hydrophobic interaction and Coulombic forces. Figure 1.11 illustrates a gel system composed of polymer networks connected by covalent or noncovalent interactions and swollen with solvent. Based on the solvent used, gels could be classified as organogels when formed in organic solvents and hydrogels when formed in water. Gels with disordered particulate networks include those composed of colloidal spheres or fibrils. As noted in the last section, the metallo-supramolecular gels prepared from penta(ethylene glycol) endcapped by MeBIP ligand (Figure 1.9) proved to be gels formed from colloidal spheres. Gels formed from fiber networks are also common since the high aspect ratio of fibers enables them to interact with each other in the solution state even at very low concentrations.

Some fibrillar gel networks are formed from supramolecular fibers, i.e. fibers constructed via self-assembly of small molecules through noncovalent interactions. Compared with the conventional polymeric gels, supramolecular gels based on supramolecular interactions have certain particular advantages. Since the supramolecular interactions are weak, the gel structures and thus properties are sensitive to external stimuli, including solvent composition, temperature, chemical additives, electric fields, light and mechanical forces. This makes such gels multi-stimuli-responsive. Because of their sensitivity to external stimuli gels have potential applications as sensors. Some specific advantages of the mechano-responsive character of supramolecular gels are self-healing capability, and improved processability by enabling reversible gel-sol transformation through application of heat or mechanical forces.
Hydrogels, which have water as the continuous solvent component, are particularly suited for applications in personal care products (including toothpaste and shampoo), in foodstuffs, in drug delivery and in tissue engineering. For example, the possibility exists that drugs dispersed in supramolecular gels could be released at a specific location and at a controllable rate by applying localized external stimuli to disrupt the gel structure. Hydrogel networks can be decorated with bioactive molecules that promote cell-growth and differentiation. In this thesis (Chapter IV) we will investigate the structure, properties and gelation mechanism of a supramolecular hydrogel based on metal ion coordination to guanosine derivatives which has potential applications in tissue engineering.
Figure 1.11. Schematic presentation of polymeric gels by covalent or noncovalent interaction.

1.3 Interactions between Metal Ions and Naturally-Occurring Macromolecules

Naturally-occurring macromolecules include proteins, nucleic acids (RNA and DNA) and polysaccharides. Metal ions can interact with these biomacromolecules and alter their structures and conformations, in ways critical to their functional activity. For example, metal ion-ligand complexes play a key role in the function of many enzymes, the most celebrated example being hemoglobin, in which the interaction of an iron-porphyrin complex with its protein host controls the binding of oxygen.\textsuperscript{78-81} Polysaccharides with carboxylic acid or amine functionalities are efficient binders of metal ions. For example, chitosan, a linear polysaccharide, composed of randomly distributed $\beta$-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetylD-glucosamine (acetylated unit), derived via deacetylation of chitin, a structural polysaccharide found in the exoskeleton of crustaceans, efficiently binds transition metal ions.\textsuperscript{82} Here, we focus principally on a brief survey of the role of metal ions in influencing the structure and conformation of polynucleotides.

Metal ions influence or determine almost all functions of nucleotides in biological systems.\textsuperscript{83-84} These metal ions may change the secondary (conformation) and tertiary structures of nucleotides, and lead to stabilization, destabilization or mutation. The phosphate groups carry negative charges (primarily on the oxygens) under physiological conditions, so metal cations usually bind to the oxygens through electrostatic interaction. Cruciform RNA are a common structural element in RNAs.\textsuperscript{85-91} In the presence of Mg\textsuperscript{2+} ions, such cruciform elements can adopt different folded conformations through a process.
known as coaxial stacking, a phenomenon which is of critical importance in enabling them to execute chemical and biological functions. One example is that the hairpin ribozyme of the tobacco ringspot virus, which contains a perfect cruciform structure. The enzymatic activity of the hairpin ribozyme can be modulated by changes in the conformation of the cruciform.

An unexpected feature of the human genome relates to the abundant regions that do not appear to carry any useful information. These “non-coding” regions, also called intervening sequences or introns, interrupt coding regions (exons), and thus, to use the data stored in our genes, the boundaries of introns must first be correctly recognized, and then the introns must be removed to generate an uninterrupted genetic message. This process, referred to as splicing, is performed by an enormous cellular machine known as the spliceosome, which contains five snRNPs (small nuclear ribo-nucleo-proteins), named U1, U2, U4, U5 and U6, and a number of non-snRNP associated proteins. Spliceosomal function is of crucial importance: 50% of all human genetic diseases, a large number of cancers relate to errors in splicing. Valadkhan and coworkers have demonstrated the ability of an in vitro-assembled base-paired complex of U6 and U2 snRNAs to remove an intervening sequence from a pre-mRNA-like construct in a two-step reaction that is chemically identical to a splicing reaction. Despite the fact that the reaction is very inefficient, and that appropriate caution should be exercised in assuming the validity of such simple model systems, it was concluded that the U6/U2 complex might indeed succeed in recapitulating the function of the spliceosomal catalytic core, i.e. it may function as a minimal model spliceosome. The model spliceosome has a cruciform structure, and, in Chapter V, we describe a series of studies using small angle
X-ray scattering and molecular modeling, designed to deduce which of four possible folded conformers exists under catalytic conditions.

1.4 Thesis Outline

This thesis describes the structural characterization of several four macromolecular species stabilized by metal ion binding:

CHAPTER II The Structure and Gelation Mechanism of Tunable Guanosine-Based Supramolecular Hydrogels

This chapter investigates the structure and gelation mechanism of hydrogels formed in aqueous KCl, by mixing a gelator (guanosine, $\text{G}$) with a nongelator ($2',3',5'$-tri-O-acetylguanosine, $\text{TAcG}$), using small angle neutron scattering (SANS), small angle X-ray scattering (SAXS) and polarized (VV) and depolarized (VH), static and dynamic, light scattering (SLS and DLS). From the resulting gelation mechanism an explanation is proposed for the unusual rheological behavior of these gels.

CHAPTER III Structure of a Class of Metallo-Supramolecular Polymeric Gels

This chapter investigates the structural transformation which accompanies the formation of transparent metallo-supramolecular polymeric gels through self-assembly in mixed solvent systems of a ditopic ligand consisting of OMeBIP end-capped oligoethylene glycols in the presence of Zn$^{2+}$. The effects of oligoethylene glycol core length and solvent composition are explored.

CHAPTER IV Studies of the Self-assembly of Metallo-supramolecular Polymers in Solution.

This chapter investigates the structure of metallo-supramolecular polymers self-assembled in solution from a ditopic macromer consisting of OMeBIP end-capped
polytetrahydrofuran in the presence of Cu$^{2+}$ and Cu$^+$. Our data shows that the macromonomer binds Cu$^{2+}$ in a 2:1 ratio and Cu$^+$ in a 2:2 ratio in solution. We evaluate the potential to use this redox-active pair to switch between metallo-supramolecular polymers of high and low molecular weight.

CHAPTER V Small Angle X-Ray Scattering Analysis of the Base-paired Complex of U6 and U2 snRNAs Points to a RNA Chaperone Role for Proteins in Assembly of the Spliceosomal Active Site

This chapter utilizes the SAXS technique to identify the Mg$^{2+}$-driven equilibrium folding conformation of a small RNA complex (model spliceosome) that catalyzes an RNA splicing reaction. To facilitate this process, SAXS data for the wild-type model spliceosome is compared against the corresponding data for three mutant RNAs. An atomistic structure for the equilibrium conformation is proposed.
1.5. References


CHAPTER II

The Structure and Gelation Mechanism of Tunable Guanosine-Based Supramolecular Hydrogels

2.1 Introduction

Polymeric and supramolecular hydrogels are being increasingly investigated for drug or gene delivery systems, tissue engineering, smart devices. Supramolecular hydrogels, assembled from low molecular weight gelators, form a network structure via noncovalent interactions such as hydrogen bonding, $\pi-\pi$ stacking, hydrophobic interactions and metal-ligand coordination. Because of the weak noncovalent interaction and low molecular weight gelator, supramolecular hydrogels have certain advantages over normal polymeric hydrogels, particularly in applications in drug delivery, tissue engineering, stimuli-responsive materials and sensors. The low molecular weight gelators facilitate much clearing of the hydrogels from the body. Further, the weak noncovalent interactions anchoring the supramolecular hydrogel network makes it mechano-responsive, and a gel-sol reversible transition is possible in response to pH, light, chemical or enzymatic stimulus under physiological conditions. Guanosines are attractive building blocks for supramolecular architectures, not only because they can form helical ribbons or columnar structures depending on conditions, but also because the side chains of guanosine can be functionalized by different groups to tune their properties. In the presence of certain metal ions, such as potassium or sodium ions, guanosine derivatives self-assemble into planar cyclic quartets, which then can associate further into columnar stacks. One problem with hydrogels formed by guanosine
and a number of its derivatives is their limited life-time stability on account of crystallization. Yu et al.\textsuperscript{21} reported stable gels can be obtained in KCl/Tris buffer solution by mixing guanosine (G) and guanosine 5'-monophosphate (GMP). In order to develop potentially useful guanosine hydrogels, one possibility is to mix the gelator, guanosine, with a nongelator which contains the same motif, but which disrupts the perfect stacking between quartets and inhibit crystallization while preserving enough supramolecular order for gelation to occur.

Hydrophobic groups may also be incorporated in the nongelator to introduce physical crosslinks (key to gel formation) between columnar stacks. It is expected that the properties of gels formed by such mixtures would be tunable by changing the ratio between guanosine and nongelator. Recently, L. E. Buerkle and S. J. Rowan prepared stable and transparent hydrogels by mixing guanosine (G) with a non-gelator of similar structure, namely 2’,3’,5’-tri-O-acetylguanosine (TAcG) (Figure 2.1).\textsuperscript{22} They further demonstrated that the thermomechanical properties of the resulting hydrogels could indeed be tuned by changing the ratio between G and TAcG.\textsuperscript{22}

Knowledge of the structural organization as well as the self-assembly and gelation mechanism of supramolecular hydrogels is critical to understand how to further control the properties of G/TAcG gels. First, we obtained insight into the structure of the columnar stacks of G/TAcG quartets via detailed analysis of G/TAcG gels of varying composition, using Small Angle Neutron Scattering (SANS). By combining these data with Atomic Force Microscopy (AFM), Differential Scanning Calorimetry (DSC), and Nuclear Magnetic Resonance (NMR) studies of L. E. Buerkle, it became possible to develop a structural model for the fibrillar network formed by the G/TAcG columnar
stacks. Second, we sought to obtain information on the mechanistic steps by which the supramolecular aggregates are formed, and hence gain more insight into the gelation mechanism. This was accomplished by carrying out static light scattering (SLS), polarized and depolarized dynamic light scattering (DLS), synchrotron small-angle X-ray scattering (SAXS) and viscosity experiments performed on G/TAcG mixtures in aqueous 0.354 M KCl over a range of concentrations encompassing the gelation transition.

Prior investigations of the self-aggregation behavior of sodium, potassium and ammonium salts of guanosine 5′-monophosphate (GMP) and deoxyguanosine 5′-monophosphate (DGMP) in aqueous solution have been reported, employing DLS, NMR, and SAXS. Small rodlike aggregates composed of guanosine quartet stacks were found in solution. In concentrated KCl solution, cylinders of DGMP were found to associate into a hexagonal phase. Analogous to the fibrillar structures seen in AFM images of G/TAcG gels, SEM images of GMP in alkali metal salts display long straight or curved rods composed of bundles of stacks of guanosine quartets. By way of contrast, we note that G/TAcG mixtures gel at much lower concentrations (0.8 wt%) than GMP or DGMP, and so our experiments are necessarily carried out at higher dilutions than these earlier experiments.
Figure 2.1. Structures of (a) guanosine (G), (b) 2',3',5'-tri-O-acetylguanosine (TAcG), and (c) a G-quartet.

2.2 Experimental

2.2.1 Materials and Sample Preparation

Guanosine was purchased from Sigma-Aldrich. The synthesis of 2’, 3’, 5’-tri-O-acetylguanosine (TAcG) was performed according to literature procedures\textsuperscript{30} and has been reported elsewhere.\textsuperscript{22} For SANS measurements, stoichiometric amounts of G/TAcG were dispersed in D\textsubscript{2}O, requiring several sonication and heating cycles to obtain a finely-divided dispersion. KCl was then added at a concentration of 0.708 M to produce a solution containing 2 wt\% of the guanosine moiety in 0.354 M KCl. The mixture was heated until a transparent solution was obtained, after which cooling to room temperature resulted in formation of a transparent, thermo-reversible gel within a few minutes. SANS experiments were performed on three hydrogel specimens, having weight ratios of G/TAcG of 60%/40%, 50%/50%, 40%/60%, encompassing the range where crystallization was inhibited, and transparent gels formed. Each sample was prepared in
0.354 mol\(^{-1}\) KCl at a concentration of 2 wt%. 50/50 G/TAcG was chosen to investigate the gelling process. A series of concentrations (0.05 wt% to 1 wt%) were prepared in deionized \(H_2O\) with the KCl concentration kept constant at 0.354M for SAXS, SLS, DLS, differential refractive index increment determination, and viscosity measurements. Hot solutions were passed through polycarbonate filters (pore diameters 400 nm or 1 \(\mu m\)) to remove possible dust contamination, and it was confirmed via UV spectroscopy that no solute was lost during filtration.

2.2.2 SANS Measurement

SANS measurements were conducted on the time-of-flight small angle diffractometer at the Intense Pulsed Neutron Source facility of Argonne National Laboratory, IL. The accessible range of scattering vectors \(q (q=4\pi\sin(\theta/2)/\lambda, \text{where } \theta \text{ was the scattering angle})\) was very wide (0.08-14 nm\(^{-1}\)). The neutron scattering length density of D\(_2O\) was 5.76\(\times\)10\(^{-4}\) nm\(^{-2}\), whereas that of TAcG and G was in the range of 1.2-1.6\(\times\)10\(^{-4}\) nm\(^{-2}\). The prepared hydrogels were heated and dissolved so that they could be transferred into the sample cells. During the experiment, the hydrogels were contained in a quartz cell maintained at the temperature of 25 °C. Data acquisition time was 6 hours. The signal of D\(_2O\) in quartz cell was subtracted as the background. Then the data of hydrogels was extracted and analyzed. The concentration of G/TAcG 60/40 was actually slightly lower than 2 wt% because we lost some materials and added some D\(_2O\) when filling the cell.

2.2.3 SAXS Measurement

Small angle X-ray scattering was carried out at the BESSRC CAT 12-ID beam line of the Advanced Photon Source, Argonne National Lab, Argonne, IL. The setup used
a sample-detector distance of 4 m and CCD detector. The sample holder was a Quarzkapillaren quartz capillary with a diameter of 2 mm purchased from the Charles Supper Company. The X-ray energy was 12keV, corresponding to a wavelength of 0.1 nm. Small angle X-ray scattering measurements were conducted on solutions of G/TAcG (50/50) at different concentrations. The samples were equilibrated for several hours prior to measurement. The exposure time was 1 s, and five exposures were averaged to obtain better signal quality. The signal from 0.354 M KCl solution was subtracted as background.

2.2.4 Viscosity Measurement

Cannon-Ubbelohde semi-micro viscometers were used to determine the viscosity of G/TAcG (50/50) solutions at different concentrations (0.05 wt% to 0.5 wt%) following instructions on the company website. The viscometer was maintained at 25 °C in a water bath. The ratio of the flow times of solution to solvent is an approximate measure of the relative viscosity $\eta_r = \eta/\eta_s$. The reduced specific viscosity may be viewed as a measure of the apparent hydrodynamic volume, $V_h^{\text{app}}$, divided by the molar mass, M, of the dissolved particles:

$$\eta_s \cdot c = (\frac{\eta}{\eta_s} - 1)/c \approx 2.5N_A \frac{V_h^{\text{app}}}{M}$$  

2.2.5 Differential Refractive Index Increment Measurement

To obtain molecular weights from static light scattering experiments, it is necessary to know the differential refractive index increment (dn/dc) of 50/50 G/TAcG solutions. This was determined according to web-posted instructions written by P. Russo, using a Brice-Phoenix differential refractometer as described later. The method
measures the deflection of light passing through a split cell, one half of which contains \textbf{G/TAcG} solution, the other solvent (aqueous 0.345 M NaCl). The deflection is calibrated against NaCl solutions of known refractive index.

\textbf{2.2.6 Static Light Scattering}

A Stabilite Ar\textsuperscript{+} laser emitting vertically-polarized light at $\lambda = 514.5$ nm with a power output of 1–1.5 W was used for static light scattering experiments. The laser was coupled to a BI 200 SM photometer-goniometer (Brookhaven Instruments) and a BI-9000 correlator. In static light scattering, the excess scattered intensity, $\Delta i(q,c)$, recorded by subtracting the solvent signal from the solution signal, was measured as a function of \textbf{G/TAcG} concentration, c, and scattering vector q, defined as

$$q = \frac{4m}{\lambda} \sin \frac{\theta}{2}$$

where $n$ is the solvent refractive index, $\lambda$ the laser wavelength in vacuo, and $\theta$ the scattering angle. The scattering angle was increased in 5\textdegree increments between 45\textdegree and 135\textdegree, corresponding to a q range from $1.25 \times 10^{-2}$ to $3.01 \times 10^{-2}$ nm\textsuperscript{-1}. The excess Rayleigh ratio, $\Delta R(q,c)$, was determined using toluene as the reference scatterer:

$$\Delta R(q,c) = \frac{[i_{\text{solution}}(q,c) - i_{\text{solvent}}(q,c)] R_{\text{toluene}}(q)}{i_{\text{toluene}}(q,c)} \times \frac{n^2(\text{toluene})}{n^2(\text{solvent})}$$

where the Rayleigh ratio of toluene is $3.2 \times 10^{-5}$ cm\textsuperscript{-1} at 514.5 nm. For dilute polymer solutions, at small scattering vectors, $qR_g < 1$, where $R_g$ is the radius of gyration of the polymer, the excess Rayleigh ratio satisfies the following equations:

$$\frac{Kc}{\Delta R(q,c)} = \frac{1}{M_w} (1 + q^2 R_g^2 / 3) + 2A_c c + 3A_c^2 c^2 + ...$$
\[ K = \frac{4\pi^2 n^2 \left( \frac{dn}{dc} \right)^2}{N_A A^4} \] (5)

where \( N_A \) is Avogadro’s number, and \( A_2 \) and \( A_3 \) are the second and third osmotic virial coefficients. At finite concentration, double extrapolation of eq 4 to \( c = 0 \) and \( q = 0 \), provides an apparent weight average molecular weight \( M_{w\text{app}} \):

\[ \frac{Kc}{\Delta R(0, c)} = \frac{1}{M_{w\text{app}}} = \frac{1}{M_w} + 2A_2c + 3A_3c^2 + \ldots \] (6)

and apparent z-average radius of gyration:

\[ \frac{Kc}{\Delta R(q, c)} = \frac{Kc}{\Delta R(0, c)} \left(1 + \frac{q^2 \left( R_g^{app} \right)^2}{3} \right) \] (7)

When the condition \( qR_g < 1 \) cannot be satisfied, a Guinier plot at low \( q \) provides an estimate of \( R_g^{app} \):

\[ \ln \frac{Kc}{\Delta R(q, c)} = \ln \frac{Kc}{\Delta R(0, c)} + \frac{q^2 \left( R_g^{app} \right)^2}{3} \] (8)

2.2.7 Polarized and Depolarized Dynamic Light Scattering

Polarized and depolarized dynamic light scattering (DLS) experiments were carried out at scattering angles from 30° to 90° in increments of 10° using an instrument equipped with an Ar⁺ Spectra Physics 2020 laser with power output 1.5-2W, a BI goniometer, a RCA Model 7265 photomultiplier, an ALV-5000 correlator, and a Newport RSP-2T polarizer. With this instrument, in addition to the intensity of scattered light, \( i_s(q, t) \), the normalized time correlation function of the scattered intensity, \( g^{(2)}(q, \tau) \), is determined:

\[ g^{(2)}(q, \tau) = \frac{\langle i_s(q,t)i_s(q,t+\tau) \rangle}{\langle i_s(q) \rangle^2} \] (9)

39
where the angular brackets indicate a statistical average. The normalized electric field correlation function, $g^{(1)}(q, \tau)$, is related to $g^{(2)}(q, \tau)$ by Siegert’s relation:

$$g^{(2)}(q, \tau) = \alpha \left| g^{(1)}(q, \tau) \right|^2 + 1$$  \hspace{1cm} (10)

where $\alpha$ is spatial coherence factor which is treated as a fitting parameter and $B$ is the baseline. Experiments were conducted at scattering angles in the range $30^\circ$ to $90^\circ$, corresponding to $q$ from $8.42 \times 10^{-3}$ to $2.30 \times 10^{-2}$ nm$^{-1}$. The electric field correlation function can also be expressed as a Laplace transform of the normalized distribution of decay rates, $G(\Gamma)$, which is equivalent to the distribution of translational diffusion coefficients, $D_t$, since $\Gamma = Dq^2$:

$$g^{(1)}(\tau) = \int_{0}^{\infty} d\Gamma G(\Gamma) \exp(-\Gamma \tau)$$  \hspace{1cm} (11)

Several methods are available to extract information regarding $G(\Gamma)$. For example, $G(\Gamma)$ can be determined via an inverse Laplace transform of $g^{(1)}(\tau)$ by an algorithm such as CONTIN$^{33}$ Here, however, we use a fitting procedure developed by Strelezky and coworkers$^{34-36}$ referred to as line shape analysis, which involves fitting $g^{(1)}(\tau)$ to a sum of stretched exponentials decays, using nonlinear-least-squares simplex based minimization:

$$g^{(1)}(q, \tau) = \sum_{i=1}^{N} A_i \exp(-\theta_i \tau^{\beta_i})$$  \hspace{1cm} (12)

where $N$ is the number of relaxation modes; $A_i$ is the amplitude of the $i_{th}$ mode; $\theta_i$ is the decay pseudorate for the $i_{th}$ mode; and $\beta_i$ is the stretching parameter. The RMS error and stability of fitting parameters indicate the goodness and reliability of the fit. We found that the experimental data could be fitted to two modes and that the RMS error was very
small. Spectral time moments\textsuperscript{24} analysis was then applied to the resulting best fit functions, and spectral time moments $M_{ni}$ computed for the $i_{\text{th}}$ mode. The zeroth time moment for the $i_{\text{th}}$ mode is calculated as:

\begin{equation}
M_{0i} = \int_{0}^{\infty} d\tau \exp(-\theta_i \tau^{\beta_i}) = \frac{\gamma(1+1/\beta_i)}{\theta_i^{\beta_i}}
\end{equation}

where $\gamma(1+1/\beta_i)=(1/\beta_i)!$ is the Gamma function. For each decay mode, the mean relaxation time, $<\Gamma_i>$, is equal to the zeroth time moment. So the mean decay rate and mean diffusion coefficient, $D_i$, for the $i_{\text{th}}$ mode can be calculated as follows:

\begin{equation}
\langle \Gamma_i^{-1} \rangle = M_{0i}
\end{equation}

\begin{equation}
D_i = \frac{\langle \Gamma_i \rangle}{q^2}
\end{equation}

For VH scattering, the mean rotational diffusion, $\Theta$, is determined by extrapolation of the mean decay rates to $q = 0$, since:

\begin{equation}
\langle \Gamma_i \rangle = D_i q^2 + 6\Theta_i
\end{equation}

The line-shape analysis method enables accurate determination of the mean decay rate and the mean diffusion coefficient without requiring exceptionally low noise levels in the intensity correlation function and hence exceptionally long signal accumulation times, needed when applying programs that use algorithms such as CONTIN. In addition to line shape analysis, the normalized decay rate distribution $G(\Gamma)$ was evaluated via an inverse Laplace transform of $g^{(1)}(\tau)$ using CONTIN\textsuperscript{33} and produced similar results, viz. the correlation functions feature two relaxation modes with mean relaxation times comparable to those determined by the above-described line shape analysis.
For monodisperse rods in dilute solution, the polarized and depolarized light scattering electric field correlation functions $I_{VV}$ and $I_{VH}$ (proportional to $g^{(1)}(\tau)$ in eq 10) are related to the rod translational and rotational diffusion coefficients ($D$ and $\Theta$, respectively) via eqs 17 and 18:

$$I_{VV}(q, \tau) = \langle N \rangle \kappa_{\text{iso}}^2 \exp(-q^2 D \tau) + \frac{4}{45} \langle N \rangle \kappa_{\text{aniso}}^2 \exp[-(q^2 D + 6\Theta)\tau]$$

(17)

$$I_{VH}(q, \tau) = \frac{1}{15} \langle N \rangle \kappa_{\text{aniso}}^2 \exp[-(q^2 D + 6\Theta)\tau]$$

(18)

Here, $\langle N \rangle$ is the average number of rods in scattering volume. $\kappa_{\text{iso}}$ is the isotropic part of the polarizability tensor and $\kappa_{\text{aniso}}$ is the molecular optical anisotropy. The second term of eq 17 is generally not important. $D$ is determined from the $q^2$-dependence of the VV decay rate, and $\Theta$ is obtained from the VH decay rate extrapolated to zero $q$.

2.3 Results and Discussion

2.3.1 Structural Analysis of Hydrogels: Small Angle Neutron Scattering (SANS)

In Figure 2.2, we show the SANS scattering profiles, measured over a wide range of scattering vectors, $0.07 \text{ nm}^{-1} \leq q \leq 14 \text{ nm}^{-1}$, of gels derived from mixtures of Guanosine (G) and 2’, 3’, 5’-Tri-O-Acetylguanosine (TAcG) having three different compositions. In Figure 2.3 these data are replotted in the form of a Holtzer plot, i.e. $qI(q)$ versus $q$. The plateau regime in the intermediate $q$ range ($0.12-0.6 \text{ nm}^{-1}$) is a characteristic indicator that the scattered intensity decays as $I(q) \sim q^{-1}$, i.e. that long rigid-rod segments exist in the gel. The sharp decay and increase of intensity at large $q$ reflects diffractive scattering from the rod cross-section, and thus is also emblematic of the presence of rigid rod structure. Of particular interest in Figure 2.3 is the fact that the width of the plateau regime clearly decreases with increase in TAcG content, indicative a
corresponding decrease in the length scale of the rod-like character of the columnar stacks. This conclusion is amplified in Figure 2.4, which displays plots of log(I(q)) versus log(q), together with orientation lines showing the scaling \( I(q) \sim q^{-1} \). Clearly the range of q-vectors where the \( q^{-1} \) scaling applies diminishes with increased TAcG content.

**Figure 2.2.** SANS scattering profiles of gels formed by G and TAcG in D\(_2\)O containing 0.354 mol\(^{-1}\) KCl with three different compositions 60/40, 50/50 and 40/60 as indicated in the figure.
Figure 2.3. Holzer plot of SANS scattering from G/TAcG gels having G/TAcG weight ratios of 60/40, 50/50 and 40/60.

Figure 2.4. Plots of log I(q) versus log q using SANS data in the scattering vector range 0.012-0.08 Å⁻¹ for three gels having G/TAcG weight ratios of 60/40, 50/50 and 40/60; the solid lines have a slope of -1.
In D₂O, the labile hydrogens in TAcG and G are exchanged with deuterium which reduces the contrast between the guanine units and solvent. However, the hydrogens on the acetyl groups of the ribose ring do not exchange (Figure 2.5). Hence the non-labile hydrogens in D₂O, which decorate the exterior of the cylindrical stacks, make the dominant contribution to the scattered intensity.

Figure 2.5. Core shell model assumed to simulate scattering from the G/TAcG gels (core atoms are in black font, shell atoms in red font).

