CHARACTERIZATION OF OPTICALLY ACTIVE BIOPOLYMERS

A dissertation submitted
to Kent State University in partial
fulfillment of the requirements for the
degree of Doctor of Philosophy

by

Steven E. Fiester

May, 2011
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>x</td>
</tr>
<tr>
<td>CHAPTER I. General Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Biopolymers</td>
<td>1</td>
</tr>
<tr>
<td>Biopolymers as liquid crystals</td>
<td>5</td>
</tr>
<tr>
<td>Exploitation of biopolymer liquid crystalline properties</td>
<td>20</td>
</tr>
<tr>
<td>Characterization of liquid crystal biopolymers</td>
<td>23</td>
</tr>
<tr>
<td>General overview and approach</td>
<td>26</td>
</tr>
<tr>
<td>Dissertation rationale and hypotheses</td>
<td>28</td>
</tr>
<tr>
<td>References</td>
<td>31</td>
</tr>
<tr>
<td>CHAPTER II. Liquid Crystalline Properties of <em>Salmonella</em> Flagella</td>
<td>38</td>
</tr>
<tr>
<td>Abstract</td>
<td>38</td>
</tr>
<tr>
<td>Introduction</td>
<td>38</td>
</tr>
<tr>
<td>Methods</td>
<td>42</td>
</tr>
<tr>
<td>Flagella harvesting, purification and recovery</td>
<td>42</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

### CHAPTER I. General Introduction

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Arrangement of molecules in solids, liquids and liquid crystals</td>
<td>8</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Arrangement of molecules in calamitic liquid crystals</td>
<td>10</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Arrangement of molecules in discotic liquid crystals</td>
<td>11</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Arrangement of amphiphilic molecules \textit{in vitro}</td>
<td>13</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Arrangement of threaded rods in chiral nematic liquid crystals</td>
<td>16</td>
</tr>
</tbody>
</table>

### CHAPTER II. Liquid Crystalline Properties of Salmonella Flagella

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 6</td>
<td>Western blot of flagellin</td>
<td>46</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Mass spectroscopy of flagellin</td>
<td>47</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Infrared spectra of flagellin</td>
<td>48</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Liquid crystalline properties of \textit{Salmonella} flagella</td>
<td>49</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Phase diagrams for flagella from \textit{Salmonella enterica} serovar Typhimurium SJW 1103, SJW 1665 and SJW 2869</td>
<td>50</td>
</tr>
</tbody>
</table>

### CHAPTER III. Optical Properties of a Self-Assembling Viral Coat Protein

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 11</td>
<td>Nanoscale structures of g8p</td>
<td>64</td>
</tr>
</tbody>
</table>
Figure 12. Illustration of the micron-scale structure of self-assembled g8p molecules and the textures at the edge and interior of a dried droplet of the suspended material at room temperature. 65

Figure 13. Self-assembling g8p filaments resemble those found in bent-core thermotropic liquid crystals, where the filaments are made from concentric layers of bent-core protein molecules, such as g8p. 67

Figure 14. Temperature dependence of g8p birefringence. 68

CHAPTER IV. Detection of Immune Complexes using Chiral Nematic Chitin Fibrils

Figure 15. Transmission electron microscopy images of chitin fibrils. 82

Figure 16. Chiral nematic arrangement of liquid crystalline chitin. 83

Figure 17. Number of defects introduced in the chitin liquid crystal by streptavidin-coated bead immune complexes. 84

Figure 18. Number of defects introduced in the chitin liquid crystal by immune complexes composed of Salmonella cells. 85

Figure 19. Immune complexes at the front and back of glass cassettes. 90

CHAPTER V. Application of Biopolymers of Varying Flexibility for Biosensing

Figure 20. Liquid crystalline texture of chitin and defects in that texture due to the introduction of immune complexes. 102

Figure 21. Immune complex-induced defects in the liquid crystalline textures of flagella and fd bacteriophage. 103
Figure 22. Comparison between the mean diameter of immune complexes and the associated defects caused by the immune complexes in the liquid crystalline textures of chitin and \textit{fd} bacteriophages……………………………………………………………………….104

CHAPTER VI. General Conclusions

Figure 23. Fibers composed of g8p…………………………………………………………………….116
LIST OF TABLES

Page

CHAPTER IV. Detection of Immune Complexes using Chiral Nematic Chitin Fibrils
Table 1. Sizes of streptavidin-coated bead and Salmonella immune complexes and the associated defects in liquid crystalline chitin.................................................................86

CHAPTER V. Application of Biopolymers of Varying Flexibility for Biosensing
Table 2. Mean diameter of streptavidin-coated bead immune complexes at an initial bead concentration of $10^7$ and the associated mean diameter of defects in biopolymer liquid crystals.................................................................101

CHAPTER VI. General Conclusions
Table 3. Comparison of liquid crystals for biosensing..............................................118
ACKNOWLEDGEMENTS

I would like to acknowledge NSF DMR0645461, the Kent State Graduate Student Senate and the W.M. Keck foundation as funding sources. I would like to acknowledge the members of my doctoral dissertation committee, Drs. Chris Woolverton, Doug Kline, Don Gerbig, Tony Jákli and Scott Bunge for their invaluable input leading to the completion of my dissertation work. I also acknowledge my labmates, James Redfearn, Jim Ferrell and Mike Shilling, undergraduate researchers, Simon Evangelista, Ben Millard, Alvin Das, Salvatore Coppola, and Jonathon Wilson and colleagues and friends, Dr. Shannon Helfinstine, Dr. Roberta Redfern, Jeanette Killius, Liou Qiu, Laurie Broadwater, Stephan Woods, Dan Adams, Kurtis Eisermann, Jessie Guinn, Jessie Francl, Shandilya Ramdas, Dr. Darren Bade, Dr. Sean Veney, Dr. A.J. Snow, Dr. Pete Wickley and Dr. Derek Damron for assistance with techniques and useful scientific debate. I would like to thank my mother, Linda Fiester, for her continued support and encouragement.
Biopolymers

Biopolymers, such as polysaccharides, nucleic acids and proteins, are assemblages of biological monomers. Biopolymers possess properties that cannot be attributed to individual monomers. While biopolymers have numerous functions in vivo, they have been reported as having various applications in vitro, as well.

Polysaccharides are biopolymers composed of monosaccharide monomers linked with glycosidic bonds. Hexoses, such as sucrose or galactose make up several polysaccharides. The glycosidic bonds form between hydroxyl groups of monosaccharides, which are usually cyclic structures containing five (a pentose) or six (a hexose) carbon atoms. Polysaccharides are named according to the location of the glycosidic bond. For example, if a glycosidic bond exists between the first carbon and the fourth carbon of α-glucoses or β-glucoses, the resulting polysaccharides are referred to as α-1,4 or β-1,4, respectively. Polysaccharides are uncharged unless they are functionalized with carboxyl groups, phosphate groups and/or sulfuric ester groups. The numerous possibilities for the placement of glycosidic bonds and functionalization allow the potential for numerous polysaccharides. Depending upon the placement of glycosidic
bonds and the functionalization, the resulting polysaccharides can have numerous functions.

Polysaccharides are used in vivo as storage and structural molecules. Storage polysaccharides are maintained in cells until energy is needed, and then they are metabolized into monosaccharides and functional groups by the cell. Plants typically use starch as a storage polysaccharide, while animal cells use glycogen (Garrett and Grisham 1999). Polysaccharides involved in structural stability in vivo include cellulose found in plant cell walls, chitin in the cell walls of fungi and the exoskeleton of crustaceans, insects and spiders (Garrett and Grisham 1999) and peptidoglycan found in bacterial cell walls.

Nucleic acids, such as deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are also biopolymers. Nucleic acids consist of repeating units referred to as nucleotides. Nucleotides consist of a five carbon sugar, a phosphate group and a nitrogen containing base. The linkage of these nucleotides through phosphodiester bonds results in a nucleic acid, a biopolymer. Polymers of nucleotides containing deoxyribose form DNA, which carries the genetic code for RNA and proteins. Briefly, sequences of DNA nucleotides, referred to as genes, are transcribed into messenger RNA (mRNA) by RNA polymerase. Messenger RNA is then translated into proteins, another type of biopolymer.

Proteins are biopolymers of amino acids linked by peptide bonds. There are 20 naturally occurring amino acids, and proteins vary in the sequence of these amino acids. The order of amino acids in a protein is referred to as the primary structure of the protein and ultimately determines the overall three dimensional structure of the protein.
Hydrogen bonding between N-H and C=O groups within the polypeptide backbone of proteins forms the secondary structure of the protein. β-sheets and α-helices are secondary protein structures that form when hydrogen bonding occurs between neighboring polypeptide chains or when hydrogen bonding occurs between every fourth peptide bond resulting in a regular helix, respectively. Hydrophobic side groups are positioned to the interior of a protein giving rise to the tertiary protein structure. The three dimensional structure of proteins composed of more than one polypeptide chain is referred to as the quaternary structure. Individual proteins can assemble with one another forming even larger polymers. Examples of multi-protein biopolymers include actin, collagen fibrils, elastin and Salmonella flagella. Different proteins have numerous functions based on their structure, charge and hydrophobicity. Proteins can function as enzymes, antibodies, transporters, ion binders, hormones, toxins, repressors, globins and structural fibers in vivo.

The interactions between biological monomers lead to polymers whose final three dimensional conformations impart functionality. For example, silk owes its tensile strength to hydrogen bonding and tight packing of the monomer fibroin. Hydrogen bonding within fibroin monomers results in strong β pleated sheets and the high percentage of glycine in fibroin allows for tight packing of fibroin monomers. The tensile strength exploited in vivo in spider webs can be attributed to the large amount of hydrogen bonding found in silk. When a stretching force is applied to silk, it has a high resilience against breakage, because the force can be distributed among the high number of hydrogen bonds. When silk fibers are woven together, tensile strength will increase,
because forces can be distributed among multiple fibers and thus a larger number of hydrogen bonds.

Another example of structure imparting functionality is observed in flagella filaments produced by *Salmonella enterica* serovar Typhimurium. α-helices at the C- and N-termini of flagellin (the protein monomer that polymerizes to form flagella filaments) polymerize through coiled-coil interactions. Resultant flagella then self-assemble into flagellar bundles. Flagella bundles have a more rigid structure than individual filaments thus allowing the bundles to be used as a rigid “propeller” for bacterial locomotion.

Proteins and carbohydrates have many applications *in vitro*, as well. Hyaluronan (a copolymer of β-(1–4)-D-glucuronic acid and β-(1–3)-N-acetyl-D-glucosamine) is found in rooster combs, bovine vitreous humor and umbilical cords (Rinaudo 2008). Hyaluronan and its derivatives can also be used in tissue engineering, viscosupplementation in joint diseases, drug delivery and filling of facial wrinkles (Rinaudo 2008). Alginates (polysaccharides produced by brown algae) are used in foods as thickeners, molded casts of teeth in dentistry, tissue engineering, drug delivery and matrices for the regeneration of cartilage, bone, liver and nerves (Rinaudo 2008). Red algae produce galactose-based carrageenans and agars. Agarose and agarose mixtures are used in electrophoresis, chromatography and as a growth medium for microorganisms (Rinaudo 2008). Carrageenans are used to thicken ice cream, clarify beer and for drug release (Rinaudo 2008). Bacterial polysaccharides, such as xanthan and gellan have been used for controlled drug release, as well (Rinaudo 2008).
Proteins have been reported as having many applications in vitro. Silk, for example, is a multi-protein polymer of fibroin produced by spiders and some insects in vivo for cocoons and prey capture (McGrath and Kaplan 1997). In vitro, silk is used for textiles, sutures, wound coatings, artificial tendons, bone repair and tissue repair (McGrath and Kaplan 1997). Another multi-protein biopolymer is collagen. Collagen can be used for drug delivery, wound dressings and tissue engineering (Lee et al. 2001). Many carbohydrate and protein biopolymers, such as xanthan (Sato et al. 1990), carrageenans (Chronakis and Ramzi 2002), fibroin (Vollrath and Knight 2001) and certain types of collagen (Giraud-Guille 1987) can retain their molecular alignment while in a fluidic state. This property defines a fourth state of matter known as the liquid crystal. Biopolymers with liquid crystalline properties may offer alternatives to traditional liquid crystals which are synthetically produced chemicals, like those used in display devices.

**Biopolymers as liquid crystals**

As mentioned, liquid crystals (LCs) compose the fourth state of matter, a fluidic suspension with anisotropic properties. Anisotropy refers to the arrangement of molecules along a common direction. Biopolymers, such as collagen, silk, bacterial flagella, myosin, hemoglobin, trypsin, deoxyribonucleic acid, ribonucleic acid and chitin form liquid crystalline structures (Brown and Wolken 1979). Biopolymers transition into liquid crystalline structures to meet energetic requirements. Exploring the liquid crystalline properties of biopolymers in water therefore offers an in vitro system to study
the arrangement of biopolymers into their most energetically favorable alignment. The molecular alignment of liquid crystals is energetically favorable, because it maximizes the amount of space available for particle movement and minimizes the steric repulsion between component molecules. Studying the preferred arrangement of pure biopolymers with one another in vitro can be used to draw insights into the organization of molecules, as well as the self-assembling properties of biological structures.

While LCs do not possess positional order as in solids, they do retain some orientational order. Positional order refers to the specific space that individual molecules occupy in a crystalline solid; orientational order refers to the way in which these individual molecules are constrained in their arrangement with respect to one another. When a solid melts to create a liquid, both positional and orientational orders are lost; when a solid melts to create a liquid crystal, the positional order may be lost, but the orientational order remains intact. Thus, liquid crystals are able to move freely as in a fluidic state while orienting with each other in a specific direction (where the average number of molecules align in the same direction, known as the director, over at least a micrometer distance). The term anisotropic is often attributed to liquid crystalline materials due to their ability to maintain orientational order; liquids are simple isotropic.

The second law of thermodynamics states that it is a universal tendency for systems to become disordered. Biological systems, however, must retain order to maintain homeostasis. Since the disorder of a system is spontaneous, energy must be input to counter this process. Biological systems operate so as to minimize this energy
expenditure. One of the ways that biological systems minimize energy expenditure is by utilizing self-assembly.

If transition into a LC phase results from melting a crystalline solid, the LC is referred to as thermotropic; if transition into a LC phase occurs due to changes in solvent parameters, the LC is referred to as lyotropic. Energetically speaking, latent heat measurements indicate that LCs are more similar to liquids than solids (Collings 2002). In other words, less energy is required to transition from a LC to a liquid than is required to transition from a solid to a LC (Fig. 1A, 1B and 1C). Latent heat values are typically 250 J/g for the solid to LC transition and 5 J/g for the LC to liquid transition (Collings and Hird 1997). Examples of thermotropic LCs include: cholesteryl myristate, 4-n-pentyl-4’-cyanobiphenyl (5CB) and cyanobiphenyl mixtures (E7). Cholesteryl myristate exhibits solid, liquid crystal and liquid states all with respectively decreasing amount of orientational order. The solid state of cholesteryl myristate exists at room temperature (Collings 2002). Once cholesteryl myristate is heated to 71°C, it transitions to a cloudy liquid crystal (Collings 2002), where the individual molecules are aligned along a common direction. The direction of alignment of a LC is commonly referred to as the director of the liquid crystal (Collings 2002).

