Dynamic Mechanical Properties of Cockroach *(Periplaneta americana)* Resilin

by

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Resilin is a cuticular protein found in a variety of insects. It can stretch up to 300% of its natural length without any creep or relaxation. Further, it operates across a wide frequency range from 5 Hz in locomotion to 13 kHz in sound production. Both the protein sequence and composition of natural resilin as well as the dynamic mechanical properties vary substantially across species. This suggests that mechanical properties may be evolutionarily tuned for specific functions within an insect. Here, samples of resilin obtained from the tibia-tarsal joint of the cockroach, *Periplaneta americana*, were tested using a custom built dynamic mechanical analyzer. The material properties in compression are obtained from the rubbery to glassy domain with time-temperature superposition (2^oC to 50^oC) and time-concentration superposition (0 % to 93% ethanol by volume in water). At low frequency the storage modulus was found to be 1.5 MPa increasing to about 5 MPa in the transition zone. The glass transition frequency at 22^oC in complete hydration was found to be 200 kHz. The data shows that cockroach resilin is less resilient than dragonfly resilin at low frequencies, returning about 79% of the elastic strain energy at 25 Hz compared to 97% for dragonfly resilin. However, at the glass transition (200 kHz) the material returns about 47% of the elastic strain energy compared to 30% in dragonfly (2MHz). The resilin pad in cockroach is a composite structure, acting as a compressive spring to passively extend the tibia-tarsal joint during cockroach locomotion. Its mechanical properties are more similar to the composite locust pre-alar arm than to the pure resilin dragonfly tendon, suggesting that macroscopic structural influences may be as important as molecular sequence differences in setting properties.

Acknowledgements

I shall like to thank my committee members, Dr. John Lesko and Dr. Robert Moore for their guidance and helpful discussions throughout the project. I appreciate Kathy Lowe's help with the Transmission Electron Microscopy images. Lastly, I am very grateful to my advisor Dr. Dan Dudek for his support and guidance throughout my studies at Virginia Tech.

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Introduction

Resilin is a cuticular protein first described in literature by Torkel Weis-Fogh in 1960 as elastic tendons in dragonflies and elastic wing-hinges in locusts(Weis-Fogh 1960). It can be strained upto 300% in tension and atleast 70% in compression a 10 fold variation in length(Weis-Fogh 1961). No material flow in creep and relaxation was observed even after weeks of straining and it snapped back to the original length on release of load. These amazing mechanical properties inspired further research leading to identification of resilin as elastic energy storage device in the salivary pump of assassin bugs(Edwards,1960),the feeding pump of Rhodinus proxilus (Bennet-Clark 1963),the spring mechanism propelling flea jumping (Bennet-Clark 1967) ,the wing folding mechanism of Dermapetra (Haas et al. 2000) and in the tibia tarsal joints of cockroaches (Neff et al. 2000) to name a few. This diverse functionality operates over a broad frequency range in nature from 6Hz in the tibia tarsal joint of cockroaches (Kram et al. 1997 ; Neff at al. 2000) to 1000 Hz in fleas which releases strain energy for jump in less than 1ms (Bennet-Clark 1967) and over 13 KHz(Fonseca 1998)in sound producing tymbal mechanism of cicada. Naturally occurring resilin is highly fatigue resistant and is used for millions of cycles in nature without failure. Resilin developed in the pupal stage of the fruit fly *Drosophila* survives for its entire lifetime of adult insects operating at 720,000 cycles per hour(Lehmann and Dickinson 2001; Elvin 2005).

Neff et al.(2000)identified resilin patches in the cockroach (*Periplaneta americana*) in the tibial-tarsal joints and the Ta4-Ta5 limb segments as an elastic structure assisting in locomotion. Data on the dynamic mechanical properties of resilin encompasses investigations by Anderson on locust resilin(Anderson 1964) and King on dragon fly resilin(King 2010). While the locust resilin was investigated in a range of frequencies from 10-200 Hz, a complete master curve of dragon fly resilin was produced by King. These data show that that

the dragon fly resilin is more resilient than locust resilin at low frequencies and has a larger rubbery plateau with frequency independent characteristics . Further, Anderson(1964)showed that the resilience of locust resilin begins to decrease at 100 Hz which implies the onset of the glass transition. Hence, if resilin in different insects had similar properties it would not have been possible for Cicada to use it at 13 KHz (King 2010).This necessitates investigation of the properties of other natural resilins to understand their particular function in insects. Moreover, resilin in nature can be either a pure polymer or a composite of resilin and chitin .It was suggested that being a composite was a driving factor in decreased resilience of locust resilin (King 2010). It may be used as a compressive structure, bending structure or in dynamic loading. Further, the static material properties of resilin differ in compression and tension.

The amazing static and dynamic mechanical properties of resilin provide a framework to understand the structure-property relationship of natural biomaterials as well as the role of passive energy storage mechanisms in nature. Resilin has the potential for use in biomedical applications like spinal disc implants, tissue generating scaffolds, and vocal tissue implants. This requires understanding its properties at different temperature, hydration and frequency levels. Further, long fatigue life and resilience of resilin provides a template for designing new polymer composites. Recombinant resilin from different insects is expensive to produce. On the contrary, natural resilin provides a cheaper and accessible alternative for mechanical studies as well as providing a basis to develop synthetic resilin from a particular insect tuned to perform in a particular engineering environment. This paper will investigate the dynamic mechanical properties in compression of cockroach tibia tarsal resilin, a composite structure(as evident from TEM images in Figure11) similar to locust and compare them with dragonfly and locust resilins. Further, six primary factors affecting the resilience of resilin: frequency, temperature, hydration, mechanical structure, chemical composition and influence

of compressive and tensile stress will be studied to understand the structure-property relations and differences in properties of naturally occurring resilin.

Resilin is largely used as a passive elastic storage device in insects without active neural control; a strategy that can be exploited in designing efficient terrestrial robots. I shall also discuss the specific use and the advantages of using resilin as a primary energy storing mechanism for cockroach locomotion.

Methods

Theory

Being a polymeric material, the technique of time temperature superposition (Ferry 1980) can be used to investigate the material properties of resilin. Typically, experiments are conducted in a small frequency range between 1-200Hz at different temperatures to obtain the storage modulus, loss modulus and tan δ(ratio of loss to storage modulus) as a function of temperature and frequency. The curves are then shifted to a reference temperature to form a smooth master curve spanning multiple decades of frequency(Ferry 1980).The resilience or the percentage of energy stored is calculated from

$$R = 100e^{\left(-\frac{\pi}{2}\tan\delta\right)} \quad , \tag{1}$$

Dolittle had demonstrated the dependence of viscosity (η) for liquids to follow the following equation:

$$log\ \eta = log\ A + B(v - v_f)/v_f, \tag{2}$$

where A and B are constants and v is specific volume and v_f is free volume per gram. This relation was extended by William-Landel-Ferry to the form (Ferry 1980):

$$log\ a_{12} = B(1/f_2 - 1/f_1)\ , \tag{3}$$

where a_{12} is the ratio of two viscoelastic relaxation times at temperatures T_1 and T_2 and $f = v_f/v$. It can be recast in the following equivalent form of WLF equation :-

$$log\ a_T = \frac{-(B/2.303f_g)(T_i - T_g)}{\frac{f_g}{\alpha_f} + T_i - T_g}\ , \tag{4}$$

where T_g is the reference temperature, f_g is the fractional free volume at T_g, α_f is the relative free volume expansion coefficient and B is a constant. In practice the factors $B/2.303f_g$ is taken as a constant C1 and f_g/α_f as constant C2 determined experimentally for different polymers. The universal values of C1 and C2 followed by a number of polymers are 17.1 and

51.6 ^{o}C respectively. The validity of this equation forms the basis of performing time-temperature superposition by assuming the properties of a polymer at temperature T and frequency ω_T has the same value at a lower temperature T_0 and lower frequency ω_0,or

$$G'(T, \omega_T) \equiv G'(T_0, \omega_0), \quad (5)$$

where G' is any polymer property and $a_T = \omega_0/\omega_T$. .

This superposition principle can be expanded to use changing solute concentrations, polymer blending ratios and stress levels instead of temperature (Ferry 1980).The hygral shift factor is found to follow Equation 6,

$$\log a_c = 1/f_2 - 1/f_c, \quad (6)$$

or,

$$\log a_c = -\frac{\left(\frac{1}{2.303 f_2}\right)(1 - c/\rho_2)}{\frac{f_2}{\beta'} + 1 - c/\rho_2} \quad (7)$$

where, the subscript 2 refers to the pure polymer and subscript c is the concentration of diluted system,ρ_2 is the density of pure polymer and β' is a coefficient related to the depression of glass transition temperature.

It can be concluded from Equations (2) and (6) that viscoelastic properties are a function of temperature and solvent concentration and mechanical dilatation (Knauss et al. 1981, King 2010)

or ,

$$G' = G'(T, c, \theta, fr), \quad (8)$$

where G' is any property is temperature is solvent concentration, and θ is mechanical dilatation, and *fr* is frequency.

Experimental limitation in using time-temperature superposition on fully hydrated natural resilin comes from the fact that it is possible to test it only in a range of 0^{o}C to

80 ^{o}C(King 2010).When too cold, the water molecules freeze if one goes below zero causing solid ice particles to induce errors in measurement. When too warm, chitin supporting the resilin structures starts to disintegrate at 80^{o}C which provides upper limit to the experimental conditions in water. Hence, it is essential to use time-concentration superposition at room temperature along with time temperature superposition to obtain the whole range of properties from the rubbery to the glassy domains.