As noted by Buhler et al.⁴¹ the scattered intensity in the intermediate q⁻¹ rod scattering range, i.e. the plateau regime in the Holtzer plot (Figure 2.2: 0.12-0.6 nm⁻¹) can be described by 
\[ I(q) \sim (\Delta \rho)^2 \times M_L \times \pi / q, \]
which enables a determination of \( M_L \), the molecular weight per unit length of the columnar stacks of G/TAcG quartets. Specifically, the scattered intensity can be expressed as the sum of a form factor \( P(q) \) and a structure factor \( S(q) \) as indicated in equation 19:

\[
I(q) = (\Delta \rho)^2 (\varphi_{\text{vol}} V_{\text{chain}} P(q) + S(q))
\]

where \( V_{\text{chain}} \) is the volume of one chain (rod). We neglect \( S(q) \), which reflects inter-rod interferences, and write \( P(q) \) as

\[
P(q) = \frac{\pi}{qL_c} + \frac{2}{3q^2L_cL_r L_c}
\]
where $L_c$ is the contour length of the rods, and $L_T$ is the total persistence length (i.e. the sum of the conformational and electrostatic contributions). In the intermediate $q^{-1}$ range, the second term in equation (20) can also be neglected. Hence, inserting equation (19) into (20), we obtain equation (21):

$$I(q) = \frac{\pi \phi_{col} (\Delta \rho)^2}{N_A \delta} \frac{M_L}{q}$$  \hspace{1cm} (21)

where $N_A$ is Avogadro's number; $\delta$ is the density of the gelator, and we have used the relationships $V_{chain} = M_n / \delta$, where $M_n$ is the number-average molecular weight of the rod, and $M_L = M_n / L_c$. The scattering intensity mainly comes from the contrast of the shell, so using the data in Figure 2.4 and the above relationship, the average mass per unit length $M_L$ (considering the shell only) can be calculated and it leads to determination of the average distance $d$ between quartets (Table 2.1). The resulting value of $d$ is larger than that generally accepted as the typical stacking repeat of guanosine-based quartets (3.4 Å). A comparable result has been reported for a similar gel formed by a lipophilic guanosine derivative and indicates a looser structure such as a continuous helical strand. 2D wide angle X-ray scattering on the gels was attempted to confirm a helical structure. Here, the key requirement is to obtain diffraction on a specimen in which the fibers are uniformly aligned. However efforts to align the fibers were not successful despite trying different sample preparation methods, including drying gel specimens over the ends of a U-shaped piece of paper clip; drying gel specimens between metal supports and extruding the gel from a syringe.
**Table 2.1.** Mass per unit length and distance between quartets for gels of three compositions

<table>
<thead>
<tr>
<th>G/TAcG</th>
<th>40/60</th>
<th>50/50</th>
<th>60/40*</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M_L$ (g/mol/Å)</td>
<td>200.2±14.5</td>
<td>175.9±11.5</td>
<td>130.0±7.2*</td>
</tr>
<tr>
<td>$d$ (Å)</td>
<td>4.2±0.3</td>
<td>4.4±0.3</td>
<td>5.6±0.3*</td>
</tr>
</tbody>
</table>

*Concentration is overestimated, so $M_L$ is smaller and $d$ is larger than true value.

For gels composed of rigid cylindrical rod segments, the intensity at larger $q$ is dominated by the interferences due to the finite width of rods, and can be described using equations 22 and 23:

$$I(q) = \phi_{\text{vol}} (\pi \Delta \rho)^2 \frac{2}{q} \exp\left(-\frac{q^2 r^2}{4}\right)$$

(22)

$$q = \frac{4\pi}{\lambda} \sin\left(\frac{\theta}{2}\right)$$

(23)

Here $\phi_{\text{vol}}$ is gelator concentration (volume fraction of rods), $\Delta \rho$ is the scattering contrast; $r$ is the radius of the rodlike scatterer and the cross-sectional radius of gyration is $R_c = r / \sqrt{2}$. Rearranging Eq. (22) into Eq. (24), evidently, the cross-sectional radius can be determined from a plot of $\ln(I(q) \times q)$ versus $q^2$ (Figure 2.6).

$$I(q)q = 2\phi_{\text{vol}} (\pi R_c \Delta \rho)^2 \exp\left(-\frac{q^2 R_c^2}{2}\right)$$

(24)

The values of $R_c$ for the three gels are listed in Table 2.2, and indicate a progressive increase with increased $\text{TAcG}$ content. The increase of $R_c$ with increased $\text{TAcG}$ content is to be expected since the non-labile hydrogens of the $\text{TAcG}$ groups make an increasingly larger contribution to the scattered intensity.
Figure 2.6. Plots of \( \ln(I(q)\times q) \) versus \( q^2 \) enable determination of the cross-sectional radius, \( R_C \), of the three \( G/T\text{AcG} \) gels (60/40: \( R_C = 7.2 \) Å, 50/50 \( R_C = 8.8 \) Å, and 40:60: \( R_C = 9.5 \) Å).

We used SANS Analysis software package v3.0 from the NIST Center for Neutron Research to fit the experimental scattering profiles on Igor Pro 6.0. In D\(_2\)O, labile hydrogens in \( G/T\text{AcG} \) will be substituted by deuterium, which results in a gradation in scattering density length and hence makes the use of a core shell cylinder model plausible (Figure 2.5). The scattering length density in the core is close to that of the solvent, and so the model approximates that of a hollow cylinder.

We utilized the form factor expression developed by Livsey\(^50\) for a monodisperse cylinder, having a circular cross-section, with a core-shell scattering length density profile:

\[
P(q) = \frac{\text{scale}}{V_{\text{total}}} \int_0^{\pi/2} f^2(q, \alpha) \sin \alpha d\alpha \quad (25)
\]
where

\[
f(q, \alpha) = 2(\rho_{\text{core}} - \rho_{\text{shell}})V_{\text{core}} j_0(qH \cos \alpha) \frac{J_1(qr \sin \alpha)}{qr \sin \alpha}
\]

\[+ 2(\rho_{\text{shell}} - \rho_{\text{solvent}})V_{\text{total}} j_0[q(H + t) \cos \alpha] \frac{J_1[q(r + t) \sin \alpha]}{q(r + t) \sin \alpha}
\]

(26)

where \(j_0(x) = \sin(x)/x\), \(V_{\text{core}} = \pi r^2 L\), and \(V_{\text{shell}} = \pi (r + t)^2 L_{\text{total}}\), where \(r\) is the radius of the core of the cylinder, \(t\) is the thickness, \(L\) is the length of the core, \(H = L/2\), \(L_{\text{total}} = 2H + 2t\), and \(J_1(x)\) is the first order Bessel function \((J_1(x) = \frac{x}{2}[1 - \frac{(x/2)^2}{2(1!)^2} + \frac{(x/2)^4}{3(2!)^2} - \frac{(x/2)^6}{4(3!)^2} + ...])\).

\(\alpha\) is defined as the angle between the cylinder axis and the scattering vector, \(q\). The integral over \(\alpha\) averages the form factor over all possible orientations of the cylinder. The shell thickness, \(t\), is considered to be uniform over the entire surface of the core. The form factor is normalized by the total particle volume such that \(P(q) = \frac{<f^2> / V_{\text{total}}}{\text{scale} + \text{bkg}}\), where \(f\) is the scattering amplitude, \(<\>\) denotes an average over all possible orientations of the cylinder, and scale and bkg are fitting constants. Utilizing the core-shell model as expressed in eqs 25 and 26, excellent least-squares fits to the experimental SANS profiles of the three gels over the entire range of q-vectors can be obtained. In generating these fits the SANS analysis software package v3.0 from the NIST Center for Neutron Research on Igor Pro 6.0 was used, inputting the computed scattering length densities of the core, shell and solvent, and constraining the fit to reproduce the position of the high-q peak in the SANS profile. The latter peak, evident in Figure 2.2 near 3 nm\(^{-1}\), arises from the cross-sectional form factor of the rod, and from the position of the peak the cylinder diameter can be estimated. The resulting least-squares fits to the
The cross-sectional radius derived from a plot of $\ln(I(q)q)$ versus $q^2$ lies numerically between the value of the core radius $R_{\text{core}}$ and total radius ($R_{\text{core}} + R_{\text{shell}}$), and is evidently determined not only by the cross-sectional dimensions but also by the scattering length densities of core and shell. A shell rich in $\text{TAcG}$ has a larger scattering contrast to the core than a shell rich in $\text{G}$. Thus, the radius determined from the plot of $\ln(Iq)$ versus $q^2$ increases relative to the total cross-sectional radius, as the amount of $\text{TAcG}$ increases.

Figure 2.7. The experimental data of three gels and fitting curve using core shell cylinder model (fitting curve in black).

In summary, analysis of the SANS profiles of hydrogels formed through self-assembly of Guanosine (G) and 2',3',5'-Tri-O-Acetylguanosine (TAcG) in D$_2$O containing K$^+$ ions indicates $q^{-1}$ scaling of the scattered intensity in the intermediate q-vector range,
indicative that the hydrogels are composed of highly rigid fibers. The width of the $q^{-1}$ scaling regime decreases with increase in TAcG content indicative of an increase in flexibility associated with the increasing insertion of TAcG groups into the columnar aggregates, which interferes with the stacking interactions between quartets. From the height of the plateau region in the Holtzer plot, an estimate of the mass per contour length, $M_L$, is obtained. The results yield values significantly larger than the expected stacking repeat of guanosine-based quartets, suggesting a looser organization of the G/TAcG quartets. At higher $q$, interferences from the cross-section of the columnar aggregates allow an estimation of the cross-sectional radius of gyration, $R_c$. The resulting values increase with the TAcG content of the columns, consistent with the conclusions that the SANS profiles can be accurately fit by a core-shell cylinder model, where the guanine-quartet macrocycle, with its labile hydrogens, formed via self-association with $K^+$, is assumed to constitute the core, and the pendant triacetylribose groups with non-labile methyl groups constitute the shell.

**Table 2.2.** Comparison of SANS cross-sectional radius of gyration versus dimensions of core shell model.

<table>
<thead>
<tr>
<th>G/TAcG</th>
<th>ln(I(q)q) vs. $q^2$</th>
<th>Core-shell cylinder model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Radius (nm)</td>
<td>Core radius (nm)</td>
</tr>
<tr>
<td>60/40</td>
<td>0.72±0.02</td>
<td>0.71±0.02</td>
</tr>
<tr>
<td>50/50</td>
<td>0.88±0.01</td>
<td>0.90±0.02</td>
</tr>
<tr>
<td>40/60</td>
<td>0.95±0.01</td>
<td>0.73±0.02</td>
</tr>
</tbody>
</table>

The SANS results provided insight into results of rheological experiments...
performed on \(G/T\text{AcG}\) gels by L. E. Buekle.\textsuperscript{22} Specifically, it was observed that the decrease in cylinder length with increased \(T\text{AcG}\) content of the gels derived from the fits to the SANS profiles (Table 2.2) correlates to a decrease in yield stress of the gels with increasing \(T\text{AcG}\) content (Figure 4(a) in ref. 22).\textsuperscript{22} This is consistent with the expectation that an increase in rigidity of the fibrils which comprise the gel network would result in an increase in modulus and yield stress of the gel. A complication arises, however, in that the yield stress was determined by a strain sweep using oscillatory shear at a frequency of 10 rad/s, and the temperature-dependence of the gel storage modulus \(G'(\omega)\), also measured at a frequency of 10 rad/s, shows an unusual stepwise decrease from a low temperature plateau value, which decreases with increasing \(T\text{AcG}\) content, in agreement with the SANS results, to a high temperature plateau, whose value shows an increasing trend with \(T\text{AcG}\) content (Figure 10 in ref. 22).\textsuperscript{22} Analysis of the frequency dependence of the storage and loss moduli shows that \(G'(\omega)\) features a high frequency predominantly elastic plateau where \(G' > G''\), with a crossover at lower frequency, \(\omega \sim 2\) rad/s, to a frequency regime where \(G'' > G'\), and then a second crossover at even lower frequency \((\omega \sim 10^{-1}\) rad/s), below which \(G' > G''\) again (Figure 11(b) in ref. 22).\textsuperscript{22} This behavior implies that, in addition to the permanent network, gel structure features a transient network component with a relaxation time \(\tau \sim 1/\omega \sim 0.5\) s. Moreover, the temperature dependence of \(G'\) and \(G''\) indicates that the transient network component disappears when the temperature is increased, but the permanent network remains, which explains the two-step feature in the temperature dependence of \(G'(\omega)\), measured at \(\omega = 10\) rad/s: the low temperature modulus (and yield stress) reflect the total (transient + permanent) network elasticity, the high temperature modulus reflects only the elasticity of the permanent
network structure. It follows that the transient network modulus decreases with increasing TAcG content, whereas the permanent network modulus increases with TAcG content, at least at higher temperatures. Moreover, the above-referenced good correlation seen between the composition-dependence of the SANS data and the low-temperature yield modulus at $\omega = 10$ rad/s indicates that the columnar stacks formed by G/TAcG quartets are a key component of the transient network. To gain further insight into the structural organization of the G/TAcG gels, experiments were undertaken to elucidate the mechanism by which G/TAcG gels form.

Finally, Macromodel software (http://www.schrodinger.com/ProductDescription.php?mID=6&sID=8) was used to predict the molecular conformation of the planar quartet complex self-assembled via association of G and TAcG in the presence of potassium ions. Macromodel uses the force field method to compute the potential energy as a function of distance and angles between atoms. The conformation having the lowest global potential energy is shown in Figure 2.8, and has a core diameter of 13 Å, which is very close to the values determined above using SANS (13.8 Å – 14.6 Å). The total diameter of the column is 24.4 Å, again comparable to that determined by SANS (29.4 Å – 30.8 Å). The core is rigid because of hydrogen bonding and ion-dipole interactions, but the shell is quite flexible and can adopt various conformations. Thus, it is to be expected that the simulated dimension for the core will compare more favorably with the SANS values than for the shell. The dimensions deduced for our analysis are very consistent with literature values for similar structures, determined either from X-ray crystallography\textsuperscript{51} or from structural models developed using density functional theory.\textsuperscript{52-54}
Figure 2.8. The simulation of the self assembly of 2’,3’,5’-Tri-O-Acetylguanosine and Guanosine in the presence of potassium ion. (a. side view, b. overlook)

2.3.2 The Gelation Mechanism of G/TAcG Hydrogels

A 50/50 G/TAcG ratio was chosen to investigate the process of gel formation. From previous dynamic light scattering (DLS) data generated by L. E. Buerkle, the gel point was found to occur at a concentration of around 0.8 wt%. Thus, a concentration series of 50/50 G/TAcG in the range of 0.05 wt% to 1 wt% as well as a 2 wt% gel were prepared for characterization using scattering techniques and viscometry.
2.3.2.1 Small Angle X-ray Scattering (SAXS)

SAXS data was collected as a function of concentration up to 1.0 wt% in order to encompass the sol-gel transition. A satisfactory signal was obtained for concentrations higher than 0.5 wt% after background subtraction. Thus, the following analysis is described for G/TAcG compositions of 0.5 wt%, 0.8 wt% and 1 wt%. The accessible lower q range was similar to the earlier SANS studies (0.07 nm\(^{-1}\))\(^{22}\) but was truncated at higher q (1.5 nm\(^{-1}\) versus 14 nm\(^{-1}\)). As demonstrated in Figure 2.9(a), and consistent with our earlier SANS experiments, in the intermediate q regime (0.13-0.6 nm\(^{-1}\)), the scattered intensity I(q) of SAXS was observed to decrease proportionally to q\(^{-1}\), indicative of scattering from rod-like species. Likewise, Holtzer plots of qI(q) versus q of the SAXS data (Figure 2.9(b)), also exhibit similar behavior to the SANS results, i.e., they feature a plateau regime in the intermediate q range (0.13-0.6 nm\(^{-1}\)), again a strong indication of scattering from long rigid rods.
Figure 2.9. (a) Plots of log I(q) versus log q using SAXS data in the scattering vector range 0.12-0.6 nm\(^{-1}\) for G/TAcG 50/50 at three concentrations of 0.5 (orange squares), 0.8 (red circle), 1 (green triangle) wt% in aqueous 0.354 M KCl solvent. Black lines are linear fits and have a slope of -1. (b) Holtzer plots (log qI(q) versus log q) of SAXS data from G/TAcG 50/50 at three concentrations of 0.5 (orange squares), 0.8 (red circle), 1 (green triangle) wt% in 0.354 M aqueous KCl. Observation of I(q) ~ q\(^{-1}\) and of a plateau in qI(q) indicate a rigid rod structure within the samples.

Following the same procedure for the SANS data analysis, according to equation 24, the cross-sectional radius of gyration, R\(_c\), of the rods can be determined from a plot of ln(I(q)\times q) versus q\(^2\) at large scattering vectors, q = 1.0 – 1.4 nm\(^{-1}\) (Figure 2.10). The resulting R\(_c\) values in Table 2.3 are in the range 1.05-1.09 nm, and are a little larger than the R\(_c\) value (0.88 nm) deduced from the corresponding fits of the SANS scattering of a 2 wt% gel. In contrast to the SANS experiments, the electron density of the core here (K\(^+\)) is similar to that of the shell.\(^{55}\) Thus the values of the cylinder radius, r = 1.48 – 1.54 nm, deduced from the above SAXS R\(_c\) results via r = R\(_c\)\sqrt{2}, are numerically comparable to the
corresponding radius \( r = (R_{\text{core}} + T_{\text{shell}}) = 1.47 \pm 0.02 \) nm, deduced from the SANS data via core-shell cylinder fits. From these considerations, it further follows that a solid circular cylinder form factor \( P(q) \) is found to accurately fit the SAXS patterns:

\[
P(q) = \frac{\text{scale}}{V_{\text{total}}} \int_0^{\pi/2} r^2(q, \alpha) \sin \alpha \, d\alpha
\]

where

\[
f(q, \alpha) = 2(\rho_{\text{cylinder}} - \rho_{\text{solvent}}) V J_0[qL \cos \alpha / 2] \frac{J_1[qR \sin \alpha]}{qR \sin \alpha}
\]

and \( V = \pi R^2 L \), where \( R \) is the radius of the cylinder, \( L \) is the length, and \( J_1(x) \) is the first order Bessel function \( (J_1(x) = \frac{x}{2}[1 - \frac{(x/2)^2}{2(1!)} + \frac{(x/2)^4}{3(2!)} - \frac{(x/2)^6}{4(3!)} + ...]) \). \( \alpha \) is defined as the angle between the cylinder axis and the scattering vector, \( q \). The integral over \( \alpha \) averages the form factor over all possible orientations of the cylinder. Using \( R_c \) from ln(I(q) \times q) versus \( q^2 \) plot, the least squares fits shown in Figure 2.11 leads to the cylinder lengths listed in Table 2.3. The lengths from the fits are comparable to those determined via SANS for the same \( \text{G/TAcG} \) composition at 2 wt\% (32.0\pm1.0 nm) in Table 2.2, and do not increase with concentration, consistent with the expectation that the columnar stacks of quartets have reached their asymptotic size at these concentrations.
Figure 2.10. Plots of $\ln(I(q) \times q)$ versus $q^2$ enable determination of the cross-sectional radius, $R_c$, of the G/TAcG 50/50 at three concentrations of 0.5 (orange squares), 0.8 (red circle), 1 (green triangle) wt% in 0.354 M aqueous KCl. The calculated radii are shown in Table 2.3.

Figure 2.11. SAXS scattering profiles of G/TAcG (50/50) at three concentrations of 0.5 (orange squares), 0.8 (red circle), 1 (green triangle) wt% in 0.354 M aqueous KCl, and their fits to circular cylinder model (in black).
Table 2.3. Dimensions from ln(I(q)×q) versus q² plot and circular cylinder model fit to SAXS scattering profiles of G/TAcG (50/50) at three concentrations

<table>
<thead>
<tr>
<th>C (wt%)</th>
<th>0.5</th>
<th>0.8</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>R_c (nm)</td>
<td>1.04±0.01</td>
<td>1.09±0.01</td>
<td>1.07±0.01</td>
</tr>
<tr>
<td>L (nm)</td>
<td>39.9±0.3</td>
<td>39.5±0.2</td>
<td>39.7±0.1</td>
</tr>
</tbody>
</table>

2.3.2.2 Viscosity Measurement

Figure 2.12. Concentration dependence of specific viscosity for G/TAcG (50/50) in 0.354 M aqueous KCl. A sudden increase in viscosity occurs at 0.3 wt%.

The concentration dependence of the specific viscosity of G/TAcG (50/50) is exhibited in Figure 2.12. Solutions at concentrations up to 0.2 wt% have viscosities indistinguishable from that of the solvent within experimental error, but a dramatic increase in \( \eta_{sp} \) occurs at \( c \geq 0.3 \) wt%. Evidently, therefore, a transition point exists between 0.2 and 0.3 wt% where a rapid growth in molecular hydrodynamic volume, \( V_h \), occurs and where the growth in \( V_h \) is much larger than the corresponding growth in molar
mass. This would be consistent with the sudden appearance of rod-like particles, for which $V_h \sim M^3$, and hence $\eta_{sp} \sim cV_h/M \sim n_pM^3$, where $n_p$ is the molar concentration of particles.

2.3.2.3 Differential Refractive Index Increment Measurement

As noted earlier, the deflection of light passing through the split cell (S) in the differential refractometer is proportional to the refractive index difference between solution and solvent. Calibration was performed at three wavelengths (589, 564, 436 nm), using aqueous KCl solutions at several concentrations (1, 5, 10, 20, and 30 g/L) (whose known wavelength-dependent $dn/dc$ values are listed in Table 2.4). The wavelength dependence of $dn/dc$ of G/TAcG 50/50 aqueous solution was then determined using concentrations of 0.15, 0.2, 0.25, 0.3 wt%. First, as shown in Appendix A, the slope of a plot of displacement versus refractive index of KCl solutions (Figure A1.1) generates values of $dS/dn$ for the instrument at three wavelengths (Table A1.1). Next, the slope of a plot of displacement versus concentration for 50/50 G/TAcG solutions (Figure A1.2) gives $dS/dc$ values for the unknown mixture (Table A1.2). Using $dS/dn$ of the instrument (Table A1.1) and $dS/dc$ for G/TAcG mixture (Table A1.2), the $dn/dc$ was calculated at three different wavelengths (Table 2.5) and plotted in Figure 2.13. From the wavelength dependence of the differential refractive index, $dn/dc$, of 50/50 w/w G/TAcG solutions in 0.354 M aqueous KCl, we determined $dn/dc = 0.179 \pm 0.004 \text{ mL/g at } \lambda = 514.5 \text{ nm}$. 

60
Table 2.4: Differential refractive index increment of KCl at 25°C and three different wavelengths.

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>436</th>
<th>546</th>
<th>589</th>
</tr>
</thead>
<tbody>
<tr>
<td>dn/dc</td>
<td>0.135660</td>
<td>0.131033</td>
<td>0.129966</td>
</tr>
</tbody>
</table>

Table 2.5: Differential refractive index increment of G/TAcG 50/50 at 25°C and three different wavelengths.

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>436</th>
<th>546</th>
<th>589</th>
</tr>
</thead>
<tbody>
<tr>
<td>dn/dc</td>
<td>0.171±0.002</td>
<td>0.176±0.004</td>
<td>0.191±0.005</td>
</tr>
</tbody>
</table>

Figure 2.13. Wavelength dependence of dn/dc of G/TAcG (50/50) in 0.354 M aqueous KCl. From the fitted line, at \( \lambda = 514.5 \) nm (\( 1/\lambda^2 = 3.78 \times 10^{-6} \) nm\(^{-2} \)), we determined dn/dc = 0.179 ± 0.004 mL/g.

2.3.2.4 Static Light Scattering (SLS)

To further investigate the onset of G/TAcG quartet self-assembly, static light scattering (SLS) of 50/50 G/TAcG was performed in aqueous 0.354 M KCl at a series of
concentrations between 0.05 to 0.8 wt%, the latter concentration representing the gel point previously determined by L.E. Buerkle, using dynamic light scattering measurements. The concentration- and angle-dependence of the scattered intensity is displayed in the form of a Zimm plot in Figure 2.14 (see equations 4-7). The weak intensity data at the two lowest concentrations show no systematic angle dependence within experimental error, indicating solute species smaller than 1/20 of the laser wavelength. Averaging the intensities at all scattering angles yields apparent weight-average molecular weights, $M_w^{\text{app}}$, of $667 \pm 45$ g/mole (0.05 wt%) and $833 \pm 185$ g/mole (0.1 wt%), each substantially smaller than the mass of a 50/50 G/TAcG quartet ($M = 1385$ g/mole) but larger than that of either component ($G$: 283.24 g/mole and TAcG: 409.35 g/mole). At these two concentrations, it therefore appears that species smaller than G/TAcG tetramers are present, e.g. an equilibrium mixture of monomers, oligomers and quartets. If we assume there is only an equilibrium between monomers and quartets, and that, $2G + 2\text{TAcG} \rightleftharpoons \text{Quartet}$, then based on the measured apparent overall molecular weights, $M_w^{\text{app}}$, determined via SLS, the resulting apparent overall binding constant is estimated to be $1.4 \pm 0.4 \times 10^9$ (L/mol)$^3$ (i.e. the mean of $1.76 \times 10^9$ (L/mol)$^3$ for 0.05 wt%, and $1.01 \times 10^9$ (L/mol)$^3$ for 0.1 wt%). For 0.2 wt% G/TAcG, where significant stacking of quartets already occurs, the mass fraction of quartets is estimated from this value of the overall binding constant to be $66.7 \pm 9.0 \%$, where the error reflects the relatively high uncertainties in the two measured molecular weights.

At higher concentrations, as seen in Figure 2.14, the scattered intensity has significant angle dependence, indicating the appearance of particles whose sizes are large compared to the laser wavelength. The values of $Kc/\Delta R(0,c)$ at each concentration were
determined by extrapolation to $q^2 = 0$ of plots of $K_c/\Delta R(q,c)$ versus $q^2$ (Figure A1.3) and the results are presented in Figure 2.15. Furthermore, apparent weight-average molecular weights can be directly calculated as the reciprocals of $K_c/\Delta R(q=0)$ values and are listed in Table 2.6. The Zimm plots at 0.2 wt% clearly show significant angle dependence and yield a much higher $M_w^{\text{app}} = 2.5\pm0.8 \times 10^4$ g/mole, suggesting that the critical concentration where the onset of aggregation occurs falls between 0.1 wt% and 0.2 wt%. This result can be compared with the relative viscosity measurements in Figure 2.12, which show a sudden increase at 0.3 wt%. Evidently, the SLS technique is more sensitive to the appearance of the rod-like columnar stacks of quartets than shear viscosity.

**Figure 2.14.** Zimm plots of G/TAcG 50/50 in 0.354 M aqueous KCl. Inset enlarges plots for the four higher concentrations. The weak intensity data at the two lowest concentrations show no systematic angle dependence within experimental error, indicating solute species smaller than 1/20 of the laser wavelength, while at higher concentrations, the scattered intensity has significant angle dependence indicating the appearance of particles whose sizes are large compared to the laser wavelength.
With further increase in concentration, $M_w^{\text{app}}$ increases between 0.2 wt% to 0.3 wt%, and then reaches a plateau at $1.5-1.6 \pm 0.15 \times 10^5 \text{ g/mole}$.

Apparent values of the z-average radius of gyration $R_{g,z}^{\text{app}}$ were also determined from the slopes and intercepts of least squares fits to the q-dependent data for each concentration in the Zimm plots of Figure 2.14 (the fits are shown in Figure A1.3 of Appendix A), and the resulting $R_{g,z}^{\text{app}}$ values are given in Table 2.7. Note these values are relatively imprecise since the intercepts have quit large errors, and in fact depend on the number of angles points included in the fits. Moreover, the products $qR_{g,z}^{\text{app}}$ for data at 0.2% and 0.4% are much larger than unity making the results unreliable, since the use of Zimm plots requires $qR_{g,z}^{\text{app}} \ll 1$. Thus the values listed, based on fits to relatively few points at the smallest angles are to be viewed as upper bound estimates.

