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MRI: Development of Advanced Dielectric Spectrometers and Chemical Techniques for the Detection, Characterization, and Cataloging of Peptide and Protein Structural Motifs.

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Project Summary

The project will develop novel instrumentation and chemical techniques to detect and resolve the dielectric responses, derived from the permanent dipole moments, of the intramolecular secondary structure and structural motifs of peptides and proteins. It will develop a set of custom dielectric spectrometers to encompass the frequency range of 1μHz to 2.5GHz. Ubiquitous Test and Measurement (T&M) equipment, i.e., signal generators and oscilloscopes, will be connected to custom preamplifiers and high-resolution temperature-controlled capacitive sample cells managed by custom highly-advanced software for noise-rejection, instrument automation, and data analysis to create highly-sensitive multi-sample dielectric spectrometers. Directed use of the Protein Databank will ensure that well-characterized model peptide and protein systems are selected for the study.

Current dielectric spectrometers apply and observe a monochromatic, single frequency, response of a material or sample. Complex systems, such as proteins, exhibit more complex behavior, as evidenced by stretched-exponential random walks of amorphous polymers. Modern T&M equipment have the potential to apply and observe the most complex behaviors of systems. However, this capability has not been realized or developed to study such complexity. Therefore, a primary goal of this project is to develop polychromatic excitation and/or response dielectric spectrometers to invoke and observe the "dance" of these complex systems, in particular proteins.

The early goals will decrease undesired effects, such as electrode polarization in an aqueous environment, fringe, stray, and parasitic effects, and the Maxwell-Boltzmann distribution due to thermal agitation. High temperature studies, >20°C, will be performed under the denaturation of the protein. These studies should partially melt the protein's core, thus reducing its internal viscosity and allow dielectric measurements of its intramolecular structural motifs. Low temperature studies, <20°C, will reduce the noise due to thermal agitation and provide insight into the interactions of embedded water molecules and proton migration within the protein. Further studies of proteins dissolved in low-dielectric gels will be used to create highly-viscous semi-solids to rotationally and translationally immobilize the protein macromolecules.

The intellectual merit encompasses four primary goals: (i) improve upon commercial instrumentation, (ii) develop polychromatic dielectric spectroscopy, (iii) improve the chemical and environmental techniques required to observe and resolve the intramolecular dielectric responses within peptides and proteins, and (iv) increase the empirical knowledge of the intramolecular charge distributions and polarizabilities of the secondary structural motifs, as provided by (i) – (iii). For instance, high temperature studies will reveal enthalpies and activation energies of the inter-motif dampening forces and of the molten core. Also, low temperature studies will reveal relaxation and resonance processes of internal hydration layers and proton migration within peptides and proteins.

The broader impact of the project has three primary goals: (i) advance current and commercially-available dielectric spectroscopy, (ii) provide a greater understanding of the intramolecular dielectrics, dynamics, and energetics, the polychromatic "dance", of peptides and proteins, and (iii) enzymatically manipulate proteins using externally applied electric fields. For instance, the before mentioned intramolecular empirical data can dispel myths of dielectric continuum models and support charge heterogeneity of the molten core in proteins. Additionally, the empirical data will provide valuable activation energy, motif flexibility, inter-motif dampening, and relaxation data for further theoretical models leading to inter-motif and inter-chain interactions, and thus protein folding energetics and dynamics. Accurate theoretical studies would promote the fields of biophysics, biochemistry, and proteomics. Ultimately, it is conceivable that an external electric field can impose complex waveforms on proteins to manipulate their enzymatic activity, therefore affecting microbiology, molecular cell biology, immunology, endocrinology, pharmacology, bioengineering, and nanotechnology.

Project Description

a. Research Activities

The proposed research will design, develop, construct, and calibrate advanced dielectric spectrometers and develop environmental and chemical techniques for the purpose of detecting and characterizing dielectric responses of intramolecular sub-domains, which form the components of the net dipole moment, and ultimately the intra- and inter-chain dielectric responses of intramolecular secondary structure and structural motifs of peptides and proteins.

The potential of the proposed spectrometers have broad impact. For this project, the spectrometers will be specifically used to study peptide and protein dielectric responses, as outlined in this submission, but the spectrometers will have much more generalized use and impact in the Test and Measurement (T&M) industry, in electronics and materials research, i.e., ceramics, polymers, etc., and in electronic development, production, and quality assurance. Therefore, the development of such advanced spectrometers have commercial, research, and academic implications.

The foundations of the proposed dielectric spectrometers are based on commercially-available T&M equipment, i.e., signal generators, electrometers, and digital oscilloscopes. The advancement of the proposed spectrometers will be to amalgamate the latter equipment into multi-channel, multi-sample, highly-sensitive, and stable broadband dielectric spectrometers. Modern electrical design techniques for electronic interfaces and sample cell design, and most importantly, highly sophisticated cutting-edge software will be used in the proposed construction. Three spectrometers are proposed: low frequency, 1μHz – 10Hz, intermediate, 1mHz – 500MHz, and high frequency, 250kHz – 2.5GHz; herein labeled Dielectric Spectrometers 4 through 6 (DS4 – DS6), respectively, with a 17 decade range. All three spectrometers will be capable of time- and frequency-domain dielectric spectroscopies (TDDS and FDDS, respectively), with advanced noise-rejection, signal stabilization, and averaging computationally performed with the proposed custom software.

It is only in recent years that all of the necessary tools have become available, with mature and stable technology, for the complete success of this project. This includes the development tools, electronics, digital T&M and temperature equipment, computers, software development capabilities, and the Protein Databank (PDB) [1] as outlined in the following description. In short, the project includes the before mentioned highly-sensitive multi-sample dielectric spectrometers spanning 1μHz to 2.5GHz, protein studies at highly-resolved temperatures, with a range of –88 to 300°C and stability of 0.01°C, pH variations for side R-group perturbations, hydration studies of proton migration of surface bound and intramolecular water, and rotational immobilization using semi-solid low-dielectric gels.

a.1. Measurement of Dielectric Signals

Theoretically, the measurement of any physical quantity requires the mapping from a source set of an empirical domain onto image elements [2] which provide an accurate representation of the physical quantity. Practically, a dielectric spectrometer will electronically measure changes of an applied electric field, which correspondingly represents electric polarizations of the sample. The capacitive sample cell, and its corresponding electronic support, is the basis of the proposed spectrometers. The electronic support will consist of extremely sensitive, precise, and accurate commercially-available electronics that have never before been used together for studying the intramolecular dielectric responses of peptides and proteins, herein regarded simply as proteins.

Technically, the proposed electronics will act as the bridge between the dielectric sample cell, where the actual physical measurement is conducted, and the final reproducible data set. This

proposal encompasses three dielectric spectrometers, the DS4 to DS6. Each proposed spectrometer has different frequency ranges and full-spectrum run-times. Although the spectrometers have different capabilities, they will be used to find and resolve "areas of interest" within each sample of protein. Since high-resolution temperature and frequency studies are proposed, it will be most efficient to first find "areas of interest" using low-resolution techniques and fast acquisition methods of TDDS. Then, once potential "areas" are identified, use the slow high-resolution steady-state methods of FDDS, which will be more sensitive and accurate, to further resolve and confirm the low resolution techniques.

All three spectrometers will be capable of performing either TDDS or FDDS (time- or frequency-domain dielectric spectroscopy). TDDS applies a broadband voltage pulse, i.e., step, square, or noise, to excite the sample to all states simultaneously. The charging or discharging transient current is then measured and processed into a dielectric spectrum. TDDS has the ability to: (i) observe transient phenomena, (ii) greatly reduce the data acquisition and run-time, (iii) rapidly scan for "areas of interest" in new samples, and (iv) provide accurate noise profiles. The disadvantage of TDDS is its limited ability to reject noise, thereby the sensitivity of the instrument is dependent more on hardware implementation than on computational software.

FDDS applies a monochromatic sinusoidal voltage to excite a single steady-state mode of the sample. The complex current through the sample is then measured and processed into a dielectric spectrum by incrementally stepping, or sweeping, through a frequency range. FDDS has the ability to: (i) observe steady-state phenomena, (ii) reject noise using lock-in methodologies, and (iii) greatly resolve dielectric responses with high frequency tolerances. The disadvantage of FDDS is it is impractical at frequencies less than 100μHz and cable impedance and inhomogeneities mask the sample impedance at frequencies greater than 10 MHz. The details of both proposed TDDS and FDDS are forthcoming.

a.1.i. Time-Domain Dielectric Spectroscopy (TDDS)

TDDS is based on applying or removing a voltage pulse with fast rise- or fall-time, respectively, and acquiring the charging or discharging, respectively, transient current through the sample cell versus time. Many long-term cycles can be used to average the charging/discharging profiles to reduce the effect of random noise. TDDS was pioneered by Robert H. Cole [3 4 5 6 7], refined with decreased acquisition times by his successor Saturo Mashimo [7 8 9], and applied to biological systems by Yuri Feldman [10].

