Liquid Crystalline Elastomers: Effects of Substrate Anisotropy on Cell Behavior

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Cell Behavior

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Abstract

One of the major challenges in tissue engineering today is inducing organization at the cellular level in vitro. Most biological tissues exhibit anisotropic behavior. They perform differently and have different mechanical properties in different directions. This environment is difficult to mimic with traditional cell culturing methods. The development of anisotropic cell substrates may have the potential to encourage the organization required at the cellular level to induce in vitro tissue formation, an exciting prospect for the advancement of the field of tissue engineering.

It is well known that liquid crystalline elastomers (LCEs) are highly organized polymers with temperature-dependent properties. Previous work has indicated that upon straining, mesogen chains within the material shift so that they are aligned in the direction of strain. This thesis addresses the hypothesis that this mesogen alignment results in the development of anisotropic modulus in LCEs. A newly developed procedure in rheology was developed to determine the degree of anisotropy experienced by LCEs fixed at 0, 50, and 100% strain. It was found that the LCEs did exhibit strain-induced anisotropic moduli. Furthermore, the degree of anisotropy, or the ratio of the longitudinal modulus to the transverse modulus, increased with increasing amounts of strain.

Based on the mechanical anisotropy seen in naturally occurring biological tissues, it was then hypothesized that strained samples would have an effect on cell alignment when used as a substrate in cell culture. No cell alignment was observed on any of the strained samples, but differential cell adhesion was observed. On unstrained samples, cells tended to adhere more readily around the edges, whereas cells on samples with 50% strain were more uniformly distributed, and very few adhered to the samples with 100% strain at all. Analysis of the reasons for these differences is beyond the scope of this thesis, but may be explored further in future work.
Table of Contents

Acknowledgements......................................................................................... i

Chapter 1: Introduction.................................................................................. 1
  Tissue Engineering...................................................................................... 1
  Liquid Crystalline Elastomers (LCEs)....................................................... 5
  Scope of the Work Herein......................................................................... 10

Chapter 2: Synthesis and Characterization Methods for Liquid
  Crystalline Elastomers.................................................................................. 15
  POBA.......................................................................................................... 15
  Mesogens.................................................................................................... 16
  5H/5tB LCEs............................................................................................... 18
  Oriented Indentation Rheology................................................................. 21

Chapter 3: Thermal and Mechanical Characterization of LCEs.............. 35
  Thermal Stability....................................................................................... 35
  Transition Temperatures.......................................................................... 36
  Modulus vs Temperature.......................................................................... 40
  Material Selection..................................................................................... 42
  Mechanical Anisotropy............................................................................. 42

Chapter 4: Cell-Material Interaction.......................................................... 56
  Sample Preparation for Cell Plating......................................................... 56
  Cell Plating............................................................................................... 58
  Cell Staining............................................................................................. 59
  Cell Imaging............................................................................................... 59
  Cell Behavior............................................................................................ 60
  Summary and Conclusions...................................................................... 62

Works Cited.................................................................................................. 68

Summary........................................................................................................ 70
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Chapter 1: Introduction

Tissue engineering is a burgeoning area of research in the field of bioengineering. Encouraging cells to organize into their respective tissues outside their natural in vivo environment has been a challenge faced by tissue engineers since the recognition of tissue engineering as its own field. New and innovative cell culturing methods have been evolving since the field first emerged, and novel approaches continue to be developed and explored, as a means of accomplishing this goal of in vitro tissue development. The introduction of new types of biomaterials into the tissue engineering process is an exciting area of research with the potential to shed light on the matter of substrate effects on cell behavior.

Tissue Engineering

Cells are extremely sensitive to changes in their microenvironment during culturing. There are currently several techniques employed in tissue engineering to induce the development of certain cell properties, including cell alignment and differentiation. Some of these techniques involve specific ions and other chemical cues, others depend on the cell-cell interactions for the appropriate lineage development, several apply mechanical loading to the material on which the cells are growing, while still others alter the topography or stiffness of the culture substrate.

Tapp and colleagues examined the potential for adipose-derived mesenchymal stem cells (ASC) to differentiate into the difficult-to-culture cells that compose most orthopedic tissues. They examined the effectiveness of each of
the methods described above using ASCs. ASCs cultured in media with the appropriate osteogenic, chondrogenic, and adipogenic growth factors were found to differentiate into osteogenic, chondrogenic, and adipogenic lineages, respectively. Collagen and polyester scaffolds were used to determine the chondrogenic potential of ASCs, and it was concluded that the final differentiation state was ultimately dependent on the concentration and type of bone marrow protein used to promote binding, and the origin of the stem cells. When TGFβ, another growth factor, was added, osteogenesis was suppressed and the stem cells underwent chondrogenesis. In other words, cell behavior was influenced much more by the composition of the surrounding media than by the structure of the scaffold on which they grew.

ASCs have shown great potential for differentiation into the desired type of cell by co-culture with that type of cell. Intervertebral discs contain cells that are notoriously difficult to culture because the cells making up the discs are not as well characterized as those found in bone or cartilage, and the process by which they develop is not very well understood. Nevertheless, when co-cultured with disc cells, BSCs (bone marrow stem cells) were found to develop many major cellular components of disc cells.

Work with cells that compose tendons and ligaments has been limited, but BSCs have been found to differentiate into collagen bundles when injected into tendon and ligament defects. Unfortunately, often after ligament injury, cells in the healing site are randomly oriented, and the collagen matrix is consequently significantly less organized. This breakdown in matrix organization negatively
affects the mechanical properties of the tissue, leaving a weakened area in the ligament. Many methods of in vitro culturing have been developed to produce oriented cell growth to alleviate this problem.

For many load-bearing tissues, including those found in skeletal tissues, mechanical cues have been found to be extremely effective in directing cell behavior because of “the cell’s ability to feel and respond to its matrix”. In the case of in vivo tissue repair, often times injured tissue loses mobility and thus the tissue growth that is intended to repair the damaged area has slightly different properties than the original tissue; i.e., scar tissue forms. The cell response is inappropriate for its location because the mechanical stimulus is inappropriate. For instance, cells don’t sense the target modulus of the tissue in need of repair.

Cells have been found to differentiate more appropriately when exposed to cyclic loading conditions similar to those to which the damaged tissue is typically exposed. For instance, it has been found that cyclic loading helps maintain levels of certain important transcription factors in the differentiation process and in active remodeling of the ECM. For example, Liao and colleagues conducted a study in which it was found that cyclic strain promotes differentiation of MSCs into smooth muscle cells.

Several studies have found that cells will elongate and align in the direction of grooves in a substrate surface, where cells on a smooth surface are randomly oriented. Lam and colleagues found that this alignment process is reversible. Specifically, cells continuously adapt to changing substrates and become randomized again once the grooves are removed. Additionally, once
randomly aligned, cells were found to orient themselves if grooves are introduced later. In other words, cells have been found to continuously remodel their cytoskeletons in response to changing external, environmental cues like mechanical stimuli. The grooves can also influence cell migration, as cells tend to “crawl” along the length of the groove. Cyclic loading has also been found to induce cell orientation along the axis of loading, but no connection to cell migration was observed.

Anisotropic substrate stiffness may be another means of directing cell orientation, as actin filaments have been found to align themselves along the axis of greatest stiffness. These anisotropic rigidity studies were performed at the micrometric scale, and though stress fibers aligned themselves in the direction of greatest stiffness, mitotic direction did not seem to be affected. In other words, cell orientation was easily controlled by anisotropic stiffness, but the direction of cell proliferation was still randomly directed.

The nuances of cell behavioral responses in all of these substrate manipulations give testament to the specificity of the cell response. Even extremely small changes to the cell’s environment can induce significant changes in cell behavior, morphology, or even lineage. The cell is continuously adapting to and interacting with its surroundings and is sensitive to even the smallest changes in culturing environments. New substrate materials with distinctly different properties from those currently utilized could reveal a new aspect of cell development that was previously unknown to science.
Traditionally, agar dishes and suspension cultures have been used to grow cells *in vitro*. Currently, three-dimensional scaffolds are frequently employed in cell culture for tissue engineering applications. Those with research interests in biomaterials have been working to develop scaffolds with unique surface coatings, varying degrees of porosity, different rates of degradation for those scaffolds used *in vivo*, and mechanical properties tailored to match those of the tissue for which it is being utilized. Interestingly, many different materials have been used to make such three-dimensional scaffolds, including but not limited to collagen, PGA (polyglycolic acid), PLA (polylactic acid), and PCL (polycaprolactone), as well as co-polymers containing any or all of these building blocks.

The need to tailor material properties to emulate the tissues for which the cells are being cultured has motivated a search for new biomaterials that may play a vital role in pushing the frontier of tissue engineering. Liquid crystalline elastomers have been proposed as a candidate for this critical role. However, LCEs are not commercially available and must be synthesized using very precise techniques. Under the right conditions, these materials have the potential to become an attractive alternative for cell culture in tissue engineering applications. This thesis examines such potential.