![Figure 2.15.](image) Concentration dependence of $K_c/\Delta R(q=0)$ from Zimm plots of low q data for $G/TAcG$ (50/50) in 0.354 M aqueous KCl.
Table 2.6. Apparent weight average molecular weight of series concentration of G/TAcG 50/50 from Zimm plots and their angle dependence.

<table>
<thead>
<tr>
<th>c (wt%)</th>
<th>Kc/ΔR_{θ=0} (mol/g)</th>
<th>Apparent M_w (g/mol)</th>
<th>Angle dependence</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 wt%</td>
<td>1.5<em>10^{-3} ± 9.9</em>10^{-5}</td>
<td>667±45</td>
<td>No</td>
</tr>
<tr>
<td>0.1 wt%</td>
<td>1.2<em>10^{-3} ± 2.9</em>10^{-4}</td>
<td>833±185</td>
<td>No</td>
</tr>
<tr>
<td>0.2 wt%</td>
<td>4.0<em>10^{-5} ± 2.0</em>10^{-5}</td>
<td>2.5<em>10^{4} ± 8</em>10^{3}</td>
<td>Yes</td>
</tr>
<tr>
<td>0.3 wt%</td>
<td>6.5<em>10^{-6} ± 7.2</em>10^{-7}</td>
<td>1.5<em>10^{5} ± 1.5</em>10^{4}</td>
<td>Yes</td>
</tr>
<tr>
<td>0.4 wt%</td>
<td>5.5<em>10^{-6} ± 1.1</em>10^{-7}</td>
<td>1.8<em>10^{5} ± 3.6</em>10^{3}</td>
<td>Yes</td>
</tr>
<tr>
<td>0.5 wt%</td>
<td>6.5<em>10^{-6} ± 2.3</em>10^{-7}</td>
<td>1.5<em>10^{5} ± 5.4</em>10^{3}</td>
<td>Yes</td>
</tr>
<tr>
<td>0.8 wt%</td>
<td>6.2<em>10^{-6} ± 2.0</em>10^{-7}</td>
<td>1.6<em>10^{5} ± 5.0</em>10^{3}</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 2.7. Apparent z-average radii of gyration of series concentration of G/TAcG 50/50 from Zimm plot. *: not reliable since q R_{g,z}^{app} is much larger than 1.

<table>
<thead>
<tr>
<th>R_{g,z}^{app} (nm)</th>
<th>0.2 wt%*</th>
<th>0.3 wt%</th>
<th>0.4 wt%*</th>
<th>0.5 wt%</th>
<th>0.8 wt%</th>
</tr>
</thead>
<tbody>
<tr>
<td>q R_{g,z}^{app}</td>
<td>2.9-4.3</td>
<td>1.4-1.7</td>
<td>1.6-2.3</td>
<td>1.1-1.4</td>
<td>0.9-1.2</td>
</tr>
</tbody>
</table>

Since qR_{g,z}^{app} is greater than unity using R_{g,z}^{app} values determined via Zimm plots, Guinier plots (Appendix A, Figure A1.4), ln(Kc/ΔR(q,c)) versus q², were used to determine more accurate values of Kc/ΔR(0,c), whose concentration dependence is presented in Figure 2.16. Apparent weight average molecular weights, M_w^{app}, were determined from Kc/ΔR(0,c) = 1/M_w^{app}, and from the slopes of plots of ln(Kc/ΔR(q,c))
versus $q^2$, apparent z-average radii of gyration $R_{g,z}^{\text{app}}$ were calculated (c.f. equation 8). The results are presented in Table 2.8 and, as for the results from Zimm plots, indicate a dramatic increase in $M_w^{\text{app}}$ from 833±185 g/mole at 0.1 wt% to $1.78\pm0.03 \times 10^4$ g/mole at 0.2 wt%, indicative that the critical concentration for stacking of G/TAcG quartets falls between 0.1 wt% and 0.2 wt%.

![Graph showing concentration dependence of $K_c/\Delta R$](image)

**Figure 2.16.** Concentration dependence of $K_c/\Delta R$ (at $q = 0$) from modified Guinier plots of low q data for G/TAcG (50/50) in 0.354 M aqueous KCl. The molecular weight and apparent z-average radii of gyration calculated from this data are shown in Table 2.8.

**Table 2.8.** Apparent weight average molecular weight and apparent z-average radii of gyration for series of concentrations of 50/50 G/TAcG obtained from Guinier plot.

<table>
<thead>
<tr>
<th>G/TAcG Conc. (wt%)</th>
<th>0.2%</th>
<th>0.3%</th>
<th>0.4%</th>
<th>0.5%</th>
<th>0.8%</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M_w^{\text{app}} \times 10^3$ (g/mol)</td>
<td>0.178±0.003</td>
<td>1.27±0.02</td>
<td>1.45±0.04</td>
<td>1.78±0.05</td>
<td>1.55±0.04</td>
</tr>
<tr>
<td>$R_{g,z}^{\text{app}}$ (nm)</td>
<td>100.4±0.8</td>
<td>69.4±0.9</td>
<td>68.4±1.7</td>
<td>65.8±2.0</td>
<td>47.8±2.1</td>
</tr>
<tr>
<td>$qR_{g,z}^{\text{app}}$</td>
<td>1.5-1.8</td>
<td>1.1-1.3</td>
<td>1.1-1.4</td>
<td>1.1-1.2</td>
<td>0.8-1</td>
</tr>
</tbody>
</table>
It should be noted that the measured $R_{g,z}^{\text{app}}$, while smaller than the values estimated via Zimm plots, are still too large to be numerically consistent with the measured $M_w^{\text{app}}$, using accepted values of the mass per unit length $M_L$ of columnar stacks of quartets. One source of the discrepancy arises because $R_{g,z}^{\text{app}}$ is a z-average quantity, while $M_w^{\text{app}}$ is a weight-average. Trace levels of large particles contribute more significantly to $R_{g,z}^{\text{app}}$ compared to $M_w^{\text{app}}$. Another source of error comes from the fact that $M_w^{\text{app}} = M_w/(1+2A_2M_wc+3A_3M_wc^2)$, where $A_2$ and $A_3$ are the second and third osmotic virial coefficients. Since $A_2$ and $A_3$ are typically positive in good solvents, this also tends to make $M_w^{\text{app}}$ too small. Recalling that the specific viscosity measurements show a sudden increase at 0.3 wt%, it appears that the number, $n_p$, and/or hydrodynamic volume, $V_h$, of the columnar aggregates of quartets are not large enough to significantly impact the viscosity at 0.2 wt%. Thus, SLS provides a more accurate estimate of the critical concentration for quartet association. With further increase in concentration, $M_w^{\text{app}}$ increases between 0.2 wt% to 0.3 wt%, and then reaches a plateau at $1.27-1.78 \pm 0.05 \times 10^5$ g/mole. Accompanying the observation of a plateau in $M_w^{\text{app}}$, we find that the apparent z-average radius of gyration, $R_{g,z}^{\text{app}}$, progressively decreases, from 100 nm at 0.2 wt% to 48 nm at 0.8 wt%. This suggests that, at these concentrations, SLS is probing intermolecular correlation length scales, which decrease with increase of concentration, i.e. that we are in the semidilute concentration regime. More detailed insight into the supramolecular organization of the columnar aggregates is obtained via DLS analysis described in the next section.

2.3.2.5 Polarized and Depolarized Dynamic Light Scattering

Polarized (VV) and depolarized (VH) dynamic light scattering experiments were
carried out on 50/50 G/TAcG in 0.354 M aqueous KCl as a function of concentration and scattering angle. VV and VH signals from dissolved G/TAcG only appeared for concentrations of 0.3 wt% and higher, and the corresponding electric field correlation functions $g_{VV}(1)(\tau)$ and $g_{VH}(1)(\tau)$ were each characterized by two discrete decay modes denoted as “fast” and “slow”. Intensity correlation functions for VV and VH scattering together with their fits to a sum of two stretched exponential decays (c.f. equation 12) are shown in Figures 2.17 and 2.18, respectively, and illustrate that, whereas the VV correlation functions show strong angular dependence, the VH correlation functions are essentially angle-independent. This is consistent with the former originating in scattering from translational diffusive modes, while the latter probes rotational diffusion. Hence a translational diffusion coefficient can be obtained from the decay rates of VV scattering and a rotational diffusion coefficient by extrapolating the VH decay rates to zero scattering vector. CONTIN multi-exponential analysis gave bimodal distributions with asymmetric distributions of decay rates, consistent with the stretched exponential decay profile of the correlations functions, and yielded similar mean decay rates to those evaluated via line shape analysis. The values of the stretched exponential exponents, $\beta$, were found to fall between 0.15 and 0.94. The VH fast mode $\beta$ values (ranging between 0.15 and 0.55) are substantially smaller than the VV fast mode $\beta$ values (ranging from 0.51 and 0.94), suggesting the former have a broader decay rate distribution. This may stem from the fact that the VH decay rates scale with particle volume whereas VV decay rates are related to particle radius. Also for both VV and VH there appears to be a trend toward smaller $\beta$ values at higher concentration, suggesting the width of the decay rate distribution increases with concentration. The concentration dependence of intensity
correlation functions at 90° scattering angle and their fits are shown in Figure 2.19.
Figure 2.17. Stretched exponential fits (black lines) to polarized DLS (VV) intensity correlation functions at scattering angles from $30^\circ$ to $90^\circ$ for G/TAcG (50/50) solutions, having wt% concentrations of (a) 0.3, (b) 0.4, (c) 0.5, (d) 0.6 and (e) 0.8, in 0.354 M aqueous KCl. The VV correlation functions show strong angular dependence of their decay rates, suggesting translational diffusive modes.
Figure 2.18. Stretched exponential fit (black lines) to intensity correlation function of depolarized DLS (VH) from 40° to 90° for 0.5 wt% G/TAcG (50/50) 0.3-0.8 wt% in 0.354 M aqueous KCl. Correlation functions and their fits were scaled for clarity by different factors: 0.1 for 90°; 0.2 for 80°; 0.3 for 70°; 0.4 for 60°; 0.6 for 50° and 0.8 for 40°. The decay rates of the VH correlation functions are relatively angle-independent.
suggesting rotational diffusion modes.

Figure 2.19. Stretched exponential fits (black curves) to the DLS intensity correlation functions at 90° scattering angle for G/TAcG (50/50) in 0.354 M aqueous KCl at concentrations from 0.3 wt% to 0.8 wt%: (a) VV scattering (b) VH scattering. In each case, the relative amplitude of the slow mode, which reflects scattering from microgel particles, increases strongly with increasing G/TAcG concentration.
For each concentration, the dependence of the fitted decay rates on the scattering vector $q$ was investigated. As an example, we show in Figures 2.20(a) and 2.20(b) the $q$-dependence of the mean decay rates of the fast and slow modes in VV and VH scattering, respectively, for $\text{G/TAcG (50/50)}$ in 0.354 M aqueous KCl. It was observed that the VV fast mode decay rates were indeed proportional to $q^2$, indicative of scattering from translational diffusive modes. In contrast, the VH decay rates show no systematic dependence on $q$-vector and it is evident that the computed decay rates are relatively imprecise, reflecting the relatively weak VH scattering intensity. It is also evident that, at large $q$-vectors, the VH decay rates fall below the VV values, in contrast to theoretical prediction (c.f equation 15 and 16). This is due to the fact that, as noted above, the VH relaxation time distributions are much wider than VV, and get wider with increasing $q$. Specifically, for 50/50 $\text{G/TAcG}$, as $q$ increases, the $\beta$ values decrease from 0.92 to 0.57 and from 0.46 to 0.31 for VV and VH fast modes respectively. The smaller VH $\beta$ values result in smaller mean decay rates, especially for higher $q$. The slow modes in both VV and VH have a more complex $q$-dependence, which reflects the fact that it is more difficult to obtain reproducible results for the slow modes. It was difficult to obtain reliable information from the correlation functions at very long decay times, due to the low signal to noise ratio and the need for longer acquisition times. However, a trend towards increasing rates with increasing $q$-vector appears to be representative of the VV slow mode, and $q$-independence for the decay rate of the VH slow mode (Figure 2.20(b)). Thus the balance of evidence suggests that these slow modes also represent translational (VV) and rotational (VH) modes.
Figure 2.20. (a) Scattering vector dependence of fast mode’s decay rates for VV (solid triangles) and VH (solid circles) of 0.5 wt% G/TAcG (50/50) in 0.354 M aqueous KCl. (b) Scattering vector dependence of slow mode’s decay rates for VV (solid triangles) and
VH (solid circles) of 0.5 wt% G/TAcG (50/50) in 0.354 M aqueous KCl. The data suggests a translational contribution to both the fast and slow modes in VV, while a rotational contribution exists in both modes of VH.

The amplitude of the slow mode in VV increases substantially relative to the fast mode as scattering angle decreases, whereas the relative contribution of each mode in VH is angle independent (shown in Figure 2.21). It is also apparent for both VV and VH that the slow mode becomes more and more dominant as concentration increases (shown in Figure 2.22). In principle, the slow mode may originate in microgel domains or hindered molecular motion of the rod-like aggregates, each of which reflect the approach of the system to gelation. The above static light scattering analysis indicates that we are in the semidilute concentration regime above the critical concentration (0.2 wt%). According to the Doi-Edwards\textsuperscript{56} and similar theories\textsuperscript{57-59} for the dynamics of rods in semidilute solution, the fast mode in VV represents unhindered longitudinal translational diffusion, and the fast mode in VH represents rotational diffusion of the rods. The scattering vector dependences of the fast modes in VV indeed exhibit the characteristics of dominant translational diffusion. In semidilute solution, the rotational diffusion process should be more hindered and increasingly slower at higher concentrations. However, the VH fast mode is observed to be concentration independent, which suggests that it originates in scattering from rotational diffusion of rod-like aggregates in dilute solution domains. It is therefore concluded that, above the critical concentration, the system consists of coexisting dilute and microgel domains, the former giving rise to the fast modes in VV and VH dynamic light scattering, via the translation and rotation, respectively, of rod-like aggregates, while translational and rotational diffusive scattering from the microgel
domains gives rise to the slow modes in VV and VH scattering, and also makes the dominant contribution to the static light scattering intensity.

(a)

(b)
Figure 2.21. (a) Scattering vector (angle) dependence of relative amplitude of slow mode for VV of G/TAcG (50/50) at 0.3, 0.4, 0.5, 0.6 and 0.8 wt% in 0.354 M aqueous KCl. (b) Scattering vector (angle) dependence of relative amplitude of slow mode for VH of G/TAcG (50/50) at 0.3, 0.4, 0.5, 0.6 and 0.8 wt% in 0.354 M aqueous KCl.

Figure 2.22. Concentration dependence of relative amplitude of slow modes at 90 degrees (q=0.023 nm\(^{-1}\)) for VV (black squares) and VH (red circles) of G/TAcG (50/50) in 0.354 M aqueous KCl. The amplitude of the slow mode in VV increases substantially relative to the fast mode as scattering angle decreases, whereas the relative contribution of each mode in VH is scattering angle independent. For both VV and VH that the slow mode becomes more and more dominant as concentration increases.

With regard to the aggregates whose scattering gives rise to the fast modes in VV and VH, it is clear that the sizes are substantially larger than those computed for the individual columnar stacks of quartets deduced from the SAXS analysis. Thus, it appears that the individual columnar entities coexist with larger aggregates. From the prior atomic
force microscopy (AFM) of 40/60 and 60/40 G/TAcG gels, the network fibers visualized have a height of 3 nm, a width of approximately 75 nm (±25 nm) and a persistence length of 300-600 nm. Also, from prior SANS analysis and SAXS data presented here, the height of 3 nm is identical to the diameter of the individual columnar stacked structures formed by the G/TAcG quartets. It thus appears that lateral interactions cause the individual rods to aggregate further into these larger fibrillar structures. Additionally, it is hypothesized that a spreading process occurs in AFM sample preparation, presumably due to attraction between the individual rods and the mica surface. Using the measured fiber width of 75 nm after spreading, and assuming individual rods laterally associate into larger cylindrical structures, we estimate a diameter of 16.9 nm (±3.0 nm) for these larger cylinders.

The Broersma\textsuperscript{60-62} equations relate $D$ and $\Theta$, respectively, the translational and rotational diffusion coefficients of long rigid rods in dilute solution, to their cross-sectional diameter $d$ and length $L$.

\begin{align*}
D &= \frac{k_b T}{3 \pi \eta L} \left[ \delta - \frac{1}{2} (\gamma_{//} + \gamma_{\perp}) \right] \quad (29) \\
\delta &= \ln \frac{2L}{d} \quad (30) \\
\gamma_{//} &= 0.807 + \frac{0.15}{\delta} + \frac{13.5}{\delta^2} - \frac{37}{\delta^3} + \frac{22}{\delta^4} \quad (31) \\
\gamma_{\perp} &= -0.193 + \frac{0.15}{\delta} + \frac{8.1}{\delta^2} - \frac{18}{\delta^3} + \frac{9}{\delta^4} \quad (32) \\
\Theta &= \frac{3k_b T}{\pi \eta L^3} (\delta - \xi) \quad (33)
\end{align*}
\[ \xi = 1.14 + \frac{0.2}{\delta} + \frac{16}{\delta^2} - \frac{63}{\delta^3} + \frac{62}{\delta^4} \] (34)

Equating the fiber diameter to that computed from the AFM images, the Broersma relations for translational and rotational diffusion coefficients in dilute solutions were employed for concentrations above the critical value to extract the contour length of the rods from the fast decays. The results are displayed in Figure 2.23, and indicate numerically comparable values were obtained from VV and VH DLS. The contour length falls between 300-500 nm and is consistent with persistence lengths (300-600 nm) estimated from AFM images. It is evident that, within experimental error, the contour length remains independent of concentration above the critical concentration, which implies that the formation of microgel domains and ultimately gelation follows because of an increase in the number density of fibrils.

In summary, the SLS and DLS results suggest the coexistence of dilute (sol) and microgel domains above the critical concentration. The scattering amplitude of the microgel domains increases as the concentration increases towards the gel point (~ 0.8 wt%).
Figure 2.23. Fibrillar lengths from Broersma’s relation using translational and rotational diffusion coefficients from fast mode’s decay rates of VV (black squares) and VH (red circles) respectively for G/TAcG (50/50) in 0.354 M aqueous KCl.

It is apparent from the above results, that the SAXS and SANS techniques probe the same short length scale characteristics of the individual columnar stacked structures formed by the G/TAcG quartets, which are cylindrical in shape, with a radius \( r = \sqrt{2} R_c = 1.51\pm0.03 \) nm and a length \( L = 39.7\pm0.3 \) nm. These techniques did not detect higher level structure because the scattering vector range did not access small enough values. The SLS technique probes an intermediate length scale associated with fibrillar aggregates of the quartet stacks and intermolecular distances within microgel domains. Utilizing the fibrillar diameter determined via AFM (16.9±3.0 nm), the polarized and depolarized DLS technique provides dimensional information on the mean length (411.2±45.9 nm) of the fibrillar aggregates formed via lateral association of the columnar stacks of quartets (Figure 2.23). Evidently, the system is heterogeneous, featuring a sol
phase with individual columnar assemblies of $\text{G/TAcG}$ quartets and fibrillar agglomerates, and microgel domains, with the volume fraction of the latter progressively rising as the concentration increases through the macroscopic gel point.

The information gained from these SLS and DLS experiments provides further insight into the molecular origin of the unusual viscoelastic properties of $\text{G/TAcG}$ gels, which, as noted above, feature a transient network contribution that disappears with increasing temperature.\textsuperscript{22} It was observed that the yield stress and equilibrium modulus of the network, measured by oscillatory strain sweep at a frequency $\omega = 10$ rad/s, decreased with increasing $\text{TAcG}$ content, which correlated with the observed decrease in rod length of the individual columnar stacks, measured by SANS. At $\omega = 10$ rad/s, the room temperature equilibrium modulus is determined by total elasticity of the gel, including the transient network contribution. Our present results indicating that a sol phase, containing individual columnar stacks and fibrillar aggregates of these stacks, coexists with microgel domains, the volume fraction of the latter increasing with concentration, suggests that it is likely that the gel network at lower temperatures consists of individual columnar quartet stacks in equilibrium with the fibrillar aggregates of these stacks which comprise the permanent gel network. It follows that the transient network contribution to the high-frequency storage modulus originates from the elastic response of the motionally-restricted columnar stacks entangled within the fibrillar network, explaining the correlation between the high-frequency modulus and the rod length. It is also likely that the disappearance of the transient viscoelasticity at higher temperatures occurs because these stacks are incorporated into fibrils via hydrophobic association. The increase in modulus of the high temperature plateau with $\text{TAcG}$ content thus reflects the fact that the
thickness and presumably rigidity of the permanent fibrous gel network increases with
the propensity toward hydrophobic association, which of course increases with TAcG
content. A schematic model for the gelation mechanism of G/TAcG gels is proposed in
Figure 2.24. In the first sketch, which is below the critical concentration, monomer and
quartet exist. In the second sketch which is above the critical concentration, the quartets
form columnar stacks and the columnar stacks aggregate into much thicker and longer
fibers. Here, microgel particles coexist with a dilute sol phase. With increased
concentration, as illustrated in the third sketch, the volume fraction of the microgel phase
increases and becomes continuous, leading to gelation.

![Figure 2.24. Schematic model for the gelation mechanism of G/TAcG gels.](image)

### 2.4 Conclusions and Future Work

In summary, SANS and SAXS measurements were performed on G/TAcG gels to
probe the structure of individual columnar assemblies of G/TAcG quartets. Both indicate
the structure is described by a rigid cylinder, the SANS profiles indicating a core-shell
structure, the SAXS profiles a homogeneous cylinder. The two measurements are
reconciled by considering the fact that the SANS intensities are influenced by the fact
that there is a radial contrast variation due to deuteration of the labile hydrogens in the core, whereas the SAXS data reflect that the electron density of the cylinder is uniform. Together, the two techniques produced a self-consistent picture of the cylinder dimensions. SLS experiments on 50/50 w/w mixtures of Guanosine (G) and 2’,3’,5’-Tri-O-Acetylguanosine (TAcG) in aqueous 0.354 M KCl indicate a sudden increase in apparent molecular weight between 0.1 and 0.2 wt%, indicating the critical concentration for self-association of G/TAcG quartets into columnar assemblies is below 0.2 wt%. Guinier plot analysis of the SLS data above the critical concentration indicate a radius of gyration that decreases with concentration and smaller molecular weights than expected, suggesting that the technique is probing the internal structure of microgel particles. SAXS measurements on the same solutions further indicate that individual columnar assemblies exist in solution at concentrations above the critical concentration for quartet self-assembly up to the gel point, and that the length of these rigid cylindrical assemblies is independent of concentration. Polarized and depolarized DLS analyses indicate bimodal correlation functions whose properties suggest the techniques probe translational and rotational diffusion of a bimodal distribution of particles, respectively. The fast modes appear to originate from fibrillar agglomerates of columnar assemblies of G/TAcG quartets, and the slow modes from microgel domains. Collectively, these experiments are consistent with the interpretation that dilute sol and microgel phases coexist in the solution below the macroscopic gel point, and that the sol phase contains individual columnar stacks of G/TAcG quartets (dimensions Lxd = 39.7±0.3 nm x 3.02±0.06 nm) and fibrillar aggregates (dimensions Lxd = 411.2±45.9 nm x 16.9±3.0 nm) formed via lateral aggregation of these columnar assemblies. With increasing
concentration, the resulting increase in number density of columnar assemblies leads to a progressive increase in fibrillar agglomeration and hence to an increase in the volume fraction of microgel domains, which ultimately leads to macroscopic gelation. Prior observation of a transient network contribution to the gel elasticity at low temperatures is attributed to the presence of motionally-restricted individual columnar stacks entangled within the fibrillar gel network.

Future work could be directed to further characterization of the gels structure by imaging techniques such as transmission electron microscopy (TEM). Supporting rheological evidence that needs to be checked would be to determine the composition dependence of the yield stress at low frequencies, since it should exhibit an opposite trend to that seen at high frequencies. Thixotropic properties of these gels would be also very interested to study using, for example, creep experiments.
2.5 References


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CHAPTER III

Structural Studies of a Class of Metallo-Supramolecular Polymeric Gels

3.1 Introduction

Recently, great attention has been focused on supramolecular polymers assembled from low molecular weight precursors via labile noncovalent interactions such as hydrophobic interactions,\textsuperscript{1} hydrogen bonding\textsuperscript{2} and metal-ligand binding.\textsuperscript{3} The labile nature of the interaction anchoring the polymeric structure makes the polymers mechano-responsive, since applied stresses can break down the polymeric structure, which subsequently reforms on removal of the stress. Metallo-supramolecular polymers formed via metal-ligand binding are of particular interest, because they combine the mechano-responsive properties of the supramolecular polymer framework with additional stimuli-responsive characteristics endowed by the functional properties of the inorganic metal ion.\textsuperscript{4-6} Here we focus on a particular class of metallo-supramolecular polymers,\textsuperscript{7-9} in which a ditopic ligand, consisting of a 2,6-bis-(1'-methylbenzimidazolyl)-4-oxopyridine moiety (O-Mebip) attached to either end of a penta-(ethylene glycol) core, self-assembles with transition metal ions (e.g. \(\text{Zn}^{2+}\)) and/or lanthanide ions (e.g. \(\text{La}^{3+}\)) to form polymer chains as illustrated in Figure 3.1. Multi-stimuli-responsive gels are obtained\textsuperscript{10-11} and their structure and properties investigated by W. Weng et al.,\textsuperscript{12-13} in a series of experimental studies using diverse techniques: optical and confocal microscopy, dynamic light scattering, wide-angle X-ray scattering and rheology. The gels were initially formed by dissolving the ditopic ligand in acetonitrile at high temperature, and then cooling from
the hot transparent sol to room temperature. The gels formed are turbid, indicating that phase separation plays a role in the gel-forming mechanism. Optical and confocal microscopy and wide-angle X-ray diffraction demonstrated that the gel morphology consists of large, semicrystalline, globular particles, and thus the gelation process is essentially colloidal in nature. By cooling the gels in a sonication bath, the particle size is reduced and mechanically robust gels with excellent mechanically-responsive (thixotropic) properties are obtained.

Later, it was discovered that transparent gels can be obtained by dissolving the ditopic ligand and metal ions in a mixture of a good solvent (DMSO) with either a poor solvent (ethylene glycol (EG)), or a non-solvent (water), and again cooling from the hot sol. Taking as example the ditopic monomer (see Figure 3.1 where \( m = 4 \)), the optical properties of gels based on the metallosupramolecular polymeric complex \( 2:Zn \) can be tuned from turbid to transparent by controlling the ratio between good solvent and poor or non-solvent. At the gel concentration of 50 mg/mL, the gels in DMSO/water are turbid at 60/40 (v/v), translucent at 70/30 and transparent at 82.5/17.5; and the gels in DMSO/EG are turbid at 40/60, translucent at 50/50 and transparent at 60/40 (Figure 2 in ref. 9). As transparency increases, the gel strength and mechanical responsiveness are maintained (Figures 9, 11, 13 in ref. 9). From microscopic studies, particle size decreases as the transparency increases and is finally below the resolution of optical microscopy. Consistent with the decrease in particle size, the gel modulus increases with increasing DMSO content to a maximum value, and then decreases (Figure 9 in ref. 9). The latter decrease is attributed to the fact that the sol content decreases when the DMSO content is sufficiently high. As noted above, the particle sizes in the turbid gels are decreased by
sonication and in fact reach sizes comparable to those in the transparent DMSO-rich mixed solvents, without decreasing the turbidity, suggesting that the optical clarity of DMSO-rich gels derives from a reduction in the refractive index contrast between the gel particles and the mixed solvent.

Figure 3.1. Schematic representation of the formation of metallo-supramolecular polymeric aggregates using ditopic ligand end-capped monomers with metal salt, Zn(ClO$_4$)$_2$.