DS5, with an intermediate frequency range of 1mHz – 500MHz, is based on the schematic shown in Figure 1a. It comprises a Tektronix AFG3252 arbitrary signal generator capable of delivering a fast rise- or fall-time as controlled from the PC. A maximum of four simultaneous sample cells can be studied as limited by the four analog channels of the detecting oscilloscope. Each isolated and active sample channel will consist of: (i) a buffer amplifier (voltage follower) to isolate its impedance from the other sample channels, (ii) a parallel-plate capacitive sample cell with guard rings, and (iii) a current-to-voltage (I-to-V) inverting amplifier with gain ranging from 10^3 to 10^9. A buffer amplifier will divide and isolate the excitation voltage without affecting its amplitude or phase due to superposition of the other sample impedances. Guard rings ensure fringe effects of the capacitive plates are not included in the measurement. The output of each sample channel will be connected to an input channel of the digital oscilloscope. The data from all four channels are encapsulated into a huge memory block and shunted to the PC via gigabit Ethernet. The proposed custom software will then process the data into dielectric spectra.

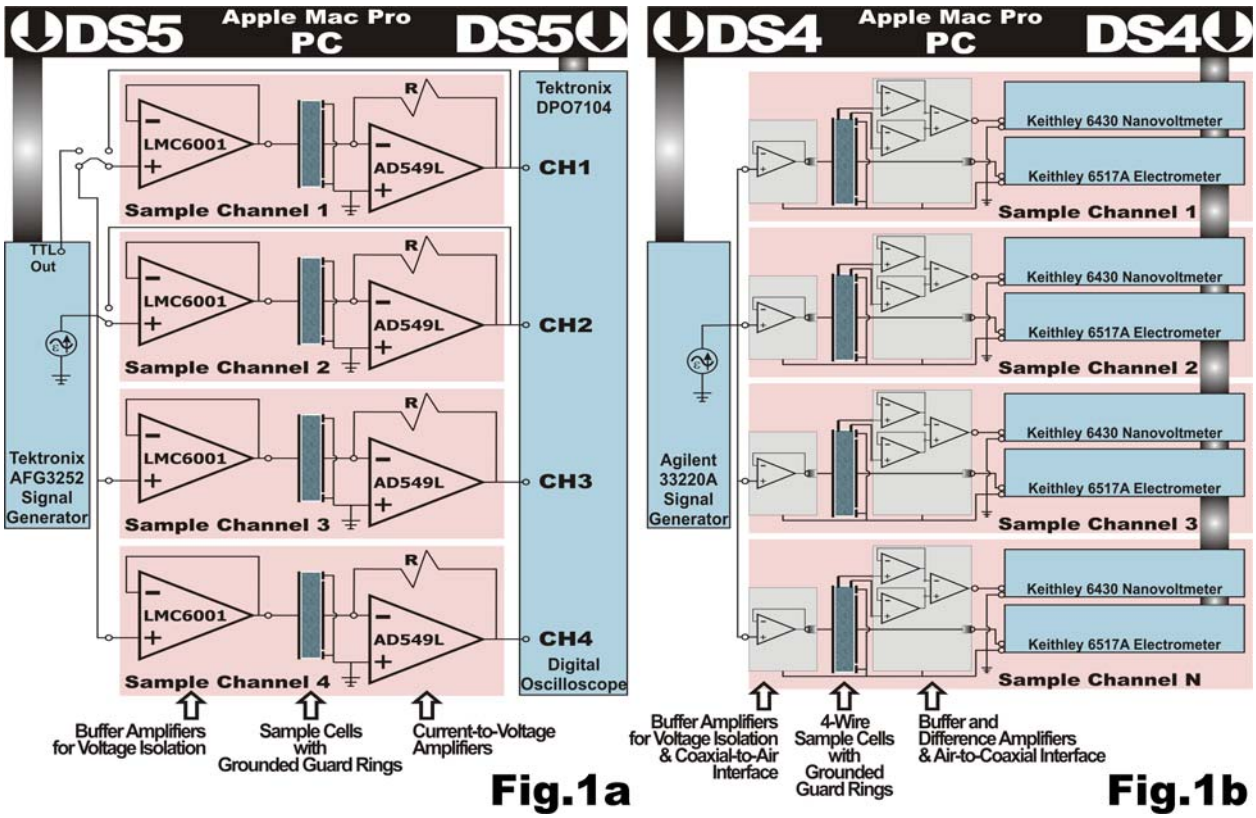

Fig.1a **Fig.1b**

Another approach to the above standard method of pulsed TDDS is to introduce a broad energetic bandwidth using white or Fourier-synthesized pseudorandom noise, as originally developed by Akiyoshi Wada [11 12 13 14]. This method has the advantages of: (i) overcoming the low-frequency propagation limits in coaxial lines, (ii) immunity to thermal drift of the DC conductivity, (iii) further reduce the acquisition time over pulsed TDDS, (iv) ensures linearity of the dielectric response immediately upon excitation, and (v) does not require high voltage pulses. The latter is especially important for biomolecular studies.

DS4, with a low frequency range of 1μHz – 10Hz, is based on the schematic shown in Figure 1b and will employ the above noise TDDS method because of its superior capabilities at ultra-low frequencies. Appropriate noise is applied to the sample cell using an Agilent 33220A arbitrary signal generator. A sample channel will consist of: (i) coaxial-to-air line coupler with an integrated buffer amplifier for multi-sample channel isolation, as discussed above, (ii) a four-wire parallel-plate capacitive sample cell with guard rings, (iii) an air-to-coaxial coupler with buffer and differential amplifiers for simultaneous voltage and current measurements, (iv) a Keithley 6430 voltmeter, and (v) a Keithley 6517A current-sensitive electrometer. The sample channel is designed to reduce parasitic capacitance at low frequencies, hence the incorporation of air lines. The four-wire sample cell reduces the measured electrode polarization at low frequencies by an order of 3 [15 16 17]. Essentially, two electrodes, in the four-wire technique, are used to measure the current through the sample cell, while the other two electrodes simultaneously measure the voltage potential across the sample cell. Buffer amplifiers, with infinite input impedance, for each voltage electrode minimize current drawn from the sample cell for the additional voltage measurement. The sample cell will incorporate Pt-Black coated Pt electrodes to further reduce the electrical double layer dependent polarization of the electrodes. The expected current sensitivity is 10^{-16}A (0.1fA) and the number of sample channels is limited by the availability of the voltmeters and electrometers.

DS6, with a high frequency range of 1kHz – 2.5GHz, is based on the above pulsed TDDS method and requires the application of microwave techniques and thus coaxial line reflectometry.

The impedance of the cables increases over 10MHz due to parasitic cable inductance. Reflectometry includes the impedance of the cables in its measurement. High frequency amplifiers, power dividers, and directional couplers replace their low frequency analogs. Shielded precision air or semi-rigid Teflon [18] lines will be implemented to ensure reproducibility and reduce signal instabilities due to cable inhomogeneities. Using an Agilent E4438C signal generator, a fast rise-time step voltage is applied to the system in which the transient current pulse is measured upon reflection of a coaxial terminus filled with the sample. A fast digital oscilloscope, the Tektronix DPO7254, will record and process the transient reflection and convert it to a dielectric spectrum. This method can extend to 20GHz with the addition of faster commercially-available oscilloscopes.

Both the DS5 and DS6 will use two digital oscilloscopes in tandem. Oscilloscope A will acquire the first waveform and then scope B will immediately acquire the second waveform, thus allowing scope A to shunt its memory block to the PC through a gigabit Ethernet. The scopes can be cascaded in this fashion to acquire extremely long continuous waveforms while maintaining a high sampling rate. The PC will then concatenate the shunted waveforms into a single continuous waveform for further processing. This method has been recently successful by research groups at NASA and the DOE and will extend the frequency range of the proposed spectrometers.