*Liquid Crystalline Elastomers (LCEs)*

Liquid crystalline elastomers are very unique polymers composed of crosslinked chains of rigid-rod molecules called mesogens connected by flexible
spacer molecules. They display interesting mechanical properties related to this distinctive structure. These properties include a two-way shape memory cycle,\(^\text{21}\) excellent shape-fixing and recovery properties, low and tunable moduli, low and tunable transition temperatures, and anisotropic modulus under strain.\(^\text{21,22}\) This research focuses on the strain-induced anisotropy of these materials, but a brief overview of all properties listed is given here.

LCE properties are inherently linked to the material’s chemical structure. There are different ways in which to arrange the mesogen and flexible spacer components in an LCE, as shown in Figure 1-1, adapted from Ingrid Rousseau’s dissertation.\(^\text{22}\) For instance, the mesogens can be connected to a flexible backbone via other flexible spacers, as is the case in side-chain LCEs. Alternatively, the polymer can consist of alternating mesogens and flexible spacers, with one flexible spacer connecting two adjacent mesogens. This second scenario is the case for the main-chain LCEs used in this research. Additionally, in each of these cases the spacers can potentially attach to either the center or the ends of each rigid-rod mesogen. All LCEs used in this research were main-chain LCEs, and thus the mesogens were connected in an end-to-end fashion via flexible spacers.

There is a very specific, yet thermally labile, order within LCEs and it is this precise ordering that imparts such unique shape memory properties on the materials. Shape memory is traditionally associated with materials composed of a crosslinked network that is easily deformable above a certain temperature. This temperature is either the glass transition temperature (\(T_g\)) if fixation is a result of network vitrification, or melting temperature (\(T_m\)) if it results from partial
crystallization of crystalline domains. The LCEs used in this research also have temperature-dependent properties, as the order of the mesogens varies dramatically with temperature. Below $T_g$, the mesogens vitrify, or transition into their glassy state, within their smectic layers. This vitrification results in a marked increase in modulus below $T_g$. Once heated through $T_g$, the mesogens soften and some of the material’s soft elasticity is restored. As the material continues to be heated through $T_c$, or clearing temperature, the mesogens become randomly oriented as they enter an isotropic phase. In summary, as the material is heated, the mesogens gradually change from stiff rods arranged with strict smectic order within their domains, where they are aligned in layers of similar orientation, to softer rod-like molecules with isotropic order, where they are randomly oriented and allow for dramatic softening of the material.

The domains discussed above are crosslinked together, but do not naturally adopt any order with respect to each other. Order can, however, be induced by uniaxially straining the sample. This has been confirmed in previous research by x-ray analysis. Figure 1-2, as adapted from the dissertation by Ingrid Rousseau shows a representation of the systematic increase in order imparted on the LCEs with increasing strain. In the relaxed state, the domains are randomly oriented, though the mesogens they contain have smectic order. As strain in the sample is increased, the domains begin to shift so that their average orientation is along the direction of the force applied. When strain is increased beyond the point at which the domains are fully aligned, the polymer chains
contained in each domain begin to exhibit pure elastic behavior since no higher
degrees of orientational order can be achieved.\textsuperscript{22}

Properties inherent to the material, like modulus and transition
temperature, are mostly dependent on LCE composition. The LCEs used in this
research were copolymers consisting of different percentages of 1,4-bis[4-(4-
pentyloxy)benzoyl]hydroquinone (\textit{5H}) and 2-methyl-1,4-bis[4-(4-
pentyloxy)benzoyl]hydroquinone (\textit{5tB}) mesogens. Increasing the percentage of
\textit{5H} relative to \textit{5tB} in a given LCE film tends to increase the transition
temperatures of the material.\textsuperscript{22,23} Transition temperatures tend to decrease when
the flexible spacer molecules in the LCE become longer and more flexible.\textsuperscript{22} By
altering the \textit{5H}/\textit{5tB} ratio and type of spacer used, the transition temperatures for
the LCE can be shifted to within a cell-tolerable range.\textsuperscript{23,24} Increasing \textit{5tB}
percentage to 20 and 30\% of the total mesogen composition has been found to
decrease $T_g$ to 36.1 and 32.8 °C, respectively.\textsuperscript{24} Cells are traditionally cultured at
human body temperature: 37 °C.

In addition to the possibility of experiments involving property changes
related to these degrees of orientation, the main-chain LCEs used in this research
allow for the completion of cyclic loading experiments. They possess the
extremely rare potential for two-way shape memory (TWSM), and also for the
slightly more prevalent but also useful property of one-way shape memory. In
TWSM, a material can reversibly deform so that it assumes one shape above the
clearing temperature ($T_c$), and a different shape below $T_c$, so long as a load is
applied continuously.
In other words, whole-material changes result from microstructural changes caused by temperature changes.\textsuperscript{22} This allows for spontaneous reversible actuation in which macroscopic changes occur, in principle, with no added stress to induce deformation because the molecular reorganization that causes them is simple rotation of the mesogens.\textsuperscript{22} TWSM is important in order to be able to cyclically stretch and relax the substrate on which cells can be cultured, which would subsequently cyclically load and unload the cells. The importance of cyclic loading on cell differentiation was described above.

Additionally, the LCEs can be used as effective one-way shape memory materials. This process is described simply and clearly by Rousseau in her dissertation: “changes in conformation undergone while deforming are fixed below $T_g$, [the glass transition temperature]. Upon heating back above $T_g$, the energy stored after deformation and fixing is released and the sample recovers its original, equilibrium chain conformation and, thus its original shape.”\textsuperscript{22} One-way shape memory is an important factor in the longevity of the LCEs in their final application. While it is possible to manipulate and fix the materials for different cell culture experiments, they can be easily reheated above their $T_g$ to recover their original shape and properties. This allows the same sample to be reused for multiple experiments, minimizing differences in uncontrollable variables between individual experiments.
Scope of the Work Herein

The combination of unique properties displayed by LCEs lead to their incorporation into cell behavior studies. First, the properties of the LCEs used needed to be tailored to fit the purpose of cell culture. The transition temperatures had to occur within a cell-tolerable range, and it was also desirable, though not required, for the modulus to resemble that of in vivo tissue. In order to obtain the appropriate properties, LCEs were synthesized using various 5H/5IB ratios, as well as different ratios of HPDMS/BDMSB flexible spacers, and two different crosslinkers (tetrakis(dimethylsiloxysilane) or CL(4H), and tris(vinyl(dimethyl)siloxysilane) or CL(3V). The range of materials synthesized and characterized is listed in Table 1-1.

It is hypothesized that an LCE composition can be found that is thermally stable through the upper temperature limit of cell culture, with one or both transition temperatures within a cell-tolerable temperature range. It is also proposed that this material will display strain-induced mechanical anisotropy due to mesogen alignment that has the potential to direct cell behavior. The syntheses of the LCEs and their components is discussed in the “Syntheses and Methodology” chapter of this thesis.
Table 1-1. Materials synthesized and characterized throughout the work to follow. It is meant to show the parameters that were held constant while others were varied in order to find the composition best suited for the cell culture experiments to be performed as described in Chapter 3.

<table>
<thead>
<tr>
<th>Mesogen ratio</th>
<th>Crosslinker type</th>
<th>Crosslinker percentage</th>
<th>Percent HPDMS</th>
<th>Percent BDMSB</th>
</tr>
</thead>
<tbody>
<tr>
<td>(70-5H/30-5tB)</td>
<td>4H</td>
<td>8</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>(70-5H/30-5tB)</td>
<td>4H</td>
<td>15</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>(70-3H/30-3tB)</td>
<td>4H</td>
<td>15</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>(100-5tB)</td>
<td>4H</td>
<td>20</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>(100-5tB)</td>
<td>4H</td>
<td>20</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>(100-5tB)</td>
<td>4H</td>
<td>20</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>(100-5tB)</td>
<td>4H</td>
<td>20</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>(100-5tB)</td>
<td>4H</td>
<td>20</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>(50-5H/50-5tB)</td>
<td>3V</td>
<td>10</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>(50-5H/50-5tB)</td>
<td>3V</td>
<td>20</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>(80-5H/20-5tB)</td>
<td>3V</td>
<td>26.7</td>
<td>100</td>
<td>-</td>
</tr>
</tbody>
</table>

The thermal stability of each of the resulting LCEs was determined using thermogravimetric analysis (TGA). Transition temperatures were found with differential scanning calorimetry (DSC). Storage and loss moduli were analyzed with respect to changing temperature using dynamic mechanical analysis (DMA). The anisotropy of a strained LCE sample was evaluated using a novel rheologic procedure described in the “Mechanical Analysis” chapter of this paper.
Once a LCE with the appropriate physical properties was synthesized, the cell-material interaction was examined. Previous studies have analyzed the effect of anisotropic rigidity on the micrometric scale where modulus anisotropy was the result of the surface profile of the material. Oblong pillars were created on the material surface that made the substrate stiffer along the longitudinal direction (parallel to the long axis of the oval pillars). They have found that actin filaments tend to align themselves along the axis of greatest stiffness. This effect on actin orientation was more significant than the same material’s effect on motility. The anisotropy did not, however, appear to have any affect on the direction of mitosis. It is hypothesized that increasing the degree of mechanical anisotropy will have an effect on cell behavior: namely, cells will align along the axis of greater modulus without the effects of material topography. The LCEs in this study use no topographic changes to impart anisotropic stiffness. The specific procedure for the analysis of this small-scale smooth-surface anisotropy effect on cell behavior is detailed in the “Cell-Material Interaction” chapter of this thesis.
Figure 1-1. Adapted from dissertation by Ingrid Rousseau, this cartoon displays the different connectivities of mesogens and flexible spacers in LCEs. The end-to-end MC-LCPs are the type of LCE used in this research.
Figure 1-2. Adapted from dissertation by Rousseau, the above pictorial representations clearly show the systematic increase in domain order as strain increases.
Chapter 2: Synthesis and Characterization Methods

for Liquid Crystalline Elastomers

Because the main-chain LCEs used in this study are entirely new materials that have not, to our knowledge, been made or characterized before, it was necessary to synthesize the elastomers in our lab. Additionally, the mesogens used in the elastomer synthesis are not commercially available, and needed to be made and purified in our lab as well. This chapter provides a detailed explanation of how these syntheses and purifications were performed.