Most recently, the effect on gelation of changing the core length of the ditopic monomer$^{10}$ was investigated. The properties of three monomers, 1, 2 and 3, derived, respectively, from tetra-, penta-, and hexaethylene glycols, were compared in the presence of Zn$^{2+}$, using the mixed solvent systems DMSO/Water and DMSO/Ethylene glycol. The changes in core length lead to substantial variations in the gel properties. Specifically, under equivalent conditions of solvent quality and polymer concentration, the storage modulus of gels formed by 2:Zn is several orders of magnitude higher than those of gels formed by 1:Zn or 3:Zn (Figure 12 in ref. 10$^{15}$). This striking variation in mechanical strength was attributed to solubility differences between the corresponding MSP, in the order 1:Zn $<$ 2:Zn $<$ 3:Zn. Thus, rheological measurements of the gelation kinetics indicate 3:Zn has the slowest and 1:Zn the fastest rate. Moreover, the rheological evidence and optical microscopy of the change in morphology during
gelation indicates $1:Zn$ phase separates by a spinodal mechanism in which over-swollen gel particles are formed initially and subsequently densify via redistribution of solvent.\textsuperscript{16} Thus, it was deduced that the lower modulus of $1:Zn$ is due to its lower solubility, which results in smaller, more dense colloidal particles and hence a lower particle volume fraction. On the other hand, $3:Zn$ gels also have a lower modulus than $2:Zn$ because of their higher solubility, which results in a larger sol fraction under the equivalent solvent conditions.

The monomers evidently have different structure-forming tendencies, since monomer 1 consists of crystalline flakes, 2 is a semi-crystalline powder, and 3 is a soft amorphous solid. To investigate this issue in more detail, we apply below differential scanning calorimetry to probe the thermal transitions in the monomers. Another question arises related to the fact that, as the transparency of the gels increases, the particle size decreases below the range accessible to optical microscopy. Dynamic light scattering tells us that nanoscale particles remain in the diluted gels, but no clues are given as to the change in structure responsible for the decrease in refractive index contrast which gives rise to transparency. Wide-angle x-ray diffraction (WAXD) indicates that the crystallinity present in the turbid gels is essentially eliminated in the gels formed in DMSO/Water and DMSO/Ethylene glycol. In what follows we apply the small angle X-ray scattering (SAXS) technique in an effort to gain the insight into the structural organization of the colloidal particles in the transparent gels.

3.2 Experimental

3.2.1 Materials and Sample Preparation
Acetonitrile, chloroform, anhydrous dimethyl sulfoxide (DMSO), anhydrous ethylene glycol (EG), and Zinc perchlorate hexahydrate were used as received from Fisher or Aldrich. Deionized water was obtained from Nanopure ultrapure water system. The synthesis of monomer 1, 2 and 3 has been reported in the literature.\textsuperscript{12-15} The metallo-supramolecular polymers 1:Zn, 2:Zn, 3:Zn were first prepared as gels in chloroform and acetonitrile. The gel was then covered and placed in an oven (90 °C) for 1 h to remove solvent. The solids were further dried in a vacuum oven (80 °C) overnight. Addition of prescribed quantities of DMSO and water, or DMSO and EG, to the solid material, followed by heating to 120 °C in a sealed vial enables the solids to dissolve. Gels were formed by allowing solutions to cool to room temperature.

\textbf{3.2.2 Differential Scanning Calorimetry}

Differential scanning calorimetry (DSC) was conducted on a Perkin-Elmer Pyris 1 instrument. Samples of the ligand monomers 1, 2, and 3, each about 10 mg in weight, were accurately weighed, then placed in hermetically-sealed cells. The heat flow was monitored as the samples were heated from 20 °C to 110 °C at heating rate of 10 °C/min. All measurements were performed under a nitrogen atmosphere.

\textbf{3.2.3 Small Angle X-ray Scattering}

Small-angle X-ray scattering (SAXS) measurements were carried out using an in-house instrument equipped with a rotating anode X-ray generator (Rigaku RU 300, 12 kW) and two laterally-graded multilayer optical elements in a side-by-side arrangement, giving a highly focused parallel beam of monochromatic Cu K\textalpha radiation($\lambda = 0.154$ nm). The beam, operated at 50 kV and 100 mA, was collimated using three pinholes and the beam diameter at the sample position was approximately 700 mm. Two dimensional (2D)
SAXS patterns were collected using a Rigaku SMax3000 pinhole SAXS camera, equipped with a 2D gas-filled multiwire detector, having a spatial resolution of $1024 \times 1024$ pixels. The X-ray exposure times for the SAXS patterns were typically 4 hours. The sample-to-detector distance was 1.5 m and the scattering vector $q$ was calibrated using a Silver Behenate (AgBe) standard, with (001) peak position at $q= 1.076 \text{ nm}^{-1}$. A beamstop, placed in front of the area detector, allows monitoring of the direct beam intensity. Based on the direct beam intensity in the presence and absence of the sample, all SAXS images were corrected for background scattering and sample absorption. Fresh gel samples were directly loaded into quartz capillaries.

Small angle X-ray scattering was also carried out at the BESSRC CAT 12-ID beam line of the Advanced Photon Source, Argonne National Lab, Argonne, IL. The setup used a sample-detector distance of 4 m and CCD detector. The sample holder was a Quarzkapillaren quartz capillary with a diameter of 2 mm purchased from the Charles Supper Company. The X-ray energy was 12 keV, corresponding to a wavelength of 0.1 nm. The gels were heated to the solution state, and then were transferred into quartz capillaries. The solutions were cooled to room temperature to form gels and equilibrated for 1 hour prior to the measurement. The exposure time was 1 s, and five exposures were averaged to obtain better signal quality.

3.3 Results and Discussion

3.3.1 Differential Scanning Calorimetry

As noted above, the length of the ethylene oxide core of the ditopic ligand has a significant effect on the physical state of the monomers 1, 2 and 3. When precipitated from a solvent mixture of dichloromethane and diethyl ether, 1 precipitates as crystalline
flakes, 2 as a semicrystalline powder, and 3 as a soft amorphous solid, suggesting an increase in disorder with increase of core length. To confirm this interpretation, differential scanning calorimetry analysis was conducted on the three monomers and is shown in Figure 3.2. For 1, during the first heating scan, a glass transition with aging relaxation peak is observed at 92 °C, followed by a prominent recrystallization endotherm at 126 °C, and a very large melting transition at approximately 246 °C. During the second heating scan a barely discernable T_g transition is seen at 83 °C, a small recrystallization endotherm again at 146 °C, and the large melting transition is seen at 243 °C. For 2, a large T_g transition is seen around 70 °C with a small aging relaxation peak in both the first and second heating scans. A broad recrystallization peak is seen in the first heating scan, near 170 °C, followed by a strong melting transition at 223 °C; during the second heating scan, no recrystallization peak is seen, and only a small melting transition at 223 °C. For 3, a large T_g transition is seen at 62 °C, with no evidence of a recrystallization or melting transition in either the first or second heating scans. The DSC results are thus consistent with the physical appearance of the solids. Specifically 1 rapidly crystallizes and forms a highly crystalline solid, 2 crystallizes slowly, and has relatively modest crystallinity, and 3 appears to be completely amorphous. Evidently, the variation in crystallinity of the monomers correlates to the solubility sequence (1:Zn < 2:Zn < 3:Zn) and variation in gelation kinetics (1:Zn > 2:Zn > 3:Zn) of the metallosupramolecular polymers, suggesting that the different tendencies for intermolecular ordering of monomers 1, 2, and 3 plays a role in the properties of the metallosupramolecular polymers derived from them via ligand-metal binding.
Figure 3.2 Differential scanning calorimetry (DSC) analysis of the ligand monomers (a) 1, (b) 2, (c) 3. Heating and cooling rates are 10 °C/min, nitrogen atmosphere.

3.3.2 Small Angle X-ray Scattering

To gain further insight into the structural changes in the metallosupramolecular gels, which accompany the addition of a good solvent, SAXS studies were initially performed using the departmental SAXS instrument on six gel systems: 2:Zn in DMSO/water at ratios of 60/40, 70/30, and 82.5/17.5 v/v and in DMSO/ethylene glycol at ratios of 40/60, 50/50, and 60/40 v/v, in each case the gel concentration being 50 mg/mL. For each solvent system, the three compositions reflect changes in optical quality from opaque, to translucent, to transparent, as well as morphological changes in optical microscopy from large spherulites to smaller spherulites to featureless. Figure 3.3, presents the SAXS results for all six systems. In Figure 3.3(a) for the gels in DMSO/water, we find that at 60/40 DMSO/water composition, where the gel is turbid,
the SAXS profile exhibits a smooth decay; at 70/30 DMSO/water, corresponding to a translucent gel, there is the appearance of two peaks in the SAXS profile; in the transparent gel at 82.5/17.5 DMSO/water, the two peaks become more prominent. The appearance of the two SAXS peaks indicates that the onset of transparency is accompanied by a transition to a new structure in the colloidal particles. A similar evolution is seen in the SAXS profiles of the DMSO/EG gels as the DMSO content of the solvent increases, except that, in the turbid gel at 40/60 DMSO/EG, there is evidently already the onset of the new structure in the SAXS profile. The positions of the two peaks, located near $q = 0.8 \text{ nm}^{-1}$ and $1.6 \text{ nm}^{-1}$, i.e., a q-spacing of 1:2, indicates that the new structure is lamellar in nature, with a $d$-spacing of $\sim 8.0 \text{ nm}$.  

The nature of this lamellar structure is not clear, but noting that the periodicity is $\sim 3$ times the length of a fully stretched ditopic monomer, it appears to involve nanosegregation of the mixed solvent within a metallo-supramolecular polymer framework. Several possibilities can be envisaged. One recognizes the surfactant-like amphiphilic character of the monomer, with its highly hydrophobic ligand end groups and highly polar core, and envisages segregation into metallo-supramolecular polymer layers, in which water or ethylene glycol preferentially swells the pentaethylene oxide cores, separated by layers of the more organophilic solvent, DMSO, in contact with the apolar ligands. Another possibility may be chain-folded metallosupramolecular polymer layers, in which the lamellar surfaces are pentaethylene oxide folds, again separated by solvent layers. A third possibility relates to the fact that increasing the DMSO content of the solvent leads eventually to the formation of a sol phase. Thus, it seems possible that
the lamellar organization might consist of phase-separated metallo-supramolecular gel layers, separated by layers of a dilute sol phase.

Figure 3.3. Small-angle X-ray scattering (SAXS) of monomer 2 (m=4 in Figure 3.1) formed in (a) DMSO/water and (b) DMSO/ethylene glycol mixtures, at different solvent
compositions. The ratio of DMSO to cosolvent (v/v) is indicated in the figure; the gel concentration is 50 mg/mL.

To date, our studies suggest that all three metallo-supramolecular polymeric species, $1:Zn$, $2:Zn$ and $3:Zn$, prepared in DMSO/water and DMSO/EG solvents, have similar structural characteristics. From microscopic studies, all gels are composed of colloidal particles, and wide angle X-ray scattering suggested these particles are highly disordered and likely to be swollen with solvent. It is thus of interest to investigate whether $1:Zn$ and $3:Zn$ gels exhibit similar lamellar structures to those observed above in $2:Zn$, when the content of the good solvent DMSO is increased into the transparent gel region. In Figure 3.4, we compare the SAXS patterns of transparent $1:Zn$, $2:Zn$, and $3:Zn$ gels (50 mg/ml) in 80:20 DMSO/water and 60:40 DMSO/EG. Evidently, gels $1:Zn$ and $2:Zn$ have similar patterns, featuring peaks at $q = 0.8 \text{ nm}^{-1}$ and $1.6 \text{ nm}^{-1}$, consistent with the previously identified lamellar organization. The SAXS pattern of $3:Zn$ is substantially weaker than the others, suggesting it has a lower electron density contrast, presumably reflecting that the phase separated particles have a different mixed solvent content. However, it also appears to feature a distinct small-angle peak at a scattering vector $q = 0.6 \text{ nm}^{-1}$, somewhat smaller than that for DMSO/water system. Thus, it appears clear that all three gels exhibit a similar structural organization, but the lamellar d-spacing is larger in $3:Zn$, perhaps indicative that the lamellae are more swollen by solvent. Similar SAXS patterns were obtained from the transparent gels formed in 60:40 DMSO/ethylene glycol, except that the scattering contrast was even weaker for $3:Zn$, so that no small-angle peaks could be discerned.
Figure 3.4. Lab source SAXS patterns from gels formed by 1:Zn, 2:Zn, and 3:Zn in 80/20 (vol/vol) DMSO/water (a) and in 60/40 (vol/vol) DMSO/EG (b) at the concentration of 50 mg/ml.
Synchrotron SAXS experiments were also performed on 1:Zn, 2:Zn, and 3:Zn gels (50 mg/ml) in 80:20 DMSO/water and 60:40 DMSO/EG (shown in Figure 3.5). The gels were heated to the sol state, and transferred into quartz capillaries. The solutions were then cooled to room temperature to form gels, and equilibrated for 1 hour prior to the measurement. The q range of the synchrotron detection system is shifted towards lower q (0.03 to 0.17 nm\(^{-1}\)) compared with the departmental SAXS instrument, but the higher intensity of the synchrotron source yields much greater signal-to-noise ratio in the detected SAXS patterns. The synchrotron SAXS profiles are similar in shape and peak positions to those generated by the departmental SAXS, but some clear differences also exist. 1:Zn gels in both DMSO/water and DMSO/EG show monotonic decreases increasing q, but with a slight but discernable shoulder centered around q = 0.5 nm\(^{-1}\) but do not show the distinct peaks seen in the patterns generated by the departmental SAXS (Figure 3.4). 2:Zn gels in both DMSO/water and DMSO/EG each show similar patterns in synchrotron SAXS to those seen using the departmental SAXS, with peaks located at q \~ 0.8 nm\(^{-1}\). In synchrotron SAXS, 3:Zn gels exhibit scattering intensities comparable to those of 1:Zn and 2:Zn whereas, with the departmental SAXS, the patterns were much weaker than those of 1:Zn and 2:Zn. Moreover, 3:Zn in DMSO water exhibits a shoulder at lower q than the peak in 2:Zn (Figure 3.5a) which appears consistent with the observation of an admittedly weak peak at lower q using the Departmental SAXS (Figure 3.4a). In DMSO/EG, 3:Zn exhibits a peak in synchrotron SAXS at q \~ 0.8 nm\(^{-1}\), similar to that seen in the patterns of 2:Zn. In departmental SAXS, the pattern of 3:Zn was too weak to identify any such features. The various discrepancies can be rationalized on the basis that the colloidal structures generated on cooling into the gel state are metastable
structures, and are therefore sensitive to details of the cooling history. This is particularly
evident in prior studies,\textsuperscript{15} which demonstrated that the phase separation of \textbf{1:Zn} is
spinodal in character and slowly evolves towards the equilibrium morphology via
redistribution of solvent. Thus it is deduced that all three gels feature a lamellar structural
organization which involves partitioning of the mixed solvent into the colloidal particles,
but that the extent of partitioning is sensitive to the thermal history. Thus the difference
between the synchrotron and departmental SAXS patterns of \textbf{1:Zn} gels indicates that the
gel studied by synchrotron SAXS after 1 h has a less fully developed lamellar
organization than the more extensively annealed gel (after one day) studied using the
departmental SAXS. For \textbf{3:Zn}, the strong intensity seen in synchrotron SAXS relative to
that in departmental SAXS can likewise be explained as indicative that the gel structure
developed in the former experiment is further from equilibrium, presuming that the
solvent distribution in the equilibrium lamellar structure allows better refractive index
matching (more transparency, less scattering intensity (Figure 3.4)).
Figure 3.5 Synchrotron source SAXS patterns from gels formed by 1:Zn, 2:Zn, and 3:Zn in 80/20 (vol/vol) DMSO/water (a) and in 60/40 (vol/vol) DMSO/EG (b) at the concentration of 50 mg/ml.

3.4 Conclusions and Future Work

Metallo-supramolecular polymers prepared from the three ditopic ligand monomers, 1, 2 and 3, in the presence of transition metal ion Zn$^{2+}$ form mechanically-robust, highly thixotropic gels, via a process of phase separation and crystallization, and the formation of colloidal particles.\textsuperscript{12-15} DSC results show the increasing solubility of the metallosupramolecular polymers generated by 1, 2, and 3 in the order 1:Zn < 2:Zn < 3:Zn correlates to a decreasing crystallinity of the monomers, which implies a decreasing tendency for ordering. Comparison with the viscoelastic properties of the resulting gels indicates that for optimal mechanical behavior, an intermediate ordering tendency of the
monomer is required. Too high a tendency results in smaller, denser colloidal particles, and hence a smaller colloidal volume fraction; too small a tendency results in less extensive gelation (a greater sol fraction).

Utilizing mixed solvent systems consisting of a good solvent (DMSO) with either a nonsolvent (water) or poor solvent (ethylene glycol), gels varying in turbidity from highly opaque (water- or ethylene glycol-rich) to highly transparent (DMSO-rich) can be obtained. SAXS studies indicate that the metallosupramolecular polymers generated from all three monomers form similar lamellar structures during the gelation process. The lamellar repeat distances are larger than the fully extended length of the monomers, indicating that solvent distribution within the lamellar structure occurs. The precise value of the lamellar repeat appears to show an increasing tendency with the duration of isothermal annealing in the gel state, which appears to reflect the time-dependent progression of solvent redistribution within the lamellar structure. The incorporation of solvent into the lamellar structure results in reduced refractive index contrast between the colloidal particles and the solvent, and provides an explanation for the increase in gel transparency with increasing DMSO content. Considering the evidence from optical microscopy and dynamic light scattering that colloidal particles persist in the transparent gels, it appears that solvent-swollen, onion-like or platelike lamellar structures may be the colloidal elements that form the gel network.

The SAXS of gels shows a lamellar structure with a periodicity of about 8 nm, about 3 times the length of a fully stretched ditopic monomer. It therefore appears that nanosegregation of the mixed solvent is involved within the metallo-supramolecular polymer framework. Presumably water or ethylene glycol being more concentrated in
regions where the ethylene glycol core is located, and excluded from regions where the ligand is located. Planar lamellar structures and concentric lamellar spheres (onion structures) are both possible. These possibilities cannot be distinguished based on the current experimentation. Future work could involve the application of freeze fracture transmission electron microscopy (freeze fracture TEM) to attempt to image the structure of the particles in the gels, especially in the transparent gels.
3.5 References


CHAPTER IV

Studies of Self-assembly of Metallo-Supramolecular Polymers in Solution

4.1 Introduction

Metallo-supramolecular polymers (MSP), formed by self-assembly of small molecules via noncovalent interactions, are a relatively new area of research interest, where much effort has been devoted to the investigation of the self assembly process. Many techniques have been tested to determine the molecular weight of MSP, including gel permeation chromatography,\textsuperscript{1-7} light scattering,\textsuperscript{8-9} mass spectrometry,\textsuperscript{2,7,10} viscosity measurement,\textsuperscript{5,11} analytical ultracentrifuge\textsuperscript{5} and vapor pressure osmometry.\textsuperscript{7} These methods have their individual advantages and disadvantages, and use of a combination of techniques offers the best approach to fully characterize samples. One of the major challenges is that the self assembly process can be kinetically dynamic, and the degree of association can be influenced by composition, concentration, temperature, solvent and other external parameters. In such a case many conventional characterization techniques lose the ability to accurately determine basic structural information such as molecular weight. One approach is to utilize a chain stopper to block the concentration dependence of the molecular weight of a supramolecular polymer prior to characterization.\textsuperscript{9} A drawback of this method is that the stopper itself may influence the molecular weight distribution of the supramolecular polymer: addition of a smaller amount of stopper leads to larger molecular weights.
The terdentate 2,6-bis(1'-methylbenzimidazolyl)-4-pyridine ligand (MeBIP) is capable of binding to a variety of transition and lanthanide metal ions. The metal-ligand interaction can be used to create polymeric structures. Since facile routes are available to obtain functionalized MeBIP moieties, including the possibility to generate liquid crystalline behavior,\textsuperscript{12} this opens up the possibility to create functional metallosupramolecular polymers. Rowan and coworkers have explored the use of ditopic monomers in which the MeBIP ligand is attached to either end of an oligomeric core, to generate metallosupramolecular gels which exhibit multiple stimuli-responsive behaviors.\textsuperscript{13-15} Here we investigate the self-assembly behavior of ditopic macromonomers, in which the MeBIP ligand is attached to either end of a core which itself is a polymeric entity.

Based on known crystal structures, divalent metal ions such as \( \text{Cu}^{2+} \) and \( \text{Zn}^{2+} \) can chelate to MeBIP ligands in a mole ratio of 1:2,\textsuperscript{16} while monovalent metal ions such as \( \text{Cu}^+ \) can chelate to MeBIP ligands in a mole ratio of 2:2.\textsuperscript{17} If we assume a similar binding stoichiometry holds in solution, then, for ditopic ligands based on MeBIP, as illustrated in Figure 4.1, when the mole ratio between \( \text{Cu}^{2+} \) and MeBIP ligands is 1:2, i.e. the \( \text{Cu}^{2+} \) to ditopic macromonomer mole ratio is 1:1 (one macromonomer has two ligands, one at the either end), there are exactly the right number of metal ions to link all monomers having MeBIP ligands at their ends, then the highest molecular weight MSP are expected. If more \( \text{Cu}^{2+} \) ions are added, it is possible that MeBIP - \( \text{Cu}^{2+} \) - MeBIP complexes will dissociate to form two MeBIP - \( \text{Cu}^{2+} \) complexes, which are thermodynamically more stable, provided the binding process is reversible. It follows that the high molecular weight MSP species will then decomplex. Conversely, when the ratio between the \( \text{Cu}^+ \)
and MeBIP ligands is 1:2 (i.e. the macromonomer:Cu$^+$ mole ratio is 1:1), because two Cu$^+$ ions are needed to bind two MeBIP ligands, there is insufficient Cu$^+$ present to bind all ligands, and low molecular weight MSP based on 2:2 complex formation will be present. Now, if more Cu$^+$ ions are added until the ratio between the Cu$^+$ and MeBIP ligands is 2:2 (or 1:1), i.e. the Cu$^+$:macromonomer mole ratio is 1:2, high molecular weight MSP species will be formed. Based on the above discussion, it appears that switching between high molecular weight and low molecular weight MSP species is possible by chemically or electrochemically switching between the two oxidation states Cu$^{2+}$ and Cu$^+$ while keeping the mole ratio between macromonomers (i.e. MeBIP ligands) and Cu (Cu$^{2+}$ or Cu$^+$) the same. The switching mechanisms at two different ligand-metal ion ratios are illustrated in Figure 4.1.

![Figure 4.1](image)

**Figure 4.1.** Redox switching between metallo-supramolecular polymer (high molecular weight) and oligomer (low molecular weight) in a system containing Cu ions and a ditopic macromonomer end-capped with the MeBIP ligand system at two different ligand-metal ion ratios.

With the goal of developing redox-active supramolecular polymers, we initiated a study of the self-assembly of macromonomers based on the MeBIP ligand with Cu$^{2+}$ and
Cu$^+$ ions in solution, and investigate the structure of the resulting metallo-supramolecular polymers. MeBIP capped polytetrahydrofuran (BIP-pTHF-BIP = BTB) of two different molecular weights ($M_n=4200$ g/mol and 7000 g/mol based on UV end-group titration) were chosen as the macromonomers to study (Figure 4.2).

![Figure 4.2. Structure of MeBIP-capped polytetrahydrofuran (Bip-pTHF-Bip = BTB).](image)

(\(M_n=4200\) g/mol and 7000 g/mol from UV end-group titration)

4.2 Experimental

4.2.1 Materials

All reagents were from Aldrich Chemical Co. and used without further purification. Both Cu$^+$ and Cu$^{2+}$ salts had triflate as the counterion. Solvents were distilled from suitable drying agents. The synthesis of the ditopic macromonomer Bip-pTHF-Bip (BTB) (Figure 4.1) was performed by Justin Kumpfer and has been reported.$^{18}$

4.2.2 SAXS Measurement

Small angle X-ray scattering was carried out at the BESSRC CAT 12-ID beam line of the Advanced Photon Source, Argonne National Lab, Argonne, IL. The setup used a sample-detector distance of 4 m and CCD detector. The sample holder was a
Quarzkapillaren quartz capillary with a diameter of 2 mm purchased from the Charles Supper Company. The X-ray energy was 12keV, corresponding to a wavelength of 1 Å. Small angle X-ray scattering measurements were conducted on solutions of BTB monomer and its Cu$^{2+}$, Cu$^{+}$ complexes solutions at different concentrations (1, 5 and 10 g/L). The samples were equilibrated for several hours prior to measurement. The exposure time was 1 s, and five exposures were averaged to obtain better signal quality. The signal from the solvent used was subtracted as background.

The radius of gyration $R_g$ of macromonomers and resulting MSPs was calculated using two independent methods: (i) from the slope of a Guinier plot of $\ln(I(q))$ versus $q^2$, using the low-q range of the SAXS scattered intensity $I(q)$, which can be related to $R_g$ via equations (1) and (2):

$$\lim_{q \to 0} I(q) = I(0) \exp\left(-\frac{R_g^2 q^2}{3}\right)$$  \hspace{1cm} (1)

$$\ln I(q) = \ln I(0) - \frac{R_g^2}{3} q^2$$  \hspace{1cm} (2)

and (ii) calculation using equation (4) from the pair distance distribution function $P(r)$, computed via Fourier transform of the scattering pattern (equation (3)), using GNOM:

$$P(r) = \frac{1}{2\pi^2} \int_{q_{\text{min}}}^{q_{\text{max}}} I(q) qr \sin(qr) dq$$  \hspace{1cm} (3)

$$R_g^2 = \frac{\int_{0}^{d_{\text{max}}} r^2 P(r) dr}{\int_{0}^{d_{\text{max}}} P(r) dr}$$  \hspace{1cm} (4)

GNOM was used to perform regularized indirect transforms of the scattering data to obtain the pair distance distribution function $P(r)$. For each scattering curve, GNOM was run with different values of the upper integration limit $d_{\text{max}}$. The proper $d_{\text{max}}$ was
chosen so that the fit to the scattering curve was optimized, and the resulting P(r) function was smooth and positive. Comparison of the two methods indicated that the Fourier transform approach provides better statistical certainty than the use of Guinier plots, as expected, since it incorporates data from all scattering vectors into the calculation.

4.2.3 Viscosity Measurement

Cannon-Ubbelohde semi-micro viscometers were used to determine the viscosity of BTB monomer and its complexes with Cu$^{2+}$, Cu$^+$ solutions following instructions on the company website.$^{20}$ The viscometer was maintained at 25 °C in a water bath. The ratio of the flow times of solution to solvent is an approximate measure of the relative viscosity \( \eta_r = \eta / \eta_c \). The reduced specific viscosity may be viewed as a measure of the apparent hydrodynamic volume, \( V_{h,app} \), divided by the molar mass, \( M \), of the dissolved particles:

\[
\eta_s \rho / c = (\eta / \eta_c - 1) / c \approx 2.5N_A V_{h,app} / M
\]  

(5)

4.2.4 Differential Refractive Index Increment Measurement

To obtain molecular weights from static light scattering (SLS) experiments, it is necessary to know the differential refractive index increment (dn/dc) of BTB as well as its complexes with metal ions. This was determined following web-posted instructions published by P. Russo (Louisiana State University)$^{21}$ using a Brice-Phoenix differential refractometer, which measures the deflection of light passing through a split cell containing solution in one compartment and solvent in the other.