Each sample channel in all three proposed spectrometers, DS4 – DS6, will have the capability of computer-controlled switching between (i) an open circuit, (ii) a close circuit, (iii) a calibrated reference capacitor, or (iv) a sample cell containing an unknown protein sample. This calibration technique was introduced by Grosse and Tirado [19] [20] [21] and can also be used with the following FDDS.

a.1.ii. Frequency-Domain Dielectric Spectroscopy (FDDS)

FDDS is based on applying a monochromatic sinusoidal voltage and acquiring the steady-state current response through the sample cell. This method differs from TDDS in that the steady-state current persists and thus allows the software to focus on the frequency imposed on the sample. This forms the foundation of lock-in methodology. A dielectric spectrum is derived by incrementally stepping, or sweeping, through a range of frequencies with the signal generator and measuring each frequency response with the detector. Most of the hardware between the TDDS and the FDDS methods are the same. The primary differences are (i) the form of the applied voltage, i.e., pulse or monochromatic sine wave, (ii) the control of the hardware for the two different modes, and of course, (iii) the software, which maintains the largest difference between the TDDS and FDDS modes.

DS4, with a low frequency range of 100μHz – 10Hz, and DS5, with an intermediate frequency range of 10mHz – 240MHz, are based on the schematic shown in Figures 1b and 1a, respectively. They will comprise similar hardware as under TDDS, therefore all of the hardware techniques for noise-rejection and increased sensitivity will also benefit each under FDDS. Converting voltmeters into highly sensitive lock-in amplifiers was first proposed by Albertini *et. al.* [22]. The essence of which has been modernized by our group for a preliminary DS5 and is discussed further under "b.2. Current Research and Instrumentation". This work supports a proposed current sensitivity of $5x10^{-14}$ to 10^{-13}A (50 to 100 fA) for DS5 delivering a superior sensitivity than the leading commercial-available spectrometer by at least a factor of six. Under FDDS, DS4 will have a comparable current sensitivity of 10^{-16}A (0.1 fA) as under the proposed TDDS mode.

DS6, with a high frequency range of 1MHz – 2.5GHz, is also based on microwave techniques however a different hardware setup is required as compared to TDDS reflectometry. This is because standing waves from the monochromatic sinusoidal voltage overwhelm the reflected impedance signal of the sample. Therefore, DS6 under FDDS will comprise sample cells and cabling techniques for network analysis. Essentially, network analysis involves the measurement of the reflected and

transmitted energy from and through the sample cell. Lost energy is due to absorption from the sample. Dielectric responses are calculated from the reflected versus transmitted ratios. The lock-in software of the DS5 will extend to the DS6 network analyzer, and thus deliver comparable current sensitivity as proposed in the latter paragraph for DS5.

And finally, all three spectrometers under FDDS will be capable of measuring the response at multiple frequencies, herein called the polychromatic response. Any function can be estimated by a Fourier series. Single term Fourier series is the basis of lock-in methodology. Lock-in techniques inherently reject noise. Therefore, developing software with a combination of these three concepts will return a clean polychromatic response function. This technique may prove essential because, for instance, consider a three frequency response that may occur if the primary mode stimulates a side response due to energy propagation along the protein backbone or other interactions of the inter-motif dynamics. The proposed polychromatic response will detect these additional excitations, whereas current or commercially-available spectrometers are incapable of measuring these side responses. Additionally, a sweep of the applied frequency range would produce an applied frequency versus response frequency versus intensity contour map. Even further advancements would both excite and measure polychromatically and thus provide new methods and insight of intramolecular protein modes, dynamics, and inter-motif interactions. The proposed hardware of all three spectrometers is capable of polychromatic excitations and responses, wherein the primary development lies in the software, its control of the instrument, and processing of the raw data.

The innovations to the above proposed designs are: (i) TDDS and FDDS modes using similar hardware, (ii) isolated and active sample cells, (iii) multiple simultaneous sample channels per instrument, (iv) combinations of monochromatic and/or polychromatic excitations and responses to study the "dance", through advanced Fourier analysis, of the intramolecular interactions and dynamics of the protein sample or generalized device under test, and (v) scalable software that is transferable to any commercially-available T&M equipment, i.e., signal generators, electrometers, and oscilloscopes. Commercially-available dielectric spectrometers, i.e., NovoControl, have active, but not isolated, sample cells. The isolation will allow simultaneous study of multiple sample cells, thereby greatly decreasing the total time to search for "areas of interest" or perform high resolution frequency or temperature studies on proteins. Additionally, commercially-available dielectric software is designed for only their proprietary counterpart hardware. The proposed software will be designed to run on ubiquitous T&M equipment, i.e., Agilent, Keithley, LeCroy, Tektronix, etc., and thus allow researchers and developers from all industries to convert their already available T&M equipment into highly-sensitive multi-sample impedance and dielectric spectrometers. Thereby, bringing this fundamental and essential measurement and instrumentation to a vast constituency.

a.2. Computer Control and Data Acquisition

Each of the three dielectric spectrometers, DS4 – DS6, will interface to an Apple Mac Pro personal computer (PC). Each PC will interface to the signal generators and detectors, i.e., voltmeters, electrometers, and oscilloscopes, using a General Purpose Interface Board (GPIB), fast or gigabit Ethernet, or Universal Serial Bus (USB), depending on the capabilities of the equipment. Switching mechanisms within the signal channels will be controlled by digital interface boards. To reduce cost, each PC will use the wholly stable Apple OSX Operating System, based on Linux, with National Instruments LabView Full Development System, version 8.2. All electronic and instrument control will be with custom LabView applications. Real-time data acquisition, as well as, digital filtering, lock-in amplification, statistical, least-squares regression and fitting, and graphical analysis, and dielectric spectral display and storage will also occur within the LabView applications. In summary, LabView running on an Apple Mac Pro will interface, control, and process the data from each of the three spectrometers, for both TDDS and FDDS methodologies.

a.3. Sample Cells

The sample cell is where the physical quantity begins its mapped representation to the collected data. Since the electric polarization of matter is based on the separation of charge, leading to dipoles and multi-poles, it will be affected by an applied electric field. The applied field is the experimental probe used to study the electric polarization within any sample or material. Changes in the electric field represent changes in the electric polarization and charge distribution within the material, hence the physical nature of impedance, admittance, and dielectric spectroscopy.

The sample cell will consist of an insulating body and two conducting electrodes, separated by a controlled distance. At any particular instant, one electrode will be positively or negatively charged to a certain magnitude. The second electrode will have the opposite charge and equal magnitude, thus an electric field is created between the space of the two electrodes. If an insulating material, a dielectric, is placed between these charged plates, then the applied electric field will cause polarization or charge separation of the material, thus increasing the measured displacement field.

Dielectric spectroscopy has been developing since 1897 [23] and five polarization phenomena have been observed: (i) Maxwell-Wagner [24 25] for heterogeneous phases or interfacial polarizations, (ii) electrode polarization which is due to the pseudo-ordering of ions on the electrode surfaces to form an electrical double layer [26 15], (iii) orientational due to permanent dipole moments in polar molecules, and inductional due to (iv) atomic and (v) electronic polarizabilities. The latter two occur at extremely rapid rates and are far beyond the frequency range for dielectrics of structural motifs; therefore they will not be discussed further. The primary goal of the proposed project is to decrease the effects of (i) and (ii) while increase those of (iii).

The proposed work will employ a number of techniques to reduce or negate the effect of electrode polarization. Four well-established techniques are proposed herein: (i) four-terminal electrodes [15 2 24], (ii) adjusting the ratio of electrode separation versus the electrode area [20 26], thereby increasing the bulk response, (iii) surface etching and roughening of the electrode [27], and (iv) coating the electrode with Pt-black [15 28 21 19 20], in which Tirado [21] reports a decrease of electrode polarization by 3 orders of magnitude. Additionally, since electrode polarization predominates at low frequencies, it is expected that the noise TDDS method of DS4, as previously discussed in "a.1.i. Time-Domain Dielectric Spectroscopy (TDDS)", will further disrupt this electrode effect while maintaining the dielectric response of the proteins.

The applied electric field will experience boundary conditions at the edges of the electrodes. These fringe effects will be present but not measured by incorporating grounded guard rings into the sample cells' detector-side electrode, as shown in Figure 1. Essentially, this electrode will be smaller than the power-side electrode and will comprise an extra insulating ring and an outer conducting guard ring. The power-side electrode will create the fringe fields and the guard ring will shunt them to ground. Therefore, the inner detector-side electrode is only absorbing the uniform electric field through the bulk sample. Grounded guard rings were initially presented in "a.1.i. Time-Domain Dielectric Spectroscopy (TDDS)".