Furthermore, an innovative rheological method used during mechanical characterization to be described in Chapter 3 was developed and is explained in depth here. This method was used as a means of determining relative moduli to quantify the degree of anisotropy displayed by strained LCE samples.

POBA

4-pentenyloxybenzoic acid, or POBA, is the molecule from which the mesogens used in this research are synthesized. Thus, it is first necessary to obtain POBA in its pure form to ensure the best results in mesogen, and ultimately LCE, synthesis. POBA was prepared in a one-pot reaction as depicted in Scheme 2-1. Below, a specific reaction to make the POBA used in our LCE syntheses is described in detail.

First, a 45-weight-percent KOH/H₂O solution was prepared by slowly adding 40 mL water to 32.7 g KOH in a beaker, stirring continuously. A small excess of 5-bromo-1-pentene (1.05 mol bromopentene : 1 mol hydrobenzoic acid)
was used in the reaction to ensure that all of the hydroxybenzoic acid was consumed. 100 mL methanol was used to dissolve 20.9 g of hydroxybenzoic acid before adding 34 mL of the 45-weight-percent KOH/H₂O solution. Finally, 25.0 g of 5-bromo-1-pentene was added and the solution was allowed to reflux at 85 °C for 48 h.

Once removed from heat, 500 mL deionized water was added to reduce the basicity of the solution and help pull any solid POBA salt remaining into solution since it is not very soluble in methanol. Four extractions were performed with 100 mL hexanes each, re-extracting the bottom (aqueous) layer containing POBA salt each time and setting the top (organic) layer aside. The POBA in the aqueous layer was protonated using hydrochloric acid (37%), causing it to crash out of solution as a white, powdery precipitate. The precipitate was filtered and washed with deionized water until the pH was about 6. To remove any remaining impurities, the POBA was recrystallized using 300 mL ethanol for every 8 g dry POBA.

Mesogens

The mesogenic dienes, rigid rod-like molecules, used in the synthesis of the LCEs examined in this research were synthesized from POBA in a one-pot reaction at room temperature as depicted in Scheme 2-2. Specifically, for 5tB synthesis, 2.4 mol POBA, 0.464 g DMAP, 1 mol tert-butylhydroquinone and 2.4 mol DCC were added to 120 mL DCM for every 12 g POBA. Likewise, the only difference in 5H synthesis was the use of hydroquinone rather than tert-
butylhydroquinone. The solution was stirred at room temperature for 24 h before the mesogens were purified. In order to isolate the mesogens synthesized, the impure mesogen solution was run through a silica gel column with an eluent that is dependent on the mesogen being isolated as shown in Table 2-1.

Table 2-1. Different eluents were used for column chromatography of each mesogen, simple filtration was used to isolate $5H$ and thus no eluent was necessary.

<table>
<thead>
<tr>
<th>Mesogen</th>
<th>Eluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>$5tB$</td>
<td>7 hexane:1 ethyl acid (vol:vol)</td>
</tr>
<tr>
<td>$5H$</td>
<td>N/A</td>
</tr>
<tr>
<td>$5Me$</td>
<td>DCM</td>
</tr>
</tbody>
</table>

In the case of $5H$, a column need not be run because $5H$ could be easily crystallized and isolated simply by filtering, followed by rotovapping to yield a solid mixture of $5H$ and POBA. Washing this solid with ethanol dissolved away the POBA, leaving pure $5H$ to be dried under vacuum at 70 ºC.

For $5tB$ and $5Me$, as the mesogenic solutions were run through the column with their respective eluents (Table 2-1), fractions of about 20 mL each were collected in test tubes. Each fraction was then tested for purity using thin layer chromatography run with a DCM mobile phase. Those fractions found to contain pure mesogen were combined by rotovap and the resulting crystals were confirmed pure by $^1$H-NMR of a mesogen-CDCl$_3$ (deuterated chloroform) solution. Deuterated chloroform was used as solvent so that the $^1$H-NMR would not show peaks for those hydrogens associated with the solvent.
**5H/5tB LCEs**

The main-chain LCEs studied here have several components: mesogenic dienes, flexible spacers, and crosslinkers. The main-chain LCEs used in this research feature the rigid rod-like mesogens attached at each end to flexible spacers and are arranged as shown in Figure 2-2. As this research explored the effects of varying the ratios and types of mesogens, spacers, and crosslinker on the LCEs’ properties, a nomenclature system was developed to make the identification of each LCE easier. The nomenclature used henceforth in this paper is as follows:

\[
E(\text{mesogens}) - \text{crosslinker} - [\text{spacers}]
\]

\[
E(x-5H/y-5tB) - z\%CL(ab) - [d-HPDMS/e-BDMSB]
\]

where

- \(x\) = mol-% 5H
- \(y\) = mol-% 5tB
- \(z\) = mol-% crosslinker
- \(a\) = 3 or 4 (number of arms on crosslinker)
- \(b\) = H or V (hydride-terminated or vinyl crosslinker, respectively)
- \(d\) = mol-% HPDMS (hydride-terminated poly(dimethyl siloxane))
- \(e\) = mol-% BDMSB (1,4-bis(dimethylsilyl) benzene)

The structures of the flexible spacers and crosslinkers used are shown in Figure 2-1. A platinum catalyst (platinum(0) - 1,3-divinyl - 1,1,3,3-tetramethyldisiloxane in xylene) was used to catalyze the hydrosilation reaction between HPDMS and the mesogens and crosslinker. This catalyst is water- and air-sensitive, so all glassware used was flame-dried and purged with nitrogen gas prior to beginning the reaction. Additionally, all transfers were completed via syringes.
A dry DCM atmosphere was established in the curing apparatus by connecting a 250 mL Erlenmeyer flask containing about 30 mL of dry DCM to the apparatus via a long, flexible needle, as shown in schematically in Figure 2-3. Again, both the flask and the apparatus were flame-dried and nitrogen purged before setting up the system. A vacuum was established in the curing apparatus to aid in the diffusion of dry DCM gas from the flask to the apparatus and the system was left to equilibrate for at least 20 min. Once the DCM atmosphere was established, the connection could be removed by removing the end of the needle from the stopper of the curing apparatus.

The synthesis of E(50-5H/50-5tB)-10%CL(3V)-[100-HPDMS] was completed as follows. The 71.4 mg 5H and 78.3 mg 5tB were first added to a prepared Schlenk tube, which was subsequently re-purged (as it needed to be “unstoppered” for the addition of the crystals) for 20 min. 0.38 mL dichloromethane solvent was added by syringe and the mixture was allowed to stir in a 40 °C oil bath for 10 min before transferring it to a 0 °C icebath to stir an additional 10 min. While the solution continued to stir at 0 °C, one drop of platinum catalyst was added, followed by 15 μL CL(3V) and 230 μL HPDMS, all of which were added by syringe. This solution was quickly transferred to one of the curing apparatus pictured in Figure 2-4 using a syringe that had been chilled for at least 15 min using a cooler of dry ice. This was a precaution against crosslinking in the Schlenk tube or in the syringe, either of which would make transferring the LCE to the curing apparatus impossible.
The apparatus shown in Figure 2-4b was designed by Kelly Burke and machined by the physics department at Syracuse University after it became necessary to produce larger samples for testing, and when some of the LCEs proved difficult to remove from the bottom of the 10 mL beakers (Figure 2-4a) in one piece. The problem of the LCEs sticking to the bottom of the 10 mL beakers in which they cured was addressed at first by coating the beakers with a silane release layer, as is now described. After cleaning in base bath, the beakers were prepared by soaking them in pirhana solution (1:3 volume ratio H_2O_2 (30%) : H_2SO_4 (conc.)) for at least an hour. Extreme caution was used while working with pirhana solution as to avoid contact with eyes, skin, and clothes. After removing the beakers from the pirhana solution, they were washed thoroughly with water and flame-dried. They were then coated with a release layer (hexamethyldisilazane), which makes the surface hydrophobic to prevent sticking, but is stable enough that it doesn’t affect the chemistry of the LCE. The release layer was prepared by mixing a 5.6 wt-% solution of hexamethyldisilazane in hexanes (20 mL hexamethyl in 400 mL hexanes) into which the beakers were dropped. The whole system was then refluxed overnight at 70 °C. After 12 hours, the beakers were removed from the liquid, rinsed with hexanes, and dried in the hood for 5 minutes before being placed in the convection oven at 50 °C for 1 hour, or until used. If the beakers were not used that day, they were placed in a desiccator to remain dry.