The clear liquid state of cholesteryl myristate occurs above 85°C (Collings 2002). Examples of lyotropic LCs include: phospholipids, disodium cromoglycate (DSCG), cetylpyridinium chloride (CPCl) and cesium pentadecafluorooctanoate (CsPFO) (Chandrasekhar and Madhusudana 1980; Woolverton et al. 2005). Lyotropic LCs are composed of two or more compounds (Chandrasekhar 1977), such as a polymer and a
**Figure 1.** Arrangement of molecules in solids, liquids and liquid crystals. Solids have both positional and orientational order (A). Liquid crystals do not have positional order but do have orientational order (B). Liquids lack both positional and orientational order (C).
solvent (Samulski 1990). Changes to the solvent proper, such as ionic strength and pH, are often used to induce liquid crystalline properties of lyotropic materials.

The optical textures of liquid crystals (e.g. their appearance using cross-polarized light microscopy) can vary depending on the molecular structure of the liquid crystal itself (Singh 2000). Typically, liquid crystals result from the alignment of (1) rod-shaped molecules (referred to as calamitic liquid crystals) or (2) disk-shaped molecules (also known as discotic liquid crystals) (Bruce 2000; Singh 2000). Calamitic liquid crystals, such as CPCl (Berret et al. 1994), can form nematic (Fig. 2A) or smectic phases (Fig. 2B and 2C), while discotics such as DSCG (Nastishin et al. 2004) form nematic (Fig. 3A) or columnar phases (Fig. 3B) (Bruce 2000). Disk shaped molecules stack upon one another into columns to form columnar phases characterized by the arrangement of columns with respect to one another (Bruce 2000). The nematic phase possesses orientational order in one dimension and no positional order (Bruce 2000). Cholesterol derivatives are mostly known for forming cholesteric liquid crystals. Since there are many molecules that are cholesteric but have no association with cholesterol, the cholesteric phase is more properly referred to as chiral nematic, a subclass of nematic LCs. Chiral nematic LCs consist of layers of aligned molecules, each with its own nematic order. While most molecules with chiral nematic properties are flat, the protrusion of side chains upwards and hydrogen atoms downwards from individual layers of the chiral nematic phase, lead to a shift in the adjacent layer (Brown and Wolken 1979). The protrusion of side chains and hydrogen atoms cause layers to arrange at an angle with respect to one another.
Figure 2. Arrangement of molecules in calamitic liquid crystals. Calamitic liquid crystals can form (A) nematic, (B) smectic A and (C) smectic C phases.
Figure 3. Arrangement of molecules in discotic liquid crystals. Discotic liquid crystals can form nematic (A) and columnar (B) phases.
oriented along a helical axis (Chandrasekhar 1977). Smectic phases contain one-dimensional orientational order and additionally stack into layers. This two dimensional order increases the viscosity of smectic LCs (Bruce 2000; Singh 2000). Smectic LCs are sub-classified as smectic A through smectic G, depending on the order of molecules within individual layers (Brown and Wolken 1979).

Much of the research conducted on biological liquid crystals has been in systems consisting of amphiphiles, such as cholesterol and phospholipids, in water (Brown and Wolken 1979). Amphiphilic molecules consist of a charged end that is hydrophilic and a hydrocarbon end that is hydrophobic (Collings 2002). Amphiphilic phospholipids have been studied and reported to form liquid crystals with numerous arrangements. These arrangements of phospholipids exclude the hydrophobic hydrocarbon chains of phospholipids from water, similar to the way in which phospholipids order in living systems. Phospholipids can assume lamellar (Fig. 4A), micellar (Fig. 4B), hexagonal (Fig. 4C) and cubic (Fig. 4D) structures in vitro (Collings 2002). Phospholipids sometimes order into spheres which pack into a cubic structure that is optically isotropic (Collings 2002). Phospholipid head groups are on the outside of spheres in aqueous solvent (Collings 2002). Head groups are external in hexagonal arrangements as well, but the molecules organize into cylinders instead of spheres (Collings 2002). Micellar and gel arrangements of phospholipids are not liquid crystalline in nature, but they are a common arrangement of phospholipids that requires explanation. Phospholipid micelles can be spherical or cylindrical in shape, but they lack the long-range order of cubic or hexagonal ordered phospholipids (Collings 2002). The most common arrangement of
Figure 4. Arrangement of amphiphilic molecules *in vitro*. Amphiphilic molecules can form lamellar (A), micellar (B), hexagonal (C) and cubic (D) structures, *in vitro*. 
amphiphilic liquid crystals and perhaps the most biologically relevant is the lamellar structure (Collings 2002). Phospholipids with lamellar ordering form double layers (similar to biological membranes) that pack parallel to one another (Collings 2002). In aqueous solutions the head groups of phospholipids always point outwards and are in contact with the aqueous environment. Inverse arrangements of phospholipids do however exist where hydrophobic hydrocarbon chains are in contact with solvent, when the solvent is organic. The ordering of phospholipids in aqueous solutions can be evaluated to predict the arrangement of proteins with hydrophobic cores (amphipathetic proteins), such as trans-membrane proteins, that arrange into unique structures to exclude hydrophobic cores from the aqueous environment.

The organization of DNA in biological systems that lack classical histones (proteins around which DNA wraps to package itself) is liquid crystalline, as well (Leforestier and Livolant 1993). *In vivo*, DNA concentrations in viruses, sperm heads, chromosomes and bacterial nucleoids can range from 50 to 800 mg/mL (Kellenberger *et al.* 1986; Leforestier and Livolant 1993). DNA forms an isotropic liquid below this concentration range (Leforestier and Livolant 1993). DNA molecules possess chiral nematic order (Livolant 1984; Livolant 1986) and columnar hexagonal order (Livolant *et al.* 1989; Livolant 1991) as concentration increases (Leforestier and Livolant 1993). Chiral nematic DNA arrangement is found in dinoflagellate chromosomes (Livolant 1984; Rill *et al.* 1989) and bacterial nucleoids (Leforestier and Livolant 1993), including bacterial plasmids (Reich *et al.* 1994). Columnar hexagonal order, such as that found in bacteriophages (Lepault *et al.* 1987), refers to DNA strands organizing into columns
which have a hexagonal arrangement with respect to one another (Fig. 4C). The liquid crystalline phases of isolated DNA in vitro are useful for modeling the self-ordering of DNA in vivo (Leforestier and Livolant 1993).

Lars Onsager, a Norwegian-born chemist and recipient of the 1968 Nobel Prize in Chemistry, explained the formation of a nematic LC phase resulting from rigid, rod-shaped molecules in the 1949 article, “The Effects of Shape on the Interaction of Colloidal Particles” (Onsager 1949). Onsager predicted that rod-like particles, such as tobacco mosaic virus, will form a lyotropic nematic phase at a high concentration (Onsager 1949; Fatkullin and Slastikov 2005). His model also predicted that longer rods will result in a more ordered liquid crystal phase, than shorter rods (Onsager 1949). Onsager’s theory stated that the long profile of rod-shaped molecules and the steric repulsion between them are the primary factors influencing the formation of calamitic liquid crystals (Wang et al. 2004). Onsager found that the most energetically favorable arrangement of rod-shaped particles in solution is an aligned one where molecules are parallel to one another. Here, repulsive forces are minimized (Van Roij and Mulder 1996; Barry et al. 2006). Parallel arrangement of hard rods is also energetically favorable, because this arrangement maximizes the amount of space available for particle movement (Dogic and Fraden 2006).

Straley extended the Onsager model to incorporate threaded rods (e.g. a helix). Straley’s model predicts that threaded rods, such as schizophyllan, poly(γ-benzyl L-glutamate), and poly ((R)-2,6-dimethylheptyl isocyanate) (Sato et al. 1998) will not align
Figure 5. Arrangement of threaded rods in chiral nematic liquid crystals. Straley predicted that the most energetically favorable arrangement of threaded rods in a chiral nematic liquid crystal is one at which the threaded rods intercalate with one another at an angle approaching parallel.
parallel to one another; they will intercalate with one another at an angle approaching parallel (Fig. 5) (Straley 1976). This arrangement has been found the most energetically favorable for threaded rods. Interestingly, bacterial flagella possess the structure of threaded rods. Flagella produced by *Salmonella enterica* serovar Typhimurium strains SJW 1103 and SJW 1665 and SJW 2869 (mutant strains of SJW 1103 with point mutations in the flagellin gene) have been reported to have liquid crystalline characteristics (Barry *et al.* 2006). Barry *et al.* found that the rod-like flagella of SJW 1665 form a nematic phase, while the helical flagella sheared from SJW 1103 and SJW 2869 form conical phases (Barry *et al.* 2006).

The arrangement of collagen in solid material *in vivo* resembles order found in liquid crystals (McGrath and Kaplan 1997). Type IV collagens are proteins found in the corneal and basal lamina that have been found to form nematic (Gathercole *et al.* 1989), chiral nematic (Giraud-Guille 1987) and smectic (Hukins and Woodhead-galloway 1977) phases *in vitro*. While solid collagenous materials have been demonstrated to have liquid crystalline order, this does not prove that this order is present during the assembly of solid collagenous materials *in vivo* (McGrath and Kaplan 1997). However, since LC order results from unique, energetically favored, self-assembly of LC molecules, the arrangement of collagen into stable liquid crystalline phases *in vitro* does strongly suggest this (McGrath and Kaplan 1997). Thus, the LC ordering facilitates the formation of three dimensional structures in which the component molecules lie along a common director.
Liquid crystalline self-assembly has been reported in egg case proteins of praying mantids (Neville and Luke 1971; Neville 1993). Gland cells of female praying mantids secrete nematic liquid crystalline egg case proteins (McGrath and Kaplan 1997). As these proteins are concentrated in the lumen of the gland, a cholesteric liquid crystal is formed (McGrath and Kaplan 1997). Dogfish egg capsule protein, a type of collagen, is also gland-secreted and exhibits liquid crystalline order (Knight and Feng 1994). It is likely that these proteins also concentrate in the gland lumen and subsequently solidify above a critical concentration.

Chitin and cellulose represent additional biological examples of polymers that transition through liquid crystalline states en route to solidification, as concentration increases. Chitin and cellulose are both structural polysaccharides (Blumenthal and Roseman 1957; de Souza Lima and Borsali 2004). Chitin and cellulose are found in arthropod cuticles and plant cell walls, respectively (Belamie et al. 2006). The confinement of newly synthesized chitin and cellulose between the cell membrane and the existing matrix of chitin or cellulose has been predicted as a means by which these molecules can be arbitrarily concentrated (Belamie et al. 2006). As the concentration of the biopolymer increases, the majority of the material may remain isotropic. A small amount, however, begins to form a metastable liquid crystal, where the concentration is only slightly above the isotropic to anisotropic phase transition, or a crystalline solid (Porter and Easterling 1984; McGrath and Kaplan 1997). A small amount of metastable liquid crystal is usually formed in lieu of a crystalline solid, because less order is required to form the liquid crystal (Papkov 1984; McGrath and Kaplan 1997). The small amount
of material with liquid crystalline order serves as a nucleation site from which more liquid crystal forms (McGrath and Kaplan 1997). The concentration of these molecules results in ordered chiral nematic liquid crystals (Belamie et al. 2006). Constant addition of biopolymer keeps the concentration of biopolymer high enough to remain in a liquid crystalline state (McGrath and Kaplan 1997). As more of the biopolymer secreted from cells, fills the confined space, the concentration increases above a threshold, and the material solidifies.

Self-assembling systems do not all require a cell to expend energy to arrange molecules. Non-self ordering systems require enzymes or energy expenditure in the form of adenosine triphosphate (ATP) hydrolysis to align and position molecules (Neville 1981; McGrath and Kaplan 1997). Self-assembling biopolymers are likely preferred in biological systems to promote organization of molecules without the expenditure of energy.

Investigation of polypeptide liquid crystals may lead to the discovery of novel materials for traditional and even new uses, in addition to the elucidation of their biological functions in vivo. Recombinant systems, such as bacteria and yeast can then be used to synthesize these polypeptides. The recombinant systems can also be used to construct hybrids of two polypeptides, such as a hybrid of silk and elastin (McGrath and Kaplan 1997). The textile technology associated with the production of silk fabric in vitro is similar to the system that silkworms utilize in vivo (Stewart 2003). The in vitro processing of silk would allow different methods of spinning the silk and the manipulation of Ca$^{2+}$ ions (Stewart 2003). In vitro processing of some polypeptides
allows for control over temperature, concentration and chemical environment (McGrath and Kaplan 1997). Controlling processing conditions could result in yields and material properties not typically seen in nature (McGrath and Kaplan 1997).

In addition to investigating the liquid crystalline properties of biopolymers to understand the process of self-assembly in vivo, the properties of biological liquid crystals can be used to develop novel biomimetic materials (Belamie et al. 2006). Research investigating biological liquid crystals in cells and tissues drew attention to polypeptide liquid crystals synthesized by Conmar Robinson (Stewart 2003). These polypeptides were precursors to rayon and other synthetic fibers (Stewart 2003). Polyesters were eventually developed as a replacement for polypeptide fibers, because polyester was more resistant to moisture and decay (Stewart 2003).

There are likely many more biopolymers that will be shown to have liquid crystalline properties, in addition to those known currently. Investigation of these liquid crystalline biopolymers can be used to determine how molecules organize in biological systems without the use ATP. Biological liquid crystals also offer biodegradable and biocompatible alternatives to synthetic chemical liquid crystals. Further investigation of biological liquid crystals can therefore be used to answer biologically relevant questions and to develop novel biomaterials.

Exploitation of biopolymer liquid crystalline properties

The unique optical properties of LCs have made them useful in various applications. One of the first applications of LCs in biology was to measure temperature
These LC devices typically used chiral nematic LCs that change color as the temperature changes (Brown and Wolken 1979). In other words, the materials are thermotropic. LCs as temperature sensors have become quite popular due to their accuracy, response time and shelf-life (Brown and Wolken 1979). LCs have also been used as chemical detectors (Shah and Abbott 2001). When chemicals diffuse into a chiral nematic LC, the pitch is affected thereby changing the color of light reflected (Brown and Wolken 1979). These detectors therefore change colors dependent upon the chemical introduced.