4.2.5 Static Light Scattering
Two laser systems were employed in static light scattering experiments. One employs a Stabilite Ar laser, emitting vertically-polarized light at $\lambda = 514.5$ nm with a power output of 1–1.5 W. The other uses a He-Ne laser, emitting vertically-polarized light at $\lambda = 632.8$ nm with a power of 15 mW. Each of the lasers was coupled to a BI 200 SM photometer-goniometer (Brookhaven Instruments) and a BI-9000 correlator. In static light scattering, the excess scattered intensity, $\Delta i(q,c)$, recorded by subtracting the solvent signal from the solution signal, was measured as a function of concentration, c, and scattering vector q, defined as

$$q = \frac{4\pi n}{\lambda} \sin \frac{\theta}{2}$$

where $n$ is the solvent refractive index, $\lambda$ the laser wavelength in vacuo, and $\theta$ the scattering angle. The scattering angle was increased in 5° increments between 45° and 135° for Ar laser, corresponding to a q range from 1.25×10^{-2} to 3.01×10^{-2} nm^{-1}. For He-Ne laser, the measurements were taken between 45° and 110°, corresponding to a q range of 1.05×10^{-2} to 2.25×10^{-2} nm^{-1}. The excess Rayleigh ratio, $\Delta R(q,c)$, was determined using toluene as the reference scatterer:

$$\Delta R(q,c) = \frac{[I_{solution}(q,c) - i_{solvent}(q,c)]R_{toluene}(q)}{i_{toluene}(q,c)} \times \frac{n^2(toluene)}{n^2(solvent)}$$

where the Rayleigh ratio of toluene is $3.2 \times 10^{-5}$ cm^{-1} at 514.5 nm and $1.02 \times 10^{-5}$ cm^{-1} at 632.8 nm respectively. For dilute polymer solutions, at small scattering vectors, $qR_g < 1$, where $R_g$ is the radius of gyration of the polymer, the excess Rayleigh ratio satisfies the following equations:

$$\frac{Kc}{\Delta R(q,c)} = \frac{1}{M_w} (1 + q^2 R_g^2 / 3 + 2A_2c + 3A_3c^2 + ...$$
where \( N_A \) is Avogadro’s number, and \( A_2 \) and \( A_3 \) are the second and third osmotic virial coefficients. At finite concentration, double extrapolation of equation 14 to \( c = 0 \) and \( q = 0 \), provides an apparent weight average molecular weight \( M_w^{\text{app}} \):

\[
\frac{K_c}{\Delta R(0,c)} = \frac{1}{M_w^{\text{app}}} = \frac{1}{M_w} + 2A_2c + 3A_3c^2 + ... \tag{10}
\]

and apparent z-average radius of gyration:

\[
\frac{K_c}{\Delta R(q,c)} = \frac{K_c}{\Delta R(0,c)} \left(1 + \frac{q^2 R_{g,\text{app}}^2}{3}\right) \tag{11}
\]

When the condition \( qR_g < 1 \) cannot be satisfied, a Guinier plot provides an estimate of \( R_{g,\text{app}} \):

\[
\ln \frac{K_c}{\Delta R(q,c)} = \ln \frac{K_c}{\Delta R(0,c)} + \frac{q^2 R_{g,\text{app}}^2}{3} \tag{12}
\]

### 4.2.6 Polarized Dynamic Light Scattering

Polarized dynamic light scattering (DLS) experiments were carried out using two instruments, each equipped with a BI 200 SM photometer-goniometer (Brookhaven Instruments) and a BI-9000 correlator respectively. One instrument has a Stabilite Ar\textsuperscript{+} laser, emitting vertically-polarized light at \( \lambda = 514.5 \) nm with a power output of 1–1.5 W. The other has a He-Ne laser emitting vertically-polarized light at \( \lambda = 632.8 \) nm with a power of 15 mW. With DLS instrument, in addition to the intensity of scattered light, \( i_s(q,t) \), the normalized time correlation function of the scattered intensity, \( g^{(2)}(q,\tau) \), is determined:
\[ g^{(2)}(q, \tau) = \frac{\langle i_s(q,t)i_s(q,t+\tau) \rangle}{\langle i_s(q) \rangle^2} \]  

(13)

where the angular brackets indicate a statistical average. The normalized electric field correlation function, \( g^{(1)}(q, \tau) \), is related to \( g^{(2)}(q, \tau) \) by Siegert’s relation:

\[ g^{(2)}(q, \tau) = \alpha |g^{(1)}(q, \tau)|^2 + 1 \]  

(14)

where \( \alpha \) is a spatial coherence factor which is treated as a fitting parameter. Experiments were conducted at scattering angles in the range 30° and 90°, corresponding to \( q \) values of \( 8.75 \times 10^{-3} \) and \( 2.39 \times 10^{-2} \) nm\(^{-1} \), respectively. The electric field correlation function can also be expressed as a Laplace transform of the normalized distribution of decay rates, \( G(\Gamma) \), which is equivalent to the distribution of translational diffusion coefficients, \( D_t \), since \( \Gamma = D_t q^2 \):

\[ g^{(1)}(\tau) = \int_0^\infty d\Gamma G(\Gamma) \exp(-\Gamma \tau) \]  

(15)

Several methods are available to extract information regarding \( G(\Gamma) \). One is called the method of cumulants: the natural logarithm of \( g^{(1)}(\tau) \) is written as a series expansion in the delay time \( \tau \), as shown equation (16):

\[ \ln g^{(1)}(\tau) = -\Gamma \tau + \frac{1}{2!} \mu_2 \Gamma^2 + \frac{1}{3!} \mu_3 \Gamma^3 + \ldots \]

where \( \Gamma = \int_0^\infty G(\Gamma) \Gamma d\Gamma \)

\[ \mu_n = \int_0^\infty (\Gamma - \Gamma)^n G(\Gamma) d\Gamma \]

(16)

\( \Gamma \) is the mean decay rate from which the z-average diffusion coefficient is obtained; \( \mu_2 \) is the variance in \( \Gamma \) values and reflects the width of the distribution of diffusion coefficients; \( \mu_3 \) is the kurtosis, which is a measurement of the skewness of the distribution. The mean
The hydrodynamic radius $R_h$ is computed from the mean diffusion coefficient via the Stokes-Einstein equation (17):

$$D_i = \frac{k_B T}{6 \pi \eta R_h} \quad (17)$$

where $k_B$ is the Boltzmann’s constant, $T$ is absolute temperature; $\eta$ is the solvent viscosity. Alternatively, as indicated in equation (18), the distribution of decay rates, $G(\Gamma)$, and hence the distribution of diffusion coefficients, $G(D_i)$, can be determined via an inverse Laplace transform of $g^{(1)}(\tau)$ using an algorithm such as CONTIN.$^{22}$

$$G(\Gamma) = \int_0^\infty g^{(1)}(\tau) \exp(\Gamma \tau) d\Gamma \quad (18)$$

### 4.3 Results and Discussion

#### 4.3.1 Bip-pTHF-Bip at 7000 g/mol

##### 4.3.1.1 Viscosity Studies

For the BTB7000 macromonomer having number-average molecular weight 7000 g/mol, a mixed solvent of CHCl$_3$ and CH$_3$OH (volume ratio of 9:1) was used as the solvent system for viscosity measurements: CHCl$_3$ is a good solvent for BTB7000 and CH$_3$OH is a good solvent for salts. Intrinsic viscosity measurement of BTB7000 yielded $[\eta] = 37.8$ ml/g (Figure 4.3), from which we determine a hydrodynamic radius of 3.47 nm, using the Einstein equation, $[\eta]M = (10\pi/3)N_AR_h^3$, where $N_A$ is Avogadro’s number and the molecular weight $M = 7,000$ g/mole. By following the change in reduced specific viscosity, $\eta_{sp}/c$, as a function of the titration of Cu$^{2+}$ and Cu$^+$, respectively, into the BTB7000 solution, it was possible to confirm the metal-ligand binding stoichiometry. Aliquots of a concentrated solution of Cu$^{2+}$ or Cu$^+$ salt dissolved in CH$_3$OH were added
to a solution of the monomer Cu complexes in CHCl₃/CH₃OH. As a result, the concentration of BTB7000 decreased from 10 g/L to 6 g/L throughout the titration process. This problem was resolved in a later titration of the BTB4200 macromonomer (Mn = 4200 g/mol), which utilized butyronitrile as a common solvent. The results are shown in Figure 4.4. With increase of salt concentration, the viscosity increases uniformly to the point where all ligands are complexed, and then decreases as excess ions accumulate and decomplexation ensues. As seen in Figure 4.4 (i), the complex of BTB7000 with Cu²⁺ reaches its maximum viscosity when the ion Cu²⁺:BTB7000 ratio is 1:1. In contrast, the viscosity of the complex of BTB7000 and Cu⁺ reaches its maximum at a Cu⁺:BTB7000 ratio of 2:1 (Figure 4.4(ii)). These results confirm the different binding stoichiometries of Cu²⁺ and Cu⁺ and molecular weight differences between the different species envisaged as shown schematically in Figure 4.1. Specifically, at a Cu:BTB7000 mole ratio of 1:1, ηsp/c ~ 0.20 g/L for Cu²⁺ (higher molecular weight MSP) and ηsp/c ~ 0.10 g/L for Cu⁺ (lower molecular weight MSP); for Cu:BTB7000 = 2:2, ηsp/c ~ 0.095 g/L for Cu²⁺ (lower molecular weight MSP), and 0.23 g/L for Cu⁺ (higher molecular weight MSP). These viscometric measurements thus support the idea that redox switching between Cu²⁺ and Cu⁺ can switch the metallo-supramolecular polymers derived from BTB7000 between high molecular weight species and low molecular weight ones.
Figure 4.3. Viscosity measurement of BTB7000 monomer in CHCl₃:CH₃OH mixed solvent (vol/vol=9/1).
4.3.1.2 Small Angle X-ray Scattering Studies

Small angle X-ray scattering was performed at the Advanced Photon Source (APS) to gain further insight into details of the macromolecular architecture of the metallo-supramolecular polymers. The synchrotron SAXS patterns of BTB7000 and its complexes with Cu\(^+\) or Cu\(^{2+}\) at three concentrations and two molar ratios, 1:1 and 1:2, were measured in mixed solvent of acetone/methanol (vol./vol.=9/1). Here chloroform was replaced by acetone in the solvent because chloroform scatters the incident X-rays too strongly. At the concentrations studied, 10, 5 and 1 g/L, no significant concentration dependence was found, except that at 1 g/L most scattering patterns had very poor statistics. From the SAXS of the BTB7000 monomer (Figure 4.5), the radii of gyration,
calculated via Guinier plots and P(r) functions computed via Fourier transform of the scattering patterns are listed in Table 4.1. It should be noted that the resulting $R_g$ values are in good numerical agreement with a value predicted based on the reported characteristic ratio of polytetrahydrofuran,$^{23}$ i.e. $C_\infty = 5.8$, yielding $R_g = 3.1$ nm. It should be noted that this result is a little smaller than the value of the hydrodynamic radius, $R_h = 3.47$ nm, deduced from intrinsic viscosity. From the results in Table 4.1, this leads to a value of the ratio $R_g/R_h \sim 1.0$, which is a little smaller than expected for a linear random coil, ($\sim 1.3$). The discrepancy is likely due to the polydispersity of the sample.

**Figure 4.5.** SAXS of BTB7000 monomer in mixed CH$_3$COCH$_3$/CH$_3$OH (vol/vol 9/1) at three concentrations: 10, 5 and 1 g/L.
Table 4.1. The radii of gyration for BTB7000 from Guinier plots and Fourier transform at 10, 5 and 1 g/L.

<table>
<thead>
<tr>
<th>BTB7000 Concentration (g/L)</th>
<th>10</th>
<th>5</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>R&lt;sub&gt;g&lt;/sub&gt; (nm) Guinier plot</td>
<td>3.4±0.1</td>
<td>3.2±0.1</td>
<td>3.7±0.3</td>
</tr>
<tr>
<td>R&lt;sub&gt;g&lt;/sub&gt; (nm) Fourier Transform</td>
<td>3.2±0.1</td>
<td>3.1±0.1</td>
<td>2.9±0.1</td>
</tr>
</tbody>
</table>

Since no significant concentration dependence was found for SAXS of BTB7000:Cu<sup>2+</sup> and BTB7000:Cu<sup>+</sup> complexes, the comparison between BTB7000 and its metal ions complexes is presented in Figure 4.6 only for a concentration of 10 g/L. The SAXS patterns of the BTB7000:Cu<sup>2+</sup> MSP clearly indicate the presence of a bimodal distribution of macromolecules: one group with R<sub>g</sub> comparable to the BTB7000 monomer and one group with R<sub>g</sub> substantially larger. Noting that, at the equimolar composition of ligand to metal ion, all Cu<sup>2+</sup> ions are bound, we deduce that macrocyclic monomers and/or dimer are present. The precise amount is quite small, however, contributing a scattering intensity at most 5% of the total, and likely much less since the intercept of the scattering curve at q = 0 cannot be accurately deduced from the present data. The evidence for a bimodal distribution of macromolecules is not so clear in the SAXS pattern of the BTB7000:Cu<sup>+</sup> MSP. However, the pattern clearly features a shoulder at scattering vector q ~ 0.4 nm<sup>-1</sup>, comparable to the q-range where the scattering pattern of the BTB7000 macromonomer begins to decrease precipitously, so it seems likely that a bimodal distribution (MSP plus a small amount of macrocycles) is indeed present, but that the MSP component has essentially a continuous distribution of sizes.
extending down to dimers. The pair distance distribution functions $P(r)$ of BTB7000:Cu$^{2+}$ and BTB7000:Cu$^+$ complexes, computed from the SAXS profile $I(q)$ using Eq. (3), are shown in Figure 4.7. Figure 4.7 (i) provides additional evidence of the presence of a bimodal size distribution in BTB7000:Cu$^{2+}$. For BTB7000:Cu$^+$ complexes, the presence of a discrete lower molecular weight species is barely discernable (Figure 4.7 (ii)) as a slight shoulder near $r \approx 5$ nm.

![Graph showing scattering intensity vs. wave number for different BTB7000 and Cu complexes](image-url)

(i)
Figure 4.6. Comparison between SAXS of BTB7000 and its complexes (i) BTB7000:Cu$^{2+}$ and (ii) BTB7000:Cu$^{+}$ in mixed solvent CH$_3$COCH$_3$/CH$_3$OH (vol/vol 9/1) at 10 g/L.

The radii of gyration, $R_g$, computed using either Guinier plots, or from the P(r) function generated by Fourier transform of the SAXS pattern, are summarized in Table 2.2. The SAXS patterns clearly show that the $R_g$ values of the BTB7000:Cu$^{2+}$ and BTB7000:Cu$^+$ MSP are substantially larger than that of the BTB7000 monomer. Moreover, $R_g$ of BTB7000:Cu$^{2+}$=1:1 is larger than that of BTB7000:Cu$^{2+}$=1:2 (Table 4.2). It is also evident from the stronger SAXS intensity of BTB7000:Cu$^{2+}$=1:1, despite containing smaller amounts of the strongly scattering metal ion that the molecular weight of BTB7000:Cu$^{2+}$=1:1 is higher than that of BTB7000:Cu$^{2+}$=1:2. In contrast, the $R_g$ of
BTB7000:Cu⁺=1:2 is larger than that of BTB7000:Cu⁺=1:1 (Table 4.2). These results are consistent with the viscometric data in Figure 4.4.
Figure 4.7. Pair distance distribution function $P(r)$ of BTB7000 Cu$^{2+}$ (i) and Cu$^+$ (ii) complexes at 1:1 and 1:2 ratios.

Table 4.2. The radii of gyration of BTB7000:Cu$^{2+}$ and BTB7000:Cu$^+$ complexes from Guinier plots and Fourier transform at 10 g/L.

<table>
<thead>
<tr>
<th>$R_g$ (nm)</th>
<th>BTB7000:Cu$^{2+}$ =1:1</th>
<th>BTB7000:Cu$^{2+}$ =1:2</th>
<th>BTB7000:Cu$^+$ =1:1</th>
<th>BTB7000:Cu$^+$ =1:2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinier Plots</td>
<td>33.7±0.6</td>
<td>27.1±0.7</td>
<td>28.3±0.2</td>
<td>37.3±0.2</td>
</tr>
<tr>
<td>Fourier Transform</td>
<td>33.5±0.2</td>
<td>26.3±0.6</td>
<td>28.9±0.3</td>
<td>32.0±0.2</td>
</tr>
</tbody>
</table>

Efforts were made to obtain information about the concentration-dependence of the molecular weight of MSP formed by the BTB7000:Cu$^{2+}$ and BTB7000:Cu$^+$ systems. One characteristic of such reversible self-assembling macromolecular systems is that the
average molecular weight increases with concentration in a manner dependent on the equilibrium constant for self-association. Such an increase can be characterized in principle by monitoring the corresponding decrease of the static light scattering quantity $\frac{Kc}{\Delta R(0,c)}$, the inverse of which provides a measure of the increase in apparent molecular weight (c.f. equation 16). BTB7000:Cu$^{2+}$ complexes indeed showed such behavior at extremely low concentrations (0.01-0.04 g/L for BTB7000:Cu$^{2+}$=1:1 and 0.1-0.3 g/L for BTB7000:Cu$^{2+}$=1:2). Assuming the contributions of virial coefficients can be neglected, i.e., that the apparent molecular weights are the true values, the degree of polymerization and apparent ligand-metal ion binding constants ions can be calculated from the slope of a plot of $\frac{Kc}{\Delta R(0,c)}$ versus concentration. However, since these measurements were performed at very low concentrations, where the solution scattering intensities were not much different from the solvent, the resulting error bars in the excess scattered light intensity made such interpretation unreliable. The same technique was also attempted on BTB7000:Cu$^+$ complexes. Here a distinct difference in behavior of the solutions was observed. It was found that light scattering intensities of the BTB7000:Cu$^+$ complex solutions of a specified concentration (BTB7000 at 0.5 g/L), prepared by diluting the solution from higher concentrations (BTB7000 at 5 g/L) did not match the intensities of solutions made up at the same composition and concentration. This suggests that the formation of the BTB7000:Cu$^+$ complexes is not as reversible as that of the BTB7000:Cu$^{2+}$ complexes. Attempts were also made to extract values of the hydrodynamic radii of BTB7000 and BTB7000:Cu$^+$ and BTB7000:Cu$^{2+}$ complexes from dynamic light scattering analysis. These attempts were not successful because of
exceedingly poor signal-to-noise ratio of the measured correlation functions, presumably due to the weak scattering intensities coupled to the limited power of the laser source available for these particular experiments (15 mW He/Ne laser).

4.3.2 Bip-pTHF-Bip at 4200 g/mol

Prior to implementing studies on BTB4200 of number-average molecular weight 4200 g/mol, we found that the mixed solvent CHCl₃/CH₃OH could be replaced by butyronitrile. This simplifies experimental procedures associated with UV-Vis titration and light scattering analysis. The UV-Vis absorption of BTB4200 complexes with Cu²⁺ and Cu⁺ in butyronitrile is displayed in Figure 4.8 shows they absorb radiation at the two available laser wavelengths, 514.5 nm and 632.8 nm. The Cu²⁺ complexes as reflected in the spectrum are green in color, and have an absorption peak around 632.8 nm and an absorption minimum around 514.5 nm. The Cu⁺ complexes are brown and absorb more strongly at 514.5 nm than at 632.8 nm. Because of the higher laser power available, it was preferred to use laser radiation of 514.5 nm wavelength for static and dynamic light scattering experiments. For BTB4200 and its Cu²⁺ complexes, laser radiation of 514.5 nm wavelength was indeed used. For Cu⁺ complexes because of the higher absorption at 514.5 nm, laser of 632.8 nm was wavelength was used for static light scattering, and radiation of 514.5 nm was attempted for dynamic light scattering due to the requirement of higher scattering intensity for DLS analysis.
Figure 4.8. UV-Vis absorption of BTB4200 complexes with Cu\(^{2+}\) and Cu\(^{+}\) in butyronitrile.

4.3.2.1 Viscosity Studies

The variation in solution viscosity was monitored while titrating 10 g/L BTB4200 (2.38 mM) with a mixed solution of 10 g/L (2.38 mM) BTB4200 and 10 mM Cu\(^{+}\) or Cu\(^{2+}\) ions. As seen in Figure 4.9 (i) the resulting viscosity exhibits a maximum value at a BTB4200:Cu\(^{2+}\) ratio of 1:1 when BTB4200 was titrated against Cu\(^{2+}\). Since one BTB4200 molecule has one MeBIP ligand at either end, these viscosity results confirm that one Cu\(^{2+}\) ion binds to two MeBIP ligands in solution (consistent with the above-reported experiments on the BTB of M\(_n\) = 7000, and with the reported crystal structure of MeBIP - Cu\(^{2+}\) - MeBIP complexes\(^{16}\)). When BTB4200 is titrated against Cu\(^{+}\), the viscosity increases and exhibits a significant change in slope when BTB4200:Cu\(^{+}\)=1:2 (again consistent with the reported crystal structure\(^{17}\)). Beyond this point, the viscosity increases very slowly in contrast to the results when titrating against Cu\(^{2+}\), and also in
contrast with the earlier result, when titrating BTB7000 against Cu\(^+\). We attribute the difference between the two titration experiments to the fact that the earlier experiment used a mixed solvent (9:1 CHCl\(_3\):CH\(_3\)OH), whereas the later experiment used a common solvent (butyronitrile). The decrease in viscosity beyond the stoichiometric equivalent ratio in the former case may reflect a higher reversibility of Cu\(^+\)-MeBIP binding in the mixed solvent, coupled with the fact that the concentration of macromonomer decreases slightly during the mixed solvent titration.

The present result suggests that binding between MeBIP and Cu\(^+\) is not as reversible as that between MeBIP and Cu\(^{2+}\), which is consistent with our above-reported observation of irreversibility in light scattering from BTB7000:Cu\(^+\) complexes. Notwithstanding the indication of irreversibility of complex formation between BTB4200 and Cu\(^+\), the viscosity results again confirm the validity of the different stoichiometries for binding between the MeBIP ligand and Cu\(^{2+}\) versus Cu\(^+\) in solution and again also support the possibility of creating a redox viscosity switch as illustrated in Figure 4.1.
Figure 4.9. Viscosity titration of Cu(OTf)$_2$ (i) and Cu(NCCH$_3$)$_4$OTf (ii) to monomer BTB4200 in butyronitrile.

In Figure 4.10, the reduced specific viscosities, $\eta_{sp}/c$, of copper complexes in butyronitrile at ratios of 1:1 and 1:2 were compared against $\eta_{sp}/c$ of BTB4200 itself. The solutions were prepared at 10 g/L and the viscosities were measured in butyronitrile at 10 g/L, and also after dilution to 8 g/L. For these solutions of low molecular weight polymers, reduced viscosity values at higher dilutions were found to give unphysical results, presumed due to systematic errors in concentration values and flow time measurements attendant to the in situ dilution process using the Ubbelohde viscometer. The resulting viscosity values fall into three groupings: BTB4200:Cu$^{2+}$=1:1 $\approx$ BTB4200:Cu$^{+}$=1:2 $>$ BTB4200:Cu$^{2+}$=1:2 $\approx$ BTB4200:Cu$^{+}$=1:1 $>$ BTB4200, which is
consistent with the expected order of high and low molecular weight MSP species illustrated in Figure 4.1. Figure 4.11 presents a comparison between the results of the viscosity titration experiments (Figure 4.9) and reduced specific viscosity values generated at 10g/L concentration for freshly prepared BTB4200:Cu$^{2+}$ and BTB4200:Cu$^+$ complexes in butyronitrile at ratios of 1:1 and 1:2 (Figure 4.10). The reduced specific viscosities of solutions of freshly-made BTB4200:Cu ($Cu^{2+}$ and $Cu^+$) complexes are in good agreement with the titration values at BTB4200:Cu = 1:1 and 1:2, respectively.

![Graph](image)

**Figure 4.10.** Change in reduced specific viscosity, $\eta_{sp}/c$, plotted versus $c$ for BTB4200, BTB4200:Cu$^{2+}$ and BTB4200:Cu$^+$ complexes in butyronitrile at ratios of 1:1 and 1:2, respectively, on dilution from 10 g/L.
Figure 4.11. Viscosity data comparison BTB4200:Cu$^{2+}$ and BTB4200:Cu$^+$ complexes between titration experiments (Figure 4.9) and newly prepared samples at 10 g/L (Figure 4.10). Stars are the viscosity data measured from newly prepared samples.

To exclude the polyelectrolyte effect in the formation of these metallo-supramolecular polymers, the viscosities of BTB4200:Cu complexes ($\text{Cu} = \text{Cu}^{2+}$ or $\text{Cu}^+$) = 1:1 and 1:2 were determined in butyronitrile containing 0.1 M inert salt: $\text{N(C}_4\text{H}_9\text{)}_4\text{PF}_6$. For BTB4200 monomer at 10 g/L in 0.1 M salts $\eta_{sp}/c$ was determined to be 0.124 g/L, essentially the same as that in the absence of inert salt. Figure 4.12 shows that for all Cu complexes, the addition of inert salt decreases the viscosity slightly through the whole concentration range studied. The decrease seems to be somewhat larger at lower concentrations, which is consistent with what one expects due to the screening of intermolecular electrostatic repulsions, so the polyelectrolyte effect appears to have a very weak effect on the self-assembly process. The viscosity of BTB4200:Cu$^+$=1:2
solutions exhibits no decrease of viscosity at 10 g/L in the presence of salts. Based on later light scattering evidence of increased instability in solutions of the BTB4200:Cu\(^+\) complexes in the presence of added inert salt, we attribute this result to the fact that these solutions contain higher molecular weight complexes which compensates the screening of polyelectrolyte effect. In total, our results confirm the expected different binding stoichiometries of Cu\(^{2+}\) and Cu\(^+\) with MeBIP, and again support the possibility to make redox-active supramolecular polymers as hypothesized in Figure 4.1.
BTB4200
- Pure Butyronitrile
- 0.1 M N(C₄H₉)₄PF₆

BTB4200:Cu²⁺=1:2
- Pure Butyronitrile
- 0.1 M N(C₄H₉)₄PF₆

η_sp/c (L/g)

c (g/L)

(ii)

BTB4200
- Pure Butyronitrile
- 0.1 M N(C₄H₉)₄PF₆

BTB4200:Cu⁺=1:1
- Pure Butyronitrile
- 0.1 M N(C₄H₉)₄PF₆

η_sp/c (L/g)

c (g/L)

(iii)
Figure 4.12. Comparison between the viscosities of (i) BTB4200:Cu$^{2+}$=1:1; (ii) BTB4200:Cu$^{2+}$=1:2; (iii) BTB4200:Cu$^{+}$=1:1 and (ii) BTB4200:Cu$^{2+}$=1:2 solutions with and without 0.1 M N(C$_4$H$_9$)$_4$PF$_6$, respectively.

4.3.2.2 Dynamic Light Scattering

Polarized dynamic light scattering experiments were carried out on BTB4200 and its Cu$^{2+}$, Cu$^{+}$ complexes in butyronitrile at scattering angles $\theta = 30^\circ$ and $90^\circ$, using high intensity laser light (Stabilite Ar$^+$ laser, emitting vertically-polarized light at $\lambda = 514.5$ nm with a power output of 1–1.5 W). The correlation functions of BTB4200:Cu$^{+}$ complexes at 632.8 nm were similar to those at 514.5 nm but had worse statistics due to the weaker laser power. Measurements at $\theta = 30^\circ$ and $90^\circ$ gave similar results and here data at $30^\circ$ are presented because the decay rate was slower with the better statistics. Three different
concentrations were studied: 1, 5 and 10 g/L. It was usually difficult to obtain useful correlation functions for 1 g/L, so here we focus on 5 and 10 g/L.