The polytetrafluoroethylene (Teflon) bottom of the new curing apparatus eliminated the need for this extensive preparation and the larger face allowed for
the synthesis of larger LCE films, which were needed as we moved forward with mechanical and biological testing. Before this new apparatus was designed, however, some success in removing the LCEs from release-layer-coated beakers was acheived by soaking the elastomer in ethanol to swell it, and gently pushing at the elastomer-glass interface with a blunt spatula.

After the LCE was removed from the curing apparatus, it was extracted in an ethanol and dichloromethane solution (50:50 volume ratio) at 37.0 °C under slight agitation. The solvent was exchanged four times to remove any uncrosslinked mesogens or spacers. Gel fractions (mass remaining after extractions / original mass) were measured to monitor how much mass was lost in this process. Higher gel fraction indicated higher crosslink density because fewer of the LCE components were able to dissolve in the EtOH/DCM solution. A low gel fraction indicates poor crosslinking or a crosslink density that is too low to effectively stabilize the LCE. After gel fractions were obtained, the sample was dried in a room temperature vacuum oven overnight before characterization.

*Oriented Indentation Rheology*

The significance of anisotropic modulus in tissue engineering was discussed in Chapter 1. It was also noted that LCEs experience a systematic increase in orientational order as strain increases. It is reasonable to hypothesize that this strain-induced alignment could result in anisotropic modulus when the material experiences sufficient strains. The material would presumably be stiffer along the stretching axis, and relatively less stiff in the orthogonal direction. This
is intuitive because it is easier to nudge a single mesogen or flexible space in one
direction or the other as opposed to dragging to entire chain along, as illustrated in
Figure 2-5.

In order to obtain data that would allow for a relative comparison of
moduli in the directions parallel and orthogonal to the axis of stretching in the
LCE samples synthesized, a new rheological method was developed. This method
was a modified indentation procedure using an oscillation time sweep designed to
mimick traditional straight-line indent-and-drag procedures. A detailed
description of the development of this procedure follows.

A record needle was attached to the top plate of a TA Instruments AR-G2
rheometer 4.90 mm from the outer radius using superglue, and was used as an
indenter tip. Using an oscillation sweep procedure with an extremely small
displacement approximates a straight-line indent-and-drag procedure. It is
important to note that an accurate modulus measurement was not the goal of this
procedure. This method was simply developed for the purpose of comparing
stiffness along longitudinal and transverse axes, establishing a proof-of-concept
that a differential does exist between them in strained samples. The oscillation
procedure chosen was a time sweep step with the parameters listed in Table 2-2.
Table 2-2. Parameters for oriented rheology oscillation time sweep are shown here.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration</td>
<td>10:00 min</td>
</tr>
<tr>
<td>Delay Time</td>
<td>00:01 min</td>
</tr>
<tr>
<td>Temperature</td>
<td>37.0 ºC</td>
</tr>
<tr>
<td>Controlled variable – Displacement</td>
<td>35.1 mm (0.001 rad)  = 35.1 µm</td>
</tr>
<tr>
<td>Angular frequency</td>
<td>6.283 rad/sec</td>
</tr>
<tr>
<td>Indentation depth</td>
<td>15 µm</td>
</tr>
<tr>
<td>Sample thickness</td>
<td>&gt; 0.5 mm</td>
</tr>
</tbody>
</table>

The samples were prepared (strained) by first relaxing the materials over boiling water. The consequently isotropic samples were then clamped into the manual stretching apparatus pictured in Figure 2-6, which was originally designed to elongate samples and check for orientation via x-ray analysis. The entire system was then heated above LCE isotropization temperature (about 75 ºC), in the convection oven. The samples were then stretched uniaxially to a prescribed strain percentage while the device was inside the oven so that they were in the isotropic phase during straining. Strain rate was approximately 50%/min, and the system was allowed to equilibrate in the 75 ºC oven for 1 min after straining was complete. Next, the system was transferred to a -8 ºC freezer to ensure that the sample was cooled well below $T_g$ and thus the shape fixed. After 5 min in the freezer, the sample was removed from the stretching apparatus and placed in a 37 ºC water bath for 10 min to allow for any recovery at this temperature before being fixed to the rheometer plate. Samples that were stretched to 100% strain recovered about 20% at 37 ºC, and those at 50% strain averaged about 10% recovery.
The strained samples were fixed to the bottom (stationary) plate of the rheometer with double-sided tape, oriented as shown in Figure 2-5. The indenter tip was then lowered until it was just touching the surface of the sample, as observed visually and indicated by resistance to rotation. The oven was then closed and the temperature allowed to equilibrate. Finally, the indenter tip was lowered another 15 µm, using the gap feature in TA Instrument’s Rheology Advantage Instrument Control AR software version V5.7.1, resulting in an experimental setup depicted in Figure 2-7. The oscillation procedure with the parameters shown in Table 2-2 was begun and data regarding apparent modulus of a sample with assumed circular geometry was collected and analyzed using Systat Software’s Sigmaplot for Windows, version 11.0.

Again, it is critical to remember that the raw data acquired in the rheological procedure detailed above was not an accurate measurement of the material’s modulus. It is also notable that an accurate measurement is not required for the goal of this analysis. The interest of this thesis is related to the degree of anisotropy displayed by LCEs under different strains. The data useful to this analysis was therefore the ratio of the apparent modulus in one direction (tangential) to that in the orthogonal direction (radial). Both measurements made the same geometric assumptions, so inaccuracies caused by these assumptions could be ignored when the ratio of the data containing. In other words, any inaccuracies were incorporated into both sets of raw data in the same way. Taking the ratio of the two data sets caused the inaccuracies to cancel each other. With this in mind, the apparent modulus measurements were normalized by taking the
ratio of tangential-to-radial apparent moduli measured at a given strain percentage. The resulting data provided a quantitative analysis of how much stiffer the strained samples were in one direction than the other, while eliminating the need to compensate for inaccuracies and calculate the “real” moduli of the samples.
Scheme 2-1. POBA was synthesized using the one-pot reaction shown here.
Scheme 2-2. Synthesis of 5tB and 5H from POBA was performed as a one-pot reaction as shown here.
Figure 2-1. These are the chemical structures of crosslinkers (a, b) and spacers (c, d) used in LCE synthesis.
Figure 2-2. a) Main-chain LCEs prepared in this study are shown schematically as rigid rods (mesogens) connected end-to-end by flexible spacers (siloxane chains). b) These chains are chemically crosslinked to form a stable network.
Figure 2-3. This schematic depicts the set-up used to establish a dry DCM atmosphere in the curing apparatus.
Figure 2-4. LCEs were cured in a flame-dried, nitrogen-purged 10mL beaker (a) and in a machined curing apparatus (b) designed by Kelly Burke. In both, a dry DCM atmosphere was established.
Figure 2-5. Mesogens and smectic domains are known to align along the axis of stress in strained LCE samples as shown in Figure 1-2. It is thought that this alignment may result in anisotropic modulus. An oriented indentation rheology method was developed to test this hypothesis. a) The samples were oriented as shown on the bottom plate of the rheometer. b) The oscillation procedure resulted in indentation and dragging in the directions depicted.
Figure 2-6. Uniaxial tensile stress was applied to LCE samples at 75 °C using the apparatus shown until the samples were stretched to the prescribed strain.
Figure 2-7. This schematic depicts the experimental setup for the newly developed rheometer oscillation time sweep procedure, which was used to obtain relative moduli measurements of mechanically anisotropic LCE samples with strain-induced orientation described in Chapter 1, viewed from the side (a) and the top (b).
Chapter 3: Thermal and Mechanical Characterization of LCEs

LCE chemical composition and structure are fundamental to the establishment of the thermal and mechanical properties characteristic of the bulk material. Thus, varying LCE chemical compositions during synthesis resulted in the modification of the physical properties expressed by the materials produced. Mesogen, crosslinker, and flexible spacer types and percentages were methodically varied as depicted in Table 1-1 in order to isolate each one’s effect on material properties. Several common methods of thermal and mechanical analyses were used to quantify these differences and establish relationships between composition and particular properties. Once these relationships were established, they were used to adjust the properties of the LCEs for optimal performance in the desired cell culture experiments.

Thermal Stability

It was important to ensure that all LCEs to be used in cell culture were thermally stable through the temperature range in which the cells grew (35-38 °C). Additionally, it was important to establish that future DSC analyses were valid and not affected by material decomposition. TGA was used to quickly and easily determine the thermal stability of each of the materials synthesized. Samples were equilibrated at 30.00 °C and data collection was begun as temperature was ramped to 600.00 °C at 10.00 °C/min. It is clear from Figures 3-1 and 3-2, that all the
samples tested far exceeded the requisite 37 °C at which cells are typically cultured, regardless of how their composition was altered. TGA experiments for materials of varying mesogen compositions were conducted in previous research\textsuperscript{24} and the same trend of extremely high decomposition temperatures was established. It was concluded that thermal stability of any of the LCEs is not of concern for the application in these experiments, and does not need to be considered during determination of the optimal composition of the materials.