Because of the unique alignment capabilities of liquid crystal materials, calamitic liquid crystals that form the nematic liquid crystalline phase are commonly found in display devices (Bruce 2000), as well. In these displays, the LC is placed between two pieces of conductive indium-tin oxide (ITO)-coated glass (Collings and Hird 1997). A material, such as polyimide, is rubbed onto the surface of the ITO-coating to promote LC alignment. The polyimide treatment is rubbed onto the surface of the two pieces of ITO-coated glass in a direction perpendicular to one another (Collings and Hird 1997). This alignment allows the LC to rotate 90° between the top and bottom piece of glass (Collings and Hird 1997). Ambient light therefore enters the top piece of glass, becomes polarized, is rotated 90° by the LC and is allowed to pass through the second polarizer in the bottom piece of glass (Collings and Hird 1997). When a voltage is applied to this system, the alignment of the LC is altered and light is not permitted to pass through the system. Depending upon the charge properties of a biological liquid crystal, biopolymer LCs may offer alternatives to some of the chemicals used in LC displays.
Liquid crystals also have been used for pathogen detection. Biosensors exist that use cross-polarized light microscopy and liquid crystals to detect bacteria in “real-time” (Woolverton et al. 2001; Helfinstine et al. 2006). These biosensors utilize antibodies as receptors to form immune complexes with the microorganism of interest. The growing immune complexes then disrupt the alignment of liquid crystals. As a result, the “defects” in the liquid crystalline texture can be detected using cross-polarized light microscopy. While immune complexes occupy enough volume to cause notable defects in liquid crystalline textures, individual antibodies and antigens do not (Woolverton et al. 2001; Helfinstine et al. 2006). In other words, once the diameter \( d \) of an immune complex exceeds a critical size (greater than \( K/W \), where \( K \) describes the elastic energy of the liquid crystal and \( W \) describes the anchoring energy between the immune complex and the LC), an optically observable defect occurs in the LC texture (Shiyanovskii et al. 2005).

One of the liquid crystalline biosensors requires the antigen of interest to be attached to the walls of the sensor proper (Guzman et al. 2005) and uses thermotropic liquid crystals (Woltman et al. 2007). The thermotropic liquid crystal typically used in this biosensing device is 5CB (Gupta et al. 1998). However, liquid crystals, such as those used in liquid crystalline displays and sensors (e.g. 5CB), can be toxic to living organisms (Woolverton et al. 2005; Dhar et al. 2008).

LC biopolymers may offer a non-toxic, biocompatible alternative to the cytotoxic ones. Toxicity is an important variable in these LC biosensors, because toxic material may alter or decrease the integrity of an antigen preventing antibody recognition.
(Woolverton et al. 2005). The toxicity of the liquid crystal must therefore be considered when antibody-antigen interactions are a sensor component. Thermotropic liquid crystals, such as 5CB and surfactant-based lyotropic liquid crystals, such as CPCl and CsPFO are toxic to certain forms of bacteria (Woolverton et al. 2005). Woolverton et al. (2005) found that 5CB (100 wt%) inhibited the germination and growth of *Bacillus atrophaeus* spores, while CPCl and CsPFO inhibited the growth of *S. aureus* and *E. coli* (Woolverton et al. 2005) after 15 minute incubations with the respective LC at room temperature. CsPFO inhibited the germination and growth of *Bacillus atrophaeus* spores, while CPCl did not (Woolverton et al. 2005).

**Characterization of liquid crystal biopolymers**

Cross-polarized microscopy is often used to examine LCs. A polarizer takes natural light, which vibrates in all directions along a line, and only allows light waves in a single plane to pass through. In cross-polarized microscopy, two polarizers are crossed with one another at a 90° angle. Therefore, when two polarizers are crossed at a 90° angle with respect to one another, light is not permitted to travel through, and a sample would appear black when viewed between crossed polarizers. If two polarizers were aligned parallel with one another, light would be allowed to pass, and a sample would appear bright. LCs are referred to as birefringent, double refractive or optically anisotropic (Brown and Wolken 1979) due to their unique optical properties. Birefringent materials allow light to pass through them at different velocities and in different directions (Brown and Wolken 1979) leading to the unique coloration of liquid
crystals when viewed with cross-polarized microscopy. When LCs are positioned between two crossed polarizers, the LCs reorient light allowing it to pass through the second polarizer. Thus, the sample does not appear dark; the LCs appear bright.

The liquid crystalline textures of biopolymers can be observed with a brightfield microscope equipped with crossed polarizers. The resolution of a brightfield microscope does not allow for the observation of individual molecules of biopolymers or the topology of liquid crystalline textures, though. The small size of individual protein and carbohydrate biopolymers and often the structure of larger assemblies of multiple biopolymers are simply not within the resolution limit for a light microscope.

Transmission electron microscopy (TEM), scanning electron microscopy (SEM) and atomic force microscopy (AFM) are therefore used to study individual biopolymers, liquid crystal topology and aggregates consisting of multiple proteins, such as collagen fibers.

Electron microscopes use electron radiation as opposed to visible light to view specimens. Since the wavelength of an electron is substantially less than the wavelength of visible light, resolution is increased. The increased resolution of electron microscopes allows smaller objects to be observed than what can be observed with a brightfield microscope. In electron microscopy, specimens are placed in the path of the electron beam under vacuum. The column through which the electron beam flows is under vacuum, as well, so as to eliminate scattering of the electron beam by air molecules. Magnetic coils aid in focusing the electron beam. Transmission electron microscopy specimens are placed on copper-coated grids and negatively stained with an electron
dense material, such as phosphotungstic acid. The electrons are scattered by the electron
dense material and pass through less dense areas resulting in an image (Raven and
Johnson 2002). Specimens for scanning electron microscopy are placed on aluminum
stubs and coated with a heavy metal, such as gold. Unlike TEM, SEM does not construct
an image from electrons passing through the specimen but instead constructs an image
from the electrons scattered from the surface of the specimen due to the heavy metal
coating (Raven and Johnson 2002).

Atomic force microscopy can be used to construct three-dimensional images of
surfaces with minimal sample preparation under physiological conditions. AFM moves a
probe (cantilever) across the surface of a specimen. The resulting changes in height of
the cantilever are detected using a laser beam. The laser beam reflects off of the
cantilever to a photodiode. The cantilever moves upwards when it encounters an area
with an increased height, as compared to its surroundings. The movement of the
cantilever upwards causes the reflected laser beam to move upwards, and an increase in
height is detected by the photodiode (Dufrene 2002).

Even imaging techniques as sensitive as TEM, SEM and AFM cannot be used to
determine the secondary structures of proteins. Structural characteristics of proteins can
be determined using Fourier transform (FT) infrared (IR) spectroscopy. FT-IR
spectroscopy uses a molecule’s absorption of IR to determine secondary structural
changes of proteins and requires a change of dipole moment upon excitation (Nolting
2006). IR spectra of proteins contain an Amide I and an Amide II band. Stretching
vibrations of the C=O bond of the amide gives rise to the Amide I band, while bending
vibrations of the N-H bond is detected as an Amide II band (Gallagher 2007). Since hydrogen bonding in the secondary structure of a protein is associated with C=O and N-H bonding, the location of the Amide I and II bands are sensitive to changes in secondary structure (Gallagher 2007).

**General overview and approach**

Paucities exist in the primary scientific literature addressing: (1) what are the liquid crystalline properties of less investigated and uninvestigated biopolymers, (2) how can biological liquid crystals be used to replace synthetic chemicals in liquid crystal devices, such as biosensors, (3) do the flexibility of liquid crystal molecules affect their sensing capacity and (4) do biological liquid crystals function as optimally as their chemical counterparts. These questions are addressed in the following studies using chitin, bacterial flagella and \textit{f\textalpha} bacteriophage.

The liquid crystalline properties of bacterial flagella have been sparsely investigated. Only two studies have reported the liquid crystalline characteristics of bacterial flagella. Yamashita \textit{et al.} (1998) exploited the LC properties of bacterial flagella to obtain oriented specimens required for structural determination with X-ray fiber diffraction (Yamashita \textit{et al.} 1998), but the LC properties of the flagella themselves were not characterized. Barry \textit{et al.} (2006) is the only report to have characterized the LC properties of bacterial flagella. Barry \textit{et al.} (2006) did not however fully purify flagella samples, produce phase diagrams for straight bacterial flagella or fully characterize the liquid crystalline textures of helical flagella. We report the use of
Salmonella enterica serovar Typhimurium strains SJW 1103 (helical flagella), SJW 1665 (straight flagella) and SJW 2689 (helical flagella), their extraction and purification, so as to better characterize the liquid crystalline properties of flagella. Differences in the secondary structure of flagella from each of the above strains were also characterized using IR spectroscopy. Phase diagrams were constructed using a system of purified flagella in sterile water. The functionality of liquid crystalline flagella in sensing was also assessed.

The liquid crystalline properties of the major coat protein (g8p) of fd bacteriophage has not been previously reported in the scientific literature. Purified g8p, due to its bent-core structure, was studied to determine its liquid crystalline properties in water. Atomic force microscopy (AFM) was used to determine the arrangement of individual g8p molecules in strongly birefringent g8p films. Scanning electron microscopy was used to determine the structure of macromolecular assemblies formed by g8p molecules. g8p samples were additionally heated to 300°C to document heat stability of g8p assemblies.

A relatively well characterized biological liquid crystal, chitin, was used to determine the efficacy of using biological LCs in liquid crystalline-based biosensing devices. Chitin (poly-β-(1→4)-N-acetyl-D-glucosamine) has been studied for its LC properties, but the application of liquid crystalline chitin for biosensing is lacking in the primary literature. Monosaccharides in the chitin polysaccharide possess an acetylamine group in place of a hydroxyl group. The acetylamine groups allow for increased hydrogen bonding, which increases the strength of the chitin matrix. The structural
strength of chitin is needed for the exoskeleton of crustaceans and insect, where it is found. Sonication of the chitin matrix results in rigid chitin fibrils.

One micron streptavidin-coated polystyrene beads incubated with 1:1000 anti-streptavidin antibodies were introduced into the chitin liquid crystal. The immune complexes formed from the streptavidin-coated beads were used as surrogates for bacterial immune complexes. Bacterial immune complexes consisting of *Salmonella enterica* serovar Typhimurium SJW 1103 and anti-*Salmonella* Typhimurium antibodies were also added to the chitin liquid crystal. Chitin liquid crystalline textures in the presence of immune complexes were observed to determine if the immune complexes resulted in disclinations detectable with cross-polarized light microscopy.

**Dissertation rationale and hypotheses**

It is the overarching aim of the following studies to determine the optical properties of chitin, bacterial flagella and the major coat protein of *fd* bacteriophage and to assess their possible applications. The proposed study evaluating the optical properties of biopolymers consists of four hypotheses:

Hypothesis 1: Bacterial flagella form varying liquid crystalline phases based on their phenotype and these phenotypic changes result in different liquid crystalline textures.

Hypothesis 2: The major coat protein of *fd* bacteriophage, g8p, has liquid crystalline and fiber forming properties due to its bent-core structure.
Hypothesis 3: *Salmonella*, in the form of *Salmonella* immune complexes, will be detected as defects in liquid crystalline chitin.

Hypothesis 4: The amount of distortion caused by the introduction of immune complexes into a LC will decrease as the flexibility of the liquid crystal biopolymer increases.

Hypothesis 1 is based on data in a prior report that demonstrated unspecified liquid crystalline properties in flagella samples. The following study aims to characterize the changes in secondary structures of flagellin monomers that result in different flagella phenotypes and to better characterize the phases formed by the different flagella phenotypes.

The second hypothesis is designed to document the arrangement of g8p molecules in liquid crystalline arrangements. Due to its bent-core structure and self-assembling properties, it was hypothesized that g8p possesses liquid crystalline properties. While synthetic bent-core liquid crystals exist, only one proteinaceous biopolymer bent-core liquid crystal, preCOL, has been characterized (Knight and Feng 1994; Knupp et al. 1998; Hassenkam et al. 2004). This hypothesis aims to document the second of only two known proteinaceous-based bent-core liquid crystals.

The third hypothesis is based on evidence that immune complexes cause defects in discotic liquid crystals (Helfinstine et al. 2006). The number of defects in the liquid crystal has been found to increase with the initial number of bacteria used to form immune complexes. This hypothesis aims to determine the sensitivity of detection of
Salmonella immune complexes when introduced into the liquid crystalline matrix of chitin, a rigid, polysaccharide, chiral nematic liquid crystal.

Hypothesis 4 takes the flexibility of biopolymers into account for sensing. Immune complexes are predicted to cause defects larger than the immune complexes themselves in liquid crystalline chitin. The sizes of defects are predicted to decrease if more flexible rods are used for the LC. Immune complexes introduced into a LC composed of rigid rods (e.g. chitin) would cause the rods to reorient around the immune complex. The reorientation of the rods would result in a defect, or disclination, in the LC that could be viewed at low magnification. Semi-flexible (e.g. fd bacteriophage) and flexible rods (e.g. S. Typhimurium SJW 1665 flagella) would have the opportunity to bend around an immune complex thereby lessening the magnitude of the disclination.

The subsequent chapters address these hypotheses and conclude with a final synopsis.
References


Collings, P. J. and M. Hird (1997). Introduction to liquid crystals chemistry and physics, CRC.


CHAPTER II

Liquid Crystalline Properties of *Salmonella* Flagella

Abstract

The liquid crystalline properties of purified flagella from three strains of *Salmonella enterica* serovar Typhimurium were characterized in this report. The purity of flagella was confirmed using mass spectroscopy. Infrared spectroscopy and crossed polarized light microscopy were used to characterize differences in the secondary structures of flagellin samples and liquid crystalline textures, respectively. The most energetically favorable arrangement of flagella in its liquid crystal state was used to predict the most favorable arrangement of flagella in concentrated biological systems, such as the surface of peritrichously flagellated bacterial cells. This study documents the chiral nematic properties of flagella from *Salmonella enterica* serovar Typhimurium SJW 1103 and SJW 2869 for the first time.

Introduction

*Salmonella enterica* serovar Typhimurium is a motile, Gram-negative, rod-shaped bacterium commonly known for its role in food poisoning. *Salmonella* owe their motility to peritrichous flagella attached to proton gradient-driven rotary motors anchored in the
cell wall (Darnton and Berg 2007). The flagella themselves are homopolymers of one protein, flagellin (Hasegawa et al. 1982). Interactions between α-helical termini of flagellin monomers result in self-assembly of a flagella filament composed of as many as 30,000 flagellin monomers (Vonderviszt et al. 1992; Yonekura et al. 2002). These filaments typically possess a left-handed chirality, are 20 nm in diameter, and as long as 15 μm in length (Hasegawa et al. 1982; Yonekura et al. 2002). Flagella are flanked by a hook that acts as a universal joint between the flagella and the rotary motor at the proximal end (Kitao et al. 2006) and a cap that rotates as flagellin monomers are added at the distal end (Yonekura et al. 2002).

*Salmonella* cells rotate left-handed flagella (helices which would move backwards if rotated clockwise) counter-clockwise to propel forward, known as a “run” (Kitao et al. 2006). The multiple flagella on the surface of *Salmonella* bundle with one another when rotated counter-clockwise (Kitao et al. 2006). This bundle of flagellar filaments acts as a “propeller” that pushes the *Salmonella* cell forward (Kim et al. 2003). Straight “runs” of *Salmonella* cells are interrupted by periods of erratic movement referred to as “tumbles.” During the complex process of “tumbling,” at least one rotary motor reverses direction (Kim et al. 2003). The reversal of the rotary motor produces a twisting force that causes a left-handed flagellum to transform into a right-handed flagellum (helix that would move forward if rotated clockwise) resulting in displacement of this flagellum from the bundle (Samatey et al. 2001; Kim et al. 2003). The displacement of individual flagellum from the flagellar bundle results in breakdown of the bundle itself. The breakdown of the
flagellar bundle results in uncoordinated movement of the bacterium, known as a “tumble.”

