Abstract
We have been investigating collagen-like model oligopeptides as molecular bases for complex ordered biomimetic materials. The collagen-like molecules incorporate aspects of native collagen sequence and secondary structure. Designed modifications to native primary and secondary structure have been incorporated to control the nanostructure and microstructure of the collagen-like materials produced. We find that the collagen-like molecules form a number of “lyotropic rod” liquid crystalline phases, which because of their strong temperature dependence in the liquid state can also be viewed as “solvent intercalated thermotropic” liquid crystals. The liquid crystalline phases formed by the molecules can be “captured” in the solid state by drying off solvent, resulting in solid nanopatterned (chemically and physically) thermally stable (to >100°C) materials. Designed sequences which stabilize smectic phases have allowed a variety of nanoscale multilayered biopolymeric materials to be developed. Preliminary investigations suggest that chemical patterns running perpendicular to the smectic layer plane can be functionalized and used to localize a variety of organic, inorganic, and organometallic moieties in very simple multilayered nanocomposites. The phase behavior of collagen-like oligopeptide materials is described, emphasizing the correlation between mesophase, molecular orientation, and chemical patterning at the microscale and nanoscale. In many cases, the textures observed for smectic and hexatic phase collagens are remarkably similar to the complex (and not fully understood) “helicoids” observed in biological collagen-based tissues. Comparisons between biological morphologies and collagen model liquid crystalline (and solidified materials) textures may help us understand the molecular features which impart order and function to the extracellular matrix and to collagen-based mineralized tissues. Initial studies have utilized synthetic collagen-like peptides while future work will also focus on similar sequences generated via genetic engineering methods.

Introduction

Hemicoids in Biology
Cholesteric phases can be obtained from highly simplified model collagen molecules and solidified to produce model “hemicoid” materials with their cholesteric orientation intact. An unexpected, but not surprising, feature of many of these model collagen molecules is the large number of smectic phases observed as the drying temperature is lowered. The collagen model molecules are very well behaved thermotropic (temperature dependent) liquid crystal mesogens in concentrated solution, generating a series of cholesteric, smectic A*, smectic C* and hexatic and crystalline phases as their drying temperature is lowered. The dried materials resulting from these phases have optical textures and surface morphologies which resemble a number of collagen-based tissues and other biological structures, many of which are difficult to rationalize in terms of simple cholesteric orientation. In a smectic liquid crystal, the molecules (or other rigid rods) are aligned and can have a helicoidal pattern of orientations. Smectic phases also

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feature molecules (or other rigid rods) arranged in regular layers, rather analogous to membranes. Thus smectics have some positional order, whereas the positions of molecules (rigid rods) in a cholesteric are uncorrelated (see for example, Fraden)\textsuperscript{2-6}. A smectic helicoid will form a material with a sinusoidal variation in orientation and a pattern of chemically distinct regions, defined by the ordered positions of groups or substructures on the rigid rod molecule.

A number of workers have investigated helicoidal arrangements of molecules, fibers, crystals, and fibrils in tissues and in structural biomaterials.\textsuperscript{7-18} The arrangement of basic units (molecular or supermolecular) in these biological helicoids is typically compared to a cholesteric liquid crystalline phase with two caveats: (1) additional features and order are apparent in the biological helicoids that are not present in cholesteric liquid crystals, and (2) the helicoids are solid state materials and not liquid crystals.

Many studies on helicoids have focused on collagen molecules and collagenous tissues. Early work in this area led to the identification of a number of collagenous tissues and collagen-based biological structures which possess geometrical features that can be considered helicoidal. In some cases microscope images suggesting complex arrangements of objects could be more simply and completely accounted for as projection through a helicoid or as helicoidal fracture surface. Reconstruction of these images using helicoidal models has provided new insights into the structure and geometry of tissues and in some cases offered an empirically demonstrated revised model for the ultrastructures of common tissues such as bone.

More recently, several studies have associated biological helicoids with liquid crystallinity – in the liquid state – of precursor molecules. Girauard Guille and coworkers have demonstrated liquid crystalline cholesteric phases in vitro for collagen fibers and triple helical molecules through a systematic series of studies\textsuperscript{12,14-16,19} which have done much to advance the idea of liquid crystalline precursor phases for helicoids. In “Biology of Fibrous Composites”, Neville has recently compiled an “atlas” of helicoids.\textsuperscript{20} He advances the possibility of blue phases (crisscrossed cholesteric cylinders) as additional helicoidal precursors.