Dynamic Mechanical Analysis

Sample Preparation

Live adult cockroaches*(Periplaneta americana)* were obtained from North Carolina Biological supply and were housed in a cage and fed with water and food. The cockroaches were euthanized by immersion in 70% ethanol and stored at 4^{o}C. Identification of resilin in the legs was done using Leica Microsystems Inc. M165FC optical microscope. Since resilin is found to fluorescence under ultraviolet light (Weis Fogh 1960; Neff et al 2000), a filter UV set for Leica MZ16 F/FA was used to identify the resilin pads(King 2010).Each cockroach leg has multiple resilin patches located at the tibia-tarsal joints and the Ta4-Ta5 tarsal segment joints(Neff et al 2000).The largest patch at the tibia-tarsal joint was used for experiments. The leg is first dissected separating the tibia from the tarsa at the tibia-tarsal joint leaving the resilin pad attached to the tibia. The resilin pad is then trimmed carefully under an optical microscope to provide a 0.35 mm long (mean) almost rectangular sample of resilin attached to the tibia for testing. The tibial segment is then separated from the main leg segment by cutting a 5-6 mm piece from the tibia with overhanging resilin pad. Figure 1a and 1b shows the location of resilin for the cockroach at the tibia –tarsal joint. Figure 1c shows the fluorescence of the resilin pad and a typical sample used for the experiments.

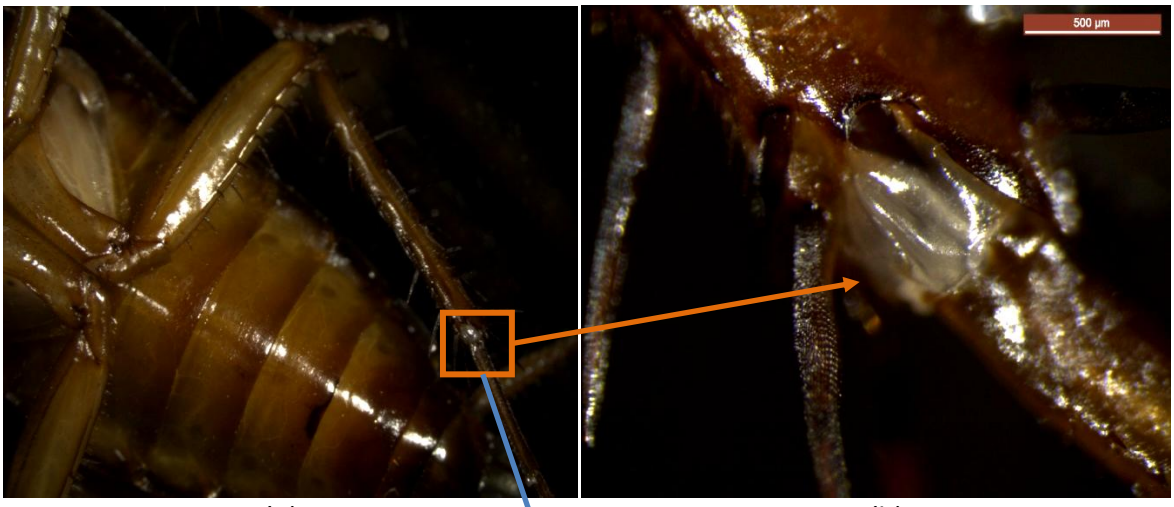

Fig 1 (a) *Fig 1 (b)*

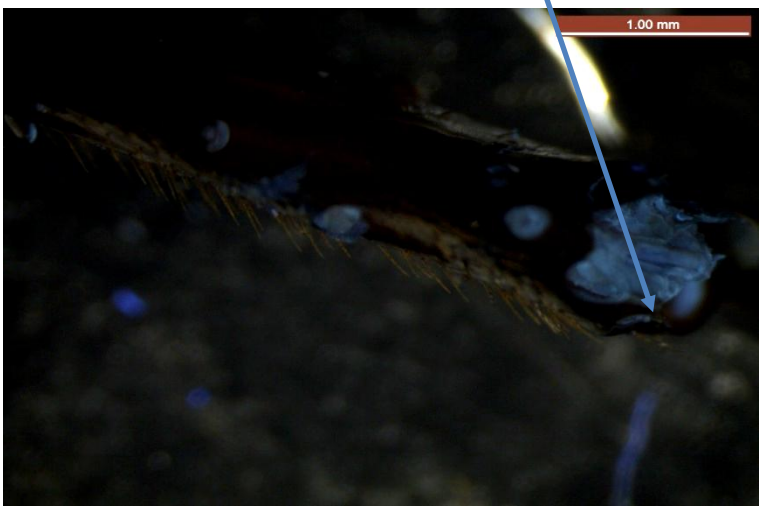

Figure 1 (c)

Figure 1(a) Resilin pad at the tibia tarsal joint of cockroach.
Figure 1(b) Enlarged view of the resilin pad
Figure 1(c) Autofluroscence of a dissected resilin pad under UV excitation.

Experimental Setup

The experimental setup(King 2010) consists of an electromagnetic oscillator(V203, LDS test and Measurement, Royston, UK) with a displacement gage attached to the oscillator shaft via a stainless steel cantilever beam, and a force gage to which the tibial segment is glued(Figure 2).The displacement gage is built on a stainless steel cantilever shaft with two backed foil gages (Vishay Micro measurements) forming a half bridge circuit. The output of the displacement is linear (7.01mV/micrometer, Rsq=0.99) and a natural frequency of

528Hz. The force gage is constructed on a stainless steel cantilever with a carbon fiber rod attached having two unbacked semiconductor gages (Micron Instruments) forming a half bridge circuit. The output is linear (0.0215N/V, Rsq=0.99)with a natural frequency of about 1000Hz.The displacement and force gage signals were amplified by Vishay Micro-measurements Strain Gage Conditioner(2120B).

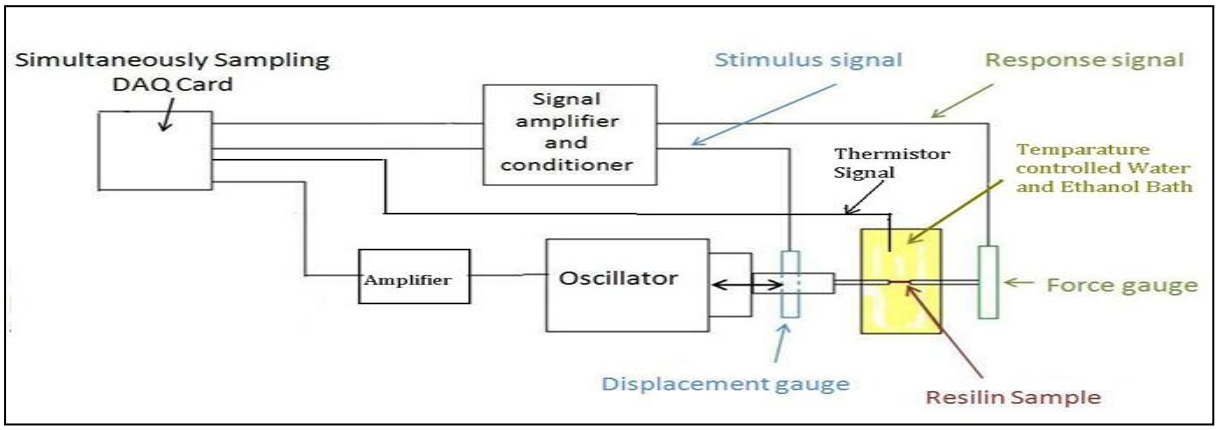

Figure 2(a)

Figure 2(b)

Figure (2a) Dynamic Mechanical Analyzer setup. Labview swept sine program provides input signal to amplifier through DAQ and drives oscillator at different frequencies. The displacement gage measures the strain and force gage measures stress at each frequency. The sample is immersed in a temperature and concentration controlled bath, the stimulus and response signal from the strain gages goes through the signal conditioner, into the DAQ and processed by LabView code.
Figure (2b) Lead indenter pressing on a dissected resilin sample immersed in water bath .

The sample attached to the force gage at one end and in contact with the lead indenter (sharpened from a 500mm pencil lead to match sample size) on the resilin end is immersed in a solution chamber .The temperature of the chamber could be controlled by using a LAUDA

RE206 temperature bath with resolution of 0.1oCand recorded by a thermistor calibrated with temperature bath. A schematic diagram of the experimental setup is shown in Figure 2.

Mounting

The sample is mounted by attaching one end of the tibial segment to the force gage tip using cyanoactrylate (Locite).A sharpened pencil lead glued to the shaft of the oscillator is used as an indenter of the resilin pad. The resilin pad was prestrained compressively by about 10 percent to ensure contact with the sample throughout the experiment.

Experimental procedure

The shaker was oscillated to produce an additional 6% strain at a frequency range of 10Hz to 90Hz using a -swept sine LabView program(National Instruments, Texas) with a custom built voltage amplifier (King 2010).The displacement and force signals from the strain gage amplifiers were recorded using a simultaneous sampling data acquisition card PCI-4461(National Instruments, TX).The time-domain signal was converted to the frequency domain to obtain complex modulus and phase difference between the force and displacement signals. Storage modulus, loss modulus and tan $\delta$is then calculated from the data for each run and plotted against corresponding frequency. Since the stiffness of cuticle is 20 GPa (Vincent and Wegst 2004) and that of resilin is 1MPa(Weis Fogh 1961) it can be assumed that changes in elongation during experiments were solely due to the resilin pad (King 2010).