The process by which left-handed flagella convert into right-handed flagella during “tumbling” is referred to as polymorphic transition (Kim et al. 2003). During this process, the flagellum transitions from left-handed to various right-handed forms with varying periodicities throughout the process of “tumbling” and eventually back into a left-handed flagellum required for “running” (Kim et al. 2003). In addition to the twisting force caused by reversal of the rotary motor, left-handed flagella can also be transformed into various right-handed forms by pH (Kamiya et al. 1982) and ionic strength shifts (Kamiya et al. 1982; Darnton and Berg 2007). Of note is the fact that single point mutations in the flagellin gene can also result in right-handed flagella (Kanto et al. 1991). The right-handed mutant flagella do not undergo polymorphic transition.

*S. Typhimurium* SJW 1665 and SJW 2869 are two strains of *Salmonella* with single point mutations in the gene encoding flagellin. SJW 1665 and SJW 2869 are both derivatives of *S. Typhimurium* SJW 1103 (Kanto et al. 1991). SJW 1103 is genetically identical to wild-type *S. Typhimurium* TM2 except that SJW 1103 only expresses one of the two structural genes for flagellin (Fujita et al. 1981; Yamaguchi et al. 1984). The two structural genes normally carried in *S. Typhimurium* for flagellin production are *fliC* (encoding phase 1 or H1 flagella) and *fljB* (encoding phase 2 or H2 flagella) (Schmitt et al. 1996). In a process known as flagellar phase variation, *S. Typhimurium* “oscillates” between the expression of *fliC* and *fljB* (Silverman et al. 1979). The biological significance of phase variation is poorly understood (Ikeda et al. 2001; Bonifield and
Hughes 2003). SJW 1103 possesses only the fliC gene (Yamaguchi et al. 1984; Kanto et al. 1991). Since SJW 1103 is the parental strain of SJW 1665 and SJW 2869, SJW 1665 and SJW 2869 do not express fljB. Flagella of SJW 1103 have a periodicity of 2.4µm and are left-handed – the handedness observed for wild-type S. Typhimurium. SJW 1665 and SJW 2869 express straight and curly (right-handed with a periodicity of 1.1µm) flagella, respectively. The structural differences between SJW 1665 and 2869 and their parental strain, SJW 1103, arise from single protein sequence changes at amino acids 449 and 427 for SJW 1665 and 2869, respectively (Kanto et al. 1991).

While strains such as SJW 1103, 1665 and 2869 have been used to study polymorphic transition (Kanto et al. 1991), they have also been used as models to study the macroscale arrangement of helical rods in a liquid crystalline state. Barry et al. (2006) studied the liquid crystalline state of flagella isolated from SJW 1103, 1665 and 2869 and the arrangement of the flagella while in a liquid crystalline state. A liquid crystal is a fluidic state of matter whose molecules orient with one another in a specific direction but are not positioned in set places in relationship to one another, as in a crystalline solid. The direction along which the molecules align is referred to as the director (Collings 2002). Two common arrangements of molecules in a liquid crystal are the nematic arrangement, where molecules are parallel to one another, and the chiral nematic arrangement, where molecules align parallel to one another within a layer with adjacent layers having alignments rotating slightly from the reference layer in a helical fashion (Collings 2002).
While studies of the liquid crystalline properties of bacterial flagella have been sparse (Yamashita et al. 1998; Barry et al. 2006), studies utilizing the information obtained from the arrangement of isolated flagella filaments with respect to one another in their liquid crystalline state to draw biologically relevant conclusions are absent from the literature. The present study evaluates purified flagella filaments isolated from SJW 1103, 1665 and 2869, to catalogue the differences in secondary structure between the three flagellin proteins and more fully investigate the liquid crystalline properties of flagella.

Methods

Flagella harvesting, purification and recovery

*Salmonella enterica* serovar Typhimurium SJW 1103, 1665, and 2869 were obtained from the Rowland Institute (Harvard University, Cambridge, MA) and grown in tryptic soy broth (BD, Franklin Lakes, NJ) at 37°C for 16 hrs at 200 RPM (Darnton and Berg 2007). Bacterial cells were pelleted by centrifugation at 3000 x g for 20 minutes using an Avanti J-26 XP centrifuge (Beckman Coulter, Brea, CA) and resuspended in sterile distilled, deionized water (ddH₂O). Flagella were sheared from bacterial cells using a vortex (Kumara *et al.* 2006). Differential centrifugation at 10,000 x g for 20 minutes separated bacterial cells from flagella (Kumara *et al.* 2006). Intact flagella were recovered by centrifugation at 50,000 x g for 1 hr (Kumara *et al.* 2006). Flagella filaments were resuspended in sterile ddH₂O and purified using DEAE weak anion

Recovery of flagellin was verified by Western blot. The concentration of flagellin was normalized using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE) with an extinction coefficient of 3.6 (Vonderviszt et al. 1989). Flagellin samples were electrophoresed on 10% polyacrylamide gels (120V for 1.5h) and transferred to PVDF membranes. Mouse monoclonal IgG antibody against Salmonella flagellin (Biolegend, San Diego, CA) was diluted 1:5000 in PBS-T for use in the Western blot (1 h) and bound antibodies were detected using chemiluminescence. Flagella suspensions were flash frozen and lyophilized using a Model 75040 Labconco freeze dryer (Labconco Corporation, Kansas City, MO). Flagellin purity was assessed with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopy (Voyager System 1160, Applied Biosystems, Carlsbad, CA).

**Characterization of flagella filaments**

Flagella stain (BD, Franklin Lakes, NJ), diluted fivefold with 0.6% (w/v) safranin, was used to visualize flagella using brightfield microscopy. Flagella length was determined by image analysis using ImageJ (National Institutes of Health, Bethesda, MD). Only polymerized flagellin can be visualized using this technique. Individual flagellin monomers are not large enough to view with traditional light microscopy due to the diffraction limit of light.
Flagellin conformational studies were performed at 20°C using a Bruker Tensor FT-IR (Bruker Optics, Inc., Billerica, MA) equipped with a specialized attenuated total reflection (ATR) objective (512 scans). Spectra were analyzed with the Bruker OPUS software using a Blackman-Harris 3 term apodization function and a spectral resolution of 2 cm⁻¹. A calcium fluoride salt crystal was used as the substrate for measurements. Flagellin samples were adjusted to 8.33 mg/mL using Centricon centrifugal filter units with a molecular weight cutoff (MWCO) of 10000 (Millipore, Billerica, MA). Pierce dialysis cassettes (Thermo Fisher Scientific, Inc., Waltham, MA) with a MWCO of 10000 were used to exchange flagellin samples with D₂O.

**Characterization of liquid crystalline flagella**

Flagella samples were observed with cross-polarized light microscopy to evaluate the liquid crystal phases formed by the three flagella phenotypes. Phase diagrams were constructed for each flagellar phenotype by the slow addition of water until samples transitioned to a biphasic, and then isotropic, liquid. The concentrations of flagellin were recorded at both of these points using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE), and an extinction coefficient of 3.6 (Vonderviszt et al. 1989).
Results

Characterization of flagella filaments

Western blot confirmed the recovery of *Salmonella enterica* serovar Typhimurium flagellin (Fig. 6). Mass spectroscopy confirmed the purity of flagellin samples (Fig. 7). Length measurements of flagella filaments were determined using image analysis. SJW 1103, 1665 and 2869 flagella are $8.5 \pm 2.8 \ \mu m$ (n=18), $8.2 \pm 4.3 \ \mu m$ (n=18) and $11.3 \pm 3.5 \ \mu m$ (n=18) in length, respectively. IR spectra suggest that SJW 1665 has less $\alpha$-helical character than either SJW 1103 or SJW 2869. SJW 2869 appears to have less $\alpha$-helical character than SJW 1103 (Fig. 8).

Characterization of flagella liquid crystals

Flagella filaments purified from *S. Typhimurium* SJW 1103 formed a chiral nematic liquid crystalline phase (Fig. 9A), as demonstrated by the presence of fingerprint textures at high concentrations (Fig. 9A, insert). SJW 1103 flagella formed a chiral nematic liquid crystal above 24 mg/mL and an isotropic liquid below 14 mg/mL (Fig. 10). SJW 1665 flagella filaments formed a nematic liquid crystal (Fig. 9B) above 16 mg/mL and an isotropic liquid below 10 mg/mL (Fig. 10). Flagella from SJW 2869 also formed a chiral nematic liquid crystal (Fig. 9C) with biphasic properties between 5 mg/mL and 27 mg/mL (Fig. 10).
Figure 6. Western blot of flagellin. (A) 5 μg of flagellin from SJW 1103, (B) 5 μg of flagellin from SJW 1665 and (C) 5 μg of flagellin from SJW 2869
Figure 7. Mass spectroscopy of flagellin. Mass spectroscopy revealed the purity of (A) SJW 1103, (B) SJW 1665 and (C) SJW 2869 flagellin.
Figure 8. Infrared spectra of flagellin. Flagellin from SJW 1665 and SJW 2869 had less α-helical structure than flagellin from SJW 1103. Flagellin from SJW 1665 had less α-helical structure than flagellin from SJW 2869.
Figure 9. Liquid crystalline properties of *Salmonella* flagella. (A) Flagella from SJW 1103 form a liquid crystal state. Scale bar denotes 100μm. (Insert) The liquid crystalline texture formed by SJW 1103 flagella is chiral nematic in nature, as demonstrated by the fingerprint texture observed with crossed polarized microscopy. Scale bar denotes 10μm. (B) Flagella from SJW 1665 forms a nematic liquid crystal. Scale bar denotes 100μm. (Insert) Bundled flagella from SJW 1665 are visible with a brightfield microscope. Scale bar denotes 100μm. (C) SJW 2869 flagella has liquid crystalline properties. Scale bar denotes 100μm. (Insert) The liquid crystalline state of SJW 2869 flagella is chiral nematic in nature. Scale bar denotes 100μm.
Figure 10. Phase diagrams for flagella from *Salmonella enterica* serovar Typhimurium SJW 1103, SJW 1665 and SJW 2869. SJW 1103, SJW 1665 and SJW 2869 flagella suspensions are liquid crystalline in nature above 24mg/mL, 16mg/mL and 27mg/mL, respectively.
Discussion

Intact flagella from SJW 1103, SJW 1665 and SJW 2869 were isolated and purified in order to characterize the liquid crystalline properties of pure flagella absent of contaminates. This report characterizes the liquid crystalline properties of fully purified *Salmonella* flagella for the first time. This report additionally elucidates the changes in secondary structure of flagellin monomers that result in flagella with varying phenotypes.

The methodology used for the isolation of flagella did not involve lysis of the cell and therefore eliminated numerous cytoplasmic contaminates. *Salmonella enterica* serovar Typhimurium SJW 1103 is known to secrete numerous proteins including various virulence factors and proteins associated with the flagellar apparatus (Komoriya *et al.* 1999). Weak anionic exchange chromatography removed these contaminates, as verified by mass spectroscopy data. Mass spectroscopy and Western blot also verified recovery and purification of flagellin proteins. Microscopy of stained flagella demonstrated polymerization of flagella.

The flagellin proteins from SJW 1665 and SJW 2869 have point mutations in the flagellin gene resulting in changes in the protein amino acid sequence at amino acid 449 and 427, respectively (Kanto *et al.* 1991). Both mutations are from alanine to valine and occur in the C-terminal α-helical region (Kanto *et al.* 1991) which is important for the polymerization of flagellin into flagella and flagella polymorphism (Vonderviszt *et al.* 1992).

Chemical bonds in structures such as α-helices and β-sheets have different vibrational frequencies. When the frequencies of infrared (IR) light and molecular
vibrations coincide, infrared light is absorbed. This absorption of infrared light can be detected using IR spectroscopy. α-helices absorb IR between 1648-1657 cm⁻¹, while β-sheets absorb between 1623-1641 cm⁻¹ (Barth and Zscherp 2003). As compared to SJW 1103, a shift in the IR spectra of SJW 1665 and SJW 2869 flagellins toward 1641 cm⁻¹ suggests that mutations in SJW 1665 and SJW 2869 result in a change of the secondary structure of flagellin in which there is a loss in the α-helical structure of the protein (Fig. 8). This change in secondary structure results in changes in flagella phenotypes which manifest as differences observed in the liquid crystalline properties of flagella in suspension.

Purified flagella from SJW 1103 and SJW 2869 were both found to form liquid crystals with chiral nematic arrangements above 24 mg/mL and 27 mg/mL (Fig. 10), respectively. These values are lower than those previously reported (Barry et al. 2006), likely due to the increased purity of the flagella used in this study or differences in the length of the flagella filaments. Filament length was characterized using a modified flagella stain. The flagella stain allows visualization of flagella, because complexes of crystal violet, tannic acid and aluminum potassium sulfate precipitate around polymerized flagellin as ethanol in the stain evaporates (BD specification sheet, 06/2003). (The majority of flagella used by Barry et al. (2006) were approximately 2 μm in length, while flagella used in this report were between 8.2 ± 4.3 μm and 11.3 ± 3.5 μm in length, on average.) The liquid crystalline textures of flagella purified from SJW 1103 and SJW 2869 are conical as previously reported (Barry et al. 2006). This study, however, evaluated more extensively these liquid crystal characteristics and revealed
chiral nematic arrangements, reported by fingerprint textures observed at higher flagellin concentrations (Fig. 9A, insert and 9C, insert).

The arrangement of rod-shaped (SJW 1665) and helical-shaped (SJW 1103 and SJW 2869) flagellin biopolymers can be determined from careful observation of the liquid crystal between crossed-polarizers. Purified flagella in a liquid crystalline state, therefore, present a novel in vitro model to study the arrangement of flagella in high concentrations (e.g. the surface of a pertrichously flagellated bacterium). The arrangement of rod-shaped molecules in a liquid crystal is predicted by the Onsager model; rod-like particles, when at a high concentration, form a nematic phase (Onsager 1949; Fatkullin and Slastikov 2005). Onsager found that the most energetically favorable arrangement of rod-shaped particles in solution is aligned parallel to one another, where repulsive forces are minimized (Van Roij and Mulder 1996; Barry et al. 2006). Straley extended the Onsager model to incorporate threaded rods, such as helices. Straley’s model predicts that threaded rods will not align parallel to one another; they will intercalate with one another at an angle approaching parallel (Straley 1976).

Experimentally, flagella from SJW 1103 and SJW 2869 are threaded rods, preferring to align with one another at angles approaching parallel, but forming chiral nematic liquid crystal textures (Fig. 9A and 9C). Flagella produced by SJW 1665 are rod-like structures, preferring to align parallel to one another, forming nematic liquid crystal textures (Fig. 9B).