*Transition Temperatures*

Differential Scanning Calorimetry (DSC) is a method used to identify glass and clearing transition temperatures. This is accomplished by measuring changes in heat flow, or how much heat it takes to increase the temperature of a sample, across a range of temperatures. Here, we used DSC to measure the phase transition temperatures of each LCE. Thus, a trend could be established relating a specific change in material composition to either an increase or decrease in transition temperature. Moreover, the degree to which these transitions were affected by each composition change could be quantified.

A traditional DSC procedure, in which there was a first and second heat and cool, was followed for all samples. The procedure involved a first and second heat and cool, with the first cycle clearing any thermal memory and the second cycle being used for data collection. Temperature was ramped to -20.00 °C, then to 100.00 °C at 10.00 °C/min, where it was held isothermally for 1 min before ramping to -20.00 °C at 10.00 °C/min. This completed the first heat/cool cycle and
cleared any thermal memory of the sample. The sample was held isothermally for 1 min at -20.00 °C before ramping again to 100.00 °C at 10.00 °C/min where it was again held isothermally for 1 min before cooling back down to -20.00 °C at 10.00 °C/min. The second heat and second cool data were plotted and analyzed using Sigmaplot (Systat Software, Inc., V 11.0).

\( T_g \) was calculated indirectly from the resulting data sets using the half-delta criterion. The drop in heat flow (endotherm) associated with \( T_g \) in the second heat cycle was defined as delta. This change in heat flow was multiplied by 0.5 to determine the value at \( T_g \). A drop line inserted into the second heat plot at this calculated heat flow was then used to identify \( T_g \), which was assumed to occur halfway through the endotherm.

The first approach considered for altering \( T_g \) in the LCEs studied in this research was to vary the ratio of 5H to 5tB. It had been previously reported in research literature concerning these materials that increasing the percentage of 5tB in the material would lower the transition temperature of the material.\textsuperscript{22,24} Since mesogen effects on transition temperatures were already well-characterized in the literature, no experiments were run on materials with varying mesogen ratios. Instead, a particular ratio of 80-5H/20-5tB was chosen. This composition was found previously to have a \( T_g \) of 36.1 °C and a 58.6 °C \( T_c \). These transition temperatures could be further tuned by altering other components of the material composition.

The next material modification explored was varying the percentage of crosslinker used. Crosslink density was controlled for both types of crosslinker by
modifying the moles of crosslinker used to account for the extra connection possible for the CL(4H) when compared to that of the CL(3V). It was postulated that the greater the crosslinker density, the higher the transition temperatures would become because of the larger amount of energy required to disrupt the structure of the LCE. However, as Figure 3-3 shows, increasing crosslink density actually lowered the glass transition temperature of the LCE studied. It should be noted that the material containing 20% crosslinker broke up during extraction and yielded relatively low gel fraction values. Thus, despite having a higher apparent crosslink density (concentration of crosslinker used in synthesis), the material’s actual crosslink density was lower than that of the one synthesized with 10% apparent crosslink density, which remained continuous during extraction and boasted high gel fraction numbers. These results also give important insight into the potential inconsistency in LCE composition despite precise synthesis procedures. Though the most reliable assessment of actual crosslink density is modulus measurement, the results suggest that gel fraction may be a more accurate representation of crosslink density than crosslinker volume used in synthesis. In conclusion, increasing the crosslink density (given that the type of crosslinker was the same), raised the transition temperatures of the LCEs.

The next approach to controlling \( T_g \) and \( T_c \) was to alter the flexible spacer used to connect to mesogens. The effects of spacer modification on LCE glass transition temperature were first examined by Rousseau.\textsuperscript{22,23} In this research, materials were first synthesized using only hydride-terminated poly(dimethylsiloxane), or HPDMS. Incorporating a certain percentage of 1,4-
bis(dimethylsilyl)benzene (BDMSB), a significantly shorter molecule, increased the transition temperatures as seen in Figure 3-4. This was to be expected, as the aromatic character of this molecule would require more energy to induce molecular motion associated with the glass transition. Increasing the percentage of BDMSB from 30 to 70% raised the $T_g$ of an otherwise identical LCE by about 20 °C.

The LCE network is extremely variable from sample to sample, even when the materials have the same composition and extreme care is taken to replicate the synthesis procedure exactly each time. The random way in which the molecules react and bind and orient themselves within the material makes every sample unique. It was important to collect information regarding the range of values that a given property, like $T_g$ for instance, could assume for materials of identical composition. For example, all of the materials shown in Figure 3-5 are E(80-5H/20-5tB) – 26.7% CL(3V) – [100-HPDMS], but all were produced from different syntheses. As is clear from a cursory examination of Figure 3-5, $T_g$ can vary up to 15 °C from synthesis to synthesis even when protocol is followed precisely in each.

In all of the above tests, the clearing transition tended to be obscured by the $T_g$, making it difficult to do any analysis other than the recognition of general trends. This sufficed for the identification of the optimal material composition, but a more precise characterization of the specific materials used in cell culture experiments was desirable. Because the glass transition and clearing temperatures are extremely close and thus near impossible to distinguish in a traditional DSC
run like those in Figure 3-5, an annealing technique was used to make the actual temperatures of the transitions more obvious. The sample was equilibrated at 100 ºC, held isothermally for 10 min, and cooled to the temperature at which a maximum was reached on the second cool of the traditional DSC. The sample was then held isothermally at this temperature for 60 min before finishing the cooling cycle to -20 ºC, where it was again held isothermally for 10 min before heating once more to 100 ºC. The result was a clear separation of $T_g$ and $T_c$, as seen in Figure 3-6. $T_g$, as found using the half-delta criterion described above, averaged 46.3 ºC with a standard deviation of almost 6 ºC. This data gives testament to the variability inherent to LCE structure even in materials of identical composition.

**Modulus vs Temperature**

Dynamic Mechanical Analysis (DMA) is a tool used to analyze the viscoelastic properties of materials using a variety of experimental procedures involving variables like stress, strain, and temperature. In this thesis, DMA was used to assess the effects of temperature on the modulus of the LCE. DMA data was also useful for establishing relationships between differences LCE composition and bulk modulus at a given temperature. Of particular interest was the material’s modulus at 37 ºC (body temperature) and how it could be affected by varying different components of the LCE, like mesogen composition, crosslink density, and spacer type. DMA results can also be used to confirm $T_g$ conclusions drawn from DSC data. Since materials experience a dramatic drop in modulus as
they are heated through their $T_g$, a spike in the Tan Delta plot can be used as a relatively accurate estimate of the material’s $T_g$.

For this analysis, the samples were loaded into the DMA and were allowed to equilibrate at -80.00 °C where they were held isothermally for 5 min. Data collection was begun as temperature was ramped at a rate of 3.00 °C/min up to 150.00 °C. This procedure was used on all LCE samples in Figures 3-7 and 3-8. It is clear from the data presented in Figure 3-7 that a modest increase in the percentage of $5H$ (30%) resulted in a dramatic increase in bulk modulus of the LCE at temperatures up to about 65 °C. At temperatures higher than 65 °C, the material with 80% $5H$ displayed a lower modulus than its 50% counterpart, and at very high temperatures ($>130$ °C), the two materials had almost identical moduli. This is thought to be a result of a comparable softening of all mesogens in the material at these high temperatures, in addition to the purely isotropic nature of mesogen orientation throughout the entire sample. Furthermore, the Tan Delta plot confirms once again that increasing the $5H$-to-$5tB$ ratio increases a material’s $T_g$.

The reproducibility of DMA data collected for each of two materials having identical compositions, but prepared in different syntheses (Figure 3-8), suggests that the mechanical properties of LCEs with identical compositions are relatively consistent despite inevitable differences in network structure and DSC data regarding $T_g$. This was encouraging for the potential applications in which these LCEs could be used because it marginalized the unpredictability of network organization despite careful execution of material synthesis.
Material Selection

Ultimately, it was decided that E(80-5H/20-5tB) – 26.7%(3V) – [100-HPDMS] samples had the most optimal mechanical properties for the cell experiments in mind. These materials have an average T_g of 46.3 ºC, as calculated from the T_g of each sample tested using DSC with annealing, the results of which are shown in Figure 3-6. Cells are typically grown at 37 ºC, human body temperature, which is far enough below the average T_g to ensure that strain imparted on the materials can be maintained with minimal recovery during cell culture. As will be discussed in the next section, samples strained to 50% recovered only 10% at 37 ºC. Those stretched to 100% strain recovered 20% at the same temperature.

In addition to an ideal T_g for flexibility in the range of cell experimental designs possible, E(80-5H/20-5tB) – 26.7%(3V) – [100-HPDMS] samples have a storage modulus of about 15-25 MPa at 37 ºC, as seen in Figure 3-8. The average Young’s modulus of mouse neural tissue has been reported to be 7-200 MPa. Tendons have been found to have an elastic modulus of 300-1200 MPa. Based on these estimates, the modulus of the LCEs to be used for mouse fibroblast culture seems appropriate.