The experiments were carried out by varying 3 parameters: frequency, temperature and ethanol concentration. The temperature sweep was performed by collecting data at 12 different temperatures ranging from -2^{o}C to 55^{0}C at fixed ethanol concentrations. The concentration sweep was carried out by changing ethanol concentration from 0% to 93 %(by volume in water) in 18 different steps. Five different resilin pads were tested in this manner and the experimental results were averaged to produce master curve for resilin at reference

temperature of 23^{o}C and concentration of 100% hydration using time-temperature and time-concentration superposition principles (Ferry 1980).

Transmission Electron Microscopy

Sample Preparation

Resilin samples were fixed overnight in solution of 5% glutaraldehyde. 4% formaldehyde and 2.75% picric acid in 0.05M sodium cacodylate buffer. It was washed several times in 0.1M Na cacodylate for 15 minutes each, then post fixed in 1% OsO_4 in the buffer for one hour. The samples were again washed in Na cacodylate buffer 2 times for 10 minutes each and dehydrated in graded ethanol solutions of 15%, 30%, 50%, 70%, 95% and 100% respectively allowing 15 minutes in each ethanol solution. The sample is then infiltrated with 50:50 solution of propylene oxide: Poly Bed 812 for 8 hours. The infiltration is completed with 100% mixture of Poly/Bed 812 for 10 hours. Freshly prepared 100% Poly/Bed 812 was used to embed the samples in flat embedding mold .The mold was cured at 60^{o}C in oven for 48 hours. Thin sections of 90 nanometers were cut using a Leica Ultracut UCT microtome with diamond knives, collected on grids and analyzed in Zeiss 10CA Transmission Electron Microscope equipped with AMT Advantage GR/HR-B CCD Camera System. Since fixatives cannot completely stabilize the protein matrix in resilin(Elliott, Huxley et al. 1965) the samples had a tendency to get wrinkled and folded. Drying for longer times helped reduce this wrinkling effect to some extent.

Results

Dynamic Mechanical Analysis

The storage modulus, loss modulus and tanδ from each DMA experiment were plotted against the frequency. Two types of experiments were conducted; one by changing the temperature at constant ethanol concentrations and another by changing the ethanol concentrations at room temperature. The glass transition frequency was determined to be the peak of the tanδ curve.

Temperature shift

Experiments were carried out at a temperature range of -2^{o}C to 55^{o}C with an increment of 4^{o}C to 6^{o}C at constant solution concentration. The data was shifted using principle of time-temperature superposition to draw the master curve. One example is shown below for shifted data in water. The test was performed in the temperature range of 3^{o}C to 55^{o}C and 100% hydration of resilin. Figure 3a shows the unshifted data for storage modulus, loss modulus and tan δ and Figure 3b shows the shifted master curve at reference temperature 23^{o}C.

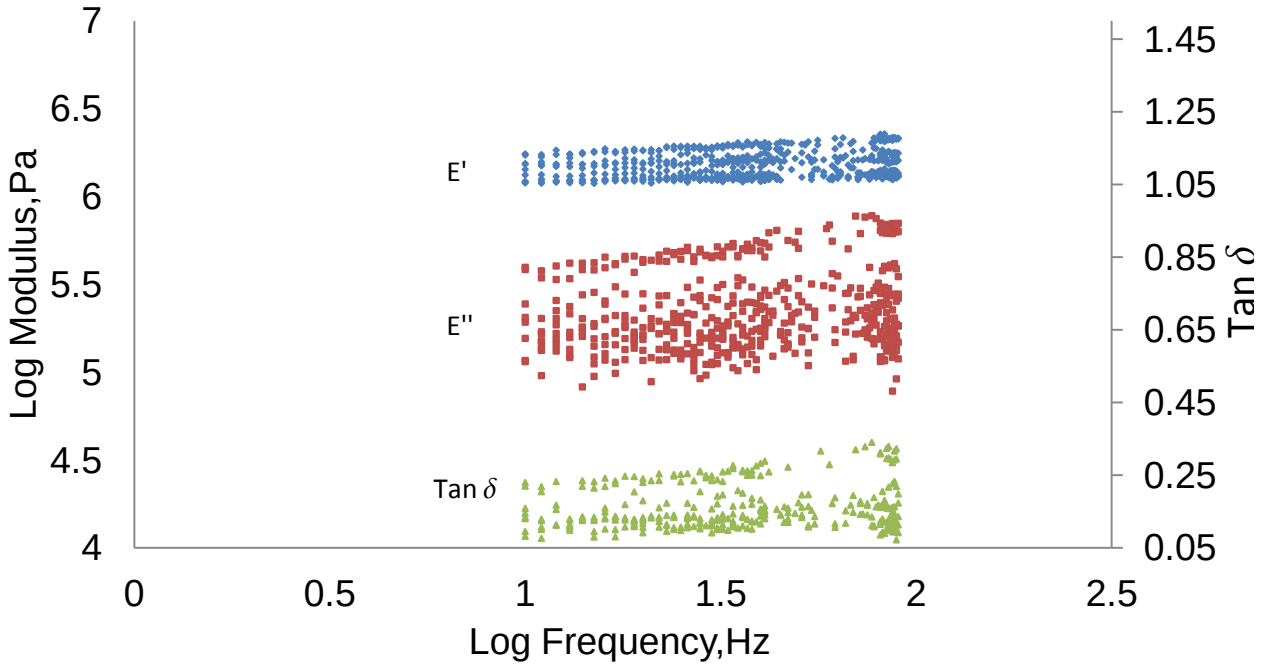

Fig 3a

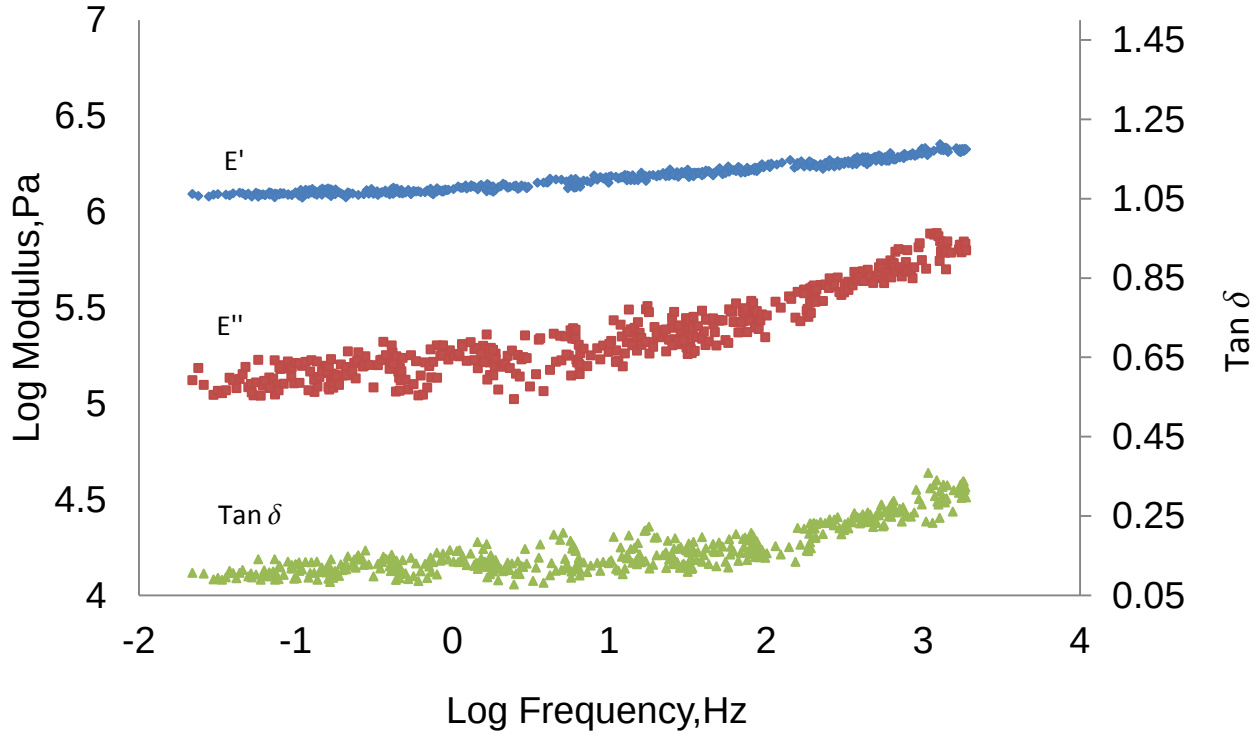

Figure 3b

Figure (3a) Storage Modulus(E'), Loss Modulus(E'') and Tanδ data collected in temperature range from 3^0C to 55^0C in water.
Figure (3b) Master curve obtained by Time Temperature Superposition at reference temperature of 23^0C

It is evident from Figure. 3 that resilin does not show much shift in water. The storage modulus indicates it is still in the rubbery plateau at about 1.6 MPa with maximum shift factor of 2 at the coldest temperature (Figure 4).Experimental limitations does not allow for performing tests at sub zero temperatures in ice. The shift factors for five different samples follows the WLF equation(Ferry 1980) verifying the validity of time temperature superposition technique. Using reference temperature at 23°C, the shift factors are plotted with temperature in Figure 4.