S. Typhimurium SJW 1103 was the most motile bacteria followed by SJW 2869 and SJW 1665 (data not shown). Bundles of SJW 1665 flagella can be observed with
light microscopy (Fig. 9B, insert). Since individual flagella are too small in diameter to be imaged with light microscopy, this indicates that these flagella pack very tightly with each other. These tightly-packed straight flagella would not have a pitch sufficient to propel a bacterium forward. Consequently, SJW 1665 was found to be non-motile.

Purified SJW 2869 flagella formed a chiral nematic phase in concentrated solutions supporting a packing of flagella with one another in an orientation approaching parallel (Fig. 9C). Right-handed curly flagella, such as flagella from SJW 2869, have been cited as non-motile in semi-solid media (Iino and Mitani 1966). While counter-clockwise rotation of these right-handed filaments would lead to disassembly of the right-handed flagellar bundle, right-hand rotation (clockwise rotation) of a right-handed flagella bundle would lead to propulsion on the bacterium. The motility of SJW 2869 is approaching 1/27th that of SJW 1103 and is predicted to be associated with right-handed rotation of the rotary motor normally associated with a “tumble.”

This report documented chiral nematic fingerprint textures formed by flagella purified from Salmonella enterica serovar Typhimurium SJW 1103 and SJW 2869 for the first time. Differences in the liquid crystalline textures of flagella from SJW 1103, SJW 1665 and SJW 2869 were attributed to differences in the secondary structure of flagellin monomers that lead to changes in flagella phenotype. The liquid crystalline textures formed by flagella were used to predict the most energetically favorable arrangement of flagella in concentrated biological systems (e.g. the surface of peritrichously flagellated Salmonella cells) for the first time. Future studies will study the response of flagella to electric fields.
References


CHAPTER III

Optical Properties of a Self-Assembling Viral Coat Protein

Abstract

Monomers of the *fd* bacteriophage major coat protein, g8p, interact to form a helical bent-core structure surrounding the viral DNA. It was hypothesized that g8p has mesophasic characteristics guiding viral coat assembly. Atomic force and scanning electron microscopy reveal that g8p monomers organize to form films. These films wrap around a central axis to form birefringent fibers that resemble the filament structure of bent-core thermotropic B7 liquid crystals. These results offer insight into the g8p self-assembly mechanism that results in the formation of new viral particles *in vivo*.

Introduction

Viruses of the family *Inoviridae*, including *fd*, M13 and *f1* bacteriophages infect *Escherichia coli* bacteria (Goldsmith and Konigsberg 1977). *Inoviridae* are 1000 nm in length, 6 nm in diameter and possess a circular single-stranded DNA genome (Welsh *et al.* 1996). The *Inoviridae* genome is surrounded by 2700 copies of the major coat protein, g8p, in addition to minor coat proteins (Welsh *et al.* 1996; Zeri *et al.* 2003; Holland *et al.* 2006). The g8p protein is a 50 amino acid α-helix approximately 7 nm in
length and 1 nm in width (Welsh et al. 1996) (Fig. 11A). The C-terminus of g8p is immobilized to the interior of the virus due to its strong interaction with viral DNA, while the N-terminus is mobile and free to interact with its environment (Colnago et al. 1987; Zeri et al. 2003). During the process of viral assembly, g8p is stored temporarily in the cell membrane of its bacterial host (Zeri et al. 2003). This appears to be necessary for viral assembly; the viral DNA extrudes through the bacterial membrane acquiring the g8p protein as it is shed from the host cell (Marassi et al. 1997). Furthermore, hydrophobic residues of individual g8p monomers interact with one another between Val21 and Ile39 (Fig. 11B) to form a stabilized helical array that surrounds the viral genome (Welsh et al. 1996; Cann 2005). Helicity appears to result from a 23° tilt angle between Lys8 and Gly38, permitting unique functional properties (Zeri et al. 2003). While filamentous bacteriophages do not possess a lipid membrane, the amphipathic nature of g8p cause the peptide to functionally resemble a lipid membrane, facilitating viral penetration through the host membrane during infection (McDonnell et al. 1993; Zeri et al. 2003).

The rigid, rod-like structure of filamentous bacteriophages and the uniformity in dimensions among viral particles has led to several studies of intact phage. The cholesteric and smectic liquid crystalline phases fd bacteriophage suspensions have been reported (Dogic and Fraden 1997), as well as the phase behavior of fd phage with varying effective diameters (Dogic and Fraden 2000) and in the presence of hard spheres (Adams et al. 1998) and Dextran (Dogic et al. 2004). g8p has even been engineered to nucleate zinc sulfide and cadmium sulfide on intact M13 bacteriophages (Mao et al. 2003) for
nanowire construction. There is however a lack of data reporting the optical properties of purified g8p apart from the intact phage.

This report investigates the optical properties of g8p attributed to its bent-core structure. Bent-core g8p has not been previously evaluated as a liquid crystal, nor compared with the novel bent-core liquid crystals recently receiving attraction due to their unique chirality and electric polarization properties (Link et al. 1997). Thus, this report marks the first time the birefringent properties of purified g8p, and its associated macromolecular assemblies, have been extensively documented.

The paucity of data evaluating the optical properties of g8p motivated us to study the structures of self-assembled g8p molecules in aqueous suspensions and as they dry out. We show that these structures strongly resemble the B7 phases of 2-nitro-1,3-phenylene bis[4-(4-alkoxyl)phenyliminomethyl] benzoates] (Pelzl et al. 1999) that form helical filaments in their isotropic melt and concentric cylindrical fibers in air (Jákli et al. 2003). These materials are similar to other biological liquid crystals, such as the mussel byssal thread proteins (Hassenkam et al. 2004).

Methods

The g8p protein was synthesized at the Molecular Biotechnology Core facility of the Lerner Research Institute (Cleveland Clinic, Cleveland, OH) by fluorenylmethoxy carbonyl chemistry on Wang resin in an Omega 396 peptide synthesizer (Advanced ChemTech, Louisville, KY) and purified by high performance liquid chromatography over a Jupiter 4 µm Proteo 90Å column (250 x 4.6 mm,
Phenomenex, Torrance, CA) in a Beckman System Gold High Performance Liquid Chromatograph (Beckman Coulter, Inc., Brea, CA). Purity was assessed with a 4800 MALDI TOF/TOF™ mass spectrometer (Applied Biosystems, Carlsbad, CA) and found to be >90% pure. The final product was lyophilized using a Freezemobile 25EL lyophilizer (VirTis-FTS/SP Scientific, Warminster, PA).

Lyophilized g8p was dispersed in sterile, 0.22 μm filtered, distilled, deionized water by heating to 90°C. Ten μL of 1.3 wt % g8p was placed on optically clear glass and observed with polarized optical microscopy (POM) until dry. Thickness of birefringent regions was measured using a Tencor Instruments Profilometer (Milpitas, CA). Tapping mode was used on a NanoScope IIIa atomic force microscope (Digital Instruments, Santa Barbara, CA) to determine the arrangement of g8p in strongly birefringent areas. Lengths of birefringent fibers were analyzed in 4 wt % g8p dispersions using Spot Software (Version 4.6, Diagnostic Instruments, Inc., Sterling Heights, MI).

For scanning electron microscopy (SEM), 1.3 wt % g8p was dried and sputter-coated with gold in an Anatech LTD Hummer VI-A sputter coater (Alexandria, VA) for three minutes at 10 mA pulses. Images were obtained with a Hitachi S-2600N SEM (Hitachi High-Technologies Europe, Krefeld, Germany) operated at 25 kV.

Ten μL of 1.3 wt % g8p was heated from 25°C to 300°C at a ramp rate of 2°C min⁻¹. Changes in birefringence were monitored per minute with an Olympus BX-60 microscope (Olympus America, Inc. Center Valley, PA) equipped with a color charge-coupled device camera.
**Results**

Birefringent fibers measuring as large as 20 µm in width and 10 µm in thickness, as determined by polarized optical microscopy (POM) and profilometry, were observed in 1.3 wt % g8p dispersions. Profilometry and POM also demonstrated the presence of birefringent films of about 30 µm thickness. Atomic force microscopy (AFM) of birefringent films revealed an organized alignment of g8p monomers (Fig. 11C) that had a periodically modulated structure of about 30 nm (Fig. 11D). Smaller scale AFM scans reveal that this structure possesses a unique topology (Fig. 11D). Based upon the dimensions of individual g8p monomers, we have inferred the 30 nm periodicity to correlate with a longitudinal aggregation of five g8p molecules.

Profilometry and SEM of g8p droplets suggested that the g8p concentration was higher at the edge of droplets (Fig. 12A). Brightfield microscopy demonstrated that suspensions of g8p were weakly birefringent, especially where the g8p concentration was high, *e.g.* at the edge of a droplet (Fig. 12B). These edge areas, however, are strongly birefringent with POM (Fig. 12C).

Similarly, brightfield microscopy demonstrated that g8p fibers possessed weaker birefringence (Fig. 13A and B) as compared to the strong birefringence seen with POM (Fig. 13C and D). Figure 13C shows that sections of the filaments appear black between cross-polarizers when the respective section is parallel or perpendicular to the cross-polarizers. Furthermore, the elongated focal conics along the cross section of the filaments show that the smectic layers are also parallel to the filament axis, indicating
Figure 11  Nanoscale structures of g8p. (A) The g8p peptide, oriented N-terminus to C-terminus (left to right). The bent-core structure of g8p is a result of the angle between Lys8 and Gly38 (highlighted). Scale bar is equal to 1 nm. (B) Hydrophobicity plot demonstrating the hydrophobic character of the bent-core region. (C) AFM image showing periodic ridges of g8p assemblies. (D) These periodic modulations are clearly seen in a smaller scale scan and indicate a periodicity of about 30nm.
Figure 12  Illustration of the micron-scale structure of self-assembled g8p molecules and the textures at the edge and interior of a dried droplet of the suspended material at room temperature. (A) optical microscopy image without polarizers; (B) POM image between cross-polarizers; (C) SEM image. All scale bars are equal to 100 µm.
concentric layer structures of the filaments (Fig. 13D), similar to thermotropic bent-core liquid crystals (Coleman et al. 2003). The lengths of birefringent fibers in 4 wt % g8p dispersions ranged from 0.1 mm to 0.5 mm with an average fiber length of 0.3 ± 0.14 mm (data not shown). SEM demonstrated that g8p fibers form layers that wrap around a central axis (Fig. 13E and F) resulting in birefringence (Fig. 13C and D). The fibers additionally have the capacity to wrap about one another helically to form larger fibers with a more complex superstructure. A strong and abrupt reduction in birefringence occurred when 1.3 wt % g8p was heated from 30°C to 42°C (Fig. 14A, B and C). This is contrasted by the optical characteristics of dried dispersions of g8p, where birefringence does not change over the range of 30°C to 230°C (Fig. 14D, E and F).

Discussion

The major coat proteins of viruses have self-assembling properties (Flint et al. 2004). The localization of coat proteins in a host cell’s membrane, or around viral DNA or RNA, raises the local concentration of the coat protein assisting in this self-assembly process (Flint et al. 2004). This is similar to the way in which an increase in the concentration of a lyotropic material leads to the formation of an anisotropic liquid crystalline texture. The viral DNA of fd phage is used as a scaffolding for the addition of g8p to form intact phage of consistent dimensions in vivo. Without viral DNA as a template for g8p addition, g8p is free to form concentric fibers of varying dimensions compared to the helical 1000 nm x 6 nm unilamellar structure found in intact phage. It is
Figure 13  Self-assembling g8p filaments resemble those found in bent-core thermotropic LCs liquid crystals. Filaments have diameters of approximately 15µm. (A and B) optical microscopy images of g8p filament without polarizers; (C) POM images of g8p filament between cross-polarizers, arrows denote regions of fibers lacking birefringence, (D) POM images of g8p filament between cross-polarizers ; (E and F) SEM images of g8p filament. Scale bars on panels A, C and E are 100 µm and panels B, D and F are 20 µm.
Figure 14  Temperature dependence of g8p birefringence. Heating of hydrated 1.3 wt % g8p from (A) 30°C and (B) 41°C to (C) 42°C resulted in a loss of birefringence. Scale bars on panels A, B and C are equal to 20 µm. A dried g8p sample retained its birefringent properties at (D) 30°C, (E) 110°C and (F) 230°C. Scale bars on panels D, E and F are equal to 100 µm.
ultimately the primary amino acid sequence of coat proteins (Flint et al. 2004), as well as their physical properties (e.g. hydrophobicity and interaction with viral DNA), that contribute to the potential for self-assembly by a virus. In addition to the inherent self-assembling properties of g8p as a viral coat protein, this report documents for the first time, that the bent-core structure of g8p results in unique molecular arrangements, similar to the smectic A_b phase observed in thermotropic bent-core materials (Sekine et al. 1997).

Early research on bent-core materials demonstrated their ability to form helical filaments (Jakli et al. 2000) and films with a smectic structure (Bailey et al.; Jákli et al. 2003). The ordered arrangement of g8p into complex films and birefringent filaments parallels those of B7 bent-core liquid crystals (Coleman et al. 2003). The dimensions of g8p are also similar to those of synthetic bent-core liquid crystals. Synthetic bent-core liquid crystal molecules typically possess dimensions of approximately 6 nm x 0.5 nm (Hassenkam et al. 2004); the dimensions of g8p are 7 nm x 1 nm (Welsh et al. 1996). While data have reported the optical and self-assembling properties of bent-core chemicals, these properties have been demonstrated by only one bent-core protein, preCOL (Knight and Feng 1994; Knupp et al. 1998; Hassenkam et al. 2004). preCOL has dimensions of 250 nm x 20 nm, an angle of 50°, and forms byssal threads in mussels (Hassenkam et al. 2004). While the dimensions and bend angle of preCOL are substantially larger than that of g8p, preCOL and g8p are both bent-core proteins. Both preCOL (Hassenkam et al. 2004) and g8p self-assemble into optically active films and fibers. g8p, like preCOL (Hassenkam et al. 2004), exists as a complex fluid that is
insoluble in water. AFM data demonstrate that g8p organizes into an ordered arrangement of birefringent areas similar to the ordered smectic phase of preCOL.

Future research will compare g8p with thermotropic bent-core liquid crystals and allow us to predict the behavior of the intact fd virus in electric fields. Among possible applications for the fiber forming capacity of purified g8p is nanowire construction. Our research demonstrates the capacity for purified g8p alone to self-assemble into proteinaceous fibers in the absence of other viral proteins and viral DNA. These g8p fibers, with varying lengths and diameters, could be used as a biological template to assemble conductive nanowires with a variety of dimensions, similar to that observed with intact virus.
References


\textit{Biomacromolecules} \textbf{5}(4): 1351-1355.