Mechanical Anisotropy

The LCE samples were strained as described in Chapter 2, using the stretching apparatus originally designed to assess molecular orientation via x-ray analysis. Each sample was stretched and fixed at 50% strain, then allowed to
recover in a 37 ºC water bath before being tested in the radial orientation as depicted in Figure 2-6 using the parameters discussed in Chapter 2. Having recovered to the extent it would at 37 ºC prior to the first test, the sample was simply repositioned on the bottom plate after the radial run and tested in the same manner in the tangential direction. After both radial and tangential tests were completed for the sample at 50% strain, the material was relaxed over boiling water. The fully recovered sample was then strained to 100%, and retested in each orientation using the same methods described for the sample at 50%. Tests were run on E(80-\textit{5H/20-5tB}) – 26.7% CL(3V) – [100-HPDMS] samples selected for cell culture based on other mechanical properties conducive to cell growth as discussed above. The strain percents were selected so that a progression could be seen in cell response to

As previously mentioned, strained samples were allowed to recover in a 37 ºC water bath for 10 min before rheology experiments were conducted. This was done to account for any recovery the samples might undergo during preparations done at 37 ºC prior to cell plating. Previous recovery experiments have indicated that for LCEs of this composition, 10 min is adequate time to allow for equilibration. In other words, holding the sample isothermally for any longer has no additional effects on shape recovery. It was found that the amount of recovery displayed by the samples increased approximately linearly with increasing initial strain, which is to be expected due to the linear increase in stored energy in a sample in response to increasing strain percentage, much like that in a spring stretched to a greater length. Those LCEs that were stretched to
50% strain lost about 10% of their strain in the 37 °C bath, making the final sample strain 40%. Those stretched to 100% lost about 20%, making their final strain 80%.

The tests were conducted at 37 °C because that is the temperature at which the cells used in future experiments were to be cultured. However, during equilibration, the actual temperature reached a maximum about 41 °C for about 10 s because of overshoot during heating above room temperature due to a delay in temperature sensing and adjustment by the rheometer. This may have induced some additional recovery prior to data collection, but the effects were assumed to be minimal because T_g of the samples tested averaged 46.3 °C, approximately 5 °C above the maximum temperature reached during equilibration. Additionally, the appearance of the sample, including sample length, did not change from pre-equilibration to post-experiment.

The oscillation procedure with the parameters shown in Table 2-2 was begun and data was collected and analyzed using Sigmaplot, the results of which are shown in Figure 3-9. It is clear from the data that the LCEs do in fact display strain-induced anisotropic moduli with the stiffer direction being that parallel to the straining direction (tangential orientation), as predicted. Once again, this raw data is a measure of the apparent moduli of the samples, assuming a circular geometry over the entire face of the bottom plate of the rheometer. The implications of this were discussed at length at the end of Chapter 2. Thus, tangential-to-radial ratios were calculated and plotted as in Figure 3-10, which clearly shows that the ratio of the tangential-to-radial modulus increases as strain
percent is increased. This trend is indicative of a systematic increase in domain alignment with increasing strain.
Figure 3-1. Thermal analysis of two elastomers with identical mesogen and spacer ratios, but different crosslinker percentage was used to ensure thermal stability of the material through cell culture temperature. Samples were equilibrated at 30.00 ºC and data collection was begun as temperature was ramped to 600.00 ºC at 10.00 ºC/min.
Figure 3-2. Thermal analysis of three elastomers with identical mesogen composition and crosslinker percentage, but different spacer ratios was used to ensure thermal stability of the material through cell culture temperature. Samples were equilibrated at 30.00 °C and data collection was begun as temperature was ramped to 600.00 °C at 10.00 °C/min.
Figure 3-3. Second heat (-50.00 °C to 100.00 °C at 10.00 °C/min) and second cool (100.00 °C to -50.00 °C at 10.00 °C/min) DSC data of two elastomers with identical mesogen and spacer ratios, but different crosslinker percentage was used to assess the effect of varying crosslink density on $T_g$ and $T_c$. 
Figure 3-4. Second heat (-50.00 °C to 100.00 °C at 10.00 °C/min) and second cool (100.00 °C to -50.00 °C at 10.00 °C/min) DSC data of three elastomers with identical mesogen composition and crosslinker percentage, but different spacer ratios was used to assess the effect of spacer length on $T_g$ and $T_c$. 
Figure 3-5. Traditional DSC [second heat (-50.00 °C to 100.00 °C at 10.00 °C/min) and second cool (100.00 °C to -50.00 °C at 10.00 °C/min)] of several E(80-5H/20-5tB) – 26.7%CL(3V) – [100-HPDMS] samples was completed and the maximum exotherm was found as each is labeled. The temperature at which this maximum exotherm occurred for each sample was the temperature at which that sample was annealed in future DSC experiments.
Figure 3-6. DSC with annealing of the same four E(80-5H/20-5tB) – 26.7%CL(3V) – 100%HPDMS [100-HPDMS] samples used in the traditional DSC shown in Figure 3-7 was used to help separate and thus differentiate between T_g and T_c. Samples were equilibrated at 100 °C, held isothermal for 10 minutes, and cooled to the temperature at which a maximum was reached on the second cool of the traditional DSC, as shown in the legend. Each sample was then held isothermal at this temperature for 60 min before finishing the cooling cycle to -20 °C. T_g averaged 46.3 °C with a standard deviation of about 6 °C.
Figure 3-7. DMA of two elastomers with identical crosslinker percentage and spacer ratios, but different mesogen compositions was used to assess the effect of varying mesogen composition on modulus-vs.-temperature behavior of the material. Samples were allowed to equilibrate at -80.00 °C, then held isothermal for 5 min. Data collection was begun as temperature was ramped at a rate of 3.00 °C/min up to 150.00 °C.
Figure 3-8. DMA of two of the E(80-5H/20-5tB) – 26.7%CL(3V) – [100-HPDMS] samples analyzed with DSC shown in Figures 3-5 and 3-6 (corresponding sample numbers) shows reproducibility of mechanical properties in samples of the same composition. Samples were allowed to equilibrate at -80.00 ºC, then held isothermal for 5 min. Data collection was begun as temperature was ramped at a rate of 3.00 ºC/min up to 150.00 ºC.
Figure 3-9. Oriented rheological analysis of strained LCE samples was performed using an oscillation time sweep procedure at 37.0 °C with an approximately linear displacement of 0.001 rad at 6.283 rad/sec. Results show that the strained samples have anisotropic moduli, being stiffer in the direction of stress application during the straining process. Additionally, increasing strain percent results in a larger differential between the two directions (parallel and perpendicular to the stretching axis).
Figure 3-10. Oriented rheological analysis of strained LCE samples was performed using an oscillation time sweep procedure at 37.0 °C with an approximately linear displacement of 0.001 rad at 6.283 rad/sec. In order to establish a relationship between increasing strain and degree of anisotropy, the ratio of tangential-to-radial apparent moduli was calculated and plotted as shown above. Results indicate that increasing strain percent results in a higher degree of anisotropy.
Chapter 4: Cell-Material Interaction

After an LCE composition was developed that yielded a film with appropriate mechanical properties was developed and fully characterized, it was successfully demonstrated that the material displayed strain-induced anisotropic modulus. Previous research has suggested that cells have the ability to “feel” and respond to differences in substrate modulus when cultured in vitro. The goal of the work outlined in this chapter was to analyze the extent to which effects, if any, of substrate anisotropy on cell alignment. This analysis was conducted by straining and sterilizing LCE samples, plating cells on the strained samples, allowing them to incubate, then staining and imaging the cells. A visual comparison of cells grown on each sample of varying strain was used to draw conclusions about the potential for directed cell growth on these anisotropic materials.

Embryonic mouse fibroblast (C3H10T1/2) cells were used to test for substrate effects on cell behavior. These cells excrete an extracellular matrix (ECM) composed mostly of collagen. These cells can readily remodel their ECM in response to external stimuli. This remodeling capability makes these cells ideal for the assessment of mechanical stimuli, such as anisotropic modulus, on cell morphology and behavior.

Sample Preparation for Cell Plating

Cell plating was accomplished using a 2-day procedure. On the first day, LCE samples were prepared for cell culture. Each film of uniform thickness was
cut into three rectangular strips at least 2.00 mm wide and 5.00 mm long using a standard razor blade. The rectangular strips were then boiled in water for 3-4 minutes each before straining in order to ensure complete relaxation of the sample and as a method of preliminary “cleaning”.

The boiled samples were then stretched using the same apparatus pictured in Figure 2-5 that was used for straining in anisotropy analysis. For each film, one strip was left as-is after boiling for a 0% strain control. A second strip was stretched to 50% strain and the third to 100%. The strain percentage was calculated by measuring the distance between the clamps of the stretching apparatus before and after stretching, and dividing the difference by the original, pre-stretched distance.

After samples were loaded into the stretching apparatus and initial measurements were obtained, a heat gun was held at a distance above the sample that allowed for a constant temperature at or above 80 °C, ensuring the material was above isotropization temperature at all times during the straining process. Concurrently, the knob on the sample was turned clockwise while the strain gauge was observed until the desired strain was reached. Strain rate was approximately 50%/min for all samples. The sample was allowed to equilibrate under the heat gun in its stretched conformation for at least 30 s before the heat was removed and final measurements for strain calculation were obtained. The entire device was then placed in the freezer at -8 °C for at least 2 min to ensure complete shape fixing. The sample was removed from the apparatus and stored in a sealed 15 mL glass vial in a cool location until use for cell culture.
Cell Plating

Strained samples intended for use in cell culture experiments were soaked overnight in a 70% ethanol solution for sterilization. Each sample then underwent UV sterilization for 30 min on each side. They were then soaked in complete cell nutrient media for 3.5 h in an incubator at 37 °C. This step was added because similar samples prepared in this way were found to be more conducive to cell adhesion than those that were not because protein adsorption from the media onto the material surface aides in cell adhesion.