Although thermoelectric coolers and heating coils are inexpensive methods for temperature control, they will emit electronic interference that will disrupt the dielectric responses of the samples. Therefore, precision liquid coolers and heaters, i.e., Julabo USA, Inc., will be employed to control the temperature of the sample cells within a range of -88 to 300°C and a stability of 0.01°C. The liquids are nonpolar derivatives of silicone oil with miniscule electrical conductivity and a dielectric constant of less than 3 [29]. Therefore a single cooler or heater should maintain temperature control for all sample channels of a single spectrometer. The controllers will facilitate extremely stable high-resolution temperature studies for the proposed cold near-frozen studies and the below protein denaturation studies where the tertiary structure is melted but the secondary structure remains intact,

as further discussed in "a.4. Peptide and Protein Samples and Studies" and "b.1.ii. Structure and Permanent Dipole Moments of Peptides and Proteins".

In summary, the sample cells will be capacitive plates separated by a discreet distance and held firmly by an insulated body. The protein samples will be between the two capacitive plates. Grounded guard rings will negate fringe effects and five techniques will be explored to reduce electrode polarization. And finally, nonpolar liquid immersion baths will be employed for accurate high-resolution temperature studies on the protein samples.

a.4. Peptide and Protein Samples and Studies

The Protein Databank (PDB) [1] is a free and searchable internet site at Rutgers University, NJ, containing over 41k submissions. The protein structures submitted to the PDB are near atomic-resolution 3D maps characterized by either X-ray crystallography or nuclear magnetic resonance (NMR). The PDB is an established library of 3D protein structures. The PDB will be extensively searched to find model proteins for this research. The proteins chosen for the study will be previously characterized by complimentary methods and commercially-available, i.e., Sigma-Aldrich. The initial studies will comprise proteins with a high content of pure α-helices, preferably with only a few sub-domains which contain no ligands or porphyins. Such model systems will facilitate the observation and assignment of the intramolecular structural motifs.

Initial protein samples will be studied in dry powdered and in pure aqueous forms. Powdered protein samples have the advantage of having constrained rotational movement, which will have the effect of decreasing the dielectric response from the molecular net dipole moment. Dry samples will also produce negligible electrode polarization effects because mobile charge or ion carriers will not exist, as previously found studying hemoglobin with the current DS2.

Salt and pH variations on aqueous solutions will confirm previously reported studies of the molecular net dipoles of proteins and further characterize electrode polarization effects. The permanent dipole moments of the protein's structural motifs will be perturbed by the localized acidic or basic R-groups of the amino acids whose backbone atoms create those motifs. These perturbations will be studied by varying the pH of the solution. At least the three extremes can be evaluated; acidic, neutral, and basic solutions. Such pH variations of the solution will charge or uncharge the side R-groups accordingly, depending on the exposure of the R-group to the solution, its pK_a value, and the pH of the solution.

The most important studies of this project may be the high-resolution high-temperature experiments below the denaturation point of the protein where the tertiary structure is partially or fully melted and elastic, flexible, or fully open, but while the secondary structural motifs remain fully intact. Since the α-helical and β-sheet motifs have extensive hydrogen-bond lattices and as such have a high enthalpy, the hydrophilic/hydrophobic interactions compressing the tertiary molten core structure should release at a lower temperature than the structural motifs. This is the "area of interest" for these studies. Stable resolved warming of the protein should soften the tertiary structure and molten core, thereby allowing the intact secondary structural motifs some torsional degrees-of-freedom in which to respond to the applied field. These studies will be performed using the Julabo liquid heaters as described in "a.3. Sample Cells". Since these studies are temperature dependent, activation energies of the tertiary melt should lend information on the degree of crystallization of the molten core, through an Arrhenius approach, while proving the strengths, relaxation rates, and heterogeneity of the permanent dipole moments of the intramolecular structural motifs of proteins.

The molecular net dipole will probably mask the dielectric responses of the intramolecular motifs. It is conceivable that the torque and rotational inertia of the whole protein will require immobilization to decrease the net dipole which will, in turn, enhance the dielectric responses of the

intramolecular structural motifs. Therefore, it is proposed to rotationally immobilize the proteins with three techniques: (i) semi-solid chemical matrices, (ii) thermal effects, and (iii) molecular alignment using magnetic fields in conjunction with the latter two techniques. Essentially, these techniques will increase the viscosity of the medium, thereupon "freezing" the macromolecules rotational inertia.

The first technique is to embed the protein in a highly-viscous semi-solid chemical matrix to impede the rotational motion of the protein. Gelatins and agaroses, collectively known as gels, form semi-solid aqueous environments that are commonly used in biochemical research, i.e., electrophoresis and chromatography. The latter techniques use gels to slow the translational mobility for protein separation and purification. It is proposed to use these gels in higher concentrations to also inhibit the rotational inertia of the proteins. These gels are also known to have low dielectric signatures as proven by electrophoresis. Gels are the most obvious choice for the proposed protein study because they imitate *in vivo* environments. Other highly viscous substances may also be investigated, such as ionic or quaternary ammonium liquids. Prior to protein studies, various commercially-available high-purity gels will be characterized with the proposed spectrometers.

The second technique will use low temperature studies, in the range of –88 to 0°C, in conjunction with trehalose, a sugar commonly known as "nature's antifreeze". Studies below the freezing point of water will reduce its thermal agitation on the proteins, thereby decreasing the normally broad Maxwell-Boltzmann distribution and thus environmental noise contributing the dielectric response of the protein. This combination of low temperatures and trehalose should create a pseudo frozen, highly viscous solution that is not crystallized, to reduce the agitation due to the water while simultaneously constraining the protein under study.

If required, we will also study the effects of magnetic alignment of the protein's net dipole on their dielectric responses. Scheraga *et.al.* [30] suggest that dipoles of the structural motifs, most notably α-helices, have a general alignment to the net dipole of the folded protein. Therefore, by aligning the net dipole of the protein, this should increase the dielectric signal from a greater population of motif aligned dipole moments. The magnetic alignments may also be used in conjunction with the gels to impede the rotation once magnetic alignment is applied.

a.5. Expected Dielectric Responses, Interpretation, and Publication

The phenomenological response of the complex dielectric is shown in Figure 2. Peter Debye [31] first interpreted this response as the reaction of electric dipoles with the applied electric field of polar molecules. The frequency dependence is based on the relaxation rate of the dipole due to a viscous environment or other dampening forces. The early work of Debye centered on simple polar molecules with first-order responses.

Dielectric responses became increasingly more complex as the molecular complexity grew with the system. For example, amorphous polymers and biological macromolecules present broadened and skewed dielectric loss peaks which have been successfully fit to the phenomenological equation of Havriliak and Negami [32 33 25], or

$$\varepsilon^*(T,P,\omega) = \varepsilon_\infty(T,P) + \frac{\varepsilon_0(T,P) - \varepsilon_\infty(T,P)}{\left(1 + (i\omega\tau(T,P))^\alpha\right)^\beta} \qquad \text{Eq. 1}$$

where $\varepsilon^*(\omega)$ is the complex dielectric response measured at frequency, $\omega=2\pi f$, ε_∞ is the high frequency dielectric, ε_0 is the static or low frequency dielectric, i is $\sqrt{-1}$, τ is the relaxation time for a particular dielectric transition, and α and β is the symmetric and asymmetric broadening of the relaxation, respectively. This relation represents the most generalized form for the Debye [31 34] relation occurs with $\alpha=1$ and $\beta=1$, Cole-Cole [35 36 37] occurs with $\beta=1$, and Davidson-Cole [38 39 3]

occurs with α=1. Schönhals and Schlosser [40 41 25 42 43 44] presented a physical interpretation for α and β in that α is correlated with the intermolecular dynamics and $\alpha\beta$ relates to the local intramolecular dynamics. Additionally, $\Delta\varepsilon=\varepsilon_\infty-\varepsilon_0$ relates to the magnitude of the dielectric response and ultimately the magnitude of the permanent dipole, i.e., the persistence length of the α-helix, and τ relates to the rotational inertia and viscosity of the local environment, i.e., local dampening or frictional forces, at a particular temperature T and pressure P. Other fitting relations exist, such at the stretched-exponential of the Kohlraush-William-Watts (KWW) [45 46 47] relation, however, the Haviliak-Negami relation maintains its prevalence.