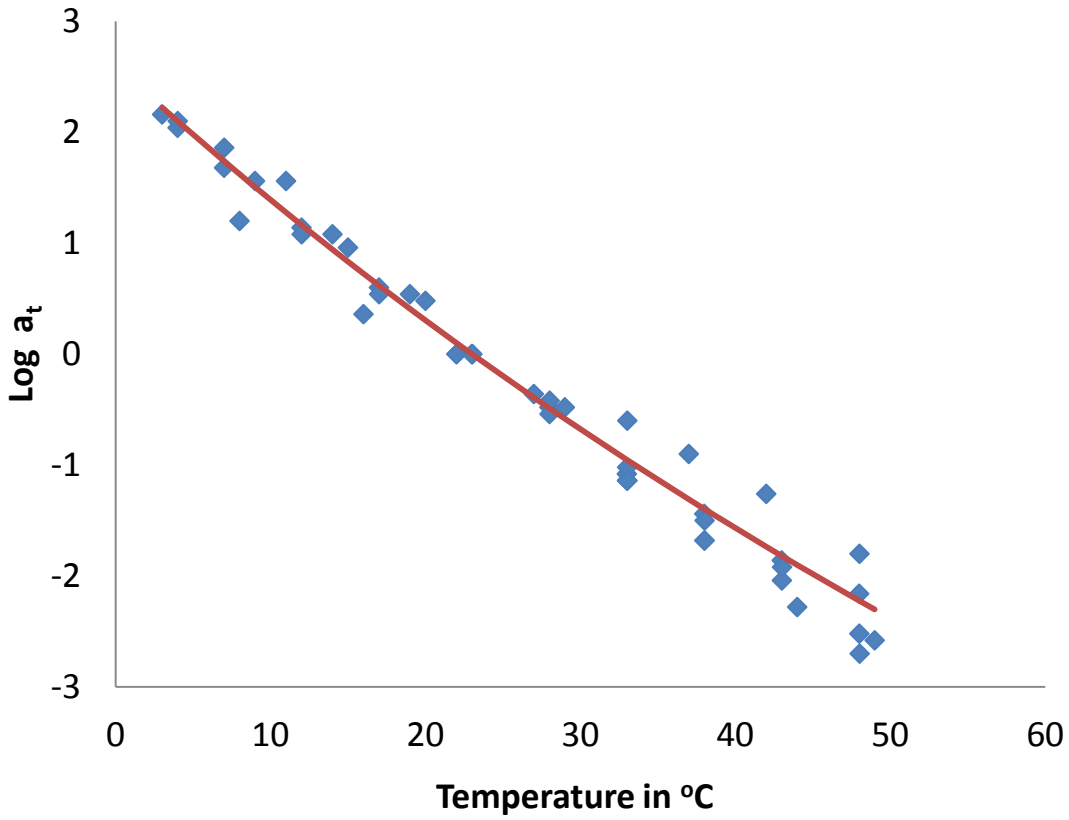

Figure (4) WLF fit to Log a_t values with C1=20, C2=200 °C at reference temperature 23°C

In order to drive the material through the glass transition and obtain the complete range of viscoelastic properties, hydration levels need to be controlled in addition to temperature. It can be achieved by performing temperature sweep at reduced concentrations of water .Four different ethanol-water solution viz. 65%, 73%, 80% and 84 % ethanol by volume in water were used and temperature sweep was carried out at each concentration. Figure (5) shows one such experimental master curve obtained by time temperature superposition from a test performed in the temperature range from 6°C to 48°C.

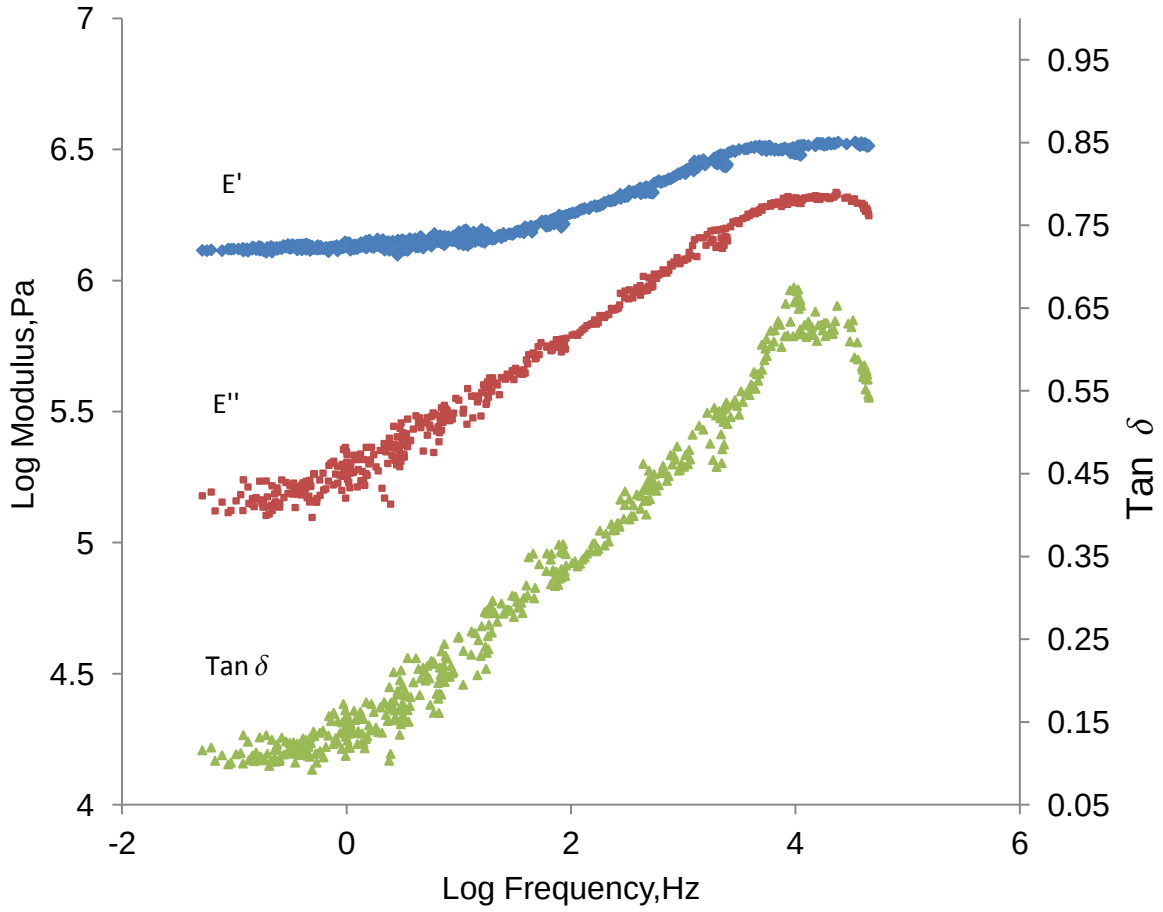

Figure (5) Storage Modulus, Loss Modulus and Tanδ Master Curve in 73% Ethanol (by volume in water) by time temperature superposition at reference temperature 23^{o}C

Here the material just reaches glass transition at $10^{3.7}$ Hz with storage modulus of 5.0MPa. It is evident that performing test at higher concentration of ethanol can drive resilin through glass transition at room temperature for reasons described later in the paper. Further, performing time-concentration superposition will be necessary to obtain the complete master curve of resilin. Table 1show the C1 and C2 values of the material at different ethanol concentrations obtained by fitting the shift factors in WLF equation as required by the principle of Time temperature superposition with reference temperature at 23^{o}C. The shift factors are valid upto the glass transition.

Table 1: WLF constants for different ethanol-water concentrations and temperature ranges at 23°C reference temperature

Ethanol Concentration	Temperature Range(°C)	C1	C2 (°C)
0%	3 to 49	20	200
65%	-2 to 48	21.5	205
73%	0 to 48	25	200
80%	9 to 53	24	210
84%	13 to 55	25	180

Hygral Shift

The superposition theory can be extended to perform time concentration superposition (Ferry 1980) and obtain master curve for resilin at room temperature (23°C).Figure(6a) shows ushifted storage modulus data from one such test by varying ethanol concentration from 0% to 90% by volume in water in 18 steps at 23°C.The data is then shifted to 0% ethanol concentration (full hydration)to form a master curve at 23°C describing the storage modulus, loss modulus and tanδ from rubbery to glassy domain(Figure 6b).