The possibility of helicoids from more highly oriented precursor phases, such as smectic and hexatic phases, has been largely ignored. Smectic phases are more varied and complex than simple nematics and cholesterics and many of the general compilations and treatises on liquid crystals do not cover the smectics fully. Furthermore much of the published characterization of smectic phases relies on a variety of methods to characterize order and orientation. With a few notable exceptions, the literature on helicoids has been dominated by polarizing optical microscopy and electron microscopy and tomography studies. Thus comparisons between the biology oriented studies and the physical characterization studies available for smectics can be difficult. There is thus a need not only to establish smectic phases as likely helicoid precursors, but to also provide data that bridge the two fields and allow comparisons between helicoids (biology) and smectic phases (physics) to be made.

In the present work, a set of polarizing optical micrographs showing typical optical textures and defect patterns for biological smectics, in this case model collagens, has been combined with more quantitative and direct corroborating data on the same samples. These results provide evidence that biological rigid rod molecules can form chiral smectic liquid crystalline phases. A role for smectic phases in collagenous tissue formation is suggested. In addition, a set of optical textures from identified biological smectic
phases is provided by these studies. The model collagens studied thus provide an ideal basis to determine what a smectic-based helicoid would “look like” in terms of typical defect textures, surface morphology, fracture surfaces, anchoring and orientation effects, and overall optical texture (crossed polarizers).

Collagen-like Peptides – Sequence Designs
Repetitive peptides and polypeptides have been studied previously, by a number of workers, to elucidate very general sequence dependencies of collagen structure. Typically a simplified hexapeptide or tripeptide motif is used to build up a longer collagen-like molecule through repetition. Examples include “Gly-Pro-Pro” used to make (Gly-Pro-Pro)$_{12}$, and “Gly-Ala-Pro-Gly-Pro-Ala” as the basis for a polymer, poly(Gly-Ala-Pro-Gly-Pro-Ala). Early work by Scatturin, Traub, and others established minimum conditions for triple helix formation and stability. These include a high proportion of imino acid residues and the presence of glycine at every third position in the sequence. The second point has recently been confirmed in single crystal studies on short collagen-like peptides. Early workers also established sequence pattern – triple helical conformation – thermal stability trends. Recent work by a number of groups, most notable those led by Goodman and by Fields, have established the thermal and concentration dependent stability for triple helices in solution for a large number of sequence patterns and lengths. A large quantity of data now exists, and the helix melting point for simple repetitive collagens can in many cases be predicted from these data. Our models utilized the wealth of available sequence-stability data in sequence design. The hexapeptide motifs (Gly-Ala-Pro-Gly-Pro-Ala), (Gly-Val-Pro-Gly-Pro-Pro), (Gly-Ala-Pro-Gly-Pro-Pro), (Gly-Ser-Pro-Gly-Pro-Pro), and (Gly-Pro-Ala-Gly-Pro-Pro) were chosen to provide a range of comparable sequences which would allow sequence dependent effects on morphology to be identified.

Experimental
Peptides containing six repeats of each hexapeptide motif were synthesized at the Tufts Protein Core facility at the Sackler School of Medicine. All of the motifs were synthesized with glutamic acid ends as solubilizing blocks. The glutamic acid solubilized oligopeptides which were synthesized thus had sequences:

- $E_5(GAPGPA)_6E_5$ Peptide “CPE1”
- $E_5(GAPGPP)_6E_5$ Peptide “CPE2”
- $E_5(GVPGPP)_6E_5$ Peptide “CPE3”
- $E_5(GSPGPP)_6E_5$ Peptide “CPE4”
- $E_5(GPAGPP)_6E_5$ Peptide “CPE6”

In addition variants of two of the collagen forming sequence motifs were synthesized with polar asparagine solubilizing ends instead of glutamic acid. The polar interaction between asparagine residues is expected to be attractive, and to encourage triple helix formation. The glutamic acid ends can be repulsive (under conditions where they are charged) or attractive depending on their degree of ionization. The two additional oligopeptides synthesized and studied were:

- $N_5(GAPGPP)_6N_5$ Peptide “CPN2”
- $N_5(GPAGPP)_6N_5$ Peptide “CPN6”

The synthetic oligopeptides were found to be soluble to concentrations in excess of 200 mg/ml in pure water. The oligopeptides are also ethanol soluble to concentrations up to 100 mg/ml, depending on the individual peptide sequence. Providing temperature, solvent, drying geometry and substrate are controlled, the phase behavior observed is insensitive to concentration changes for concentrations from 100–200 mg/ml. At very low starting concentrations of oligopeptide, different optical textures are observed. The solid dried
films from low concentration solutions are extremely thin and surface effects may dominate the textures formed. Strong surface orienting effects have been observed in some of our previous work on collagens. Very high starting concentrations also produce unusual optical textures. Solution concentrations of 100-200 mg/ml in water and ethanol were used to prepare solid films. Droplets (10 µl) were cast onto glass slides and either dried uncovered in a dust free location, or covered with a coverslip (but not sealed). Samples were dried at temperatures in the 0-5°C range, focusing on temperatures bracketing 0.7, 1, 2, and 3°C where changes in morphology have been observed to occur.