Samples tended to float in cell media, and thus needed to be anchored to the bottom of the Petri dishes in which they were incubated. A 3% GTG agarose mixture was thus used as a “cement” to hold the samples in place without affecting normal cell processes. The agarose powder was added to Milipore water (4 g agarose to 100 mL water) and the solution was autoclaved at 121 °C for 5 min to sterilize before entering a 20 min cool-down cycle. One drop of the agarose solution (about 10-20 µL) was used to secure the samples in place.

The C3H10T1/2 line of mouse fibroblast cells was cultured on a monolayer plate and used for all cell-material interaction experiments. The bond between the cells and the plate on which they are grown were cleaved using an enzyme called trypsin. After the bonds were cleaved, a media/cell suspension with trypsin formed. Trypsin is harmful to cells in large quantities and/or for long periods of exposure. The mixture was centrifuged to yield a pellet of cells and a supernatant containing trypsin that had been inactivated by other enzymes in the complete cell nutrient media. The supernatant was removed via vacuum and a
new, trypsin-free cell/media suspension was prepared. This suspension was transferred to the anchored LCE samples and cells settled to the bottom for attachment in about an hour. The cultures were allowed to incubate at 37 °C for 3.75 h before imaging.

Cell Staining

The Fluorescence Microscopy Protocol for LIVE/DEAD® Viability/Cytotoxicity Assay Kit from Molecular Probes, Inc. was used to stain the cells plated on the LCEs prepared as explained above. This assay allowed the orientation to be viewed under 5x or 10x magnification. Additionally, live cells (green) were easily distinguishable from dead (red) cells. Stains were prepared in the concentrations suggested in the protocol. The cells were incubated at 37 °C for 20-30 min after stain application before viewing under the microscope.

Cell Imaging

A Leica DMI-4000 B Microscope and Leica Application Suite (Leica Microsystems, Version 3.5) were used to capture images of the stained cells on the LCEs. Since the materials were opaque and fluorescence used was emitted from the bottom platform, the samples had to be flipped over so that the side on which the cells were attached faced downward. Fluorescent light shone up from the bottom of the instrument, and excited fluorescence in the dye, which shone back down, where the camera captured the image. Light settings for Calcein AM (515 nm) and Ethidium heterodimer-1 (635 nm) fluorescence were used to image
the live and dead cells, respectively. In both cases, the excitation wavelength was 495 nm.

Images were captured in grayscale, and exposure was adjusted in Leica Application Suite so that the display showed no under- (dark) or over- (white) exposed areas. On average, exposure times were set to 1 s for dead cell image capture and 250-500 ms for live cell image capture. Once grayscale images were acquired, the appropriate colors were applied using the software (green to 515 nm wavelength light for live cells, and red to 635 nm wavelength light for dead cells).

**Cell Behavior**

For conclusions to be drawn regarding substrate effects on cell behavior, a control plate was required. A smooth polystyrene sample was chosen because it is the material typically used for culturing fibroblasts. The material is known to be nontoxic and is conducive to cell adhesion and proliferation. Cell behavior on smooth polystyrene substrates such as those used in this research, particularly fibroblast behavior, is well characterized and was considered “normal” *in vitro* behavior for the purposes of this thesis. This normal fibroblast morphology and cell density is depicted in Figure 4-1. The cells were spread out in a monolayer, with roughly spherical cell bodies averaging about 15 µm in diameter. These cell bodies were surrounded by fibrous anchors extending up to 40 µm out from the cell body. The anchoring fibers are irregularly shaped and stretch out from the cell body in apparently random directions, with some appearing more uniformly dispersed and others stretching significantly farther along one axis than another.
Cell density on these materials after 3.75 h in culture was calculated to be about 111 cells/mm$^2$ and was relatively uniform over the entire surface of the sample.

Cells were more likely to adhere to unstrained E(80-5H/20-5tB) – 26.7% CL(3V) – [100-HPDMS] films around the edges of the samples. Cell density close to the edges was calculated to be about 30 cells/mm$^2$, while average cell density in the center region of the sample was found to be about 7 cells/mm$^2$.

Though cell orientation was random, the cells did appear to have and extended fibers along one axis, as can be seen in Figure 4-2, making the cells more spindle-like than those seen on the polystyrene control. This organization may or may not have something to do with the smectic organization of mesogens within their respective domains. Further experimentation is needed to confirm or disprove the involvement of mesogen organization in the cells’ adoption of a spindle shape.

The most uniform cell adhesion on LCE samples was seen on those strained to 50%, as can be seen in Figure 4-3. The cell density on these materials was found to be about 17 cells/mm$^2$ and was only slightly lower than this in the centermost region of the sample. Cells on this material had morphologies more similar to that of those plated on the polystyrene control, but did appear to have a slightly modified anchoring network. Rather than a relatively uniform halo-like extensions from the cell body, as was seen with the control cells, the cells on this material generally seemed to have one or two strong connections with the underlying substrate far away from the cell body. The gaps between these anchor sites and the cell body were then bridged by relatively thin strips of fibers. They
did not appear to have a very extensive collagen matrix immediately surrounding the cell body. The reason for this behavior is not known.

\[E(80-5H/20-5tB) - 26.7\% \text{ CL(3V)} - [100-HPDMS] \text{ samples strained to 100\% were not very conducive to cell adhesion, as is clear from Figure 4-4. Very few cells were found to adhere to these materials, and those that did were found primarily at either end of the samples. These cells appeared to have a more dense anchoring network in the immediate vicinity of the cell body, with fewer projections branching out and anchoring far away from the cell body. Than had been seen in any of the other LCE samples or the polystyrene control. Again, further experimentation would be necessary to draw any conclusions about whether the high degree of anisotropy in these materials is a contributing factor in this morphology, or to determine what role (if any) the anisotropy plays in discouraging cell adhesion.\]

Summary and Conclusions

The hypothesis presented in this thesis had two components. First, it was hypothesized that main-chain LCEs that were known to adopt higher degrees of orientation upon straining, would experience strain-induced anisotropic modulus. A new rheological procedure was developed to confirm that the systematic increase in mesogen alignment with increasing strain did, in fact, impart anisotropic modulus on the materials. Second, it was proposed that this anisotropic modulus may have some effect on cell behavior, specifically cell
alignment, when the strained materials were used as a substrate in the culture of fibroblast cells.

It was thought that the cells would be randomly oriented on the samples with 0% strain, would show some semblance of alignment on the samples strained to 50%, and would be highly oriented on the 100% strained samples. No effects on cell alignment were noted in the preliminary cell culturing experiments conducted in this thesis. It was found, however, that the unstrained LCEs, and those strained to 100%, were much less conducive to cell adhesion than the moderately strained samples (50%) and the polystyrene control. Further experimentation is necessary before it can be determined whether or not this observation is a result of anisotropic modulus.

In conclusion, strain-induced mesogen alignment in LCEs causes the material to develop anisotropic modulus, with the axis of greater stiffness along the direction of strain. The ratio of the modulus along the longitudinal axis (in the direction of strain) to that along the transverse axis (orthogonal to the direction of strain) appears to increase linearly with increasing strain, as the ratio calculated for materials with 100% strain was about double that calculated for materials with 50% strain. It is still unclear how this anisotropic modulus may or may not affect cell behavior, but it does not appear to have any significant effect on cell alignment. The anisotropy may, however, affect cell adhesion to the materials. Further experimentation and analysis is needed to draw any conclusions regarding the reasons for the anisotropy’s effects, or lack thereof, on cell behavior.
Figure 4-1. Control C3H10T1/2 cells were plated on smooth polystyrene and stained using the LIVE/DEAD® Viability/Cytotoxicity Assay Kit from Molecular Probes, Inc. after a 3.75 h incubation period. Cell viability and morphology on this control material were the standard to which those cells on LCE samples were compared.
Figure 4-2. Cells plated on unstrained, or 0% strain, E(80-5H/20-5tB) – 26.7% CL(3V) – [100-HPDMS] samples and incubated for 3.75 h adopted a morphology similar to those cultured on the polystyrene control, but tended to be found in higher densities around the edges of the sample than toward the center.
Figure 4-3. Cells were found to adhere extremely well to samples strained to 50% and were found to be relatively evenly dispersed over the entire sample, with only slightly higher densities toward the ends of the sample.
Figure 4-4. Very few cells attached to those samples possessing 100% strain. Those that did adhere were most commonly found at either end of the strained sample.
Works Cited

Capstone Summary

Tissue engineering is a field of bioengineering that examines the possibility of culturing cells in such a way that they organize into a tissue. It is a continuously evolving area of bioengineering research, and there is currently a strong movement toward “biomimetic” tissue engineering methods. In other words, researchers are working to simulate, or mimic, the in vivo environment experienced by naturally occurring cells as closely as possible. The hope is that this biomimetic environment will encourage cells to appropriately differentiate and arrange themselves into the organized tissue structures found in the body.