BTB4200 monomer exhibited correlation functions with similar decay rates at different concentrations as shown in Figure 4.13 (i). The hydrodynamic radius was determined to be 2 nm from cumulant analysis and 1.8 nm from Contin analysis. This result is consistent with a value of the hydrodynamic radius estimated from the viscosity data. The correlation functions of BTB4200:Cu$^{2+}$ complexes did not show any concentration dependence at 5 and 10 g/L. Figure 4.13 (ii) compares the correlation functions at 10 g/L for the BTB4200:Cu$^{2+}$ =1:1 and BTB4200:Cu$^{2+}$ =1:2 complexes versus that of the BTB4200 monomer. Since the BTB4200:Cu$^{2+}$ complexes exhibit much slower decays compared to the BTB4200 monomer clearly they contain much larger particles. Moreover, BTB4200:Cu$^{2+}$ =1:1 has a slightly slower decay than BTB4200:Cu$^{2+}$ =1:2, and therefore has larger particles. The Contin package indicates a bimodal size distribution of particles as shown in Figure 4.14 (i): one centered at a size comparable to the BTB4200 monomer and the other centered at a hydrodynamic radius of 30 to 80 nm. Although the relative scattering amplitude of the fraction with the larger size appears to be enormous, their actual mass concentration is in fact quite small. This is because the intensity of light scattered by a macromolecule scales approximately as cMP(q) (the mass concentration times the molecular weight times the particle scattering function). Since P(q) has a value unity at q = 0, and decreases monotonically as q increases, at low scattering angles, q → 0, the scattering intensity scales as cM, and so the mass concentration present for each particles scales as the intensity of that species divided by its molecular weight. At large scattering angles, the weighting of the larger particles
decreases since \( P(q) \) becomes increasingly smaller. Thus, at \( \theta = 30^\circ \), the relative scattering amplitudes of each species is proportional to \( cM \); at \( \theta = 90^\circ \) where \( P(q) \ll 1 \)
for the larger particles, the relative scattering amplitudes of the two species are comparable (Figure 4.14 (ii)), confirming that the mass concentrations of the large particles are in fact quite small. Figure 4.13 (iii) indicates surprisingly that DLS analysis yields correlation functions of BTB4200:Cu\(^+\) complexes and BTB4200 monomer with similar decay rates. This implies no significant difference in the apparent hydrodynamic radii of BTB4200:Cu\(^+\) complexes and BTB4200 monomer. A possible explanation is that the BTB4200:Cu\(^+\) complexes form mainly rings of relatively low degree of polymerization. The hydrodynamic radii of such rings could be rather similar to that of the linear BTB4200 macromonomer.\(^{24}\) However, this would not be consistent with the fact that \( \eta_{sp}/c \) is substantially larger for BTB4200:Cu\(^+\) complexes than for BTB4200 monomer at 10 g/L. Assuming that \( \eta_{sp}/c \sim N_A V_h/M \), such rings would have smaller \( \eta_{sp}/c \) than that of the linear macromonomer. Comparison of macromolecular size information for polyelectrolytes derived from DLS data and viscometric data at finite concentration in the absence of added inert salt is fraught with difficulty because of the potentially strong contribution of electrostatic repulsions. Thus the fact that the hydrodynamic radii of BTB4200:Cu\(^+\) complexes and BTB4200 monomer at 10 g/L are similar, but \( \eta_{sp}/c \) is substantially larger for BTB4200:Cu\(^+\) complexes than for BTB4200 monomer at 10 g/L, is perplexing, but may simply reflect inadequate compensation for intermolecular electrostatic repulsions through dilution, a task made difficult by the weak scattering power of the solutions. The presence of strong electrostatic repulsions would normally be expected to dramatically increase the diffusion coefficient of a polion, and thus give an
erroneously low estimate of the hydrodynamic radius. Analysis of SLS data from these solutions described later suggests that this may indeed be a possibility. Comparison of DLS size data for BTB4200:Cu$^{2+}$ complexes versus the viscometric data on the same material is rendered difficult because the strong light scattering due to the presence of small quantities of large particles makes it difficult to determine accurately the size of the more abundant smaller macromolecules, which are likely to make the dominant contribution to the solution viscosity.
Figure 4.13. Normalized correlation functions of (i) BTB4200 at 5 and 10 g/L; (ii) comparison between BTB4200, BTB4200:$\text{Cu}^{2+}$=1:1 and BTB4200:$\text{Cu}^{2+}$=1:2 at 10 g/L. (iii) comparison between BTB4200, BTB4200:$\text{Cu}^{+}$=1:1 and BTB4200:$\text{Cu}^{+}$=1:2 at 10 g/L.
To further understand the scattering properties of the supramolecular polymers, DLS experiments were performed on BTB4200 and its complexes with Cu$^{2+}$ and Cu$^+$ at a macromonomer concentration of 10 g/L in butyronitrile containing 0.1M N(C$_4$H$_9$)$_4$PF$_6$ salt. As expected, the addition of 0.1 M inert salt does not significantly change the correlation function for BTB4200 macromonomer (Figure 4.15 (i)). The inert salt does produce a large change in the DLS correlation function for the BTB4200 Cu$^{2+}$ complexes: eliminating the dominant slow decay, and hence evidently destroying the large particles with hydrodynamic radii of 30-80 nm. In the presence of inert salts, the BTB4200:Cu$^{2+}$=1:2 complexes exhibit a correlation function with similar decay rate to that of the BTB4200, while the correlation function of BTB4200:Cu$^{2+}$=1:1 complexes decays slower than that of the BTB4200 and hence indicates a higher degree of aggregation, as expected. The particle size distributions derived via Contin analysis also confirm this observation (Figure 4.16). The mean hydrodynamic radii are 5.1 nm for BTB4200:Cu$^{2+}$=1:1 and 1.75 nm for BTB4200:Cu$^{2+}$=1:2 in inert salts. Thus the large particles observed in the absence of inert salt appear to be some type of cluster due to electrostatic interactions. The observation of slow modes in dynamic light scattering from polyelectrolytes at very low ionic strength has been reported in the literature. The hydrodynamic size after electrostatic interactions are screened by addition of inert salt appear to be more consistent with expectation and viscosity data.
For Cu\(^+\) complexes, the presence of inert salt results in correlation functions which decay more slowly as seen in Figure 4.15 (iii) and yields a mean hydrodynamic radius of 5.0 nm, which indicates a significant level of BTB4200:Cu\(^+\) complex formation, reinforces the conclusion that DLS detection of complex formation in the absence of salt is masked by the increase in diffusion coefficient due to electrostatic repulsions. Note that, unlike Cu\(^{2+}\) complexes, Cu\(^+\) complexes are not thermodynamically stable and DLS analysis indicates formation of very large aggregates in BTB4200:Cu\(^+\) solutions at 10 g/L after storage in excess of one week (Figure 4.17). Increased Cu\(^+\) ion concentration increases the rate of such aggregation. The addition of inert salts appears to enhance this instability, and DLS detects formation of large aggregates in these solutions after one or two days.
Figure 4.15. Normalized correlation functions of (i) BTB4200 10 g/L with and without 0.1M N(C₄H₉)₄PF₆; (ii) comparison between normalized correlation functions of...
BTB4200, BTB4200:Cu^{2+}=1:1 and BTB4200:Cu^{2+}=1:2 at 10 g/L in the presence of 0.1M N(C_{4}H_{9})_{4}PF_{6}; (iii) comparison between normalized correlation functions of BTB4200, BTB4200:Cu^{+}=1:1 and BTB4200:Cu^{+}=1:2 at 10 g/L in the presence of 0.1M N(C_{4}H_{9})_{4}PF_{6}.

Figure 4.16. Intensity amplitude integration plot of size distribution for BTB4200 Cu^{2+} complexes in 0.1 M inert salts from Contin analysis.
Figure 4.17. Time effect of DLS data for BTB4200 Cu$^+$ complexes without salt: (i) BTB4200:Cu$^+=1:1$; (ii) BTB4200:Cu$^+=1:2$. 
It is relevant to compare the DLS results in the presence of inert salt (Figure 4.15 and 4.16) with the above measurements of reduced viscosity in the presence of inert salt (Figure 4.12). For BTB4200 Cu\(^{2+}\) complexes, it is evident that consistency is seen because DLS detects the presence of particles in the size range from 2 to 25 nm in BTB4200:Cu\(^{2+}\) = 1:1 i.e. larger than the unassociated macromonomer (Figure 4.16), and the reduced specific viscosity of BTB4200:Cu\(^{2+}\) = 1:1 is also higher than that of the unassociated macromonomer (Figure 4.12), confirming the formation of complexes. For BTB4200:Cu\(^{+}\) = 1:2 complexes, consistency is also found since the diffusion coefficient is smaller than that of the macromonomer (Figure 4.16), and again the viscosity is higher than that of the unassociated macromonomer (Figure 4.12).

4.3.2.3 Differential Refractive Index Increment Determination

Prior to applying static light scattering to determining the molecular weights of the metallo-supramolecular polymers formed via complexation of Cu ions with the BTB4200 monomer, it is necessary to determine the differential refractive index increments, dn/dc, which are a measure of the light scattering contrast factor in solution. dn/dc values were determined using a Brice-Phoenix differential refractometer, which records the deflection (S) of light passing through a split cell containing solution in one compartment and solvent in the other. The deflection is then proportional to the refractive index difference between solution and solvent. Calibration was performed using aqueous KCl solutions (with known dn/dc at 25°C in Table 4.3) at several concentrations (1, 5, 10, 20, and 30 g/L), and at three wavelengths (589, 564, 436 nm), from which the wavelength dependence of dn/dc of the unknown sample was determined. The concentrations of macromonomer and its metal ion complexes were in the range 0.5 to 5 g/L. First, the
slopes of plots of displacement versus refractive index of KCl solutions (see Appendix, Figure A2.1) provide \( \frac{dS}{dn} \) values at each wavelength for the instrument listed in Table A2.1 in Appendix. Then the slopes of plots of displacement versus concentration for the solutions with unknown \( \frac{dn}{dc} \) (See Appendix, Figure A2.2) generate \( \frac{dS}{dc} \) values. Combining the \( \frac{dS}{dn} \) of the instrument with the \( \frac{dS}{dc} \) measured for the unknown samples (Table A2.2 in Appendix), \( \frac{dn}{dc} \) values for each unknown sample were calculated at three different wavelengths (Table 4.4) and are plotted in Figure A2.3 (See Appendix). From the wavelength dependence of the differential refractive index, \( \frac{dn}{dc} \) values of BTB4200, BTB4200:Cu\(^{2+}\)=1:1, BTB4200:Cu\(^{2+}\)=1:2, BTB4200:Cu\(^+\)=1:1 and BTB4200:Cu\(^+\)=1:2 were determined at \( \lambda = 514.5 \text{ nm or 632.8 nm} \) (Table 4.5). Finally, the optical constants \( K \) in equation 15 were calculated for each species (Table 4.5).

**Table 4.3:** Differential refractive index increment of KCl at 25°C and three different wavelengths.

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>436</th>
<th>546</th>
<th>589</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \frac{dn}{dc} ) (mL/g)</td>
<td>0.135660</td>
<td>0.131033</td>
<td>0.129966</td>
</tr>
</tbody>
</table>
Table 4.4. Differential refractive index for BTB4200 and its Cu$^{2+}$ and Cu$^{+}$ complexes at three wavelengths.

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>436</th>
<th>546</th>
<th>589</th>
</tr>
</thead>
<tbody>
<tr>
<td>dn/dc (mL/g)</td>
<td>BTB4200</td>
<td>0.154±0.005</td>
<td>0.149±0.004</td>
</tr>
<tr>
<td></td>
<td>BTB4200:Cu$^{2+}$=1:1</td>
<td>0.150±0.007</td>
<td>0.138±0.008</td>
</tr>
<tr>
<td></td>
<td>BTB4200:Cu$^{2+}$=1:2</td>
<td>0.156±0.007</td>
<td>0.140±0.009</td>
</tr>
<tr>
<td></td>
<td>BTB4200:Cu$^{+}$=1:1</td>
<td>0.159±0.005</td>
<td>0.147±0.006</td>
</tr>
<tr>
<td></td>
<td>BTB4200:Cu$^{+}$=1:2</td>
<td>0.153±0.007</td>
<td>0.144±0.007</td>
</tr>
</tbody>
</table>

Table 4.5. Differential refractive index increment values and corresponding optical constants for BTB4200 and its Cu$^{2+}$, Cu$^{+}$ complexes at 514.5 or 632.8 nm.

<table>
<thead>
<tr>
<th>λ (nm)</th>
<th>BTB4200</th>
<th>BTB4200 2+:Cu$^{+}$=1:1</th>
<th>BTB4200 2+:Cu$^{+}$=1:2</th>
<th>BTB4200 2+:Cu$^{+}$=1:1</th>
<th>BTB4200 2+:Cu$^{+}$=1:2</th>
</tr>
</thead>
<tbody>
<tr>
<td>dn/dc (mL/g)</td>
<td>514.5</td>
<td>0.150 ±0.005</td>
<td>0.140 ±0.008</td>
<td>0.143 ±0.009</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>632.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.139 ±0.006</td>
</tr>
<tr>
<td>K (cm$^2$ mol/g$^2$)</td>
<td>514.5</td>
<td>4.03 ±0.27$\times$10$^{-7}$</td>
<td>3.53 ±0.41$\times$10$^{-7}$</td>
<td>3.68 ±0.48$\times$10$^{-7}$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>632.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.48 ±0.31$\times$10$^{-7}$</td>
</tr>
</tbody>
</table>

Subsequently, as discussed later, to more accurately determine the molecular weight from static light scattering, the differential refractive index increment value of HO-MeBip in butyronitrile was determined against KCl (see Appendix, Figure A2.4 (i).
and A2.4 (ii)). Heat was supplied during sample preparation to increase the solubility. From the dn/dc values listed in Table 4.6 and their wavelength dependence in Figure A2.4 (iii), the dn/dc value of HO-MeBip in butyronitrile at 514.5 nm was determined to be 0.300±0.004 g/mL.

**Table 4.6.** Differential refractive index for HO-MeBip at three wavelengths.

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>436</th>
<th>546</th>
<th>589</th>
</tr>
</thead>
<tbody>
<tr>
<td>dn/dc (g/mL)</td>
<td>0.330±0.004</td>
<td>0.293±0.004</td>
<td>0.281±0.006</td>
</tr>
</tbody>
</table>

### 4.3.2.4 Static Light Scattering

To determine the apparent molecular weight and apparent radius of gyration of BTB4200 and its Cu$^{2+}$ and Cu$^+$ complexes, and to compare them with the hydrodynamic radius from dynamic light scattering discussed above, static light scattering was performed in butyronitrile. The resulting data on the concentration- and angle-dependence of the scattered intensity are presented in the form of Zimm plots for BTB4200 (Figure 4.18 (i)); BTB4200:Cu$^{2+}$=1:1 (Figure 4.18 (ii)); BTB4200:Cu$^{2+}$=1:2 (Figure 4.18 (iii)); BTB4200:Cu$^+$=1:1 (Figure 4.18 (iv)) and BTB4200:Cu$^+$=1:2 (Figure 4.18 (v)). The scattered intensity from the BTB4200 macromonomer was very weak and exhibited no systematic angle dependence within experimental error at the highest concentration, indicating it has a radius of gyration smaller than approximately 1/20 of the laser wavelength, i.e. 26 nm. The angle-dependence seen at higher dilutions is viewed as artifactual and represents some systematic error in the excess intensities, perhaps stemming from a slight increase in parasitic scattering at lower scattering angles. From
the average of all intensity values at each concentration, apparent weight average molecular weights for BTB4200 were obtained: 1715±322 g/mol (1 g/L), 2194±105 g/mol (5 g/L) and 2336±56 g/mol (10 g/L). No significant concentration dependence exists for BTB4200 except that the statistics of the data deteriorates with decreasing concentration. Thus virial coefficients do not contribute significantly to the concentration dependence of light scattering from BTB4200 in the concentration range studied. One discrepancy noticed is the apparent weight average molecular weight from static light scattering is consistently smaller than the number average molecular weight (4200 g/mol) obtained from NMR, UV titration and also proved by viscosity titration. This anomaly is due to the quite different polarizabilities and hence different effective differential refractive index increments for the end groups (MeBIP) versus the core (polytetrahydrofuran) of BTB4200 (c.f. Figure 4.2). Thus the BTB4200 monomer can be viewed as a triblock copolymer composed of MeBIP end groups and polytetrahydrofuran core. The dn/dc value of a copolymer can be expressed in terms of the dn/dc values of each component by equation 19.30 Moreover, the apparent weight average molecular weight can be formulated in terms of the molecular weights and dn/dc values of each component (equation 20).30

\[
dn / dc = \sum W_i (dn / dc)_i
\]  

(19)

\[
M_{w,app} = \frac{1}{c(dn / dc)^2} \sum c_i M_i (dn / dc)^2_i
\]  

(20)

Here \( W_i \) is the weight fraction of each component. \( c \) and \( c_i \) are the total mass concentration and mass concentration of each component, respectively. \( M_i \) is the
molecular weight of each component. Assuming BTB4200 is monodisperse with a molecular weight of 4200 g/mol, the molecular weight of the pTHF core would be 3492 g/mol considering BTB4200 has two MeBIP ligands (molecular weight = 354 g/mol). As discussed in the section on measurement of dn/dc, the dn/dc values for BTB4200 and HO-MeBIP in butyronitrile are 0.150 g/mL and 0.300 g/mL respectively at 514.5 nm. From equation 19, the dn/dc value for the pTHF core was then calculated as 0.120 mL/g. It then follows that the apparent molecular weight computed for the BTB4200 macromonomer from equation 20 is 2323 g/mol, which is quite consistent with the experimental value 2336±56 g/mol determined from light scattering at 10 g/L.
\[ K_c/(R-R_{\text{solvent}}) = \sin(\theta/2)^2 + 0.2c \]

(ii)

(iii)
Figure 4.18. Zimm plot of BTB4200 (i), BTB4200:Cu\(^{2+}\)=1:1 (ii), BTB4200:Cu\(^{2+}\)=1:2 (iii); BTB4200:Cu\(^+\)=1:1 (iv) and BTB4200:Cu\(^+\)=1:2 (v) in butyronitrile at 1, 5 and 10 g/L.
Both BTB4200 Cu\(^{2+}\) complexes, BTB4200:Cu\(^{2+}\)=1:1 and BTB4200:Cu\(^{2+}\)=1:2, show strong angle dependence indicating the existence of very large particles. BTB4200:Cu\(^{2+}\)=1:1 did not show significant concentration dependence, while BTB4200:Cu\(^{2+}\)=1:2 increases with concentration. Apparent weight average molecular weights, M\(_w^{app}\), were determined for Cu\(^{2+}\) complexes from Kc/ΔR(0,c) = 1/M\(_w^{app}\) (see Appendix, Figure A2.5), and, noting that the products q*R\(_g\) were generally smaller than 1, apparent z-average radii of gyration R\(_g^{z,app}\) were calculated from the slopes of Guinier plots of ln(c/ΔR(q,c)) versus q\(^2\), (see appendix, Figure A2.6) (c.f. equation 18). The results are presented in Table 4.7. The Guinier plots of both complexes yielded similar apparent R\(_g\) values falling between 43-58 nm. These values are much larger than expected for metallo-supramolecular polymers and probably represent some type of dense aggregate or clusters, presumably identical to those seen in DLS experiments. Thus, the R\(_g\) values are consistent with values of the hydrodynamic diameters of larger particles in DLS data (Figure 4.13 (ii) and Figure 4.14) (R\(_h=30-80\) nm). Evidently, in agreement with the DLS data, the scattered intensity in SLS comes mainly from these large aggregates. Compared with the values of the apparent radius of gyration, the apparent molecular weights determined are unphysically small. The origin of this discrepancy is presumed to relate to the fact that the scattering reflects comparable contributions from a bimodal population of particles, one present in very high concentration, but having a very small molecular weight, the other present in very low concentration, but having a large size and hence strongly angle-dependent scattering (c.f. Figures 4.18 (ii) (iii)). Thus the measured weight-average molecular weight reflects strongly the low molecular weight component, but the radius of gyration reflects only the contribution from the large
particles. The increase of apparent $M_w$ with decreasing concentration also indicates the important contribution from virial coefficients, since the aggregation should be less at lower concentrations, i.e. $M_w$ should decrease with concentration.

**Table 4.7.** Apparent weight average molecular weights and apparent radii of gyration for BTB4200:Cu$^{2+}$=1:1 and BTB4200:Cu$^{2+}$=1:2 from Figure A2.5 and A2.6.

<table>
<thead>
<tr>
<th>Species</th>
<th>c (g/L)</th>
<th>Apparent $M_w$ (g/mol)</th>
<th>Apparent $R_g$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTB4200:Cu$^{2+}$=1:1</td>
<td>10</td>
<td>$7.86\pm0.11 \times 10^3$</td>
<td>43±1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>$7.40\pm0.15 \times 10^3$</td>
<td>46±1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>$1.16\pm0.11 \times 10^4$</td>
<td>58±2</td>
</tr>
<tr>
<td>BTB4200:Cu$^{2+}$=1:2</td>
<td>10</td>
<td>$9.18\pm0.16 \times 10^3$</td>
<td>46±1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>$1.82\pm0.04 \times 10^4$</td>
<td>53±1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>$2.41\pm0.11 \times 10^4$</td>
<td>57±1</td>
</tr>
</tbody>
</table>

The SLS data for the BTB4200:Cu$^+$ complexes in Figure 4.18 show no evidence of scattering from large aggregates, consistent with the above reported DLS experiments (Figure 4.13 (iii)). The apparent weight-average molecular weights estimated at each concentration form the relation $Kc/\Delta R(0,c) = 1/M_{w,app}$ are listed in Table 4.8. The results for BTB4200:Cu$^+$=1:1 (Figure 4.18 (iv)) and BTB4200:Cu$^+$=1:2 (Figure 4.18 (v)) are self
consistent in that they generate erroneously low apparent molecular weight averages at the two higher concentrations, viz. $M_w^{\text{app}} \sim 10^3$, smaller than the measured $M_w^{\text{app}}$ of the BTB4200 macromonomer. At the lowest concentration, for both complexes, $M_w^{\text{app}}$ increases to a value comparable to that of the macromonomer. This is in fact what one expects for a polyion at low ionic strength, i.e. the large positive second virial coefficient stemming from the polyelectrolyte effect will cause $M_w^{\text{app}}$ values to increasingly deviate on the lower side from the true $M_w$ value as concentration increases. At the lowest concentration, both the extent of any aggregation and the contribution from virial coefficients are minimized. The presence of a large positive second virial coefficient could also provide a rationale for the fact that the diffusion coefficient of the BTB4200:Cu$^+$ MSP is comparable to that of the BTB4200 monomer. Even if aggregation is present so that the hydrodynamic radius increases, the contribution of the virial coefficient could mask the expected decrease in the diffusion coefficient. Our DLS results indicating that addition of inert salt decreases the value of the diffusion coefficient of the BTB4200:Cu$^+$ complexes (Figure 4.15 (iii)) provides further support for this explanation.

**Table 4.8.** Apparent weight average molecular weights for BTB4200:Cu$^+$ =1:1 and BTB4200:Cu$^+$ =1:2 from Figure 4.18 (iv) and (v).

<table>
<thead>
<tr>
<th>Species</th>
<th>BTB4200:Cu$^+$ =1:1</th>
<th>BTB4200:Cu$^+$ =1:2</th>
</tr>
</thead>
<tbody>
<tr>
<td>c (g/L)</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Apparent $M_w$</td>
<td>983±41</td>
<td>1165±58</td>
</tr>
</tbody>
</table>
DLS has shown that the large aggregates in BTB4200:Cu$^{2+}$ complexes can be destroyed in 0.1 M inert salt ($(\text{NC}_4\text{H}_9)_4\text{PF}_6$). SLS experiments were carried out to confirm this interpretation. The resulting Zimm plots for 10 g/L solutions of BTB4200:Cu$^{2+}=1:1$ and BTB4200:Cu$^{2+}=1:2$ (in which dn/dc values were assumed the same as in the absence of salt) confirmed equivalent behavior (Figure 4.19 (i)): The plots no longer show any angle dependence. The apparent M$_w$ was calculated as 4902±64 g/mol and 1947±23 g/mol for BTB4200:Cu$^{2+}=1:1$ and BTB4200:Cu$^{2+}=1:2$. Consistent with the DLS and viscosity data, performed in the presence of 0.1 M salts to screen the polyelectrolyte effect, BTB4200:Cu$^{2+}=1:1$ complexes have a higher molecular weight than BTB4200 macromonomer or BTB4200:Cu$^{2+}=1:2$ complexes. Also, the fact that the M$_w$ of the BTB4200:Cu$^{2+}=1:2$ complex is comparable to that of the macromonomer is consistent with the fact that the corresponding hydrodynamic radii measured by DLS are comparable to each other, indicating predominantly unimolecular macrocycle formation. These results are qualitatively consistent with the viscometric results (Figure 4.12) which show in the presence of inert salt the reduced specific viscosity decreases in the order BTB4200:Cu$^{2+}=1:1$ > BTB4200:Cu$^{2+}=1:2$ > BTB4200. Evidently the viscosity measurement is more sensitive than DLS to the presence of macrocycle formation in BTB4200:Cu$^{2+}=1:2$.

A similar picture is seen when comparing the light scattering data for BTB4200:Cu$^+$ complexes versus the corresponding viscometric data. DLS shows that the diffusion coefficients of BTB4200:Cu$^+$ complexes decrease to values smaller than BTB4200 macromonomer in 0.1 M inert salts, and the Zimm plot of SLS data (Figure 4.19 (ii)) also confirms this result. The apparent molecular weights for
BTB4200:Cu$^{+}$=1:1 and BTB4200:Cu$^{+}$=1:2 increase on addition of inert salt from approximately 1000 g/mol to 2242±121 and 3582±101 g/mol respectively. In the presence of inert salts, BTB4200:Cu$^{+}$=1:1 complexes have a similar apparent molecular weight to that of the BTB4200 monomer, indicating macrocycle formation, whereas the higher molecular weight of BTB4200:Cu$^{+}$=1:2 shows substantial high molecular weight complex formation. This is again qualitatively consistent with viscosity results (Figure 4.12) specifically that the reduced specific viscosity of BTB4200:Cu$^{+}$=1:2 > BTB4200:Cu$^{+}$=1:1 > BTB4200.
Finally, SLS experiments were performed for BTB4200 Cu$^{2+}$ complexes using the high power 514.5 nm laser (Figure A2.7), in an attempt to detect an upturn in the quantity $\frac{K_c}{\Delta R(0,c)}$ at very low concentrations (decrease in apparent molecular weight) associated with the dissociation of BTB4200:Cu$^{2+}$ complexes (such information becomes possible in theory because the contribution of the virial coefficients becomes negligible (see Eq. (10)). However, within experimental error no systematic decrease of $\frac{K_c}{\Delta R(0,c)}$ with increasing concentration was found.
4.4 Conclusions and Future Work

Two BTB macromonomers of \( M_n = 4200 \) (BTB4200) and 7000 \( \text{g/mol} \) (BTB7000) were employed here to study their self-assembly behavior in the presence of \( \text{Cu}^{2+} \) and \( \text{Cu}^+ \). Viscometric titrations for both macromonomers show that MeBIP ligands bind \( \text{Cu}^{2+} \) in a 2:1 ratio and \( \text{Cu}^+ \) in a 2:2 ratio in solution. This confirms the potential to use this redox-active pair to switch between metallo-supramolecular polymers of high and low molecular weight. Several significant differences were noted in the titrating the two macromonomers. One difference is that the increase in reduced specific viscosity for both BTB7000:Cu\(^+\) and BTB7000:Cu\(^{2+}\) complexes at maximum self-assembly (from \( \eta_{sp}/c \sim 0.039 \) to \( \sim 0.2 \), i.e. \( \sim x5 \)) is much higher than that of the corresponding BTB4200:Cu complexes (\( \eta_{sp}/c \sim 0.012 \) to \( \sim 0.02 \), i.e. \( \sim x2 \)). This indicates that BTB4200:Cu complexes have smaller hydrodynamic volumes than those of BTB7000:Cu complexes. While some part of this difference stems from the difference in molecular weights of the macromonomers, it may also reflect that BTB4200:Cu complexes form a substantial fraction of rings, whereas BTB7000:Cu complexes form a higher percentage of linear supramolecular polymers. A second difference is that a change in rate of increase with addition of \( \text{Cu}^+ \) is seen for BTB4200:Cu\(^+\) complexes rather than a distinct maximum as seen for BTB7000:Cu\(^+\) complexes. This suggests a measure of irreversibility in the formation of BTB4200:Cu\(^+\) complexes. The fact that irreversibility is not seen in the titration of BTB7000 is ascribed to the fact that that experiment involved titration in a mixed solvent system, which necessitated some dilution of the system during titration. The presence of irreversibility in the formation of Cu\(^+\) complexes is further supported by
the observation that light scattering measurements of molecular weight of BTB7000:Cu\(^+\) on a solution (BTB7000 at 0.5 g/L) obtained via dilution from a more concentrated solution (BTB7000 at 5 g/L) differ from corresponding measurements on a freshly-made solution at the same concentration.