CHAPTER IV

Detection of Immune Complexes using Chiral Nematic Chitin Fibrils

Abstract

Bacterial pathogen testing typically involves culture-based techniques that can take several days to report results. Liquid crystal-based biosensors have the capability of rapidly identifying bacteria, when an optical distortion of the liquid crystal occurs. Distortions occur due to antibody binding events between antibodies and antigens resulting in the formation of immune complexes. When introduced into a liquid crystal, immune complexes can cause defects in liquid crystalline textures once the diameter of the immune complexes exceed a critical size at which the anchoring energy between the immune complexes and the liquid crystal is overcome by the elastic energy of the liquid crystal. Defects in liquid crystalline textures are detectable with cross polarized microscopy. We hypothesize that chitin, a ubiquitous biopolymer, can be formulated as a sensitive liquid crystal that reports the presence of streptavidin-coated bead (as bacterial surrogates) and Salmonella immune complexes. Streptavidin-coated bead and Salmonella immune complexes were detectable as defects in liquid crystalline chitin to an initial concentration of $10^6$ particles/mL. Defects caused by streptavidin-coated bead immune complexes were larger in comparison to those formed by Salmonella immune
complexes at the same starting concentration. We conclude that the liquid crystalline phase of chitin can be utilized for sensing immune complexes.

**Introduction**

Pathogenic bacteria continue to be problematic in the food industry despite continued advances in bacteriology. The processing of food is often a multistep procedure that presents numerous opportunities for the introduction of unwanted bacterial contaminants (Hall 2002). The ability to detect bacterial contaminants in real-time has therefore become valuable in food microbiology, as confirmed by approximately 38% of pathogen detection research being rooted in the food industry (Lazcka et al. 2007). Thirty three percent of reported bacterial detection methods center upon the detection of *Salmonella* (Lazcka et al. 2007). Of the foodborne bacterial contaminants, *Salmonella* has been cited as causing the greatest number of annual deaths (Leonard et al. 2003).

*Salmonella enterica* serovar Typhimurium (*S. Typhimurium*), a common food contaminant, is traditionally identified using culture-based techniques cited to take upwards of 2 to 9 days, which make these techniques less than preferred in scenarios involving human health hazards (Golash et al. 2002; Alonso-Zaldivar 2009). Screening for food pathogens is a process that mandates rapid results in order to prevent the distribution of contaminated food to the public. The screening process correspondingly reduces human disease by limiting the ingestion of pathogens. Each year in the United States, there are as many as 81 million cases of foodborne illnesses with 91% being of bacterial origin (Ivnitski et al. 1999). Over 5 million tests are conducted yearly to screen
for the presence of *Salmonella* alone (Ivnitski *et al.* 1999) resulting in the allocation of millions of dollars for pathogen testing (Alocilja and Radke 2003). Infection rate, testing time, and monies allocated for *Salmonella* testing all contribute to the need for simple, inexpensive, real-time biosensors.

While several techniques have been developed to screen samples for microorganisms more rapidly than traditional culture-based techniques, few are economically feasible. Among the disadvantages associated with these biosensors are high complexity, a specialized staff is often required, expense, inability to automate, limited selectivity, and limited sensitivity (Ivnitski *et al.* 1999; Helfinstine *et al.* 2006). One optical liquid crystalline-based biosensor seems to eliminate many of these disadvantages. This real-time biosensor uses cross-polarized light microscopy and liquid crystals to detect bacteria (Abbott *et al.* 2001; Woolverton *et al.* 2001; Helfinstine *et al.* 2006).

Liquid crystals comprise a fourth state of matter located between solids and liquids that lose their positional order while retaining some of their orientational order. Liquid crystals are able to move freely as in a liquid state while orienting with each other in a specific direction referred to as the director of the liquid crystalline phase (Collings 2002). In the aforementioned optical liquid crystalline-based biosensor, antibodies are utilized to form immune complexes with the bacteria of interest. These immune complexes disrupt the alignment of liquid crystals. As a result, the presence of immune complexes causes defects, also known as disclinations, in the liquid crystalline texture, detectable using cross-polarized light microscopy. The number of defects can be
correlated to the amount of bacteria in a specimen. While immune complexes occupy
enough volume to cause notable defects in liquid crystalline textures, individual
antibodies and antigens such as *Bacillus* spores (Woolverton *et al.* 2001; Helfinstine *et al.*
2006), do not.

Major drawbacks to use of liquid crystalline materials are their toxicity and
expensiveness. Many liquid crystals, such as those commonly used in liquid crystalline
displays, are toxic to bacteria and eukaryotic cells (Woolverton *et al.* 2005; Dhar *et al.*
2008). The effects of liquid crystals on antigens, proteins recognized and bound by
antibodies, are paramount in liquid crystal-based biosensors. Toxicity presents a
problem, because altering or decreasing the integrity of an antigen could prevent antibody
recognition and cause the biosensor to function less than optimally (Rand *et al.* 2002).
To avoid the harmful effects of the liquid crystal on an antigen and to remain
economically feasible, this study uses the biological liquid crystal, chitin (Belamie and

Chitin, a rigid, asymmetric, rod-shaped carbohydrate polymer, is found in the
cuticle of insects and crabs (Murray and Neville 1998). Due to its structure, chitin has
been expected and later demonstrated to form liquid crystalline phases, including a
nematic phase at concentrations between 4.1 and 4.5 wt % in $10^{-4}$ M HCl (Brown and
Wolken 1979; Belamie *et al.* 2004; Belamie and Giraud-Guille 2004). Chitin has also
been suggested as a suitable functional material, because it is biodegradable,
inexpensively obtained from crab shell waste, and generally has a low toxicity (Kumar
2000). This study aims to exploit the liquid crystalline properties of chitin to detect
Salmonella enterica serovar Typhimurium.

Methods

Liquid crystal preparation

Chitin was prepared according to the methods of Revol and Marchessault (1993), Murray and Neville (1998), and Belamie and Giraud-Guille (2004) (Revol and
Marchessault 1993; Murray and Neville 1998; Belamie and Giraud-Guille 2004). Briefly, five grams of chitin flakes (Sigma-Aldrich Corporation, St. Louis, MO) were
acid hydrolyzed at 110°C in 3N hydrochloric acid (Fisher Scientific, Pittsburgh, PA) for
90 minutes to protonate the chitin. Acid hydrolyzed chitin was thoroughly dialyzed with
6000-8000 molecular weight cutoff dialysis tubing (Spectrum Laboratories, Inc., Rancho
Dominguez, CA) against sterile 16MΩ deionized, distilled water (ddH2O) to raise the pH
to 2.8. Dialyzed chitin samples were dried under sterile airstream, resuspended in ddH2O
to yield a concentration of 4.1 wt% at a pH of 2.8 and sonicated with a Fisher Scientific
sonic dismembrator model 60 (Fisher Scientific, Pittsburgh, PA) at 30W for 3 h.

Characterization of chitin

After sample preparation, 4.1wt% chitin was diluted to 0.41wt% in sterile water.
The chitin sample was placed on a 300 mesh carbon-coated copper grid and water
allowed to evaporate. The preparation was stained with 1% phosphotungstic acid and
observed with a JOEL100S transmission electron microscope (TEM). An average length of chitin fibers was calculated using TEM images.

**Preparation of immune complexes**

One micron streptavidin-coated polystyrene beads (Polysciences, Inc., Warrington, PA) were used as bacterial surrogates and incubated with anti-streptavidin antibodies (Rockland, Inc., Gilbertsville, PA) to form immune complexes. Specifically, streptavidin-coated beads at concentrations of $10^8$, $10^7$, $10^6$ and $10^5$ were incubated with anti-streptavidin antibodies at a concentration of 1:1000 for one hour at 37°C in ddH$_2$O (pH 7.4).

*S. Typhimurium* SJW 1103 was generously provided by the Rowland Institute (Harvard University, Cambridge, MA) and grown to log phase in tryptic soy broth at 37°C at 150 RPM. Cells were adjusted to concentrations of $10^8$, $10^7$, $10^6$ and $10^5$ in phosphate buffered saline (pH 7.4) and incubated with anti-*Salmonella* antibodies (Meridian Life Science, Inc., Saco, ME) at a concentration of 1:50 for one hour at 37°C to form *Salmonella* immune complexes.

**Detection of immune complexes**

Three micron polystyrene beads (Polysciences, Inc., Warrington, PA) were used as immune complex surrogates to determine if liquid crystalline chitin would distort in the presence of immune complexes. The three micron beads were mixed with chitin and loaded into 20μm polyimide (PI)-coated glass cassettes (LXD, Cleveland, OH).
Streptavidin-coated bead immune complexes were washed with sterile water at pH 2.8, and Salmonella immune complexes were washed with sterile PBS at pH 2.8. Immune complexes were centrifuged for 20 min at 17000 x g, and the supernatant was evacuated with a sterile syringe. Immune complexes were then resuspended in 4.1wt% chitin (60 μL of 4.1wt% chitin per 300 μL of streptavidin-coated bead immune complexes and 45 μL of 4.1wt% chitin per 1 mL of Salmonella immune complexes). Immune complex samples dispersed in chitin were degassed to remove air bubbles for 2 min and pulled into 20μm polyimide (PI)-coated glass cassettes (LXD, Cleveland, OH) by vacuum. These cassettes were observed under crossed polarizers using a 10x objective on an Olympus BX61 microscope (Center Valley, PA). Analysis of immune complexes and disclinations was conducted using ImageJ (National Institutes of Health, Bethesda, MD).

**Statistical analysis**

The mean number of defects ± standard deviation will be reported for each dilution and compared via one-way ANOVA. Significance will be set *a priori* at p<0.05.

**Results**

Chitin fibers were 0.34 ± 0.06 μm in length (n=10) after 3 h of sonication, as determined from TEM images (Fig. 15). Chitin fibers appeared to dry parallel to one another (Fig. 15, insert). Evaluation of chitin fibrils between crossed polarizers revealed
a chiral nematic liquid crystalline texture (Fig. 16). The chiral nematic liquid crystal, chitin, distorted in response to three micron polystyrene beads (data not shown).

While the average diameter of the streptavidin-coated beads is one micron, the average diameter of streptavidin-coated bead immune complexes was 3.6 ± 1.4 μm at a bead concentration of 10^7. The number of defects caused by streptavidin-coated bead immune complexes in the chitin liquid crystal (LC) was highest at a concentration of 10^8 and decreased in number until a concentration of 10^5 was reached. Disclinations were detectable and countable at concentrations of 10^8, 10^7, 10^6 and 10^5 (Fig. 17). The introduction of streptavidin-coated beads or anti-streptavidin antibody alone did not cause disclinations within the chiral nematic phase of chitin. The average diameter of disclination caused by the immune complexes at a bead concentration of 10^7 beads/mL was 27.4 ± 6.8 μm. There was not a statistically significant difference in the disclinations between the 10^6 and the 10^5 dilutions or between the 10^5 dilution and the bead control. There was a statistically significant difference (p < 0.05) between all other groups as determined by the Tukey-Kramer test.

*Salmonella* immune complexes caused disclinations in liquid crystalline chitin detectable at *Salmonella* concentrations of 10^7 and 10^6. The average diameter of *Salmonella* immune complexes was 6.1 ± 2.6 μm, and the average diameter of disclinations caused by immune complexes at a *Salmonella* concentration of 10^7 was 15.4 ± 2.9 μm. The number of disclinations formed from *Salmonella* at concentrations of 10^7 and 10^6 were above the baseline number of disclinations formed in the presence of bacteria alone at those concentrations (Fig. 18). The difference between the 10^7, 10^6 and
**Figure 15.** Transmission electron microscopy images of chitin fibrils. Transmission electron microscopy reveals chitin fibers with an average length of \(0.34 \pm 0.06 \, \mu m\) after 3 h of sonication. Scale bar denotes 1 \(\mu m\). (Insert) Chitin fibers appear to lie parallel to one another (arrow). Scale bar denotes 100 nm.
Figure 16. Chiral nematic arrangement of liquid crystalline chitin. Chitin fibers at a concentration of 4.1 wt% form a chiral nematic liquid crystal. Scale bar denotes 100 μm.
**LC Defects Caused by Streptavidin-Coated Bead Immune Complexes**

**Figure 17.** Number of defects introduced in the chitin liquid crystal by streptavidin-coated bead immune complexes. The number of defects decreased in number from an initial bead concentration of $10^8$ to a concentration of $10^5$. 
Figure 18. Number of defects introduced in the chitin liquid crystal by immune complexes composed of Salmonella cells. Salmonella was detectable to a concentration of $10^6$ as defects in the liquid crystalline texture of chitin.
Table 1. Sizes of streptavidin-coated bead and *Salmonella* immune complexes and the associated defects in liquid crystalline chitin.

<table>
<thead>
<tr>
<th>Initial Concentration of <em>Salmonella</em> or Beads</th>
<th>Mean Diameter of <em>Salmonella</em> Immune Complexes (μm)</th>
<th>Mean Diameter of Defects in Chitin LC Caused by <em>Salmonella</em> Immune Complexes (μm)</th>
<th>Mean Diameter of Streptavidin-Coated Bead Immune Complexes (μm)</th>
<th>Mean Diameter of Defects in Chitin LC Caused by Streptavidin-Coated Bead Immune Complexes (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^8$</td>
<td>-</td>
<td>-</td>
<td>$5.2 \pm 1.5$</td>
<td>$32.2 \pm 9.4$</td>
</tr>
<tr>
<td>$10^7$</td>
<td>$6.1 \pm 2.6$</td>
<td>$15.4 \pm 2.9$</td>
<td>$3.6 \pm 1.4$</td>
<td>$27.4 \pm 6.8$</td>
</tr>
<tr>
<td>$10^6$</td>
<td>$3.1 \pm 0.9$</td>
<td>$6.9 \pm 2.5$</td>
<td>$3.4 \pm 0.7$</td>
<td>$16.9 \pm 9.6$</td>
</tr>
</tbody>
</table>
the bacterial control groups were all found to be statistically significant \( p < 0.01 \) by the Tukey-Kramer test. At the same initial concentration of *Salmonella* cells or streptavidin-coated beads, the defects in liquid crystalline chitin caused by streptavidin-coated bead immune complexes were significantly larger than the defects caused by *Salmonella* immune complexes (Table 1).

**Discussion**

Chitin fibers aligned parallel to one another in their LC state. This is supported with TEM images of chitin fibers after drying (Fig. 15, insert). This arrangement is likely a remnant of the chiral nematic alignment of acid hydrolyzed chitin fibers. The chiral nematic arrangement in concentrated suspensions of chitin fibrils has been noted to mimic the arrangement of chitin in arthropod cuticles (Belamie *et al.* 2006). Chitin at a concentration of 4.1 wt\% was used in this study, because chitin at higher concentrations (\textit{e.g.} above 6.5 wt\%) formed a gel (Belamie *et al.* 2004). The self-assembly of increasing concentrations of chitin into a liquid crystal and subsequent fibrillar gel has been implicated as a precursor to the solid chitin cuticle in arthrods \textit{in vivo} (Belamie *et al.* 2006). The inherent biocompatibility, renewability and self-assembling characteristics of chitin suggest that it might be exploited for numerous biomedical applications (Belamie *et al.* 2006). The self-assembling properties of chitin fibers into a liquid crystal were exploited to detect *Salmonella*, in the form of *Salmonella* immune complexes.