Dried films were used for the majority of characterization studies. However a few experiments demonstrating the liquid crystalline nature of the films were also prepared. In these cases films were prepared with coverslips as above. Optical textures were observed in the liquid state, and then part of the coverslip was broken off by gentle lifting. The effect on the optical texture was observed. Comparison between the deformed liquid (coverslip lifted) and the areas still intact under the remaining piece of coverslip could be made. Samples were also allowed to dry around the edges. In this situation, when part of the coverslip was fractured/lifted off, intact and stressed contiguous regions in the wet and dry states could be compared.

**Characterization - Molecular**
A key feature of collagen triple helices is their triple helical conformation, which allows them to behave as rigid rods and to exhibit thermotropic liquid crystallinity in concentrated solution. The secondary structure of the collagens in dried samples was evaluated using wide angle X-ray measurements and FTIR spectroscopy, as described in previous work.

**Characterization – Microscopy**
Polarizing optical microscopy was used to obtain pictures of textures and to observe the angular dependence of optical extinctions within the sample. Oriented domains can be identified in this manner and a large number of liquid crystalline phases can be “fingerprinted” and identified through comparison with the liquid crystal literature. Because the measurements rely on a qualitative record of birefringence in the sample, little data is obtained regarding the optical properties and absolute (as opposed to relative) orientation of the rods. Field emission scanning electron microscopy (FESEM), a high resolution version of SEM with resolutions on the order of 1 to several nm, was used to examine the surfaces of the samples. The FESEM was used to look for evidence of crystalline spherulites, characteristic helicoidal surface and fracture textures, and to look for evidence of smectic organization in the samples such as terracing. In some cases images of individual smectic layers (in neat stacks) were obtained, allowing an estimate of the smectic layer spacing. Standard electron microscopy was also used to examine fracture and free surfaces of samples.

**Characterization – Quantitative**
Spatially resolved laser ellipsometry was used to quantitatively map the optical properties of a number of films. Small angle X-ray scattering provided direct evidence of smectic layers. In some cases the layered structures are sufficiently extensive and periodic to produce a large number of X-ray reflections which are orders of the layer spacing. In these instances the orders of the layer spacing show up in WAXS and WAXD measurements.
Results
FTIR spectroscopy indicates a shift in the Amide A vibration to higher wave numbers (higher frequencies and shorter wavelengths) as the preparation temperature for the peptides is lowered. Under conditions where cholesteric and smectic phases are observed, Amide A bands typical of triple helices are observed. The FTIR behavior of the peptides in the triple helical and non-triple helical conformations have been described previously.

Figure 1. Dried thick film of CPE4 in the smectic A* phase, 2°C. A large number of stacked, roughly 10 nm layers are visible. Two sets of layers are denoted with short black lines in the figure.

Cholesteric phases are observed for collagen-like model peptides dried at 4-5°C, and can be identified in the polarizing microscope. A detailed study of these materials was presented previously. In our prior study we demonstrated that a sinusoidally varying molecular orientation results in a solid surface with sinusoidal variations in surface chemistry. In the model collagens used, a hydrophobic triple helical center block was combined with acidic hydrophilic ends. Twisting of the molecules results in periodic availability of the ends. As reported previously, a number of the films dried in this temperature range display crisscrossed patterns or bands periodically broken by jogs. Because these occur at temperatures intermediate between the cholesteric and smectic A* phases, these textures suggest a TGBA* phase.

At 2-3°C, depending on the peptide sequence, smectic layers are observed. FESEM images of films of CPE4 and CPE6 dried at 2°C allow direct visualization of smectic layers, as shown in Figure 1 for CPE4. The resolution of the FESEM, operated at 1 kV is close to 2 nm. In the Figure, the layers are ~10 nm or 100 Å apart, a value in accord with the length of a 36 residue triple helix with a rise per residue of approximately 2.8 Å – on the order of 110 Å. Lower resolution images of flakes of CPE4 and CPE2 (3°C) reveal several length scales of sinusoidal orientation patterns and undulating layer packing. This type of hierarchical smectic A* texture has been reported previously, by other workers, in studies of smectically ordered poly(benzyl L-glutamate), an α-helix used to obtain a smectic A* phase. The formation of hierarchical length scales of sinusoidal texture can be rationalized in terms of the orientation and layer packing in a smectic A* phase and is likely a hallmark of smectic A* based helicoids.
Optical microscopy of these phases is difficult because the smectic layers will often align with the glass slide, resulting in a “pseudoisotropic” smectic phase. Here the long axes of the collagen triple helical “rods” are, on average, perpendicular to the glass slide. Unusual concentric circular optical and cracking patterns are typical, and occur along defects which develop in the liquid phase as the films dry. At 1°C a filamentous TGB* optical texture is observed (Figure 2), resulting in a solid with a distinctive “filamentous” surface texture for several of the peptides. SEM studies indicate a high surface area “filamentous” topography associated with the observed optical texture, however the light and dark stripes banding the individual filaments are an optical/orientation phenomenon. The filamentous texture occurs in a narrow temperature range and smectic C* characteristic optical textures and defects are observed at lower temperatures. Thus the filamentous phase may be a TGBC*, but further characterization is needed.