Consistent with the viscosity data of BTB7000:Cu complexes, SAXS indicated the radii of gyration for BTB7000:Cu complexes varied in the sequence BTB7000:Cu\(^+\)=1:2 > BTB7000:Cu\(^{2+}\)=1:1 > BTB7000:Cu\(^+\)=1:1 ≈ BTB7000:Cu\(^{2+}\)=1:2. Also, the SAXS data indicate that the BTB7000:Cu\(^{2+}\) complex has a bimodal size distribution, a minor component having a radius of gyration comparable to that of the monomer, and a major component with substantially larger sizes. In contrast, SAXS analysis suggests that BTB7000:Cu\(^+\) complexes exhibit a continuous size distribution, likely extending down to dimers in equilibrium with a small amount of macromonomer.

Static light scattering determined a weight average molecular weight of the BTB4200 macromonomer smaller than the measured number-average molecular weight. This discrepancy was resolved by a model calculation taking consideration of the very different polarizabilities of the ligand end-groups and polyTHF core. DLS indicates a discontinuous bimodal size distribution of BTB4200:Cu\(^{2+}\) complexes: one having hydrodynamic radii similar to the BTB4200 monomer, the other enormously larger and attributed to the formation of some type of cluster due to electrostatic interaction, since they are destroyed by the addition of inert salt. SLS experiments on solutions of BTB4200:Cu\(^{2+}\) complexes confirm this picture. Strong angle-dependence of the SLS intensity is observed coupled to a weight-average molecular weight that is unphysically
small compared to the measured radius of gyration. This is additional evidence of a bimodal distribution consisting of a trace amount of very large structures coexisting with a large population of small molecules. Because of the dominant light scattering contribution of the very large clusters formed in salt-free BTB4200:Cu$^{2+}$ solutions, quantitative structural information about the predominant population of smaller molecules cannot be obtained by SLS or DLS. Thus, for this case, the viscometric titration data provide a more reliable picture of the extent of metallo-supramolecular polymer self-assembly.

In the presence of 0.1 M added inert salt, DLS analysis indicates the hydrodynamic radius of BTB4200:Cu$^{2+}$=1:1 complexes is larger than that of BTB4200:Cu$^{2+}$=1:2 complexes, and that the latter is comparable to that of the BTB4200 macromonomer experiments with or without inert salt. SLS experiments on the same solutions indicate a weight-average molecular weight of BTB4200:Cu$^{2+}$=1:1 complexes which is approximately twice that for BTB4200:Cu$^{2+}$=1:2, the latter being comparable to that determined for the BTB4200 macromonomer. These results suggest with the formation of metallo-supramolecular polymers having a weight-average aggregation number of approximately 2.0 at the stoichiometric composition BTB4200:Cu$^{2+}$=1:1. The DLS and SLS results are qualitatively consistent with the corresponding reduced specific viscosity data which decrease in the order BTB4200:Cu$^{2+}$=1:1 > BTB4200:Cu$^{2+}$=1:2 > BTB4200. Evidently the viscosity measurement is more sensitive than DLS to the presence of a macrocycle formation in BTB4200:Cu$^{2+}$=1:2
In contrast, DLS experiments performed in the absence of added salt found no evidence of formation of large clusters for BTB4200:Cu⁺ complexes, and that the hydrodynamic radii measured at 10 g/L are comparable to those of the BTB4200 macromonomer. However, SLS measurements of the weight-average molecular weight at 10 g/L yielded values smaller than the molecular weight of the monomer, which indicates a significant contribution of electrostatic repulsions to the second osmotic virial coefficient. This implies that the hydrodynamic radii determined from DLS experiments at finite concentration will be erroneously small. In the presence of added salt, the correlation functions in DLS showed slower decays and gave hydrodynamic diameters of 10 nm for BTB4200 Cu⁺ complexes, indicating significant complex formation. SLS analysis in inert salt indicates that the apparent weight-average molecular weight of BTB4200:Cu⁺=1:2 complexes is higher than that for BTB4200:Cu⁺=1:1, the latter being comparable to that determined for the BTB4200 macromonomer. Again these data are qualitatively consistent with the corresponding reduced specific viscosity data which decrease in the order BTB4200:Cu⁺=1:2 > BTB4200:Cu⁺=1:1 > BTB4200.

In summary, it appears that the most reliable information on the self-assembly of these BTB:Cu-based MSP in the absence of added inert salt is obtained from viscometric data, compared to DLS or SLS experiments. Analysis using the latter techniques is rendered difficult because of the weak scattering intensity of these relatively low molecular weight complexes, and, at low ionic strength, by the strong thermodynamic non-ideality due to electrostatic interactions. From these results, it is clear that MSP formation occurs, and that its extent at a fixed composition of ligand to metal ion depends on the redox state of Cu. This opens up the possibility of a redox viscosity switch based
the stoichiometric compositions BTB:Cu=1:1 or BTB:Cu$^{2+}$=1:2. From viscometric measurements, it is also clear that the magnitude of the viscosity switch increases dramatically with the molecular weight of the macromonomer. The sensitivity to molecular weight (observed by comparing the two macromonomers BTB4200 and BTB7000) may reflect increased formation of linear versus ring MSP, as well increased chain length and an enhanced electroviscous effect. SLS and DLS experiments in the presence of added inert salt confirm that MSP formation occurs in these systems but that the degree of self-association is rather small, and it is anticipated that the resulting viscosity switch will therefore be rather weak unless substantially higher molecular weight macromonomers are used. In addition to exploring the use of higher molecular weight macromonomers, future work might include synchrotron SAXS studies of BTB4200 and its Cu complexes to explore in more detail the possibility of ring formation in these low molecular weight MSP.
4.5 References


21. macro.lsu.edu/HowTo/dndc.doc, website of Paul Russo's Macromolecular Group, LSU.


CHAPTER V

Small Angle X-Ray Scattering Analysis of the Basepaired Complex of U6 and U2 snRNAs Points to an RNA Chaperone Role for Proteins in Assembly of the Spliceosomal Active Site

5.1 Introduction

An unexpected feature of the human genome relates to the abundant regions that do not appear to carry any useful information. These “non-coding” regions, also called intervening sequences or introns, interrupt coding regions (exons), and thus, to use the data stored in our genes, the boundaries of introns must first be correctly recognized, and then the introns must be removed to generate an uninterrupted genetic message. Removal of the intervening sequences from pre-mRNAs is not only a pivotal and nearly ubiquitous step in the expression of most eukaryotic genes, but also an important means of generating transcriptomic diversity through alternative splicing. The spliceosome, the ribonucleoprotein complex which performs this complex reaction, is one of the largest and most complicated molecular machines in the cell, consisting of over 150 different components in human, including five small nuclear RNAs (snRNAs) named U1, U2, U4, U5 and U6.\(^1\) The snRNAs are highly conserved and play important functional roles at different stages of spliceosomal cycle. However, at the time of catalytic activation of the spliceosome, only three snRNAs, U2, U5 and U6 are present (Figure 5.1). Extensive structural and functional similarities between these snRNAs and the group II introns,\(^1-2\) self-splicing ribozymes that perform a splicing reaction mechanistically identical to that of the spliceosome, has raised the possibility that the spliceosomal snRNAs may be
descendants of group II-like ribozymes. This, in turn, suggests that the spliceosomal and group II intron active sites may have a closely similar organization of the functionally equivalent RNA elements and likely a central role for the spliceosomal snRNAs in the formation and function of the active site.

The splicing process is complicated, in which two transesterification reactions play key roles, one leading to the formation of a 29–59 bond in the intermediate species known as the lariat intron, and one creating a 39–59 linkage in the spliced product (Figure 5.1). Metal ion coordination by the 39-linked oxygen of the bridging phosphate at the 59 splice site suggests the involvement of at least one inner sphere coordinated divalent cation. Mutational studies have indicated that at least in vitro, U5 snRNA is dispensable for the first step of splicing under certain conditions. Thus, U6 and U2 are the only snRNAs that are absolutely essential for splicing based on current data. In activated spliceosomes, U6 and U2 form extensive, functionally critical basepairing interactions and existing data points to a central role for this basepaired complex in spliceosomal function (Figure 5.2(i)). It has also been shown that at least the first step of the splicing reaction occurs in the vicinity of a conserved sequence in U6, the so-called ACAGAGA box. Two other regions of U6, the AGC triad and the intramolecular stemloop (ISL), are implicated as important elements for splicing catalysis in vivo. The reacting groups of the first step of splicing, the branch site and 5' splice site, are brought to proximity at least partially through interactions with the AUGAUGU branch-bind site of U2 and the ACAGAGA sequence of U6, which are juxtaposed in the basepaired U6/U2 complex (Figure 5.2(i)). In addition to positioning the reacting groups close to each other, the interactions between the substrates and the U6/U2 complex helps
position the reacting groups close to the catalytically essential residues in U6.

The ACAGAGA sequence of U6, which interacts with the 5' splice site before and through the first step of splicing partially through basepairing,\textsuperscript{12-13,17-19} is an evolutionarily invariant sequence and point mutations in this region frequently block splicing before either the first or the second step.\textsuperscript{6,8-9} Further, phosphorothioate substitutions in several positions of this sequence block splicing.\textsuperscript{20-21} Interestingly, the phosphorothioate interference sites in ACAGAGA are located in the immediate vicinity of the 5' splice site, which is known to coordinate to a magnesium ion, raising the possibility that the ACAGAGA sequence may help in coordination of catalytic metals.

In addition to the catalytically critical residues in the ACAGAGA sequence, two other regions of U6, the AGC triad and an asymmetric bulge in the intramolecular stemloop (ISL), contain functionally critical sequences where nucleobase mutations or phosphorothioate substitutions block splicing.\textsuperscript{6} Both these sequences have direct counterparts in group II introns and mutational and phosphorothioate substitution analyses in group II introns have yielded results very similar to the ones observed in the spliceosome.\textsuperscript{22} Further, it has been shown that the asymmetric bulge in both systems binds a functionally critical metal ion. In group II introns, a similarly positioned AGC triad and asymmetric bulge, together with the functional equivalent of the ACAGAGA sequence, are juxtaposed through a complex series of tertiary interactions to form the active site including binding pockets for two metal ions.\textsuperscript{16,22}
Figure 5.1. Two-step chemical mechanism for pre-mRNA splicing. Exon 1 and exon 2 which carry coding information are separated by an intron represented by blue line. U2, U5 and U6 are small nuclear RNAs (snRNA). The so-called branch adenosine (A) and one of its hydroxyl groups (OH), involved in the splicing reaction, is identified. In the first transesterification step, the hydroxyl group on the branch adenosine (A) attacks and cleaves the phosphodiester linkage between exon 1 and the intron, making exon 1 a leaving group, and forming a 29–59 bond in the lariat intron creating a second hydroxyl group created at the end of exon 1. In the second transesterification step, a new OH group created in the first step at the end of exon 1, attacks the phosphodiester linkage between
exon 2 and the intron, creating a 39–59 linkage which ligates exon 1 and exon 2 and excising the lariat intron.

![Diagram of spliceosome](image)

**Figure 5.2.** (i) Structure of the wild type model spliceosome formed by connecting the U6 and U2 sequences. (ii) Schematic representations of the cruciform and four possible coaxial conformations.

If, indeed, as mentioned above, the active sites of group II introns and the spliceosome are highly similar, the sequences in U6 which are equivalent to those

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forming the group II intron active site should be positioned close to each other to build a related active site. Existing evidence suggests that such an arrangement may indeed occur. Chemical probing experiments have indicated the proximity of the ACAGAGA sequence and the U6 ISL in activated spliceosomes, both before and after the first step of splicing. Further, mutational complementation analyses point to an interaction between the second G in the ACAGAGA box and a U2 nucleotide located across the duplex from the AGC triad, suggesting the physical proximity of the ACAGAGA and AGC sequences. Taken together, the existing data suggests that the basepaired complex of U6 and U2 not only binds and positions the reacting groups in proximity, but also it serves to juxtapose the three critical functional elements in U6 that likely help form the spliceosomal active site. Also, it is likely that the U6/U2 complex acts as a scaffold for the rest of the spliceosomal proteins to interact with the substrates or otherwise regulate the spliceosomal catalytic function.

From the above details, defining the structure and function of the U6/U2 complex is clearly crucial for understanding spliceosomal function. Previous work has shown that incubation of in vitro-synthesized RNAs containing the sequence of the central domains of human U6 and U2 snRNAs in the presence of divalent cations leads to the efficient formation of a basepaired species. Crosslinking studies proved the presence of a long-range interaction between the second G of the ACAGAGA box and a U2 nucleotide positioned across the duplex from the AGC triad, almost identical to what had been previously described within spliceosomes in vivo. NMR studies proved that isolated U6 ISLs could bind magnesium via their asymmetric stemloops, in a further parallel to observations made within spliceosomes. Further analysis of metal binding by isolated,
in vitro-assembled U6/U2 complexes was consistent with metal binding at the ACAGAGA and AGC boxes, overlapping the sites of phosphorothioate interference observed within spliceosomes. Finally, in the presence of magnesium, the in vitro-assembled U6/U2 complex could perform splicing on short RNA oligonucleotide substrates in a two-step transesterification reaction that was identical to splicing reactions performed by group II introns and the second step of spliceosomal splicing (Figure 5.1). Together, the above data indicates that the in vitro-assembled complex of U6 and U2 snRNAs, which we term a minimal model spliceosome, is both structurally and functionally relevant to that observed in the spliceosome and thus provides an ideal means for obtaining insight into the organization and function of the spliceosomal active site.

As illustrated in Figure 5.2, NMR evidence suggests that, in the presence of Mg ions, the minimal spliceosome folds into a cruciform structure featuring a four-way helical junction, a common element in the secondary structure of many functional RNA species. In the minimal spliceosome, the arms of the four-way junction are typically labeled Helix I/III, Intramolecular Stem Loop (ISL), Helix II, and Stem I. The arm lengths are Helix I/III >> ISL ≈ Helix II >> Stem I. In such structures, the component helices may remain in the cruciform conformation, or the structure may be stabilized by pairwise coaxial stacking, in which case four distinct coaxial stacking conformers are possible, viz., parallel and antiparallel stacking of Helix I/III on Stem I with ISL on Helix II, and parallel and antiparallel stacking of ISL on Helix I/III with Helix II on Stem I. Thus, we label these conformers in terms of the pairs of stacked helices as: (a) Helix I/III ISL : Helix II Stem I, (b) Helix I/III Stem I : Helix II ISL, (c) Helix I/III ISL: Stem I
Helix II and (d) Helix I/III Stem I : ISL Helix II, as shown in Figure 5.2 (ii). Available evidence suggests that, at low concentrations of Mg\(^{2+}\), RNA junctions exist in the parallel form, at higher Mg\(^{2+}\) concentrations (~ 0.5 mM), they rotate into the antiparallel form, and at still higher Mg\(^{2+}\) concentration (~1 mM) they exist in a 90° cruciform of helical stacks, i.e. neither parallel nor antiparallel.\(^{29}\) Noting that, in the native spliceosome, it has been shown that the asymmetric bulge area of ISL and the ACAGAGA sequence are in close proximity,\(^{16}\) it has been suggested that the active site has antiparallel coaxial stacking of Helix I/III on Stem I with ISL on Helix II.\(^{28}\)

Here we seek to determine which of the possible coaxial stacking conformers of the minimal spliceosome are present in aqueous solutions under catalytic conditions. As a cruciform structure,\(^{29}\) the model spliceosome should fold in Mg\(^{2+}\) buffer into one of the four conformers illustrated in Figure 5.2(ii), or possibly as an equilibrium between different species. Since the arms of the model spliceosome vary in length, in principle it appears possible to distinguish the conformers in terms of their different SAXS patterns. In practice the differences in SAXS scattering functions between the different conformers are expected to be quite small. To enhance our experimental ability to identify different conformers, several mutant constructs were prepared, each having lengths of one arm different from the wild type (Figure 5.3 (i), (ii), (iii)). By comparing the SAXS patterns from these species with that of the wild type, it is hoped to facilitate identification of the equilibrium conformer(s).
Figure 5.3. Sequences of four mutant model spliceosomes, each with a modified helical segment: (i) ISL + 5 bp; (ii) Helix II + 3 bp; and (iii) Helix II - 7 bp. (iv) Schematic comparison of folded conformers of the Helix II -7 bp mutant.

Solution small angle X-ray scattering (SAXS), a powerful, albeit low-resolution, tool for probing the global structure of macromolecules, was used to attempt to identify the equilibrium conformer. Due to sample limitations (50 μM in ~80 μL) and to avoid aggregation, extremely low concentrations of the model spliceosome are required, necessitating use of a synchrotron source. One piece of information easily obtained from SAXS is the radius of gyration (R_g), an estimation of overall size. While, reportedly, there is no significant difference between the R_g values of folded and unfolded conformers, we anticipated that the structure of folded RNA conformers may be approximated as a cylinder, for which \( R_g^2 = \frac{L^2}{12} + \frac{d^2}{8} \) where L and d are the cylinder length and diameter, respectively, and hence that R_g will be determined by the length
rather than the width. For example as illustrated in Figure 5.3 (iv), if, in the mutant, only helix II is shorter than in the wild type species, and the same folding conformer is assumed, the fourth conformation will generate a shorter cylinder, meaning a smaller radius of gyration, while, for the other three conformations, only part of the cylinder width decreases and hence the radii of gyration will remain essentially constant, and indistinguishable from the $R_g$ of the wild type, provided $L >> d$. Thus, comparing the size ($R_g$) from SAXS of this mutant versus the wild type species, the fourth conformer can be selected or excluded. By such means, it is anticipated that the particular conformer present in solution might be identified. From Figure 5.2 (ii), it is apparent that the first and second conformers (Helix I/III ISL : Helix II Stem I and Helix I/III Stem I : Helix II ISL, respectively) cannot be distinguished in this way because a change in length of any single helical arm affects the overall size (length) in exactly the same way. So only three folded conformations are considered here: Helix I/III ISL : Helix II Stem I (or Helix I/III Stem I : Helix II ISL); Helix I/III ISL : Stem I Helix II; and Helix I/III Stem I : ISL Helix II.

Recently, in analyzing SAXS data, an ab initio modeling approach has been developed to characterize the shape of biological macromolecules by using either an angular envelope function$^{31-32}$ or an ensemble of densely packed beads$^{33-35}$ and matching the scattering data by nonlinear minimization. In spite of the limited resolution, a number of successful studies$^{36-39}$ show that solution scattering curves provide sufficient information to reconstruct molecular shapes. In addition, if a high-resolution model of fragments of the macromolecule is known from other methods such as crystallography or NMR, using the obtained shape as a basis, a more realistic atomistic model can be built.
by structure refinement against the scattering data via rigid body minimization\textsuperscript{29} or molecular dynamics and energy minimization.\textsuperscript{40}

We have here performed a detailed structural analysis of the in vitro-assembled human U6/U2 complex using small-angle X-Ray Scattering (SAXS), \textit{ab initio} electron density and atomistic resolution modeling studies. The snRNA forms a four-way junction-like structure in which the ACAGAGA-containing stemloop and the ISL are coaxially stacked, with the ISL and helix II stacking side by side. In addition to providing a first glimpse into the structure of this functionally critical snRNA complex, our data provides clues to the way spliceosomal proteins influence the structure and, thus, the function of snRNAs in activated spliceosomes.

5.2 Experimental

5.2.1 Small Angle X-ray Scattering Experiments

The snRNA constructs were provided by Prof. Valadkhan and assembled as described.\textsuperscript{11} Briefly, the U6/U2 chimeric constructs were heated at 90 °C for 2 min. followed by cooling on ice for 5 min. to break down any possible aggregates. Next, MgCl\textsubscript{2} was added to a final concentration of 40 mM and solution was placed in a heating block at 75 °C, and the RNA was allowed to refold as the solution was allowed to cool from 75 °C to room temperature. SAXS experiments were performed using the X-21 beamline of the National Synchrotron Light Source (NSLS). The scattering data was collected by a MARCCD area detector and then converted to a 1D curve. A flow cell system was employed to avoid possible radiation damage to the U6/U2 complexes. The X-ray energy was 12keV, corresponding to a wavelength of 1.033 Å.

Small angle X-ray scattering was conducted on the wild type and mutant
constructs at 2, 5, 10 and 20 μM concentrations with a 30 second exposure time. The background signal from MgCl₂ and Tris-HCl in the buffer was normalized and subtracted. Several constructs were analyzed in two different SAXS sessions several months apart to ensure reproducibility of the data. As a calibration experiment, SAXS experiments were also performed on three model helices, each constructed by combining two of the helical arms of the wild type model spliceosome. Structural analysis was also performed on these species as described below for the model spliceosome.

5.2.2 Determination of the Radius of Gyration (R₉)

R₉ was calculated using two independent methods: (i) from the slope of a Guinier plot of lnI(q) versus q², using the low-q range of the SAXS scattered intensity I(q), which can be related to R₉ via equations (1) and (2):

\[
\lim_{q \to 0} I(q) = I(0) \exp\left(-\frac{R^2 q^2}{3}\right) \quad (1)
\]

\[
\ln I(q) = \ln I(0) - \frac{R^2 q^2}{3} \quad (2)
\]

and (ii) calculation using equation (4) from the pair distance distribution function P(r), computed via Fourier transform of the scattering pattern (equation (3)), using GNOM:

\[
P(r) = \frac{1}{2\pi^2} \int_{q_{\text{min}}}^{q_{\text{max}}} I(q)qr \sin(qr) dq \quad (3)
\]

\[
R^2_g = \frac{\int_0^{d_{\text{max}}} r^2 P(r) dr}{2 \int_0^{d_{\text{max}}} P(r) dr} \quad (4)
\]

GNOM was used to perform regularized indirect transforms of the scattering data to obtain pair distance distribution function P(r). For each scattering curve, GNOM was run with different D_{max} varied in steps of 2 Å. The proper D_{max} was chosen so that
scattering curve was fit well and the P(r) function was smooth and positive. Comparison of the two methods indicated that the Fourier transform approach provides better statistical certainty than Guinier plots, as expected, since it incorporates data from all scattering vectors into its calculation.

5.2.3 Construction of the Atomistic Density Models

To determine the shape of the wild type and mutant U6/U2 complexes, we employed the 3D structure reconstruction program DAMMIN\textsuperscript{34} to compute a low resolution particulate structure in the form of a dummy atom model from the SAXS data. Specifically, DAMMIN uses a simulated annealing procedure to perform refinement against experimental SAXS data such that the resultant particulate assembly within a defined volume exhibits a scattering profile that matches the experimental one. In our study, we conducted 10 independent DAMMIN runs (with no symmetry assumption) in the “slow” mode on the SAXS scattering curves obtained at 5 μM RNA concentration.\textsuperscript{34} We used a spherical initial search volume with a diameter exceeding the corresponding experimentally measured structure size. A cylindrical initial search volume with dimensions exceeding the corresponding experimentally measured structure size also gave the same results. Ten individual 3D bead models were thus obtained for each construct and were aligned (based on their axes of inertia) by minimizing their normalized spatial discrepancy (NSD) using SUPCOMB,\textsuperscript{42} We found that the NSDs for the wild type U6/U2 complex and all mutants were consistently smaller than unity (wild type: 0.83±0.04; ISL+5: 0.69±0.05; H2+3: 0.64±0.04; H2-7: 0.90±0.06), implying that all the dummy atom models produced for the U6/U2 complexes were structurally similar and reproducible by DAMMIN. The aligned bead models were averaged using DAMAVER\textsuperscript{43}
to compute the probability maps and generate low-resolution structures with effective occupancy. The output pdb files comprising dummy atoms were finally converted to electron density maps using SFALL from the CCP4 program suite\textsuperscript{44} to generate a MTZ file, which was first converted to an XPLOR-NIH-compatible format by XPLOR-NIH and finally to a PyMOL-compatible format using the codes published by Lilley and coworkers.\textsuperscript{39} The electron density maps were displayed and compared using PyMOL.\textsuperscript{45}

5.2.4 Atomistic Structure Reconstruction

A high-resolution model for the four helical stems of the U6/U2 complex (the ISL, helix II, U2 stem I and the stem containing helices I and III and the ACAGAGA sequence) was built for both wild type and mutant constructs and also for the three model helices that each combined a pair of these four helical structures using MacroModel (Schrödinger Inc.).\textsuperscript{46-47} MacroModel performs molecular dynamics simulations on model systems at finite temperatures using mixed stochastic dynamics and Monte Carlo algorithms. The models for the four stems were built separately and underwent energy minimization (PRCG) in aqueous medium in MacroModel using AMBER molecular mechanical force field.\textsuperscript{48} The output files of the minimized conformation of the helical stems were stored in pdb format.

The minimized four helical stems of the U6/U2 complex were inserted inside the electron density map in PyMOL to provide the most satisfactory accommodation. After the helical stems were positioned, they were linked together and minimized in MacroModel under constraints to maintain the relative positions between the stems. The minimized atomistic structure of the four linked stems was used as an input in CRYSOL\textsuperscript{49} to back-calculate a 1D SAXS profile and compare with the experimental data. We found
that the deduced antiparallel conformer in which the ISL and helix II show a side-by-side stacking exhibited a back-calculated SAXS profile that was in close agreement with the experimental results. Further refinement of the fitting was performed iteratively by manual manipulation of the relative distance between different helical stems followed by energy minimization using MacroModel and calculation of 1D SAXS profile using CRYSOL until a structure was identified which closely matched the experimental SAXS data (judged by the $\chi^2$ analysis). We determined that the overall structure size was responsible for the accuracy of the fit at the lower scattering vector, while the separation distance between the ISL and helix II was critical for the closeness of the fit at intermediate and high scattering vectors (0.05-0.15 Å$^{-1}$).

5.3 Results and Discussion

5.3.1 Model Helical Constructs

To gain insight into the structural features of the U6/U2 basepaired complex in the absence of all other spliceosomal factors, we made RNAs containing the functionally essential sequences of human U6 and U2 snRNAs in vitro and allowed them to form a basepaired complex by incubating them in the presence of magnesium. Biochemical characterization of the complexes was performed in the Valadkhan laboratory. The efficient formation of the basepaired complexes was demonstrated from analysis of the reaction mixture on a non-denaturing PAGE as previously reported.$^{11}$ Psoralen-mediated UV crosslinking was performed which determined that the base-pairing interactions in vitro-assembled U6/U2 complex resemble those previously shown to form in vivo and in cellular extracts. As mentioned above, in the base-pairing arrangement observed in the spliceosome, the functionally critical sequences (such as ACAGAGA box, the AGC triad
and the intramolecular stemloop) are far apart from each other and are juxtaposed through poorly understood tertiary interactions.16

To begin to understand the organization of the spliceosomal catalytic core, it is essential to determine how the helices defined by psoralen crosslinking and chemical modifications, and by genetic interactions in vivo, are arranged relative to each other in the tertiary structure of the in vitro-assembled U6/U2 complex and how the functionally important residues in U6 are juxtaposed as a result of this arrangement. To gain insight into the three dimensional arrangement of the helices in the basepaired complex of U6 and U2 snRNAs in isolation, we resorted to the small-angle X-ray scattering (SAXS) technique, which can yield information about the shape and size of a macromolecule without the size limitations of NMR approaches or a need for crystalline samples. Thus, it was an ideally suited approach for defining the global structure of the U6/U2 complex.