Many tissues in the body display anisotropic mechanical properties. Their strength, rigidity, etc. depend on the direction in which they are tested. This anisotropy is the result of the mechanical requirements necessary to perform the basic function of that tissue in the body. These requirements are often different in one direction than in the orthogonal (perpendicular) direction. For example, bone can withstand higher stresses along its long axis (in the direction of normal loading in the body) than it can along its transverse axis (perpendicular to the direction of loading). Muscles are stiffer, or have a higher modulus, along their long axes, in the direction that experiences high stresses in skeletal movement. Blood vessels have greater circumferential strength because the pulsatile stresses that result from blood flow require that the vessels resist bursting.

Cells tend to align themselves in the direction of greatest substrate stiffness and appear to impart greater strength to the overall tissue in that direction. This is how many tissues are organized – as a network of precisely
aligned cells. Thus, one of the goals of tissue engineering is to find ways to coax cell alignment \textit{in vitro} for certain applications. However, cells in culture tend to randomly orient themselves when cultured on isotropic substrates that display the same mechanical properties in every direction. Mimicking the anisotropic properties of the \textit{in vivo} environment with the culture substrate is one proposed solution to this challenge of inducing cell alignment and ultimately, the development of an organized tissue.

There are several factors to consider when developing an appropriate anisotropic substrate for cell culture. Perhaps most obviously, the substrate must be stable at the standard culturing temperature of 37 °C (body temperature). The material must also possess an order at the molecular level that imparts on it directionally-dependent mechanical properties (i.e. anisotropic modulus). Additionally, in alignment with the principles of biomimetics, it would be referable that the material’s modulus fall within a range similar to that of body tissues with which the cells would normally come into contact \textit{in vivo}.

The liquid crystalline elastomers, or LCEs, used in this research possess all three of these properties. LCEs are polymers consisting of two types of molecules: rigid rod structures called mesogens, and flexible spacers. The rod-like mesogens are connected end-to-end by flexible spacers to form polymers that are organized into individual domains. These particular materials posses what are called smectic-C domains, meaning that the mesogens within each domain are aligned in a diagonal pattern. Though the mesogens within these domains are ordered, the domains themselves are randomly oriented within the material. The
domains are crosslinked to each other by crosslinker molecules, creating a continuous, stable network that makes up the material proper.

The materials exhibit two transition temperatures: glass transition temperature ($T_g$), and clearing temperature ($T_c$). In the context of polymers such as LCEs, $T_g$ can be defined as the temperature below which there is little movement of the domains and the material experiences a dramatic increase in stiffness. The clearing temperature can be defined as the temperature above which there is a large amount of movement and slippage among the domains, resulting in a dramatic decrease in material stiffness. Between the two, the material displays a relatively constant modulus. The exact temperatures at which these transitions occur vary with the composition of the LCE, including types and ratios of mesogens, crosslinker type and density, and spacer type. Above $T_c$, isotropization, or randomization of domain orientation, occurs. If the material is mechanically manipulated, or uniaxially stretched in the case of these experiments, above this isotropization temperature ($T_c$), the domains will shift and align themselves in the direction of strain. Cooling the material below $T_g$ will then fix the domains in this position, resulting in a fixed bulk material deformation as well as a fixed domain orientation.

It has been confirmed by x-ray analysis in previous research that the domains do align in response to uniaxial straining. It is thought that this alignment might impart greater stiffness along the axis of orientation due to polymer chain organization. This research worked to determine the accuracy of the hypothesis that anisotropic modulus was induced by domain alignment due to uniaxial
stretching of LCE films. Furthermore, once mechanical anisotropy was confirmed, its effects on cell behavior were assessed by culturing mouse fibroblast cells on the stretched, and thus anisotropic, substrate.

First, different LCEs were synthesized systematically, varying mesogen type and ratio, spacer type and ratio, and crosslink density. The effects of changes in each of these components on mechanical properties of the material were assessed using thermogravimetric analysis (TGA), differential scanning calorimetry (DSC), and dynamic mechanical analysis (DMA). TGA confirmed the thermal stability of the material, DSC was used to locate the precise temperatures at which glass and clearing transitions occurred, and DMA established the bulk modulus, or average material stiffness, assumed by the material at a given temperature. Once composition effects on these properties were established, the material’s chemical composition was altered using the established relationships to tailor LCE properties to meet the specifications required for successful cell culture.

Since there exists no traditional procedure to test for the strain-induced anisotropic modulus predicted due to domain alignment, a new rheological procedure was developed. Rheology is the study of how viscous materials behave under specific conditions. For the purpose of this research, a rheometer served to yield data that could be used to confirm the prediction of anisotropic modulus in the strained LCE samples. A rheometer is an instrument used to measure the flow behavior of a viscous material in response to applied forces. There is a traditional rheological procedure in which an indenter tip is pressed into the surface of a
material and drug a short distance. The resistance of the material to this
deformation is transduced and measured as a force by the rheometer. This force is
then translated into a modulus within the software.

Using an oscillation time sweep procedure, a method very similar to the
one currently used to obtain a modulus measurement was employed to establish
relative moduli of strained LCE samples in the directions parallel and
perpendicular to the axis of strain. Since the rheometer in our lab used rotational
methods and was thus only capable of producing angular, as opposed to straight-
line, displacements, an extremely small displacement angle (0.001 rad) was used
to approximate the traditional straight-line indent-and-drag procedure. It is
important to note that this procedure was not intended to give a true modulus, but
simply to compare the stiffness (or resistance to deformation) of the material in
the direction of strain to that in the direction perpendicular to the stretching axis.

The method described above confirmed the hypothesis that straining an
LCE sample did, in fact, impart anisotropic stiffness on the material. The samples
were found to have a higher modulus in the direction parallel to the axis of strain,
much like naturally occurring body tissues. Furthermore, it was found that
increasing the strain percentage of the material increased the anisotropy, with a
larger differential between the stiffnesses of each direction in samples stretched to
greater final lengths. This was attributed to a systematic increase in the orientation
of the domains with increasing strain. In other words, the domains gradually
aligned themselves along the stretching axis as the material was stretched to
greater lengths.
Once it was established that these materials could display anisotropic modulus, it was of interest to see whether or not cells could “feel” and respond to this anisotropic substrate stiffness. The material chosen for use in cell culture experiments had a $T_g$ above 37 °C, the temperature at which cells are incubated. This ensured minimal recovery, or loss of strain, during the cell culturing process. Strained samples that were held at 37 °C for 10 minutes lost 10-20% of their initial strain, with those samples originally stretched to greater lengths losing more than those stretched to relatively smaller strains.

Mouse fibroblast cells, precursors to those cells found in organized tissues such as tendons and ligaments, were plated and observed on samples with 0%, 50%, and 100% strain. It was thought that the cells would be randomly oriented on the samples with 0% strain, would show some semblance of alignment on the samples strained to 50%, and would be highly oriented on the 100% strained samples. It was found, however, that the strain-induced anisotropic modulus of LCE substrates had a more pronounced effect on cell adhesion than it did on cell alignment or morphology.

LCEs like those synthesized and characterized in this research could lead to breakthroughs in the tissue engineering field. Highly ordered tissues such as muscles, tendons, and ligaments, are notoriously difficult to produce in vitro because it is difficult to establish an environment conducive to cell differentiation, alignment, and networking similar to that found in the body. Mechanically anisotropic substrates, used in combination with appropriate proteins, growth factors, and cell signaling molecules, could lead to enhanced alignment and
differentiation to produce tissues \textit{in vitro} that possess mechanical properties more similar to those of natural tissues than have been previously possible.

Additionally, the LCEs synthesized throughout this research have the potential to be further explored in future work. For example, one study has shown that mesenchymal stem cell differentiation is influenced by substrate modulus, with neurological lineages found to result from cultures on 0.1-1 kPa substrates, myogenesis is most prominent on 11 kPa substrates, and osteogensis is typical of 34 kPa substrates. Additionally, relative elasticity within the substrate has been found to have a more significant effect on cell behavior than absolute stiffness does. In other words, varying substrate stiffness within a single plate has a more profound effect on cell behavior than a comparison of cells cultured on substrates with uniform stiffness, where stiffness is varied from one substrate to the next.

One of the LCEs synthesized during experimentation displays its steepest modulus change across the temperature range tolerable for cell growth. If a temperature gradient could be produced across the material to allow for a modulus gradient, it would be interesting to see how the cells would respond to this gradient. If the modulus change is just right, with the change over the media similar to that across a tissue junction found \textit{in vivo} (ex. bone-to-tendon), we may be able to create a junction of different types of cells cultured on the gradient and gain insight about their interactions.

In previous studies, differentiation was determined based on protein markers produced by each cell as a result of gene expression, as could be done here. Based on the results of the anisotropic stiffness experiments, perhaps a
system could even be designed that allows for the simultaneous application of anisotropic stiffness (using clamps to hold the sample at the required strain) and a modulus gradient, creating the potential for oriented tissue junctions. In this case, the different cell types that differentiate according to the bulk modulus of the substrate area on which they adhere, could align in the direction of strain to produce a tissue-tissue junction in which the cells of each possess a high degree of orientation. This could be a very exciting development in the field of tissue engineering.