In recent years, Georgios Floudas' work on glass-forming polymers and α-helical peptides closely mimics the proposed project. The research of Floudas recognizes that: (i) dielectric spectroscopy is the only instrumentation capable of studying the persistence length and its dynamics of the α-helical dipole of peptides [48 49], (ii) temperature studies of peptides around the glass temperature (T_g) reveals Arrhenius (β and γ processes below T_g) and non-Arrhenius, that of Vogel-Fulcher-Tammann (VFT), (α process above T_g) behaviors [48 49 50], (iii) the latter α process actually reveals multiple component processes [48], (iv) pressure is the physical variable that can distinguish the energy-landscape model from the free-volume model [44 51], (v) temperature, not pressure, dominates dynamic arrest at T_g [50], and (vi) the above Havriliak-Negami relation can be applied to and fit the dielectric responses of peptides under isothermal or isobaric conditions. These recent findings and conclusions support the concepts, foundations, and ultimate success of the proposed project.

In summary, the proposed multi-sample highly-sensitive dielectric spectrometers, along with proper protein candidates and chemical and environmental control, will detect and resolve the intramolecular secondary structure and structural motifs of peptides and proteins. It is anticipated that the success of the proposed instrumentation, sample cell, and software can be published in, for example, the *Review of Scientific Instruments*, and *Measurement Science and Technology*, as well as, any number of electrical engineering, i.e., *Proceedings of the IEEE*, and materials science journals. Additionally, the success of the intramolecular protein dielectric responses, under the various proposed conditions, can be published in, for example, *Physical Review E*, *Journal of Physical Chemistry B*, *Physical Chemistry Chemical Physics*, *Biophysical Chemistry*, *Journal of Chemical Physics*, *Journal of American Chemical Society*, *Journal of Molecular Biology*, *Molecular Physics*, *Macromolecules*, *Biomacromolecules*, *Biopolymers*, and *Bioelectrochemistry*. Dissemination of a successful project through such publications would prove advantageous to the National Science Foundation, industrialists, and academics.

a.6. Task Analysis, Time-Line, and Research Staff

Prior to funding, and after submission of this proposal, it is planned to develop the preliminary foundations for the proposed TDDS. Most of this work will include software development. Our group spent most of 2006 developing and testing the foundations for the proposed FDDS. The FDDS work developed hardware interfaces and custom software to prove noise-rejection techniques and instrument calibration. Further discussion of this work is detailed under "b.2. Current Research and Instrumentation".

After funding, the test protocol of proteins will be accumulated to act as real model systems to achieve the proposed goals. The Protein Databank (PDB) [1] will be extensively searched for protein candidates with high percentages of structural motifs. All candidates will be commercially-available, i.e., Sigma Aldrich. The initial candidates will have a high α-helical, zero β-sheet, and low hairpin turn content because of their strong inherent dipole moments. The next candidates will have a high β-sheet, parallel and anti-parallel, zero α-helical, and low turn content because of their weak dipole moments. And finally, candidates of mixed α-helical and β-sheet content will be scheduled to

gauge the gradient of responses between the α to β extremes. All protein candidates will have zero ligand and porphyrin content to reduce any potential interference of the motif's dielectric responses. Acidic and basic side R-groups, along with their relative positions to the structural motifs, will also be considered in the test models and poised for further pH studies. See section "b.1.ii. Structure and Permanent Dipole Moments of Peptides and Proteins" for more information.

After funding, it is estimated that the first 12 to 18 months will be devoted to the design and development of the sample cells, custom circuitry, and software integration with the commercially-available signal generators, voltmeters, electrometers, and oscilloscopes. The sample cells will require integration of the liquid temperature controllers, metallurgy, nonpolar and Pt-black coatings, isolation and amplification of the sample channels, and proper guarding and shielding of the sample cells and cabling. The amplifiers, wiring, sample cells, and software for the DS4 through DS6 will require about 3 to 6 months, with porting of the existing DS3 software to these three spectrometers. Therefore, the DS4 through DS6 will be built immediately after funding with the initial studies on metallurgy and nonpolar and Pt-black coatings, owing to the reduction of electrode polarization, following shortly after their development.

After the first spectrometer is built, immediate testing may commence on the protein candidates. Since a spectrum may require days to run, parallel development will continue during the initial protein tests. All in all, between the proposed high-resolution temperature studies, the semi-solid chemical matrices, and the protocol of the protein candidate, actual dielectric measurements of the proteins, in their various environments, will require approximately 24 to 36 months.

All researchers on the current and proposed team are professionals in their respective fields, spanning biophysical chemistry, molecular biology, EMI electrical engineering, metallurgy, machining, and software development. The Principle Investigator has 25 years in scientific research. Both the electrical engineer and technician have a combined 50 years experience, the metallurgist is a retired engineer with expertise in patents and product development, and both software developers have a combined 40 years professional experience.

b. Description of Research Instrumentation and Need

b.1. Rationale of Technical Feasibility and Developmental Foundations

b.1.i. Theory and Measurement of Dielectric Relaxation

The classic work "Polar Molecules" in 1929 by Peter Debye [31] formalized the complex dielectric spectra, and anomalous dispersion and absorption, of polar molecules, i.e., water. C. J. F. Böttcher later modernized the polarization of molecules in static and alternating electric fields in his two-volume publication, "Theory of Electric Polarization" [52] [53]. Essentially, the increase in magnitude and change of phase of the displacement field D, is due to the polarization of matter as compared to the *in vacuo* electric field, E, as proven by Maxwell in 1865 [54], or

$$\hat{D} = \varepsilon_0 \left[\varepsilon_\infty + \frac{\varepsilon_s - \varepsilon_\infty}{1 + i\omega\tau} \right] E_0 e^{i\omega t} \qquad \text{Eq. 2}$$

where the variables are defined under Equation 1. Therefore, the observed frequency dependent increase of the displacement field D, as compared to E, is due to the introduction of an insulating or dielectric material, as seen by the second term.

Comparing Equation 2 with the *in vacuo* electric field equation, and solving for the real and imaginary components of the complex permittivity for relaxation processes of any dielectric material yields

$$\varepsilon' = \varepsilon_\infty + \frac{\varepsilon_s - \varepsilon_\infty}{1+(\omega\tau)^2} \quad \text{and} \quad \varepsilon'' = \frac{(\varepsilon_s - \varepsilon_\infty)(\omega\tau)}{1+(\omega\tau)^2} \qquad \text{Eq. 3}$$

where ε', the real component of the complex permittivity, yields the frequency-dependent dielectric constant and ε", the imaginary component, yields the energetic absorption or dielectric loss from the applied electric field. The latter complex dielectric relaxation yields the normalized real and imaginary components, respectively, as shown below

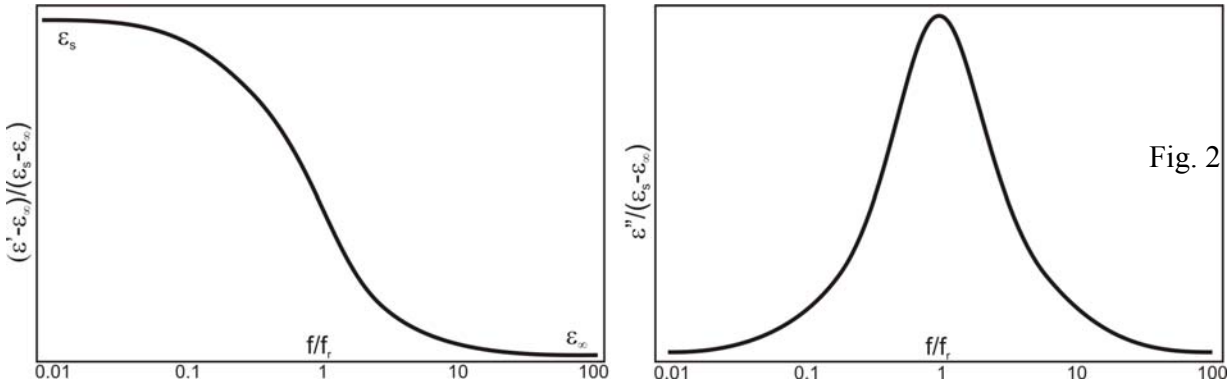

Fig. 2

where the right plot represents the anomalous dispersion, a decreasing permittivity with increasing frequency, and the left plot represents the absorption or energetic loss of the applied electric field. The product, ωτ, is unity at the center of the relaxation transition. The most generalized method to fit dielectric data was discussed under Equation 1.