In our SAXS studies, we used the chimeric U6/U2 construct described above to ensure that the signal obtained by SAXS analysis reflects the structural features of U6/U2 complexes and not that of monomeric U6 or U2 snRNAs. As the longest dimension of the three-dimensional structure of U6/U2 is likely determined by the length of coaxially-stacked helices, we first ensured that our analysis can indeed detect differences arising from different stacked arrangements of the U6/U2 helices. To this end, we designed three constructs: construct A (Figure 5.4 (i)) contained the sequences forming helix III, ACAGAGA and helix I plus the U6 ISL; construct B (Figure 5.4 (ii)) resembled the first one but ISL was replaced by helix II; in construct C (Figure 5.4 (iii)), ISL and helix II were connected to each other, forming a long stemloop. The SAXS scattering patterns of the above constructs are shown in Figure 5.5 (i) and the deduced radii of gyration ($R_g$),

189
determined via Guinier plots of the low-q SAXS intensity, are summarized in Table 5.1 and compared to two sets of values estimated from models based on dimensions of A-form RNA (diameter of double helix = 28 Å; distance between base pairs = 2.9 Å). Analysis of the concentration-dependence of $R_g$ indicated significant aggregation of constructs A and C, but not of construct B, at concentrations higher than 2 μM. The $R_g$ values of the first two constructs were closely similar, whereas that of the construct containing helix II and ISL was significantly shorter. The $R_g$ values calculated from effective cylinder lengths based on A-form RNA for the three constructs varied in a manner consistent with the experimental $R_g$ values, indicating the validity of the results. It is evident, however that the $R_g$ values estimated from dimensions of A-form RNA, both from a crude cylinder model and from a molecular model generated as described below from the SAXS data, using the Macromodel software, are consistently smaller than the experimental values from SAXS. Since the percent discrepancy in size is essentially identical for each species, so that the experimental values correlates well with the model values, it seems clear that the discrepancy originates in a structural feature missing from the models, e.g. scattering from a hydration layer and/or bound counterions. 50-51

(i)

(ii)
Figure 5.4. Structure of the three helical control sequences: (i) A: Helix I/III+ISL; (ii) B: Helix I/III+Helix II and (iii) C: ISL+Helix II.
**Figure 5.5.** (i) SAXS of three controls and the fit to experimental data of control B (Helix I/III+Helix II) from atomistic structure in Macromodel; (ii) Helix I/III+Helix II atomistic structure from Macromodel and its density map generated from SAXS data.

**Table 5.1.** Radii of gyration ($R_g$) of helical constructs from SAXS diffraction data are compared versus values from molecular models based on A-form RNA.

<table>
<thead>
<tr>
<th>R$_g$ (Å)</th>
<th>Construct A (helices I/III:ISL) (2 μM)</th>
<th>Construct B (helices I/III:II) (10 μM)</th>
<th>Construct C (ISL:helix II) (2 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinier Plots</td>
<td>52.3±2.9</td>
<td>52.1±0.6</td>
<td>34.4±4.5</td>
</tr>
<tr>
<td>Cylinder Model</td>
<td>38</td>
<td>40</td>
<td>25</td>
</tr>
<tr>
<td>Computed via MacroModel</td>
<td>47</td>
<td>47</td>
<td>29</td>
</tr>
</tbody>
</table>

Using the SAXS diffraction data, we also attempted to determine the ab initio three-dimensional structure of construct B at high concentration (10 μM). Similar to the approach developed by Lipfer and coworkers,$^{39}$ we used the three-dimensional structure reconstruction program DAMMIN to generate a low-resolution electron density map from the SAXS profile of construct B. DAMMIN uses a simulated annealing procedure to perform refinement of an initial rod or spherical model made up of small particles against the experimental SAXS data, such that the resultant particle assembly exhibits a scattering profile that matches the experimental SAXS data. As shown in Figure 5.5 (ii),
the predicted electron density map of construct B shows a rod-like structure. With the electron density map as a guideline, we applied molecular modeling using MacroModel software from Schrödinger Inc., and generated a high-resolution atomistic model. As a self-consistency check, we note that the ISL stem has a very similar structure and dimension to the ISL structure determined via NMR spectroscopy as shown in Figure 5.6. Comparison of the electron density map generated from SAXS data and the atomistic structural model for construct B showed substantial overlap (Figure 5.5 (ii)), further supporting that construct B has a cylindrical structure. The SAXS curve calculated from the atomistic model fit the experimental data quite well at middle and high q range as shown in Figure 5.5 (i). Consistent with the size comparison in Table 5.1, the atomistic structure of construct B and its calculated SAXS curve each yield a smaller $R_g$ than the experimental data. It is also true for the other two constructs. The systematic difference between the computed values and the experiment is powerful support that the SAXS technique is providing accurate values of $R_g$ and the discrepancy with the computed values reflects a real difference originating in solvation and counterion binding not included in the molecular modeling.
5.3.2 Wild Type and Mutant U6/U2 Complexes

Next, we performed SAXS on the chimeric U6/U2 complex. SAXS data on the wild type complex was compared with three mutant species. In one species, the upper base-paired segment of ISL was extended by five base-pairs (ISL+5), while the other two had either 3 base-pairs added or 7 base-pairs removed from helix II (H2+3 and H2-7, respectively). The nucleobase identity of the inserted or removed base-pairs was chosen carefully such that the existing base-pairing interactions within helices were not affected. The inserted or removed base-pairs were positioned as far away as possible from the junction of the helices or potential interhelical interaction sites to minimize any potential
impact on the stacking arrangement between helices. The three mutant constructs were tested in protein-free catalytic assays to ensure that the introduced mutations had not altered their ability to form a functional structure. Since altering the size of ISL and helix II will affect the longest diameter of the structure differently in the four possible stacking arrangements, determining the $R_g$ of the mutant constructs allows us to differentiate between the four stacking conformations.

We obtained SAXS profiles at several RNA concentrations between 2 and 20 $\mu$M in the presence of 40 mM MgCl$_2$ at room temperature. The SAXS curves of the wild type complex and three mutant forms, each at four concentrations, are shown in Figure 5.7 (i)-(iv). Guinier plots were made for each species at all concentrations and the corresponding plots at 5 $\mu$M are displayed in Figure 5.8. All $R_g$ values derived via Guinier plots are listed in Table 5.2. $R_g$ values of all model spliceosome species were independently determined via Fourier transform of the pair distribution function $P(r)$ using GNOM software. and are listed in Table 5.3.
Figure 5.7. SAXS patterns of (i) wild type spliceosome, (ii) ISL+5, (iii) Helix II+3 and (iv) Helix II-7, each at 2, 5, 10 and 20 μM.

Figure 5.8. Guinier plots of wild type spliceosome and mutants (ISL+5, Helix II+3 and Helix II-7) at 5 μM.
Table 5.2. $R_g$ values of wild type and mutant U6/U2 complexes from Guinier plots at several concentrations.

<table>
<thead>
<tr>
<th>RNA concentration</th>
<th>WT (Å)</th>
<th>ISL+5 (Å)</th>
<th>H2+3 (Å)</th>
<th>H2-7 (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 μM</td>
<td>107.2±0.8</td>
<td>78.7±1.4</td>
<td>85.3±1.5</td>
<td>71.4±0.9</td>
</tr>
<tr>
<td>10 μM</td>
<td>62.8±0.8</td>
<td>62.5±1.4</td>
<td>62.0±1.5</td>
<td>64.0±1.2</td>
</tr>
<tr>
<td>5 μM</td>
<td>49.7±2.2</td>
<td>57.0±1.3</td>
<td>57.7±3.1</td>
<td>49.7±2.8</td>
</tr>
<tr>
<td>2 μM</td>
<td>54.4±2.1</td>
<td>60.0±3.6</td>
<td>59.5±5.9</td>
<td>54.2±3.8</td>
</tr>
</tbody>
</table>

Table 5.3. $R_g$ values of wild type and mutant U6/U2 complexes obtained via Fourier transform of $I(q)$ at several concentrations.

<table>
<thead>
<tr>
<th>RNA concentration during SAXS</th>
<th>WT (Å)</th>
<th>ISL+5 (Å)</th>
<th>H2+3 (Å)</th>
<th>H2-7 (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 μM</td>
<td>100.9±0.4</td>
<td>83.5±0.6</td>
<td>89.2±0.5</td>
<td>65.5±0.1</td>
</tr>
<tr>
<td>10 μM</td>
<td>62.8±0.1</td>
<td>62.5±0.5</td>
<td>63.7±0.2</td>
<td>63.8±0.2</td>
</tr>
<tr>
<td>5 μM</td>
<td>51.9±0.5</td>
<td>56.6±0.6</td>
<td>58.0±0.8</td>
<td>51.6±1.3</td>
</tr>
<tr>
<td>2 μM</td>
<td>52.7±0.3</td>
<td>60.4±1.5</td>
<td>56.2±1.1</td>
<td>52.3±1.7</td>
</tr>
</tbody>
</table>

Comparing Tables 5.2 and 5.3, we see that, as observed for the helical constructs, GNOM analysis, which utilizes data at all scattering vector, generates similar $R_g$ values with better statistics than those from the Guinier plots, which utilize data only at low scattering vectors. Comparison of $R_g$ values after adjustment for the concentration-dependent increase in signal intensity indicate that the obtained SAXS profiles are highly
superimposable, indicating the absence of aggregation or inter-molecular interference effects up to a concentration of 5 μM. Hence we used the average of the \( R_g \) values at 2 and 5 μM to compare each species in Table 5.4.

**Table 5.4.** \( R_g \) values of wild type and mutant U6/U2 complexes calculated by Guinier plots and Fourier transform of \( I(q) \) at lowest concentration are compared.

<table>
<thead>
<tr>
<th></th>
<th>WT (Å)</th>
<th>ISL+5 (Å)</th>
<th>H2+3 (Å)</th>
<th>H2-7 (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinier Plots</td>
<td>52.1±2.2</td>
<td>58.5±2.5</td>
<td>58.6±4.5</td>
<td>51.9±3.3</td>
</tr>
<tr>
<td>Fourier Transform</td>
<td>52.3±0.4</td>
<td>58.5±1.1</td>
<td>57.1±1.0</td>
<td>51.9±1.5</td>
</tr>
</tbody>
</table>

If we assume a simple cylindrical structure for the wild type complex, with a diameter equal to twice that of a double helix strand (56 Å), the experimental \( R_g \), 52 Å (Table 5.4) suggests a length of 167 Å. In fact the electron density map of the chimeric U6/U2 complex generated by DAMMIN, shown later in Figure 5.9, indicates a cylindrical structure with a bend close to one end. The overall length of the structure is 166 Å with a minimum thickness of 29 Å, which could accommodate a single A form RNA helix, and a maximum thickness of 49 Å, which could potentially accommodate two side-by-side stacked RNA helices. To be able to unequivocally identify the individual helical elements, we turned to the mutant constructs. Table 5.4 indicates that deletion of 7 base-pairs from helix II does not alter the \( R_g \) value, which indicates that longest diameter of the structure remains unchanged, whereas addition of 3 and 5 base-pairs to helix II and ISL, respectively, results in an increase in \( R_g \) of ~6 Å which translates into an increase in the overall length of the complex of ~10 Å. These results can now be compared to
theoretical models.

Considering first a simple cylinder model based on the dimensions of A form RNA we can estimate the radius of gyration for each spliceosome species in the three different possible conformations. The results are shown in Table 5.5 together with the percent changes in predicted $R_g$ values relative to that of the wild type. Comparing these predictions with the experimental values in Table 5.4, we see that the only stacking arrangement consistent with these results is that in which the ISL and helix II are stacked side-by-side, with the stem containing helices I and III and ACAGAGA in a coaxial configuration relative to the ISL (the third structure in Figure 5.3 (iv)). Because of its non-paired bases, the ISL helix is actually predicted to be a slightly shorter than helix II by $\sim 6$ Å. This explains why addition of 3 base-pairs to helix II results in the same increment in the overall length of the molecule as the addition of 5 base-pairs to the ISL (Table 5.4).

Table 5.5. Prediction of $R_g$ of wild type and mutant U6/U2 complexes based on the assumption of a cylindrical conformation, and the predicted difference $\Delta R_g$ between mutant and wild type for the four possible stacking conformations.

<table>
<thead>
<tr>
<th>Stacking</th>
<th>Wild Type (Å)</th>
<th>ISL+5 (Å)</th>
<th>H2+3 (Å)</th>
<th>H2-7 (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HelixI/III ISL : Helix II Stem I or HelixI/III Stem I : Helix II ISL</td>
<td>41.8</td>
<td>45.5</td>
<td>41.8</td>
<td>41.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+8.9%</td>
<td>+0%</td>
<td>+0%</td>
</tr>
<tr>
<td>HelixI/III ISL : Stem I Helix II</td>
<td>41.8</td>
<td>48.1</td>
<td>44.1</td>
<td>41.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+8.9%</td>
<td>+5.3%</td>
<td>+0%</td>
</tr>
<tr>
<td>HelixI/III Stem I : ISL Helix II</td>
<td>41.8</td>
<td>41.8</td>
<td>44.1</td>
<td>36.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+0%</td>
<td>+5.3%</td>
<td>-12.1%</td>
</tr>
</tbody>
</table>
Turning to the electron density maps generated from the SAXS profiles for the three mutant constructs, ISL + 5, Helix II + 3 and Helix II – 7, we find, like that of the wild type, they are all cylindrical in shape. By manually aligning and superimposing the density maps of the model spliceosome and the mutants, we are able to identify the relative position of HelixI/III, as shown in Figure 5.9. More importantly, we found that consistent with the experimental $R_g$ values (Table 5.4), the longest dimensions of the density maps for the wild type and Helix II - 7 have similar values, whereas those of ISL + 5 and Helix II + 3 are significantly larger. These results independently exclude three of the possible folding conformations, as discussed above for the crude cylinder model, and are consistent with the conformer which has the ISL and helix II stacked side-by-side, with the stem containing helices I and III and ACAGAGA in a coaxial configuration relative to the ISL (the third structure in Figure 5.3 (iv)).

Since we have now identified the unique conformer consistent with the SAXS data, we can now proceed to develop an atomistic model for the spliceosome. As a first step, we can insert and align the four cylindrical elements, each representing the corresponding helical arms of the spliceosome, with appropriate lengths and widths, within the electron density map. Figure 5.10 (i) displays the relative direction and positions of the four arms indicated as simple cylinders within the wild type density map. To construct the atomistic models, we first developed an atomistic-resolution model for each helical stem of the basepaired structures in the U6/U2 complex, namely, the Helix I/III, ISL, Helix II, Stem I. The initial models were further refined by free energy minimization in a simulated aqueous medium using the program MacroModel, which incorporates a force field method (AMBER) to compute the potential energy as a function
of distance and angles between atoms. The generated output files of the minimized conformations of the helical stems were then inserted into the density map and ligated together. Because no divalent salts can be used in the AMBER force field, the energy minimization on the ligated four helical stems, with the electron density maps as a reference, was performed by imposing constraints to maintain the relative positions between the stems, thus minimizing mainly the structure in the ligated section. Then the relative positions of the stems were tuned until the energy minimized atomistic structures gave the best fits to experimental SAXS data. The resulting best fits to the experimental SAXS data are shown in Figure 5.10 (ii) for wild type and Figure 5.11 for mutant species.

The \( R_g \) values computed for these minimized spliceosome structures are found to be very close to the experimental results, as expected from the excellent fits seen in Figure 5.11. In addition, we found that the ISL and Helix II stems are separated from each other by a small distance (~15 Å) as represented in Figure 5.10 (i), rather than tightly packed against each other. It is noted that because priority was given to fitting the minimized structure against the experimental SAXS data, we were not surprised to find that the minimized atomistic structures contain some unphysical linkages in the ligated section. This recalls our findings in the model helix studies that the experimental \( R_g \) values are larger than those computed from A form helix dimensions, presumed due to the unknown scattering contributions of bound water and counterions. In view of these earlier findings, we felt it appropriate to obtain a more realistic atomistic representation of the wild type structure by a further minimization using MacroModel in which the unphysical bonds between different helical stems are disallowed. The result is shown in Figure 5.10 (iii).
Figure 5.9. Comparison between density maps of wild type (in white) and ISL+5 mutant (in red) to determine the Helix I/III end, indicated by the arrow.
Figure 5.10. (i) The orientations of helical arms within the wild type density map deduced from size differences between wild type and mutants. (ii) Fit to SAXS pattern of wild type spliceosome at 5 μM from atomistic structure (iii) Atomistic structure of the wild type model spliceosome after removal of unphysical bonds in the linkage regions and minimization in MacroModel.
Figure 5.11. Fit to SAXS patterns of mutant spliceosome species at 5 μM from atomistic structures. (i): ISL+5; (ii): Helix II+3; (iii): Helix II-7.

While the low resolution of structures generated from SAXS experiments does
not allow us to determine whether the helical junction between the ACAGAGA-containing stem and the ISL are indeed coaxially stacked, existing data from high-resolution structures of similar RNAs suggest that it is likely to be the case. Previous structural studies on the partial sequence of yeast U6 and U2 snRNAs have suggested that the ISL, helices I and II and U2 stem I may form a 4-way helical junction. Although genetic complementation studies suggest that the details of the base-pairing arrangements between yeast and human U6/U2 complex is different, a four-way junction-like structure is supported by existing genetic complementation data performed on a human U6/U2 complex. If this is indeed the case, the global structure of the complex of U6/U2 snRNAs will likely resemble that of studied RNA four-way junctions, in which helices two-by-two coaxially stack on each other and form an either parallel or antiparallel side-by-side stacking arrangement. Our deduced structure for human U6/U2 complex is consistent with an antiparallel stacked four-way junction, in which the ACAGAGA-containing stem and ISL stack coaxially, while the U2 stem I and helix II form the other coaxially stacked structure, juxtaposing the ISL and helix II in an antiparallel arrangement (Figure 5.10). This helical configuration is also consistent with previous data from single molecule studies, which suggest that in the majority of the yeast U6/U2 complexes, the chromophore-carrying ISL and the ACAGAGA-containing stem are positioned in a low-FRET state, with low frequency conversions to a high FRET state.

While our results are consistent with and provide a structural basis for the previous data on the helical arrangement within the U6/U2 complex, the hydroxyl radical footprinting studies in activated spliceosomes suggest that the ISL and ACAGAGA-containing stem are positioned in the vicinity of each other. Since both the ACAGAGA
and the ISL contain functional groups known to be functionally critical and likely even participate in the formation of the active site, it is expected that in the active conformation of U6, these structural elements should be juxtaposed. While the crosslinking and chemical modification data indicate that the base-paired helices formed between U6 and U2 in activated spliceosomes also form in the in vitro-assembled U6/U2 complex, the tertiary arrangement of the helices is different between the U6/U2 complex in activated spliceosomes and the one spontaneously folded in vitro in the absence of all other spliceosomal factors. Interestingly, if as the genetic evidence indicate, the human U6/U2 complex indeed has a four-way junction structure, a change in the coaxial stacking partners can juxtapose the ISL and the ACAGAGA-containing stem without the need for any further structural reorganization. A precedent for such a structural arrangement is the hairpin ribozyme, which also folds into a four-way junction structure and in which two helical structures which carry the active site elements are juxtaposed through the folding of the four-way junction. In very low concentrations of divalent cations, four-way junctions assume an open, unstacked conformation, followed by collapse into a coaxially stacked structure as the cation concentration is raised.19 Since the spontaneously folded U6/U2 complex and the one found in activated spliceosomes have different helical stacking conformations, spliceosomal factors must actively chaperone the stacking interactions of the U6/U2 complex during its formation in the spliceosomal assembly process to ensure the proximity of the ACAGAGA and the ISL.

Our data indicate that in addition to possibly helping in the formation of the U6/U2 base-paired helices, the spliceosomal proteins may play a prominent and functionally required role in ensuring the correct tertiary stacking of the base-paired
helices. Taken together, the results of SAXS analysis on in vitro-assembled U6/U2 complexes uncovers a novel role for spliceosomal proteins in chaperoning the accurate folding of snRNA complexes, and provide a first glimpse into the way proteins module the structure and thus, the function of RNAs in a large cellular RNP.

5.4 Conclusions and Future Work

Synchrotron SAXS measurements provided reproducible radii of gyration for the wild type and mutant model spliceosomes. Values computed from Guinier plots at low-q, and from the pair distribution functions generated by Fourier transform analysis of the SAXS data produced self-consistent results. Radii of gyration of the controls were systematically larger than predicted values based on crystal structures and a rigid cylinder model. Comparison of radii of gyration of the mutant species versus the wild type model spliceosome indicated a preference for a single folding conformer. With the help of a low-resolution density map generated by simulated annealing, we found energy-minimized pdb atomistic structures of wild type and mutants that fit the experimental scattering data well and agree with the chosen folded conformer. This conformer differs from that believed to be involved in formation of the active site, and involves antiparallel stacking of the HelixI/III on the Intramolecular Stemloop with the Stem I on the Helix II. Future work could involve SAXS investigation of the model spliceosome conformation in the presence of spliceosomal proteins to study the possible effect of spliceosomal proteins in modifying the folding of the model spliceosome. The SAXS scattering from the model spliceosome could be isolated from that of the proteins by using sucrose to match the scattering contrast of the proteins.54-55
5.5 References


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46. MacroModel version 9.7.211, Schrödinger, LLC, New York, NY, **2009**.


Appendix 1

**Figure A1.1.** Plot of displacement versus refractive index of KCl at three wavelengths.

**Table A1.1:** Differential displacement versus refractive index at three wavelengths.

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>436</th>
<th>546</th>
<th>589</th>
</tr>
</thead>
<tbody>
<tr>
<td>dS/dn (m)</td>
<td>8.931×10^{-4}</td>
<td>9.019×10^{-4}</td>
<td>8.955×10^{-4}</td>
</tr>
</tbody>
</table>
Figure A1.2. Plot of displacement versus concentration of G/TAcG 50/50 at three wavelengths.

Table A1.2: Differential displacement versus concentration of G/TAcG 50/50 at three wavelengths.

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>436</th>
<th>546</th>
<th>589</th>
</tr>
</thead>
<tbody>
<tr>
<td>dS/dc (m^4/kg)</td>
<td>1.702×10^{-7}</td>
<td>1.584×10^{-7}</td>
<td>1.532×10^{-7}</td>
</tr>
</tbody>
</table>

![Graph showing the relationship between q^2 (nm^2) and Kc/ΔR (mol/g). The graph is labeled with a linear equation y=0.33546x+3.11E-5.](a)
(b) $y = 0.01877x + 7.11 \times 10^{-6}$

(c) $y = 0.01816x + 5.624 \times 10^{-6}$
\[ y = 0.00918x + 5.87 \times 10^{-6} \]

\[ y = 0.00641x + 6.18 \times 10^{-6} \]
**Figure A1.3.** Plots of extrapolation of Zimm plots for G/TAcG (50/50) in 0.354 M aqueous KCl to zero scattering vector (a): 0.2 wt%; (b): 0.3 wt%; (c): 0.4 wt%; (d): 0.5 wt%; (e): 0.8 wt%.
(b) \[ y = 1606.5164x - 11.75303 \]

(c) \[ y = 1557.8496x - 11.88236 \]
\[ y = 1444.5551 \times 10^{-4} x - 12.09097 \]

\[ y = 760.8219 \times 10^{-4} x - 11.95188 \]
Figure A1.4. Modified Guinier plots of G/TAcG (50/50) in 0.354 M aqueous KCl to zero scattering vector (a): 0.2 wt%; (b): 0.3 wt%; (c): 0.4 wt%; (d): 0.5 wt%; (e): 0.8 wt%.
Appendix 2

**Table A2.1.** Slopes of differential displacement versus refractive index at three wavelengths.

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>436</th>
<th>546</th>
<th>589</th>
</tr>
</thead>
<tbody>
<tr>
<td>dS/dn (μm)</td>
<td>1033.4±27.9</td>
<td>1037.0±25.3</td>
<td>1029.4±26.1</td>
</tr>
</tbody>
</table>

**Table A2.2.** Slopes of differential displacement versus concentration for BTB4200 and its Cu$^{2+}$ and Cu$^{+}$ complexes at three wavelengths.

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>436</th>
<th>546</th>
<th>589</th>
</tr>
</thead>
<tbody>
<tr>
<td>dS/dc (μm*mL/g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BTB4200</td>
<td>158.9±2.2</td>
<td>154.8±1.3</td>
<td>151.6±2.9</td>
</tr>
<tr>
<td>BTB4200:Cu$^{2+}$=1:1</td>
<td>154.7±5.7</td>
<td>143.3±7.6</td>
<td>137.9±10.1</td>
</tr>
<tr>
<td>BTB4200:Cu$^{2+}$=1:2</td>
<td>160.9±5.5</td>
<td>145.3±8.4</td>
<td>139.4±8.2</td>
</tr>
<tr>
<td>BTB4200:Cu$^{+}$=1:1</td>
<td>164.8±3.4</td>
<td>152.2±5.2</td>
<td>145.5±4.1</td>
</tr>
<tr>
<td>BTB4200:Cu$^{+}$=1:2</td>
<td>158.2±6.3</td>
<td>149.7±6.4</td>
<td>142.9±5.2</td>
</tr>
</tbody>
</table>
Figure A2.1. Plot of displacement versus refractive index of KCl at three wavelengths.
(i)

(ii)

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(iii)

(iv)
Figure A2.2. Plot of displacement versus concentration of (i) BTB4200, (ii) BTB4200:Cu^{2+}=1:1, (iii) BTB4200:Cu^{2+}=1:2, (iv) BTB4200:Cu^{+}=1:1 and (v) BTB4200:Cu^{+}=1:2 at three wavelengths.
BTB4200: Cu$^{2+}$ = 1:1

(i)

(ii)
Figure A2.3. Wavelength dependence of dn/dc values of BTB4200 and its Cu$^{2+}$, Cu$^+$ complexes in Butyronitrile: (i) BTB4200; (ii) BTB4200:Cu$^{2+}$=1:1; (iii) BTB4200:Cu$^{2+}$=1:2; (iv) BTB4200:Cu$^+$=1:1 and (v) BTB4200:Cu$^+$=1:2.
(i) KCl

![Graph showing the relationship between concentration and dispersion for KCl.](image)

(ii) HO-MeBip

![Graph showing the relationship between concentration and dispersion for HO-MeBip.](image)
Figure A2.4. (i) Plot of displacement versus refractive index of KCl at three wavelengths; (ii) Plot of displacement versus concentration of HO-MeBip at three wavelengths; (iii) Wavelength dependence of dn/dc values of HO-MeBip.
(i)

BTB4200:Cu$^{2+}$ 1:1

$K_c(R - R_{\text{solvent}})$ (mol/g)

$\sin(\theta/2)^2$

1 g/L
5 g/L
10 g/L

(ii)

BTB4200:Cu$^{2+}$ 1:2

$K_c(R - R_{\text{solvent}})$ (mol/g)

$\sin(\theta/2)^2$
Figure A2.5. Plot of angle dependence of $K_c/\Delta R$ for BTB4200:Cu$^{2+}$ =1:1 (i) and BTB4200:Cu$^{2+}$ =1:2 (ii) to determine apparent weight average molecular weights, $M_{w,app}$, using $K_c/\Delta R(0,c) = 1/M_{w,app}$. 

![Plot of angle dependence of $K_c/\Delta R$ for BTB4200:Cu$^{2+}$ =1:1 (i) and BTB4200:Cu$^{2+}$ =1:2 (ii).]
Figure A2.6. Guinier plots for BTB4200:Cu$^{2+}$ =1:1 (i) and BTB4200:Cu$^{2+}$ =1:2 (ii) to determine the apparent radius of gyration.
Figure A2.7. $K_c/\Delta R$ data extrapolated to zero angle for BTB4200 Cu$^{2+}$ complexes at low concentrations: (i) BTB4200:Cu$^{2+}$=1:1; (ii) BTB4200:Cu$^{2+}$=1:2.
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