Chitin fibers were found to be 0.34 ± 0.06 \( \mu \)m in length, in contrast to those prepared by Revol and Marchessault (0.05 \( \mu \)m to more than 0.3 \( \mu \)m in length (Revol and
Marchessault 1993)). As the liquid crystal and immune complex suspensions are drawn into cassettes, the immune complexes are attracted to the glass as the liquid crystalline chitin flows around them. This scenario is similar to a liquid crystalline based sensor developed by Guzmán et al. (2005) in which particles are immobilized to the walls of the sensor followed by the addition of liquid crystal (Guzman et al. 2005). Large defects grow as the liquid crystal flows around the immobilized particles (Guzman et al. 2005).

Liquid crystalline chitin has been reported to form cholesteric envelopes around large foreign particles, such as dust particles (Murray and Neville 1998). In this report, immune complexes were introduced into liquid crystalline chitin as the foreign particles. Increasing immune complexes correlated with increasing defects in the parallel alignment of the chiral nematic chitin. Immune complexes (both streptavidin-coated bead and Salmonella based) caused defects in liquid crystalline chitin that were detectable to an initial antigen concentration of \(10^6\). The infectious dose for Salmonella has been reported to be between \(10^4\) and \(10^7\) (Leonard et al. 2003). The chiral nematic chitin was able to detect immune complexes when \(10^6\) or greater Salmonella are allowed to form immune complexes of \(6.1 \pm 2.6 \mu m\), this is within the detection range of the infectious dose for the bacterium.

At the same concentrations, the disclinations caused by streptavidin-coated bead immune complexes were significantly larger than those caused by Salmonella immune complexes (Table 1). The negatively charged glass (Deng and Mao 2003) of the cassettes used in this study likely attract the positively charged streptavidin-coated bead immune complexes (Fig. 19A and B). The sensitivity of this sensor could likely be
improved through the immobilization of immune complexes to the surface of the sensor itself or through the use of antibodies with a greater specificity (Helfinstine et al. 2006). Future research will focus upon improving the sensitivity of the chitin-based sensor through antibody manipulation.
**Figure 19.** Immune complexes at the front and back of glass cassettes. The number of streptavidin-coated bead immune complexes (at an initial bead concentration of $10^7$) is greater at the loading end (A) than at the non-loading end (B) of glass cassettes. Both scale bars are equal to 10μm.
References


CHAPTER V

Application of Biopolymers of Varying Flexibility for Biosensing

Abstract

The ability of liquid crystals to distort when large contaminating objects are introduced has been reported for the liquid crystalline states of disodium chromoglycate and chitin. Disodium chromoglycate and chitin are both structurally rigid, while Salmonella flagella and fd bacteriophages are flexible. The liquid crystalline textures of Salmonella flagella and fd bacteriophages were hypothesized to have a muted ability to distort in the presence of large contaminating objects (e.g. immune complexes). Immune complexes consisting of streptavidin-coated beads complexed together by anti-streptavidin antibodies were used to characterize the distortability of liquid crystals composed of fd bacteriophage and of straight flagella isolated from Salmonella enterica serovar Typhimurium SJW 1665. Streptavidin-coated bead immune complexes were introduced into the liquid crystalline textures of SJW 1665 flagella and fd bacteriophage and observed with crossed polarized light microscopy. While the introduction of immune complexes into liquid crystalline disodium chromoglycate and chitin resulted in defects significantly larger than the immune complexes themselves, the introduction of immune complexes into liquid crystalline flagella and fd bacteriophage caused defects not
significantly larger in diameter than the immune complexes. These data can be used to predict the ability of liquid crystals to distort in the presence of immune complexes based on the flexibility of the component molecules of the liquid crystal itself. A liquid crystal that significantly distorts when immune complexes are introduced could be used in a biosensing device where immune complexes are composed of bacterial cells.

**Introduction**

Biosensors are devices capable of providing quantitative data using a biological receptor and a transducer (Thévenot *et al.* 2001). A biosensor could be a device that detects a bacterium via antibody (biological receptor) and transduces that data as a signal. In other words, biosensors utilize a biological component (i.e. nucleic acid, antibody, or cell) for measurement (Harsányi 2001; Newman *et al.* 2001). Possible applications for biosensors include measuring blood glucose (Newman *et al.* 2001) and detecting enzymatic reactions, protein-binding events and bacteria (Woltman *et al.* 2007).

A biosensor has been reported that uses crossed polarized light microscopy and liquid crystals (LCs) to detect bacteria in “real-time” (Woolverton, Niehaus *et al.* 2001; Helfinstine, Lavrentovich *et al.* 2006). This biosensor utilizes antibodies as receptors to form immune complexes with the microorganism of interest. The growing immune complexes then disrupt the alignment of liquid crystals. As a result, the defects, or disclinations, in the liquid crystalline texture can be detected using crossed polarized light microscopy. While immune complexes occupy enough volume to cause notable defects
in liquid crystalline textures, individual antibodies and antigens do not (Woolverton et al. 2001; Helfinstine et al. 2006).

Another LC-based biosensor uses thermotropic liquid crystals (Woltman et al. 2007). The thermotropic liquid crystal used in this biosensing device is 4-\(n\)-pentyl-4’-cyanobiphenyl (5CB) (Gupta et al. 1998). This sensor reports macroscopic disruption of liquid crystalline order caused by microscopic binding between antibodies and antigens at the sensor surface (Gupta et al. 1998). However, liquid crystals, such as those used in liquid crystalline displays and sensors (e.g. 5CB), can be toxic to bacteria (Woolverton et al. 2005).

Toxicity, depending upon the mechanism, may alter or decrease the integrity of an antigen preventing antibody recognition (Woolverton et al. 2005). The toxicity of the liquid crystal must therefore be considered when antibody-antigen interactions are a sensor component. Thermotropic liquid crystals, such as 5CB and surfactant-based lyotropic liquid crystals (e.g. cetylpyridinium chloride (CPCl) and cesium pentadecafluorooctanoate (CsPFO)) are toxic to certain forms of bacteria (Woolverton et al. 2005). Woolverton et al. (2005) found that 5CB inhibited the germination and growth of *Bacillus atrophaeus* spores, while CPCl and CsPFO inhibited the growth of *S. aureus* and *E. coli* (Woolverton et al. 2005). CsPFO inhibited the germination and growth of *Bacillus atrophaeus* spores, while CPCl did not (Woolverton et al. 2005). Thermotropic liquid crystals also require heating to exhibit their liquid crystalline characteristics. This heating process, dependent upon the temperature required to elicit the formation of a liquid crystalline state, may damage or alter antigen or antibody integrity. This proved a
problem with one sensor designed for DNA detection, most likely damaging the sample DNA (Hoogboom et al. 2003).

The liquid crystal-based biosensor that uses lyotropic LCs for the detection of bacteria depends on solvent changes (e.g. changes in solvent content, pH or ionic strength) to elicit LC properties instead of depending on changes in temperature to elicit those properties (e.g. thermotropic LCs). This sensor utilizes free floating antibody receptors instead of surface-bound receptors to capture antigens within the liquid crystal bulk thus allowing the immune complexes to expand in three dimensions for enhanced detection (Woolverton et al. 2001; Shiyanovskii et al. 2005; Shiyanovskii et al. 2005; Helfinstine et al. 2006).

Liquid crystal based-biosensors can be improved based on which liquid crystal is used to transduce the optical signal exhibited in the presence of immune complexes. Lyotropic LCs lacking, or with limited, toxicity are preferred. Some polysaccharides, proteins, and viruses offer this advantage (Livolant and Bouligand 1978; Gathercole et al. 1989; Dong et al. 2004; Eglin et al. 2005; Lettinga et al. 2005; Barry et al. 2006; Corrigan et al. 2006).

This study aims to compare the sensing capability of various biopolymers of different lengths and flexibilities so as to access the distortability of the respective biopolymer based LC when immune complexes are introduced. The prototypical biopolymers: (1) chitin (a structural polysaccharide from arthropod cuticles), (2) flagella (a self-assembling, helical rod composed of the flagellin protein from Salmonella enterica serovar Typhimurium SJW 1665) and (3) intact fd bacteriophages are presented
to evaluate structure-function relationships. Chitin, flagella and \textit{fd} bacteriophages are all rod-shaped (calamitic) biopolymer lyotropic LCs. It is the aim of this report to compare the distortability of liquid crystalline chitin to flagella and bacteriophage LCs in the presence of immune complexes. One micron streptavidin-coated polystyrene beads, at a concentration of $10^7$, were incubated with anti-streptavidin antibodies to form immune complexes which were introduced into the biopolymer LCs. The average diameter of immune complexes was then compared to the average size diameter of defects in the LC textures.

\textbf{Methods}

\textbf{Preparation of biopolymer liquid crystals}

Chitin flakes (Sigma-Aldrich Corporation, St. Louis, MO) were processed as described in chapter 4 and flagella were sheared from \textit{Salmonella enterica} serovar Typhimurium SJW 1665 (Rowland Institute at Harvard University, Cambridge, MA) and purified following the methodology in chapter 2.

\textit{fd} bacteriophages were obtained from Prof. George Smith (University of Missouri, Columbia, MO). \textit{fd} bacteriophages were flash frozen and lyophilized using a Model 75040 Labconco freeze dryer (Labconco Corporation, Kansas City, MO). Phages were adjusted to a concentration of 20 mg/mL in distilled, deionized water with 10 mM Tris-HCl (pH 8.0) (Dogic and Fraden 1997).
Preparation of biopolymer LC and immune complex mixtures

Immune complexes of one micron streptavidin-coated beads were used to make immune complexes as described in chapter 4. Streptavidin-coated bead immune complexes were introduced into liquid crystalline chitin, as described in chapter 4. Streptavidin-coated bead immune complexes were also mixed with flagella purified from SJW 1665. Five microliters of flagella (~77 mg/mL) was mixed with five microliters of streptavidin-coated bead immune complexes at an original bead concentration of $10^7$. The LC and immune complex mixture was loaded into 20 μm capillary tubes and observed with crossed-polarized microscopy to observe disclinations.

One hundred microliters of streptavidin-coated bead immune complexes at an initial bead concentration of $10^7$ was centrifuged at 17000 x g for 20 minutes. Twenty microliters of $fd$ bacteriophage, at a concentration of 20 mg/mL, was used to resuspend immune complexes. The LC and immune complex mixture was then loaded into 100μm capillary tubes and observed under crossed polarizers.

The mean diameter of immune complexes was compared to the mean diameter of the disclinations caused in the respective LCs by the immune complexes. Significance between the mean diameter of immune complexes and the mean diameter of the associated defects was determined using a t-test. Significance was set a priori at $p < 0.05$. Comparison between the size of immune complexes and the associated LC defects allowed for the determination of which LC is more sensitive for the detection of immune complexes: rigid rods (chitin), semi-rigid rods ($fd$ bacteriophage) or flexible rods (SJW 1665 flagella).
Results

Chitin fibers, SJW 1665 flagella and fd bacteriophages all have liquid crystalline properties. Chitin fibers have chiral nematic properties at a concentration of 4.1 wt% (Fig. 20A). As reported in chapter 4, the mean diameter of streptavidin-coated bead immune complexes was 3.6 ± 1.4 μm at an initial bead concentration of 10^7 beads/mL (Table 2). The mean diameter of the defects caused by these immune complexes in liquid crystalline chitin was 27.4 ± 6.8 μm (Table 2 and Fig. 20B). The difference in the diameters of the immune complexes and the defects in liquid crystalline chitin caused by the immune complexes was statistically significant (p<0.05). SJW 1665 flagella and fd bacteriophages have nematic and chiral nematic arrangements above 16 mg/mL in ddH2O and 10 mg/mL in 10 mM Tris-HCl (pH 8.0), respectively.

Streptavidin-coated bead based immune complexes, with a mean diameter of 4.81 ± 1.4 μm, were used to determine if SJW 1665 flagella and fd bacteriophage liquid crystals would distort. When immune complexes were introduced into the flagella liquid crystal, the defects in the LC were no larger than the immune complexes themselves (Table 2 and Fig. 21A). Immune complexes of 4.81 ± 1.4 μm resulted in distortions in the chiral nematic phase (Fig. 21B) of fd bacteriophage with a mean diameter of 4.92 ± 1.4 μm (Table 2 and Fig. 21C and D). The difference between the mean diameter of immune complexes and the mean diameter of defects introduced into liquid crystalline texture of fd bacteriophages by the immune complexes was not statistically significant, as determined by a t-test (Fig. 22).
Table 2. Mean diameter of streptavidin-coated bead immune complexes at an initial bead concentration of $10^7$ beads/mL and the associated mean diameter of defects in biopolymer liquid crystals.

<table>
<thead>
<tr>
<th>Liquid crystal</th>
<th>DSCG (Shiyanovskii et al. 2005)</th>
<th>Chitin</th>
<th><em>fdl</em> bacteriophage</th>
<th>Flagella from SJW 1665</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter of streptavidin-coated bead immune complexes (μm)</td>
<td>2μm</td>
<td>3.6 ± 1.4μm</td>
<td>4.81 ± 1.4μm</td>
<td>4.81 ± 1.4μm</td>
</tr>
<tr>
<td>Diameter of liquid crystal defects (μm)</td>
<td>5μm</td>
<td>27.4 ± 6.8μm</td>
<td>4.92 ± 1.4μm</td>
<td>4.81 ± 1.4μm</td>
</tr>
</tbody>
</table>
Figure 20. Liquid crystalline texture of chitin and defects in that texture due to the introduction of immune complexes. (A) Chitin forms a liquid crystal above at or above 4.1 wt%. Scale bar denotes 100μm. (B) Immune complexes with a mean diameter of 3.6 ± 1.4μm caused defects in the liquid crystalline texture of chitin with a mean diameter of 27.4 ± 6.8μm. Scale bar denotes 50μm.
Figure 21. Immune complex-induced defects in the liquid crystalline textures of flagella and *fd* bacteriophage. (A) Liquid crystalline flagella does not amplify the presence of immune complexes in the form of defects in the liquid crystalline texture. Scale bar is equal to 10 μm. (B) Liquid crystalline phase of *fd* bacteriophage at 20 mg/mL in 10 mM Tris-HCl. Scale bar is equal to 100 μm. (C) and (D) The introduction of immune complexes into liquid crystalline *fd* bacteriophage results in defects in the liquid crystalline texture that are not significantly different in diameter from the immune complexes themselves. (C) Scale bar is equal to 100 μm. (D) Scale bar is equal to 10 μm.
Figure 22. Comparison between the mean diameter of immune complexes and the associated defects caused by the immune complexes in the liquid crystalline textures of chitin and \textit{fd} bacteriophages.
Discussion

Liquid crystal-based biosensing devices use antibodies as receptors of antigens (e.g. bacterial cells) and convert that binding event into defects in the liquid crystal itself (Shiyanovskii et al. 2005). The binding of antibody to antigen forms immune complexes which are intended to induce the formation of liquid crystalline defects larger than the immune complex itself. A functional LC-based biosensor should therefore result in immune complex-induced defects that are amplified into liquid crystalline defects that are large enough to be detectable between crossed polarizers at low magnifications.