As the drying temperature is lowered further, GPAGPP-based peptides (CPE6, CPN6) enter a smectic C* phase with very characteristic optical textures (Fig 3 – CPN6) at temperatures between 0.7°C and 1°C. Hexatic inclusions occur at the lower end of this temperature range. X-ray measurements, including synchrotron SAXS are being conducted. Preliminary data validates the layer spacing of approximately 110 Angstroms, and rods tilted 10° to the layer normal. Quantitative spatially resolved ellipsometric studies indicate very small variations in “rod” orientation as extinction lines and light and dark bands are

Figure 2. Top - polarizing optical micrograph of filamentous collagen phase from CPE6, 1°C. Bottom - Brightfield optical micrograph (without crossed polars) of the lower right hand region of the polarized micrograph.
crossed, and rod tilts (preliminary) in good agreement with the X-ray data. This result rules out cholesteric order for the optical patterns, and supports a highly aligned smectic phase. In contrast the rods in a cholesteric change their orientation by 180° between light and dark bands) in a sinusoid, and have non-sinusoidal variations in intensity at different orientations as described in detail in the existing literature on cholesteric helicoids.

Figure 3. Left (a) smectic C* zig zag texture observed for CPN6 at 1°C. Right (b) Hexatic inclusion in CPE6 at 0.7°C.

Discussion
A number of chiral smectic phases have been observed in simple model collagen oligopeptides dried from high concentration (>100 mg/ml) solutions at subambient (0–3°C) temperatures. An orderly progression from smectic A* to smectic C* and hexatic phases is observed for several of these peptides as the drying temperature is lowered. The morphology and orientation of the phases as well as the temperatures at which they occur are sequence dependent. All of the liquid crystalline phases can be dried with their optical textures intact, allowing techniques such as high resolution scanning electron microscopy to be used to directly visualize smectic layers. The dried “liquid crystal” materials formed from smectic and hexatic phases strongly resemble a number of biological helicoids which possess orderly features not well addressed by simple cholesteric models for their orientation and order. Thus collagen smectic formation is a highly biomimetic process, resulting in materials with many of the features observed in their biological analogues. Collagen fibers consist of staggered layers and are piezoelectric, suggesting a ferroelectric smectic liquid precursor phase. In both cases the collagen molecules are sufficiently well aligned to form layered chemically and physically distinct regions which exist in a regular pattern throughout the collagen material. Cholesterics, in contrast, have more disordered orientations and are characterized by slow change in average orientation of the chiral rods and do not have localized chain ends.

The observations reported here, combining optical images with ongoing corroborating molecular level details, suggest that collagen peptides are capable of well-defined supramolecular assembly when placed within appropriate environmental conditions at suitable concentrations. Furthermore, these observations at low temperatures can be easily translated to higher temperature more biologically relevant systems simply by modulating the hydroxylation content of the prolines in the sequences; a feature well-documented in the literature related to collagen triple helix formation. Changes in the overall collagen triple helix sequence length, or the polarity of the solvent used to prepare the materials also impact the liquid crystalline behavior of the model collagens and are actively being investigated as avenues to control structure.
With the design and implementation of these new collagen triblock systems, new opportunities to study relationships between collagen sequence and hierarchical assembly can be realized. This insight has major significance to fundamental concepts in liquid crystal phase formation as well as to the impact of microgravity on these phases. Furthermore, this level of understanding offers options for future model systems with which to study the impact of microgravity on materials assembly as well as tissue structure and function in vitro and in vivo. It is also important to point out that the current work has been focused on synthetically derived collagen model peptides which are necessarily limited in molecular weight and availability. Ongoing efforts are aimed at overcoming these limitations through genetic engineering in order to manipulate polymer chain length, sequences and to deal with larger levels of production.

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Literature Cited