The molecular understanding of the frequency dependent dielectric response was provided by Debye [31] and later Wada [55], in that the ability for the molecular electric dipole to follow the cyclic electric field depends on its rotational inertia, the viscosity of its surrounding environment, and the temperature of the system. The latter proves an excellent tool for study, exhibiting Arrhenius and non-Arrhenius behavior, and is further discussed under "a.5. Expected Dielectric Responses, Interpretation, and Publication".

b.1.ii. Structure and Permanent Dipole Moments of Peptides and Proteins

Early X-ray crystallographic data revealed four levels of substructure within peptides and proteins, herein known as simply proteins. The four structures are primary, secondary, tertiary, and quaternary structures [56 57 58]. The primary structure is the linear amino acid sequence of each chain within the protein. This extended structure is highly energetic and initial folding of the primary into sub-domains, called the secondary structure, occurs to form ubiquitous motifs. The most common energy reducing structural motifs are α-helices and β-sheets, for these motifs form extensive hydrogen bond lattices. For instance, if the protein is destined to have only α-helices, then the secondary structure would roughly be linear but compressed with sub-domains of spiral-staircase coils. These coils are the α-helices. The tertiary structure would form by additional folding of the non-helical domains so that the α-helices fold in on themselves in a compact 3D globular structure. Additional bonding, van der Waals, and hydrophilic/hydrophobic interactions with the aqueous environment further compresses the protein in which the interior becomes a molten core, or liquid-crystal polymeric center. Quaternary structure occurs when multiple peptide chains bond together to form a larger protein structure. Many proteins do not have quaternary structure because they are composed of only a single peptide chain.

Figure 3 was included to aid in visualizing the secondary and tertiary structures of a protein, in this case myoglobin and was directly derived from the Protein Databank (PDB) [1], entry 1A6G.

Myoglobin is an O_2 transport enzyme composed of a single peptide chain with 151 amino acids. It forms eight α-helical segments which comprise 76.16% of the total protein and surround an organic Iron-based porphyrin ring. Figure 3a is a representation of myoglobin's true atomic positions, excluding hydrogen and porphyrin atoms. The primary backbone of the protein has been highlighted by a yellow tube in which the remainder of the atoms are its amino acid side R-groups. Figure 3b is only the backbone of myoglobin. Its eight α-helical motifs are clearly depicted as spiral staircases. This is the secondary structure of myoglobin. The segments between the α-helices are either random coils or hair-pin turns and these fold to form the final 3D globular sphere. This is the tertiary structure of myoglobin.

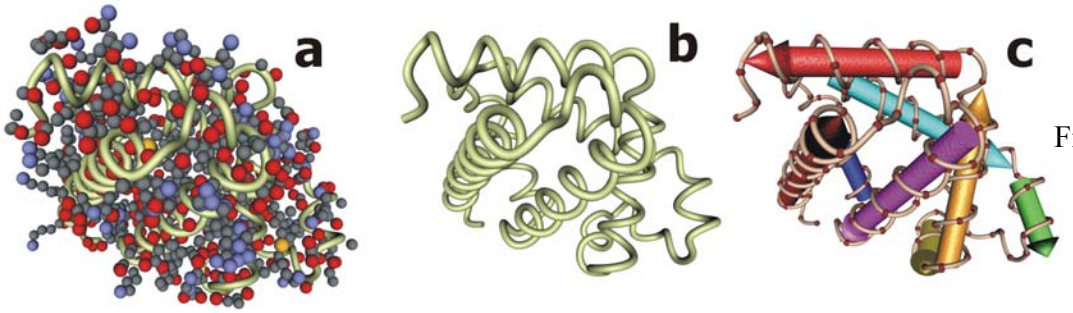

Fig. 3

The driving forces for the formation of secondary structure are steric hindrance and electrostatic interactions of inherent dipole moments. Concentrating on the latter, the typical amide or peptide bond carries a dipole moment of 3.71 Debyes (D = 10^{-18} esu cm = 3.33564x10^{-30} C m) [59] [56]. The moment of water is 1.85 D [60] [61] [62] and that of Hydrogen cyanide is 2.93 D. Therefore in comparison, the amide bond imposes a large dipole moment and thus the primary structure possesses the greatest potential energy. The reduction of the potential energy due to the amide bond dipoles contributes to the folding of the secondary structures.

Experimental and theoretical studies [55] [63] [64] [65] report that the dipole moment of an α-helix is 4 to 5 D per residue or 14.4 to 18 D per helical turn. This may seem ironic since an increase in the dipole strength per residue is apparent compared to the abovementioned 3.71 D for a peptide bond. However, these studies propose that polarization effects occur, which increase the dipole strength, while minimizing the overall potential energy due to hydrogen bond lattice formation in the folding of the α-helix. Every n^{th} residue forms a hydrogen bond with every $n+4^{th}$ residue in the helix, with the net dipole of an α-helix in roughly the direction of the primary axis. Figure 3c shows the eight dipoles that are formed due to the eight α-helical motifs for myoglobin. The dipoles range in strength from 28 to 130 D in which these large differences must be resolvable by dielectric spectroscopy. The molecular configurations of β-sheets tend to produce permanent dipole moments of near 0 D [56] [57] [58] [65].

Dielectric studies by Oncley *et. al.* [66] on native freely-suspended proteins in solution reveal relaxation times ranging from 16 ps to 2.5 μs, which translate to frequencies on the order of tens to hundreds of kHz. The high viscosity of the molten-core of globular proteins will greatly lower the observed frequency of intramolecular structural motifs, for a higher viscosity leads to an increased relaxation time and thus a lower observed transitional frequency. Therefore, it is expected to observe the transitional frequencies in the μHz to kHz range for the intra- and inter-chain structural motifs.

Conceivably, if the liquid-crystal molten core of the protein is gently melted or relaxed by thermal or chemical techniques, as detailed in section "a.4. Peptide and Protein Samples and Studies", then the proposed spectrometers should detect and resolve the individual torques and subsequent absorptions of these freer-floating motifs, especially for varying α-helices. For instance, if high-resolution temperature studies are performed below the denaturation temperature of the protein, specifically, where the tertiary structure is semi- or fully melted but the secondary structure

remains intact, then the secondary structural motifs should absorb the applied electric field at a particular frequency. This temperature range should be possible since the enthalpies of hydrogen bond lattices, that of structural motifs, is higher then those of van der Waals or hydrophilic/hydrophobic interactions. The temperature of the fractionally melted tertiary structure should lend itself to the activation energies of the molten core, through an Arrhenius approach, and the frequency of the absorption to its relaxation rate and intra-chain dampening forces. Hence the basis of this proposal and its spectrometer development.

Intramolecular electrostatics perform vital roles in the structure and function of the protein [67]. Such electrostatics contribute to, "enzyme catalysis, electron transfer, proton transport, ion channels, ligand binding, macromolecular assembly, and signal transduction." [68] Warshel *et. al.* [68] concluded on the failures of dielectric continuum models, such as the Kirkwood-Tanford treatment, that use unrealistically large dielectric constants to compensate for charged R-groups. It is apparent that continuum models are a first approximation designed to reduce computational expense, especially since simple heteroatomic organic molecules generally have π-conjugation, resonance structures, and heterogeneous electrostatic distributions and thus intramolecular permanent dipole moments. It is only reasonable that proteins mimic the same chemistry. There are recent trends of theoretical studies, both *ab initio* [65] and semi-empirical [30] using the PDB, migrating towards models of heterogeneous charge distributions and backbone structural motif dipoles in peptides and proteins.

The empirical data derived from this work would include the frequency-dependent permanent dipole moments of the structural motifs. These values will be directly applicable to theoreticians. Also, the high-resolution below denaturation temperature studies, through an Arrhenius approach, would reveal the activation energies of the melted tertiary but intact secondary structure. Further studies can disseminate the enthalpy and entropy of such mechanisms. Together, these will reveal the dampening forces and inter-motif interactions that can be directly used by theoretical and computational researchers to further our concepts and understanding of peptides and proteins.

In summary, since X-ray crystallography, NMR, and electron microscopy can not directly characterize the electrostatic properties of proteins, dielectric spectroscopy should be developed into a modern complimentary technique. Dielectric spectroscopy can provide direct empirically-based intramolecular electrostatic and dipolar data on proteins to allow for further development of more accurate theoretical models. The need for this complimentary discipline is crucial to the future of proteomics and all surrounding implications and effects, such as protein folding and energetics, enzymatic activity, and drug interaction.

b.2. Current Research and Instrumentation

The Principle Investigator (PI) has actively pursued the current research for four years. Prior theoretical and experimental results provide a strong basis for this endeavor and the PI has invested his own personal funds and time into the development of the Dielectric Spectrometers 1 to 3 (DS1 – DS3). Additionally, seven corporations, Ametek Signal Recovery, Tektronix, National Instruments, Fluke, 3M, Millipore, and Mettler-Toledo, have contributed equipment and technical assistance to the development of the DS1 to DS3.