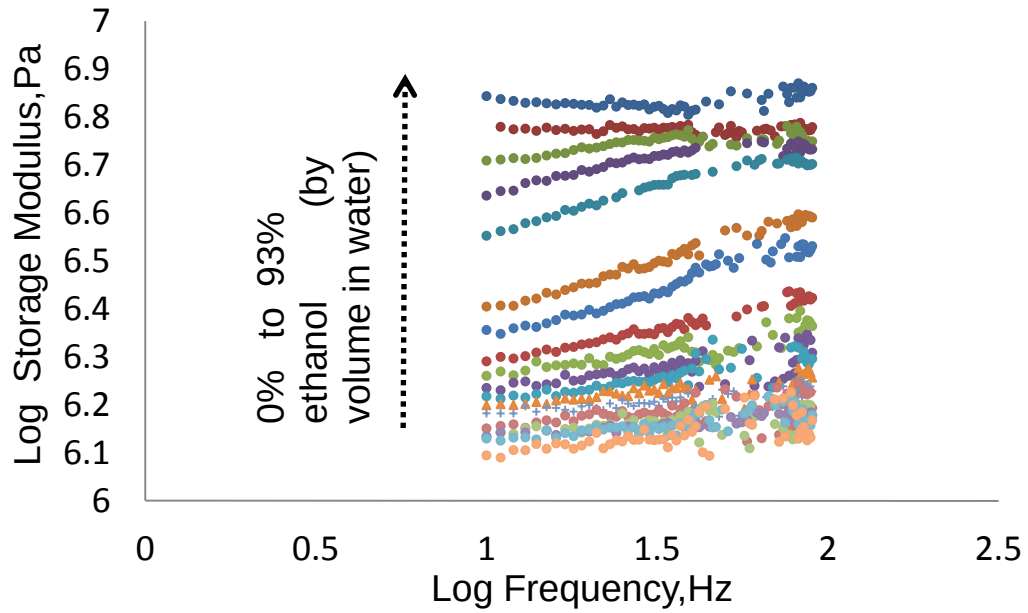

Fig 6a

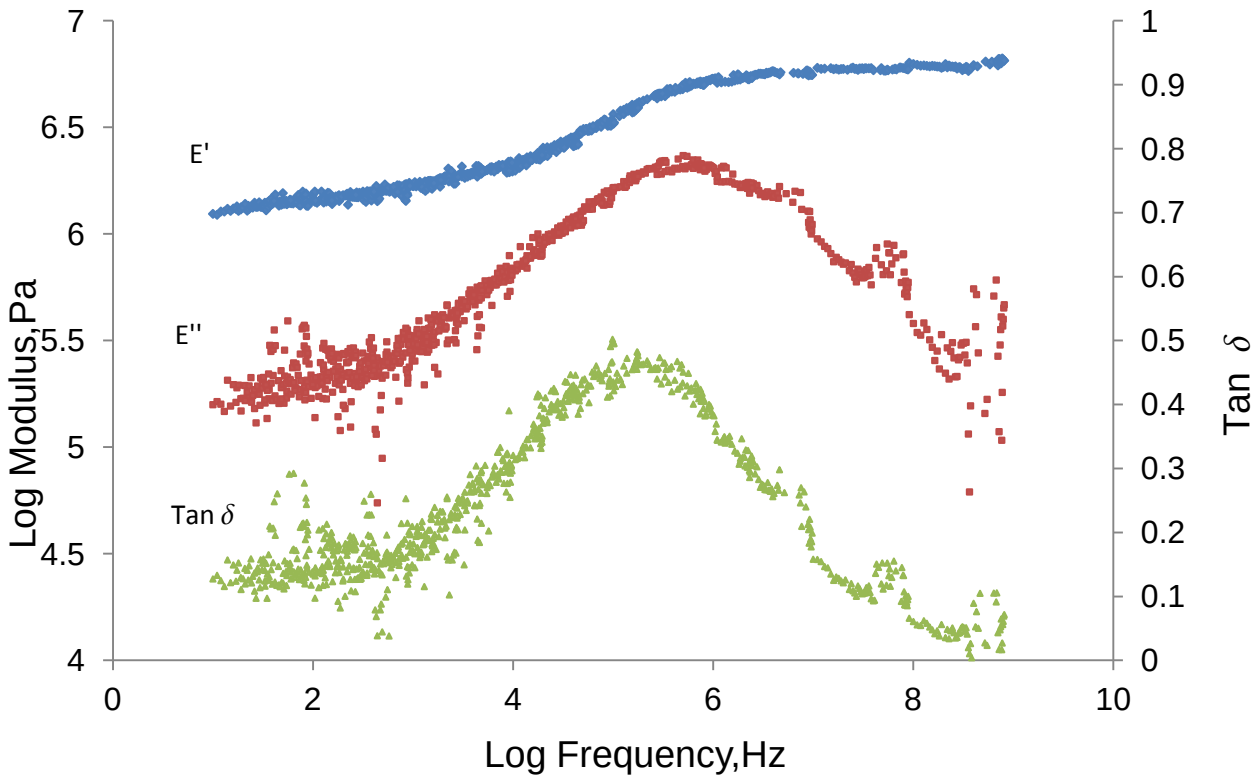

Figure 6b

Figure (6a) Unshifted Storage Modulus data obtained by varying ethanol concentrations at 23°C.

Figure (6b) Master curve for Storage Modulus, Loss Modulus and Tan δ obtained by time-concentration superposition at 23°C reference temperature and 100% reference hydration.

The data from different ethanol concentration has been shifted to reference hydration of 0% ethanol at 23°C to obtain this master curve. The glass transition frequency is about $10^{5.3}$Hz or 200 KHz as observed from the peak of the tanδ curve. The resilience at glass transition calculated from Equation (1)is about 40%, the lowest in the entire regime. Master curves from 5 different samples were averaged and plotted in an error bar plot in Figure 7.The hygral shift factors for all the 5 samples are plotted against solution concentration in Figure 8.

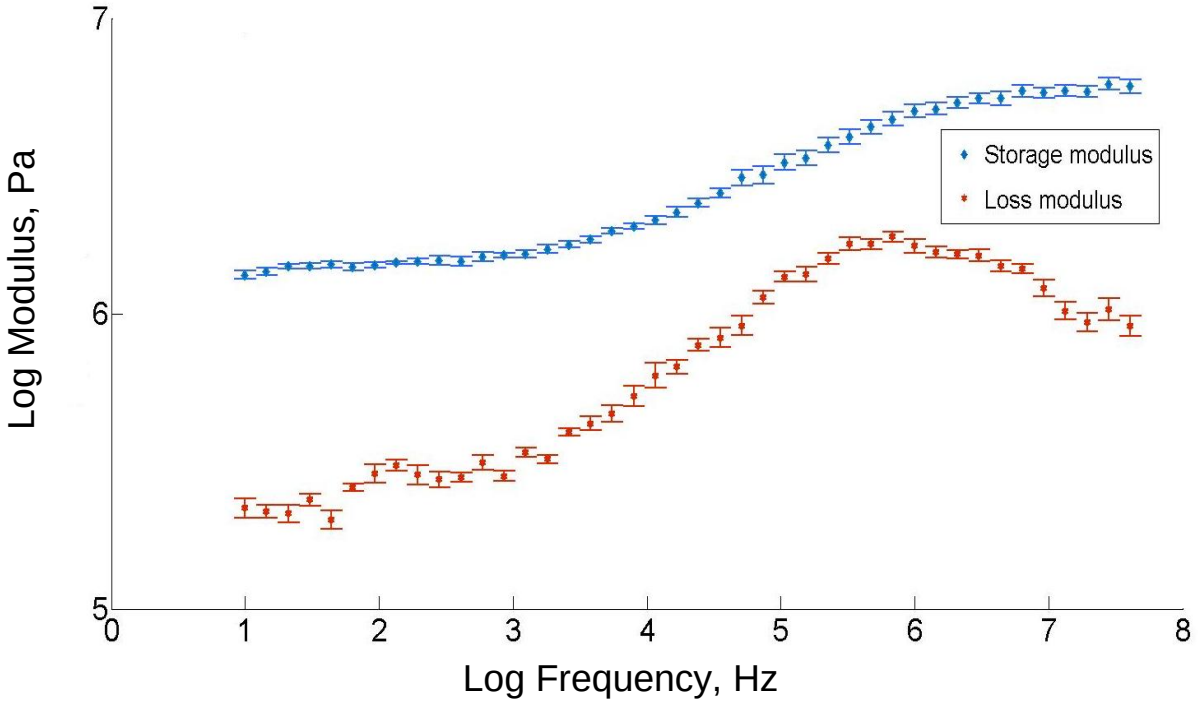

Figure 7a

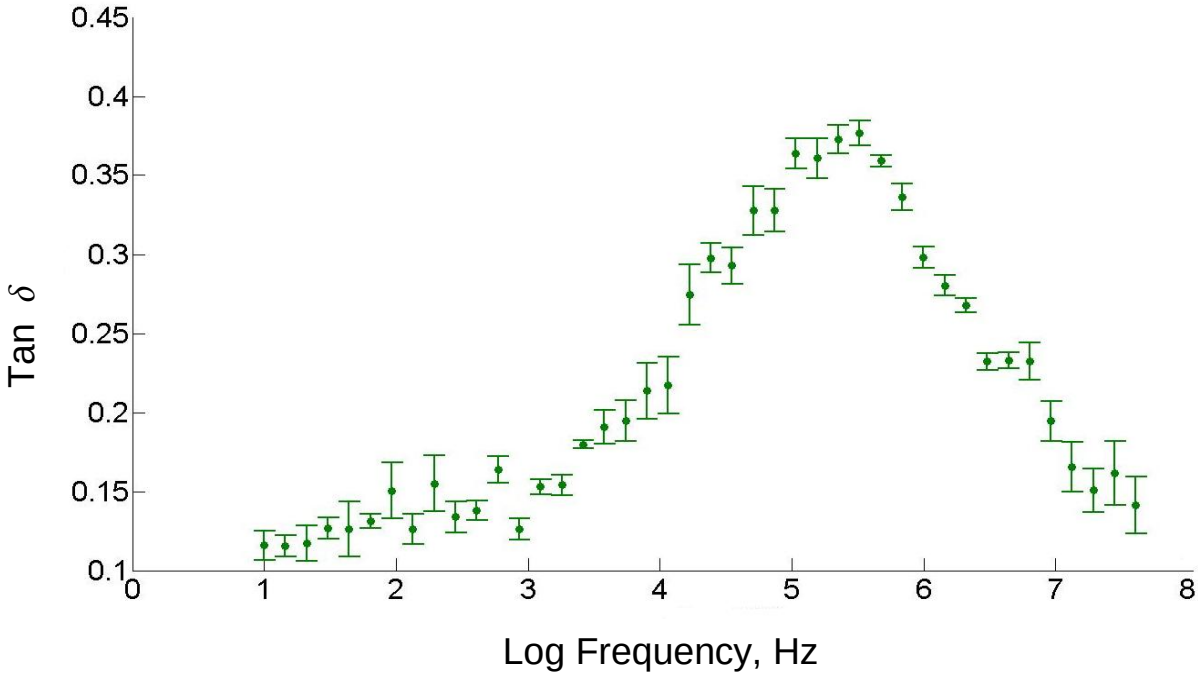

Figure 7b

Figure(7a) Storage and Loss Modulus Master Curve Error Bar plot by Time concentration Superposition at 100% hydration and 23°C temperature
Figure(7b) Tan δ (=Loss Modulus/Storage Modulus) Error bar plot .