Shiyanovskii et al. (2005) used a rigid, plank-like liquid crystal (disodium chromoglycate (DSCG)) to detect immune complexes consisting of multiple 0.56 μm streptavidin-coated beads (initial bead concentration between $10^6$ - $10^7$ beads per mL). Bead immune complexes larger than 2 μm resulted in defects in liquid crystalline DSCG larger than the immune complexes themselves (Shiyanovskii et al. 2005). Similar to DSCG, chitin is also rigid. Immune complexes introduced into liquid crystalline chitin resulted in defects in liquid crystalline chitin substantially larger than the immune complexes themselves (Table 2). Flagella and $fd$ bacteriophages, unlike DSCG and chitin, possess flexible structures. The introduction of immune complexes into the liquid crystalline phase of flagella does not result in defects any larger than the immune complexes themselves, while the introduction of immune complexes into the liquid crystalline phase of $fd$ bacteriophage results in defects, but the defects are not significantly different in diameter than the immune complexes themselves. The low
deformability of liquid crystals consisting of flexible rods (e.g. SJW flagella and *fd* bacteriophages) is likely due to the inherent flexibility of the rods muting the magnitude of the immune complex-induced distortions.

Biopolymer liquid crystals have never been used in liquid crystalline based biosensors. Biopolymers, such as proteins, are novel materials for sensing that can be manipulated. The nucleotide sequence of a gene can be altered resulting in a protein with an altered amino acid sequence and different properties from those seen *in vivo* (McGrath and Kaplan 1997). For example, changes in the amino acid sequence of subtilisin (a microbial protease) has increased the protein’s chemical stability to oxidation (Wells *et al.* 1987; McGrath and Kaplan 1997). Subtilisin can now tolerate the oxidative environment of detergents in hot water and can be used as an additive in laundry detergent to remove protein stains from clothes (McGrath and Kaplan 1997). In the same fashion, cysteine residues were added to flagellin from *E. coli* to construct disulfide-linked flagella nanotubules (Kumara *et al.* 2006). The structural properties of intact viruses can be altered, as well. *fd* bacteriophage can be sterically stabilized and the diameter of the viruses can be altered through the covalent attachment of poly(ethylene)glycol (PEG) to the surface of the virus (Dogic and Fraden 2001; Dogic and Fraden 2006). Biopolymer liquid crystals are therefore tunable and can be manipulated to change structural properties. In addition to manipulating antibodies to increase the sensitivity of liquid crystal-based biosensors, as suggested in chapter 4, the liquid crystals themselves could be manipulated to increase sensitivity. Future research
will center upon increasing sensitivity of the biosensor through the use of long, rigid rods (e.g. tobacco mosaic virus).
References


CHAPTER VI

General Conclusions

The chemical and physical properties of monomers impart functionality to the biopolymers they create (e.g. increased hydrogen bonding imparts tensile strength in silk). Some biopolymers, such as g8p molecules from the fd bacteriophage, self-assemble into structures with functions based on their arrangement with respect to one another. For example, g8p molecules are stored temporarily in the inner membrane (due to its amphipathic nature) of Escherichia coli cells during the process of fd bacteriophage viral coat assembly. As fd bacteriophage exit E. coli cells, g8p molecules self-assemble through hydrophobic interactions to form a viral coat that surrounds and protects viral DNA. The viral coat of fd bacteriophage self-assembles in a way so as to minimize energy expenditure, while creating an impenetrable viral capsid. If biopolymers self-assemble with one another along a common direction (aka, the director), their arrangement can be predicted by observing the liquid crystalline textures that are formed with cross-polarized microscopy.

The overarching aim of the preceding studies was to fully elucidate the liquid crystalline properties of relatively uninvestigated (Salmonella flagella) and previously uninvestigated (g8p) biopolymers, and to demonstrate the usefulness of biopolymers in
liquid crystal-based biosensing devices. These aims are defined and a general summary for each is stated below.

- Hypothesis 1: Bacterial flagella form varying liquid crystalline phases based on their phenotype and these phenotypic changes result in different liquid crystalline textures.

Chapter 2 of this dissertation characterizes the liquid crystalline properties of flagella produced by three strains of *Salmonella enterica* serovar Typhimurium. The three strains studied were *S.* Typhimurium SJW 1103, which produces left-handed curly flagella, *S.* Typhimurium SJW 1665, which produces straight flagella and *S.* Typhimurium SJW 2869, which produces right-handed curly flagella. Infrared spectroscopy of flagellin from SJW 1103, SJW 1665, and SJW 2869 indicated that SJW 1665 has less α-helical character than either SJW 1103 or SJW 2869, while SJW 2869 appears to have less α-helical character than SJW 1103. Differences in the secondary structure of flagellin result in flagella filaments with three different phenotypes (*e.g.* left-handed curly, straight and right-handed curly). Flagella from SJW 1103 and SJW 2869 formed liquid crystalline phases with chiral nematic textures above 24 mg/mL and 27 mg/mL, respectively. SJW 1665 flagella filaments formed a nematic liquid crystal above 16 mg/mL. Chiral nematic textures (SJW 1103 and SJW 2869 flagella) suggest that flagella filaments lie at an angle approaching parallel to one another, while nematic textures (SJW 1665 flagella) suggest that flagella filament s lie parallel with one another.
The concentrations we found at which the biphasic to liquid crystal state transition occurs for flagella filaments (SJW 1003, SJW 1665 and SJW 2869) are lower than those previously reported (Barry et al. 2006). This may be attributed to purity of the flagella used in this study compared with those used by Barry et al. (2006). This study used weak anion exchange chromatography to purify flagella, while Barry et al. (2006) did not. The presence of protein contaminants in addition to flagellin could result in errors when determining the concentration of flagellin at which liquid crystal phase transitions occur. While the majority of flagella used by Barry et al. (2006) were approximately 2 μm in length, the flagella used in this report were between 8.2 ± 4.3 μm and 11.3 ± 3.5 μm in length, on average. The flagella samples used by Barry et al. therefore only represent short fragments of the flagella filament itself. In the case of flagella from SJW 1103, 2 μm flagella filaments would be shorter than one period of the intact filament helix, which is 2.4 μm. Differences in the liquid crystal textures observed in this study and Barry et al. can be attributed to differences in the lengths of flagella filaments. Chapter 2 also documented that flagella from SJW 1103 and SJW 2869 form chiral nematic LCs, as demonstrated by fingerprint textures. The chiral nematic textures of SJW 1103 and SJW 2869 had not been previously reported by Barry et al. (2006) or others.

- Hypothesis 2: The major coat protein of fd bacteriophage, g8p, has liquid crystalline properties due to its bent-core structure.
Chapter 3 reports the optical characteristics of the major coat protein (g8p) of \textit{fil} bacteriophage. In an aqueous environment, g8p (a bent-core protein) forms filaments with a smectic structure (Fig. 23A). The smectic texture of g8p filaments suggests that g8p molecules arrange into layers where there is alignment of g8p molecules with respect to one another within layers. Scanning electron microscopy confirmed that these fibers are formed from layers of g8p that wrap around a central axis (Fig. 23B). \textit{In vivo}, g8p uses viral DNA as a template about which g8p molecules wrap forming a unilamellar structure. In the absence of the viral DNA, g8p molecules form multi-lamellar fibers. While bent-core chemicals have been reported to form helical fibers (Jakli \textit{et al.} 2000) and films with a smectic structure (Bailey \textit{et al.} ; Jákli \textit{et al.} 2003), the properties of bent-core proteins have only been reported for mussel byssal thread proteins (Knight and Feng 1994; Knupp \textit{et al.} 1998; Hassenkam \textit{et al.} 2004). Chapter 3 reports the previously unknown liquid crystal characteristics of g8p, only the second bent-core protein to be characterized as a liquid crystal.

- Hypothesis 3: \textit{Salmonella}, in the form of \textit{Salmonella} immune complexes, will be detected as defects in liquid crystalline chitin.

In chapter 4, the chiral nematic liquid crystal phase of chitin was exploited to test the hypothesis that liquid crystalline chitin can be used as a sensor to detect \textit{Salmonella}. (Revol and Marchessault 1993). The parallel alignment of fibers within layers of chiral nematic chitin was hypothesized to present an ordered matrix for immune complex
**Figure 23.** Fibers composed of g8p. (A) Fibers composed of g8p are birefringent when observed with crossed polarized microscopy. (B) Scanning electron microscopy demonstrates that g8p fibers form from layers of g8p that roll on top of one another. Scale bars are equal to 20μm.
detection. This study marks the first time that the biopolymer chitin, has been used as a LC to detect the presence of bacterial cells. Both immune complexes consisting of streptavidin-coated beads and of *Salmonella* bacterial cells, respectively, were detectable at a starting concentration $10^6$ particles/mL. This concentration is within the infectious dose range for *Salmonella* which has been reported to be between $10^4$ and $10^7$ cells/mL (Leonard *et al.* 2003). The mean diameter of the defects induced in the liquid crystalline texture of chitin by bead or bacterial-based immune complexes was significantly larger than the immune complexes themselves. Liquid crystalline chitin therefore amplified the presence of immune complexes as defects in liquid crystalline chitin when the elastic force of liquid crystalline chitin overcame the anchoring force between the LC and the immune complexes.

- Hypothesis 4: The amount of distortion caused by the introduction of immune complexes into a LC will decrease as the flexibility of the liquid crystal biopolymer increases.

The sensing potential of the liquid crystalline textures of SJW 1665 flagella and of *fd* bacteriophages was investigated in chapter 5 (Table 2). The sensing potential of flagellar and phage based LCs were then compared to the sensing capability of liquid crystalline chitin (Table 3). While the introduction of immune complexes into liquid crystalline chitin results in defects in the LC significantly larger in diameter than the
**Table 3.** Comparison of liquid crystals for sensing.

<table>
<thead>
<tr>
<th>Liquid crystal</th>
<th>DSCG (Shiyanovskii et al. 2005)</th>
<th>Chitin fibers</th>
<th>fd bacteriophages</th>
<th>Flagella from SJW 1665</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid crystal phase studied</td>
<td>Nematic</td>
<td>Chiral nematic</td>
<td>Chiral nematic</td>
<td>Nematic</td>
</tr>
<tr>
<td>Flexibility of substance</td>
<td>Rigid</td>
<td>Rigid</td>
<td>Semi-flexible</td>
<td>Flexible</td>
</tr>
<tr>
<td>Length of substance</td>
<td>-</td>
<td>0.34 ± 0.06μm</td>
<td>1μm</td>
<td>8.2 ± 4.3μm</td>
</tr>
<tr>
<td>Diameter of streptavidin-coated bead immune complexes (μm)</td>
<td>2μm</td>
<td>3.6 ± 1.4μm</td>
<td>4.81 ± 1.4μm</td>
<td>4.81 ± 1.4μm</td>
</tr>
<tr>
<td>Diameter of liquid crystal defects (μm)</td>
<td>5μm</td>
<td>27.4 ± 6.8μm</td>
<td>4.92 ± 1.4μm</td>
<td>4.81 ± 1.4μm</td>
</tr>
</tbody>
</table>
immune complexes themselves, the introduction of immune complexes into the liquid crystalline matrix of flagella and \textit{fd} bacteriophage does not result in defects significantly different in size from the immune complexes themselves (Table 3). The decreased potential of liquid crystalline flagella and \textit{fd} bacteriophage to detect immune complexes, as compared to the sensing potential of liquid crystalline chitin, appears to be attributable to the increased flexibility of the individual flagella or \textit{fd} bacteriophage fibers, as compared to the rigid nature of chitin fibers. Future studies will utilize more rigid biopolymers or biological structures (\textit{e.g.} tobacco mosaic virus) to detect immune complexes.

The optical properties of materials observed with cross-polarized microscopy can be used to infer the arrangement of the materials in self-assembling biological systems as well to assess their potential use as sensors. As another application for biopolymers, in addition to sensing, future research will focus on the effects of placing biopolymers, such as \textit{Salmonella} flagella, and whole viruses, such as \textit{fd} bacteriophages in an electric field. Electric fields can be used to rotate individual molecules of the liquid crystalline textures formed by molecular alignment, if the liquid crystalline molecules themselves are charged. However, the effect of an external electric field on a liquid crystal is dependent upon the angle at which the field is applied to the director (Collings 2002). This characteristic is similar to the way in which light (an electromagnetic wave) passes through a liquid crystalline material.

Molecules must possess a charge to be affected by an electric field. Most liquid crystals are electrically neutral except for some with bonds that result in the distribution
of slightly positive and slightly negative charges to different regions of the molecule (Collings 2002). Molecules with this charge separation are referred to as permanent electric dipoles (Collings 2002). Some liquid crystal molecules do not possess the charge separation of permanent electric dipoles. Charge separation can be induced in these molecules through the addition of an electric field. Since atoms are composed of a positively charged nucleus and negatively charged electrons, an electric field can cause a slight displacement of positive and negative charges resulting in an induced electric dipole (Collings 2002).

Many biological molecules such as nucleic acids, phospholipids, carbohydrates and proteins have an electrical charge. Nucleic acids have an overall negative charge due to the phosphate backbone. The charges of phospholipids vary depending on the head group. The major phospholipids (lipids with phosphorous-containing head groups and two hydrocarbon tails) in mammalian cells are phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine and sphingomyelin. All of these phospholipids except phosphatidylserine have one positive and one negative charge at physiological pH making them electrically neutral. Phosphatidylserine is electrically negative. Many carbohydrates making up the extracellular matrix of animal cells and the extracellular polymeric substance (EPS) of bacterial cells possess negative charges (Sutherland 2001). Proteins present a unique situation in which the charge is contingent upon the pH of the solvent. The pH at which the protein is electrically neutral is referred to as the isoelectric point of the protein. Proteins are positively charged below the isoelectric point and
negatively charged above the isoelectric point. The charge of proteins can therefore be easily manipulated through pH adjustments.

In the absence of an electric field, charged molecules in a liquid crystal possess orientational order, however, there is no alignment of the electric dipoles (Collings 2002). When an electric field is applied, the molecules rotate so that the positive and negative sides align with the electric field (Collings 2002). The orientation of the molecules is no greater in the presence of an electric field than without the electric field (Collings 2002). The effects of electric field on liquid crystalline textures can be observed with specialized microscopy techniques.

Flagella and fd bacteriophages are both charged. If flagella and phage can be made to rotate in an electric field, they offer tunable biological alternatives to the synthetic chemicals used in display devices.

Thus, this dissertation reports several biomaterials that have physical and chemical properties suggesting their potential use as alternatives for traditional LCs. Of note is the fact that these biopolymers self-assemble, are a renewable resource, are “green,” and are biodegradable. Their potential use as replacement LC materials suggests many possibilities.
References