DS1 and DS2 are based on highly sensitive commercially-available lock-in amplifiers (LIA), the Ametek 7265 and Standford Research SR830 DSP Dual-Phase LIA. Custom software written in National Instruments LabView convert these LIA into dielectric spectrometers. Their high common mode rejection inherently filters noise and extraneous signals. Their frequency range encompass 1mHz to 100kHz, a resolution of 0.1mHz, and a current sensitivity of 2fA. These are more sensitive than any commercially-available dielectric spectrometer, i.e., Agilent and NovoControl.

Our group spent most of 2006 developing the DS3. The work culminated in modern amplification techniques and software for advanced noise-rejection. The work was submitted to the *Review of Scientific Instruments*, in press and peer reviewed, and entitled, "Noise-Rejection Techniques for Impedance and Dielectric Spectrometers Using Ubiquitous Test and Measurement Equipment". The abstract is available at http://iquanta.org/news. Essentially, the DS3 produced a stable reproducible dual-phase (real and imaginary) current sensitivity of 250fA with an average phase stability of $\tan\delta<10^{-2}$. The maximum single phase current sensitivity of 60fA was realized and limited by the custom preamplifier. The sensitivity of the DS3 outperforms that of NovoControl's BDS by a factor of four and possesses inherent multi-sample features at a fraction of the cost. Currently, we are pushing the limits of the DS3 to a projected sensitivity of 100fA and $\tan\delta<10^{-3}$ and should lead to a second publication. The invaluable experience of this work is embedded in the proposed spectrometers of this NSF MRI submission.

And finally, work myoglobin and hemoglobin under (i) weakly hydrated, (ii) aqueous, and (iii) dissolved in gelatin have provided preliminary results under ambient conditions and neutral pH. Hydration studies provide insight into proton migration through the intra-molecular and surface bound water network as initiated by acidic or basic R-groups. These studies were first offered as an alternative mechanism for the observed dielectric response by Kirkwood [69] and continue to the present by Bruni [70 71 72 73]. The *in vivo* environment of most proteins is within aqueous polar water. These studies allow for observation of the net-dipole moment and its dielectric response due to pH changes [74 75 76]. The primary challenge of these studies is overcoming the electrode polarization at low frequencies [21] due to water's high dielectric constant (~80). Our initial work of dissolving proteins within a gelatin matrix has proven promising. Gelatin is a protein with miniscule α-helical content, and thus offers a negligible overall dipole moment and dielectric fingerprint. It was used to encapsulate myoglobin in a highly-viscous environment to impede its rotational motion. Studies of variable myoglobin concentration in gelatin prove its effectiveness. Development of more precise spectrometers and temperature control should confirm the latter studies.

b.3. Justification of Proposed Development Versus Commercially-Available Instrumentation

Our research of published dielectric instrumentation concerning biologically-oriented research reveals that researchers employ commercially-available network or impedance analyzers. The research groups are Samouillan *et. al.* [77], Bruni *et. al.* [78 72 73], Facer *et. al.* [79], Grosse *et. al.* [19 20], Weingartner *et. al.* [80], Bordi *et. al.* [81], Kubisz *et. al.* [82], Smith *et. al.* [83], Risuleo *et. al.* [84], and Floudas *et. al.* [85 48 86 50 49 51 87 88]. In this arena, three manufacturers are referenced; Agilent Technologies, Solartron Analytical, and NovoControl Technologies.

Agilent provides high frequency ([20,110G] Hz) analyzers with poor sensitivity (>20 μA) for the industrial purposes of electronic component testing. The analyzers are not designed for research. Solartron provides ultra-low to medium frequency ([10μ,32M] Hz) analyzers with excellent sensitivity (>10fA) however their products carry an unreliable reputation. NovoControl provides ultra-low to medium frequency ([3μ,20M] Hz) analyzers with good sensitivity (>1pA). The costs to duplicate the proposed spectrometers, with four simultaneous sample channels and similar temperature control, using NovoControl "Concept 80" would be $787,600 U.S.D. under the 2006 pricelist. Therefore, NovoControl has an exceedingly high cost, poorer sensitivity, and limited to only steady-state FDDS. Further discussion of NovoControl follows under the "Budget Justification". In short, the summary of innovations listed in the last paragraph of section "a.1.ii. Frequency-Domain Dielectric Spectroscopy (FDDS)" can not be realized with any current commercially-available impedance analyzers or dielectric spectrometers.

c. Impact of Infrastructure Projects

infinite quanta, inc. is a nonprofit I.R.S. designated 501(c)(3) organization formed for the purpose of performing pure scientific research. The Articles of Incorporation and I.R.S. tax-exempt application and ruling are located at http://iquanta.org/organization/corporate. In addition to our current collaborative efforts with academia and industry, our work will be published as discussed under "a.5. Expected Dielectric Responses, Interpretation, and Publication". These efforts may inspire new research avenues, bring the importance of dielectric instrumentation to biological research, and this work to a national view. Additionally, as discussed in "a. Research Activities", the commercial value of this work, most notably the software advancements, can progress the instrumentation and general implementation of impedance and dielectric studies to academics, industrialists, and hobbyists. In short, the impact of this work can affect the electronics and materials research and industries, dielectric understanding, and the fields of biophysics and molecular biology.

The organization is composed of cross-discipline professionals with formal training and experience ranging from molecular biology and physics, to engineering, computer programming, and instrumentation. Our organization will grow to encompass personnel and collaborations, both commercial and academic, of an innovative, intellectual, creative, and scientific spirit regardless of age, gender, ethnicity, creed, sexual orientation, or religion.

d. Management Plan

The "Task Analysis, Time-Line, and Research Staff" section is under section a.6. As indicated in this section, all research staff are seasoned professionals with specific background and skills to implement all aspects of the proposed project. The resumes of the entire research staff are included under "Biographical Sketches". The estimated costs of the project are included in the "Budget", "Budget Justification", and manufacturer quotes under "Supplementary Documents".

Stephen Lukacs is the Principle Investigator for the proposed project. He will be responsible for carrying out the research objectives, as well as, the overall coordination of the research effort. He will be in charge of the budget, the purchasing of equipment and hardware, and any fiscal audits. He will also develop the computer applications. He will chair weekly or biweekly group meetings. These will be the primary venue for measuring the project's status and progress, educating group members for mutual understanding of the project in general, and brain-storming to resolve problems and issues.

Travis Carter and Bob Macias will work together to design, simulate, fabricate, test, and calibrate any electronic components and custom circuits required for the project. They will fabricate specially designed low-noise circuits and integrate those circuits with commercially-available amplifiers and instrumentation to create the complete DS4 through DS6.

Jonathan Rose and Walter Petersen will choose the proper materials, design, machine, and construct the custom sample cells. Integration of the sample cells with the circuits, instrumentation, and fluid and temperature control systems lies at the heart of the spectrometers, therefore, all members of the team will need to collaborate during the design of the sample cells. The initial group meetings will focus on this critical aspect of the project.

Once the three proposed spectrometers are constructed, Carter and Macias will ensure that the electronics and instruments remain properly calibrated and maintained to specifications and expectations. Lukacs and Rose will develop and maintain the computer applications, prepare samples, and examine the spectra of the peptide and protein samples. Lukacs will process, analyze, and interpret the data and develop new samples, or sample characteristics, for further studies, and publish the results. Commercialization of the software can be through license and distribution agreements with Agilent or Tektronix.