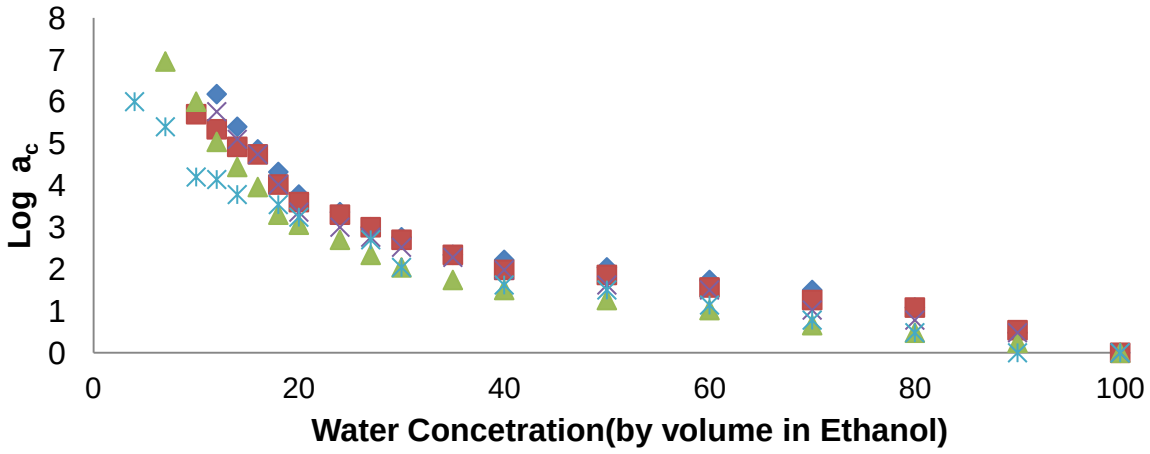

Figure (8)Hygral Shift Factors Log a_c against water concentration (by volume in Ethanol) at 100% hydration and 23 ^oC temperature.

The shift factors level off at higher concentration of water showing there is very little shift before ethanol concentration reach 60% in the solution. The total shift factor $\log a_c a_t$ for the 5 ethanol concentrations is calculated by adding the shift factors $\log a_c$ from the concentration to the corresponding shift factor $\log a_t$ from temperature sweeps.

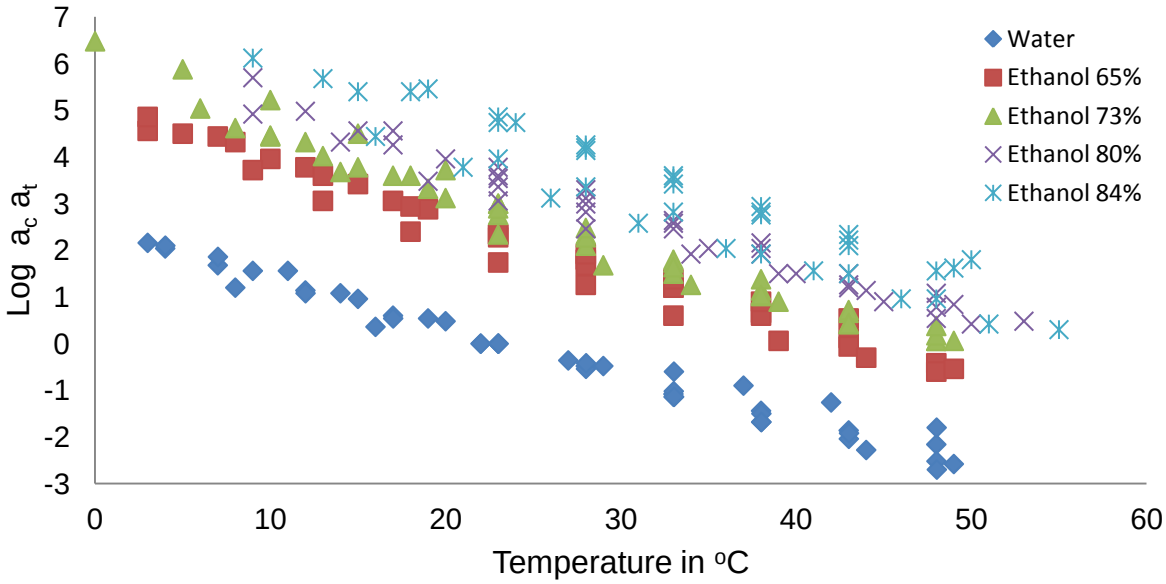

Figure (9) Total Shift factor plotted against temperature for 5 different ethanol concentrations

Using total shift factors (Figure 9) and Figure7, the value of a property at any temperature and concentration can be calculated in the experimental range.

Isoshift Factors

The data from Fig (8) can be repotted to produce isoshift curves (King 2010, Lillie 1990). For each integer value of $\log a_c a_t$, the temperature is plotted against the water content. The integer values of the total shift factors were obtained by interpolating the curves from Figure (8). This can be used to estimate horizontal shift for different combinations of temperature and solution concentrations.

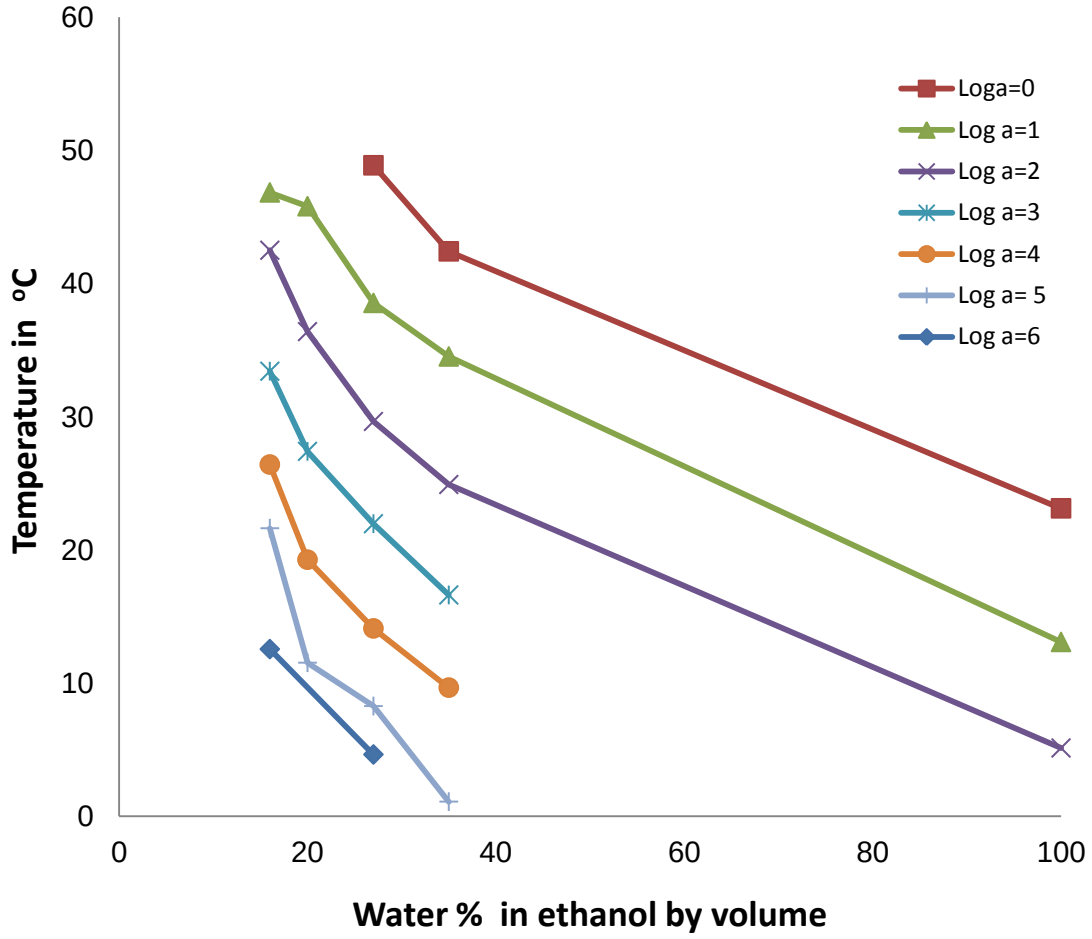

Figure (10) *Isoshift factor curves for total shift factors values from 0 to 6 at 23°C reference temperature*

These curves may be interpreted in two different ways by assigning frequencies to the isoshift curves.If curve for log a=1 is assigned a value of 1Hz, this will place the abscissa in Figure9 at 1.Hence the storage modulus is 1.6MPa.Similarly, if 1Hz frequency is assigned to

log a=5, the storage modulus will be 3.1MPa.Alternatively, if curve log a=3 is assigned a value of 10 Hz, and curve log a=4, a value of 1Hz, they will have the same storage modulus. Similarly, if curve log a=1 is assigned a value of 1 MHz, the storage modulus will be same as assigning curve log a=6 a frequency of 1Hz.

Transmission Electron Microscopy

Figure (11) TEM image of the resilin cross section

The image shows alternating dark and light layers of chitin and resilin. Resilin is devoid of any visible structure as compared to the cytoplasm from the adjacent muscle cells at the bottom of the image (Weis Fogh 1965).The thickness of the chitin sheets vary from 0.15 microns to 0.35 microns. The thickness of the resilin sheet varied from 0.6microns to

2.3microns.This implies there will be approximately 40 to 60 layers assuming uniform distribution the resilin sample being 80 microns thick. Assuming density of dry resilin to be 1.3g/cm^3(Weis-Fogh 1961) and dry chitin to be 1.425g/cm^3 (Neville1973), the fraction of resilin in the matrix can be estimated as 82%. The modulus can be estimated by the rule of mixtures for laminated composites under transverse loading as (Jones 1975)

$$E = \frac{E_f E_m}{E_f V_m + E_m V_f}, \qquad (9)$$

where, E_f and E_m are Young's modulus for matrix and fiber phases and V_f and V_m are volume fractions for fiber and matrix phases.

For the resilin-chitin composite in cockroach,

E_m = 1.0 MPa for Resilin,(Vincent and Wegst 2004),V_m = 0.82,

E_f = 15 GPa for Chitin,(Fabritius et al. (2011)),V_f = 0.18,

Hence E from Eqn (9) is 1.2 MPa.

The value is close to the storage modulus of resilin-chitin composite at low frequencies.

Glass transition temperature

From the theory of viscoelasticity, we know any property G' measured at frequency ω_T and temperature T is equivalent to G' measured at frequency $\omega_T a_T$ and temperature T_0, where a_t is the shift factor and T_0 as the reference temperature, (Ferry 1980)

If we define $\omega_T a_t$ as ω_0 ,then the shift factor a_t will be equivalent to

$$a_T = \omega_0 / \omega_T, \qquad (10)$$

and, $$G'(T, \omega_T) \equiv G'(T_0, \omega_0), \qquad (11)$$

Further, the time temperature superposition shift factors follow the WLF equation

$$Log\ (a_T) = -C1*(T- T_0)/(C2 + (T - T_0)), \quad (12)$$

where C1 and C2 are constants.

Now, from the master curve in Figure7 the glass transition frequency is $10^{5.3}$Hz for a cockroach resilin sample at 23^{o}C in water (100 percent hydration).

At the same hydration level, if the sample is oscillated at a frequency of 25Hz (frequency of use in cockroaches in nature), the shift factor required to estimate properties can be found from Equation (10) as,

$a_T = 10^{5.3}/ 25 = 7981.05$, or $\log a_T = 3.90$.

Since the shift factors are a function of temperature as derived in the WLF equation, using C1=20 and C2=200 ^{o}C as constants for WLF equation for water from Table 1 and Equation (12) with T_0 = 23 ^{o}C (reference temperature), we can get the glass transition temperature as T= - 10^{0}C

Similarly using data for dragonfly resilin in water (King 2010, Dudek et al. 2008), the glass transition frequency and temperature for dragonfly resilin under complete hydration at 23^{o}C are $10^{6.3}$ Hz and -20^{o}C at 25Hz.

Discussions

Dynamic Mechanical Properties of Resilin

Major findings

The experimental results shows that cockroach resilin is less resilient than dragonfly and locust resilin at low frequencies and has a glass transition frequency of about $10^{5.3}$Hz,which is around a decade less than that of dragon fly resilin and a decade more than the highest known frequency of resilin use in nature at 13kHz by Cicada. The mechanical properties of natural resilin can be influenced by 6 primary factors viz. frequency, temperature, hydration, mechanical structure, chemical composition and whether the experiment was conducted in tension or in compression. The following discussion will highlight influences of all these factors on cockroach resilin and compare its properties to that of locust and dragon fly resilins.

Frequency, Temperature and Hydration

Being a polymer the dynamic mechanical properties of resilin are influenced by frequency, temperature and hydration levels. While dragonfly(King,2010 ,Dudek et al2008) and cockroach resilin has a large range of frequency independent properties in the rubbery domain, locust resilin starts its transition towards glass transition at 100 Hz only (Gosline et al. 2002).However, for all the three types of resilin ,the material behaves rubberlike at its highest resilience at their respective frequency of use in nature. Temperature affects polymer properties by directly influencing the molecular mobility of polymer chains at different temperature. Tests in water revealed a very little shift for cockroach and dragonfly (Dudek et al. 2008) resilin suggesting efficient use of the material at ambient temperatures encountered by each of the insect across different habitats throughout the year. While the hydration levels

by bodily fluids in nature for 3 types of resilin are different, master referenced to 100%hydration at room temperature (Figure7a)along with the plot of isoshift factors (Figure 9) provides a common ground for analyzing the properties at different levels of hydration.

Mechanical Structure

Comparing previous data from dragon fly and locust resilin, mechanical structure seemed to play an important role in determining dynamic properties. While dragonfly resilin is a pure polymer, locust pre-alar arm is a composite of resilin and chitin arranged in alternate parallel layers (Elliot et al. 1965).A important contribution of this work is in understanding the properties independent of structure of the polymer composite. As revealed by transmission electron microscopy, cockroach resilin has structure similar to that of locust resilin, but with different mechanical properties. This suggests a significant role of factors like hydration and amino acid composition on the mechanical properties unlike to the previous hypothesis of structure being the dominant role in properties (King 2010).

To understand the role of layers of chitin in resilin, it is essential to look at mechanical models for polymers. Two basic viscoelastic models are the Maxwell element and Kelvin element consisting of a spring and dashpot in series and parallel respectively.

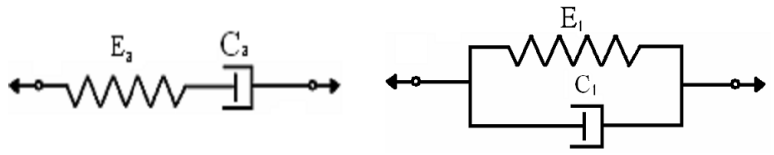

Figure (12):Left Maxwell Element Right Kelvin element, E_i is spring stiffness and C_i damping coefficient

A generalized Kelvin chain model consisting of a number of Kelvin elements in series may be used to represent dynamic properties of any polymer across a wide range of frequency.

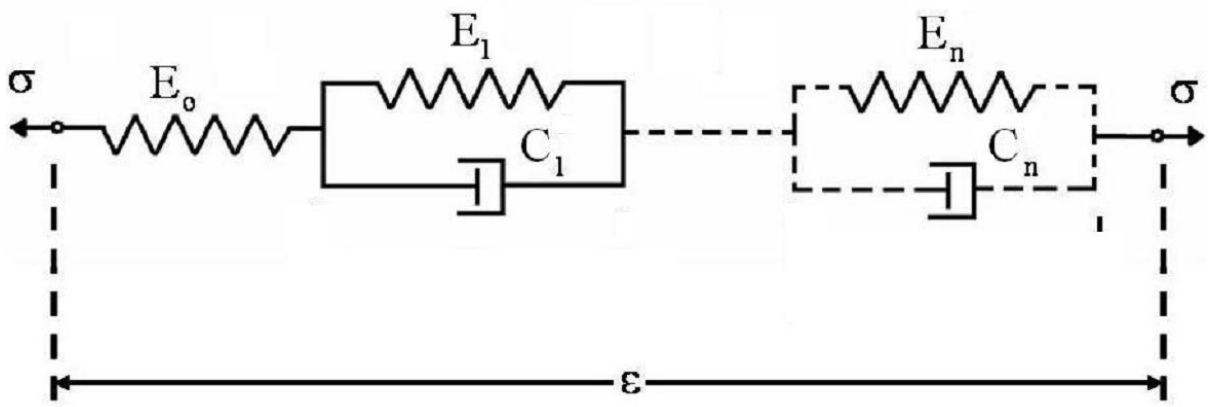

Figure (13):Kelvin chain model .E_i's are spring stiffness and C_i's are damping coefficients for n Kelvin elements in series

If we define complex compliance as D(iω) as inverse of complex modulus,

$$D(i\omega)=1/E(i\omega), \qquad (13)$$

the storage and loss compliances for the Kelvin chain can be expressed as (Ferry 1980),

$$D'(\omega) = D_0 + \sum_{i=1}^{n} D_i \frac{1}{1+\omega_i^2 \tau_i^2}\ , \qquad (14)$$

$$D''(\omega) = \sum_{i=1}^{n} D_i \frac{\omega_i}{1+\omega_i^2 \tau_i^2}\ , \qquad (15)$$

where τ is the retardation time and tan δ is expressed as,

$$\tan\delta = \frac{D'(\omega)}{D''(\omega)}, \qquad (16)$$

Since, in a laminated composite the stress remains constant across all the layers, the chitin fibers can be represented as a stiff spring added in series to the Kelvin chain in Figure. 12.

However, following Equations (14) and(15), it can be observed that this will lead to an increase in storage compliance and no change in loss compliance. Hence, the tan δ value from Equation(16) will go down representing increasing resilience. Thus, this simple mechanical model seems to prove increase in resilience with no change to loss modulus, quite contrary to our experimental results. It necessitates investigating the theory of rubber elasticity for resilin more carefully to understand the source of additional losses in a composite structure.

The high resilience and elasticity of pure resilin is attributed to two factors, random coil structure(Weis-Fogh 1961) and interaction with water (Truong et al. 2011).A random coil network is supposed to keep the polymer in a more favorable entropic state than the stretched network since it has greater number of degrees of freedom in the relaxed random state. However, incorporating structure will reduce the entropy barrier making the material less resilient. Resilin binds to chitin by the chitin binding domain where it forms a protein-carbohydrate composite in the transition zone (Guokui et al. 2010) unlike typical laminated composites where the polymer acts as the only adhesive joining parallel sheets of the fiber. Since chitin has highly ordered crystalline structure resilin needs to conform to an ordered structure to bind to chitin. Using computer simulations ,it has been suggested that (Anderson 2011) the R&R Consensus region in pro-resilin will tend to be disordered in the absence of chitin and have an increased tendency to form β-structures when it comes into contact with chitin. This might lead to loss in resilience for resilin-chitin composites due to shift to a more ordered structure from an entropically favored random structure. It is worthwhile to note that the decrease in resilience at low frequencies is less than 5% for locust resilin and about 15% for cockroach resilin when compared to pure dragonfly resilin, making the resilin-chitin composite still more resilient than polybutadiene(80%),the best known rubber.

When comparing composite structures to pure polymers, hydration level of the material itself comes into play. In our experiments, the hydration level of the solution was assumed to be the hydration level of resilin. In absence of any literature data on diffusivity in pure resilin, it was difficult to estimate the actual amount of water that enters the polymer. However, it is likely that presence of chitin layers in pure resilin will alter the amount of hydration of the material since the two materials will have presumably different diffusion coefficient. Further, diffusion in polymer-fiber composites is anisotropic with diffusion in fiber direction of composites being much faster than diffusion in transverse direction(Fahmy and Hurt 1980). Hence, to find out the extent of hydration in pure resilin compared to resilin-chitin composites further studies on synthetic resilin needs to be done. Thus, difference in properties between dragonfly, locust and cockroach resilin primarily seems to be direct influence of structure and hydration levels.

Tension/Compression

Across the insect species resilin is used both as a compression structure and a tension structure for elastic energy storage. While in fleas(Burrows et al. 2008)and cockroaches (Neff et al. 2000),it is primarily used as compressive structure ,in locusts (Weis-Fogh 1960)it is used as bending structure and it is used as dynamic loading in tension dragonfly(Weis-Fogh 1960) .To understand the effects of pre-tension and pre-compression on dynamic properties ,free volume approach to polymer viscoelasticity can be used. Free volume is directly related to the viscosity in a polymer by the Dolittle equation (Equation 2), i.e.

$\eta = Aexp(B/f)$,where f is the fractional free volume $=v_f/(v - v_f)$,from Eqn(1).

Again , free volume can be related to the stress on a polymer by (Fahmy and Hurt 1980),

$$v_{f\sigma} = v_{fo}(1 + \frac{\sigma}{4G}) \quad , \qquad (17)$$

where v_{fo} and $v_{f\sigma}$ are free volumes before and after application of stress σ, and G is the shear modulus. This implies a tensile load will increase the free volume (Bradley et al. 2000,Fahmy and Hurt 1980) and decrease the viscosity (Equation 2) whereas a compression load will have the reverse effect on a polymer. Hence, a polymer under pretension will have more free volume between its chains, resulting in greater mobility, reduced viscosity and relaxation time and an overall increase in resilience. The effect of pre tension and pre compression is evident on comparing storage modulus data from cockroach resilin with dragonfly resilin(King 2010) which has similar modulus of 1.5 MPa and 1.6MPa at low frequencies although being prestressed in compression to 10%(dynamic strain 6% on prestress) and in tension to 35% respectively(dynamic strain 2% on prestress).Whether compressive prestress might account entirely for decrease in resilience in cockroach is not fully understood in presence of several other factors as mentioned before in the paper. However, further research in obtaining synthetic resilin (Elvin et al. 2005) from dragonfly and cockroach genes will help in obtaining comparable and convenient samples and investigating effects of each of the factors individually.

Amino acid sequences

Lastly amino acid sequences play a major role in determining the mechanical properties at macroscopic level. The amino acid sequences of resilin varies widely across insect species with the amino acid sequence YGAP remains conserved (Lyons et al.2011).The resilience between mosquito and fruitfly synthetic resilins were found to vary 5% on account of differences of proline and glycine content in the resilin gene of respective insects(Dudek et al. 2008)

Functions of resilin in Cockroach and Energy Storage

Resilin was identified in cockroach at tibia-tarsal joint and Ta4-5 joint(Neff, Frazier et al. 2000).A soft elastic structure at joint can simplify locomotion by reducing body mass, lowering metabolic costs and substituting the need of active neural regulation with passive control. Further, the soft elastic structures at joints provide greater degree of freedom in movements. The localization of resilin at tarsus suggests evolutionary development as compressible elastic structures antagonist to active muscles. In the Ta4-5 segment, the resilin pad acts as a elastic backstop for disengaging claws as the retractor unguis muscle relaxes (Frazier et al. 1999).Similarly, at tibia –tarsal joint the pad acts as a compressible structure antagonist to the active muscles storing elastic energy. In addition to joints, resilin was identified by transmission electron microscopy at campniform sensilla of cockroach legs(Moran et al. 1971).Here, it allows elasticity to the cap allowing it to resume resting length after distortion by mechanical simulations.

Kram et al.(1997) provides the only literature data on the energetics of cockroach locomotion .The total kinetic energy for moving on a level surface at 21cm s^{-1}(7Hz) was estimated to be 8.24μJ per stride with the tarsus segment consuming only 7.2% or 0.6μJ of it. Considering a resilin pad of dimensions 480 mm and the area as 0.04 sq.mm, the amount of energy stored per unit volume can be calculated by,

Stored Energy= (Storage modulus) x (Max .Strain)^2 /4, (Ferry 1980) (18)

For 0.6μJ the maximum strain the pad has to undergo is about .05 mm or less than 0.5%.The contribution of potential energy change is small compared to the kinetic energy requirements. This result suggests cockroaches do not need to bend their legs at tibia-tarsal joint as much while running on level surfaces to store elastic energy, corroborating my observations on slow walking in cockroaches. However, since resilin can theoretically deform upto a

maximum of 70% of its length under compression the maximum energy that can be stored in this pad will be 3.7 μJ. This will be useful on rough terrains or negotiating obstacles where the tibia tarsal joint might need to bend to 90^{o}or more to overcome a obstacle(Watson et al. 2002).

Experimental Limitations

A major concern in the experiments was noise in the data. Two possible sources of noises were identified: electromagnetic and mechanical. Electromagnetic noise was much reduced by using shielded wires and shortening lead wires coming out from strain gages. However, the raw data always seemed to pick up 60 Hz noise from unidentified sources. Mechanical noise occurred primarily due to not attaching one end of the resilin pad to indenter by glue. Since, the pad was engulfed by glue in several attempts for rigid connection, experiments were carried out by pre straining in compression to maintain contact. However, the system is susceptible to small vibrations since the magnitude of forces was in the order of miliNewtons. The loss modulus data and tan δ data were most affected by the noise in data causing larger scatter.

High rate of evaporation of ethanol also presented a concern in maintaining constant concentration in the bath. This was partly prevented by covering the maximum amount of the solution chamber .Further, exposure to ethanol caused the resilin sample to turn a bit cloudy from the transparent appearance in water. The change in optical properties suggests some effect of ethanol on resilin altering its chemical properties.

The change of storage modulus of cockroach resilin from rubbery to glassy zone was roughly one decade in log scale. Although, most polymers show a change in modulus of around 2-3 decades, shorter change in modulus might be due to higher crosslink density of a polymer in rubbery zone(Thavamani and Bhowmick 1992) or inaccuracies of the

experimental setup in glassy domain. Similar setup for dragonfly resilin (King 2010) also produced roughly one decade change in modulus for dragonfly resilin with a force gage (AE 810) different from the custom built semi conductor gages used in the experiments on cockroach resilin. The microscopic properties leading to a small change in storage modulus presents an interesting topic for further research.

Conclusions and Future Directions

The thesis presented the dynamic mechanical properties of a third type of natural resilin from cockroach. The glass transition frequency was found to be at 200 KHz with resilience of 80% at low frequencies. Further, factors affecting variation of properties in natural resilin were identified and roles each of them in influencing mechanical properties were discussed. While locust and dragonfly resilins have comparable structure, the variation in resilience between them can be caused primarily due to difference in hydration and differences in compressive and tensile prestrain in experiments .Further, the relatively large size of resilin pad at tibia tarsal joint accounted for the fact that cockroaches can move on level surfaces with minimal bending of the joint for energy storage enabling better force transfer between leg segments.

Future research can be conducted on investigating role of resilin in locomotion of cockroaches as well as understanding material properties of resilin. The resilin pad at tibia tarsal joint is an external structure which can easily be torn on live cockroaches. By allowing cockroaches to run and climb obstacles with a dysfunctional resilin pad at the joint, the efficiency of resilin as primary energy storage mechanism at tibia-tarsal joint can be studied. Passive energy storage in compressible springs can be mimicked in robotics to simplify actual control

To understand the material properties of resilin, it is imperative to produce synthetic resilin from genes of each insect's studies. While sequences coding for dragonfly resilin is known (Dudek et al. 2009), sequences for locust and cockroach are still under investigation. Natural samples tested so far have provided preliminary understanding of the mechanical properties of resilin. However, due to presence of a number of uncontrollable factors,

synthetic resilin from dragonfly, locust and cockroach genes needs to study in details.This will give insight into a number of unanswered questions as:

1) Does amino acid sequences play an important role in deciding resilience of resilin?

2)Is crosslinking the only factor causing a short change in storage modulus from rubbery to glassy domain?

3) Do presence of chitin cause resilin to assume structure from a randomly coiled polymer network?

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