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Biographical Sketches

Stephen J. Lukacs Jr.
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EDUCATION August 1985 December 1991	Bachelor of Science degrees in Microbiology/Molecular Biology, Chemistry, Physics, and a Mathematics minor, from the University of Central Florida (UCF), Orlando, FL.
August 1993 October 1999	Ph.D. in Physical Chemistry from Rutgers University, Piscataway, NJ. Advisor: Frederick H. Long. Thesis Title: Development and Implementation of Optical Instrumentation for the Investigation of a Liquid Crystalline Random Copolyester.
EXPERIENCE May 1986 September 1986	University of Central Florida, Orlando, FL. Dr. David Kuhn. I studied master gene control via enzyme markers in developing fruit fly larvae. This work involved DNA recombination, and protein and DNA purification techniques.
August 1990 August 1991	University of Central Florida, Orlando, FL. Dr. Binayak Dutta-Roy. This position specialized in cold temperature physics, electronics, data acquisition and control, lasers, optics, and electro-optic detectors to study protein dynamics and hyperfine spectroscopy.
September 1991 December 1991	University of Central Florida, Orlando, FL. Dr. Lee Chow. This work determined the speed of light using innovative picosecond timing techniques and classical time of flight measurements.
September 1991 September 1992	Free Electron Laser (FEL) Group at the Center for Electro-Optics and Lasers (CREOL), Orlando, FL. This position specialized in high voltage electronics, CAD, ultra-high vacuum techniques, and high energy particles and lasers.
August 1992 February 1993	Rudolph Research, Inc., Flanders, NJ. This position involved the research and production of custom optical systems for ellipsometers, polarimeters, and refractometers.
February 1993 August 1993	Load Star Systems, Newton, NJ. This position involved creating a new account receivables software system through their proprietary operating system and assembler-based database language.
August 1993 October 1999	Ph.D. Candidate in Rutgers University, Piscataway, NJ. I specialized in the design and construction of laser, optical, spectrometers, and near-field microscope equipment for the study of polymer films and interfaces. In addition to the latter research, I also taught primarily college freshmen and seniors in general, analytical, instrumental, and physical chemistry.

June 1991 Present	Infinite Quantum, Inc., Yulee, FL. President and Head Developer. The foundation of this work began in 1985 as a computer consultant and programmer in Orlando, FL. This work involves the development of logistical systems for fleet maintenance. This system is based on the two-tier client/server database model using 32-bit versions of Fierbird SQL Server and Borland Delphi. This system is featured at http://www.iquanta.com.
May 2002 Present	Infinite Quanta, Inc., Yulee, FL. Founder and President. A non-profit scientific research organization that specializes in the development of scientific instrumentation and chemical techniques for the biophysical and biochemical fields.
ABILITIES Computer Development and Software	National Instruments LabView and Mathworks MatLab. Borland Delphi v1 & above, Pascal v7, and C and C++. ISIS Draw v2.2, Mathematica v2 & above, Maple v9 & above, Origin v5 & above, Igor. Nemetschek Vectorworks CAD Designer v12.
AWARDS	2nd Place in the 1985 Florida State Science Fair. 4th Place in the 36th International Science and Engineering Fair. 1998 William Rieman Award for teaching excellence at Rutgers University, with a honorable mention in 1997.
PUBLICATIONS	U.S. Copyright. Fleet Dynamics Client/Server 32. 1994 – 2006. U.S. Patent #4,772,274. Feminine Hygiene Apparatus. 1988. Lukacs, Stephen J., *Journal of Physical Chemistry B*, Temperature-Dependent Photophysical Properties of a Liquid-Crystalline Random Copolyester, 2001, 105(17), 3372-3377. Lukacs, Stephen, *Rutgers University Press*, Development and Implementation of Optical Instrumentation for the Investigation of a Liquid Crystalline Random Copolyester, 1999. Lukacs, S.L. Cohen, S.M., Long, F.H., *Journal of Physical Chemistry B*, Optical properties of a liquid-crystalline random copolyester, 1999, 103(32), 6648-6652. Burton, J.C., Sun, L., Pophristic, M., Lukacs, S.J., Long, F.H., *Journal of Applied Physics*, Spatial characterization of doped SiC wafers by Raman spectroscopy, 1998, 84(11), 6268-6273. Chow, L., Lukacs, S., Hopkins, K., *European Journal of Physics*, "Speed of light" measurement using BaF_2 scintillation detectors, 1994, 15, 49-52.

Travis J. Carter
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EDUCATION

Florida Atlantic University, Graduate Studies in Electrical Engineering – Introduction to EMC, Clayton Paul (3 quarter hours).

Florida Tech, Masters in Space Systems, 1993 – Curriculm provides a broad technical overview of space systems. Courses included Space Power Systems, Satellite Communications, Remote Sensing and Guidance Navigation Control.

Auburn University, Bachelor in Electrical Engineering, 1989 - Senior electives included Introduction to EMC, Henry Ott and Antenna Theory.

Other Training: IEEE Symposium 1993, 1994, 1995, 1997, 1998; 1999, Space Radiation Symposium 2000, "EMC: Designing Digital Equipment for Compliance", Compliance Engineering; "Conference on Lightning and Surge Protection", U. of Florida; "Lightning Protection Seminar", Dr. Peter Hasse; "Grounding and Bonding of Building Electrical Systems", NTT; "Introduction to EMC", R&B Enterprises .

EXPERIENCE

President / Licensed Professional Engineer.
Engineered Solutions of Amelia, Inc.; Amelia Island, FL.
September 1997 to Present
Providing electrical contracting and professional engineering services in the design, installation and testing of custom integrated electrical systems for residential, commercial, and industrial applications. System designs include Application Specific Industrial Control Boxes, Wired/Wireless Video, Voice and Data Networks, Distributed Audio and Medical Waste Disposal System and Lightning /Surge Protection.

December 2003 to September 2004
Engineering Manager
Unison Industries; Jacksonville, FL
Engineering manager of Technical Support Group including engineers and technicians responsible for development and certification testing of all product lines. Test support includes EMI, vibration and thermal testing in addition to operational performance testing. Support also includes failure/root cause analysis and full documentation. Role as a senior technical support staff member has remained as a function as well as the EMC/Certification expert for the company. Responsibilities also include capital equipment acquisition, long term planning and test facility management across all plant locations.

December 1994 to December 2003	**Senior Staff EMC/Electrical Engineer** Unison Industries; Jacksonville, FL EMC/Certification expert for the company. Providing EMC design and test support for all electronic products which consist of ignition systems for aerospace and industrial turbine engines. The EMC requirements include EMI , ESD, power transients and lightning protection designs for AC and DC circuits. Responsibilities also include electrical design, troubleshooting, and certification (UL/CE/FCC/CSA/ETL) of high voltage capacitive discharge products for the ignition systems. Performed as the lead engineer developing a new electrical satellite propulsion system for NASA.
April 1989 to November 1994	**EMI/TEMPEST Test Engineer.** Harris Electronic Systems Sector; Palm Bay, FL Responsible for supervising and performing EMI/EMP/ESD/TEMPEST tests on telemetry data links, communication systems, and data processing equipment. Duties included developing proposals; preparing test procedures/reports; managing cost accounts; performing EMI/EMP/ESD/TEMPEST tests; and assisting in failure analysis and redesign of non-compliant equipment. Company expert in the testing of RF transmitters and receivers for performance characteristics such as spurious responses, channel isolation, and receiver sensitivity which required extensive knowledge of RF design and test equipment. Functioned as EMI Technical Representative for test laboratory from November 1992 in addition to performing the following tasks in other Harris divisions.
September 1993 to November 1994	**Electrical Design Engineer.** Harris Government Aerospace Systems Division; Palm Bay, FL. Member of the Army's Multiple Launch Rocket System (MLRS) Improved Fire Control Panel (FCP) design team. The FCP is a customized X486 computer which includes a keyboard, display, hard drive and four circuit card assemblies (CCA). Responsibilities included electrical design of a digital and analog electrical design of a CCA and the interconnect backplane internal wiring harness. The CCA provided the following interfaces: VMEbus; SCSI bus; keyboard; serial; various discretes.
October 1991 to November 1992	**EMI Design Engineer.** Harris Government Aerospace Systems Division; Palm Bay, FL. Team member of the Audio/Video Distribution System and Automated Test Set for the NASA Space Station. Responsibilitie for EMI design of the fiber optic based audio distribution units (3), performing development testing and automated test set design.
LICENSES / MEMBERSHIPS	Licensed Professional Engineer for the State of Florida, NARTE Certified EMC Engineer, Florida State Certified Electrical Systems Contractor.
PUBLICATIONS	Switch Mode Power Supplies: An EMI Engineer's Point of View, IEEE Southcon Electronics Conference, March 1994.

Bob Macias
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EDUCATION 1960 - 1961	DeVry Technical Institute. Graduate of two year engineering program.
EXPERIENCE 1953 - 1955	Detroit News. United Press Photographer, Editorial Department.
1955 - 1959	U. S. Army Corp of Engineers. Combat photographer.
1961 - 1962	Zenith. Development of test equipment for sub-miniature audio amplifiers.
1963 - 1965	Motorola. Field Test Engineer responsible for diagnostic equipment for the hearing impaired.
1965 - 1968	Started a business venture using a novel molding process which allowed fabrication of a hearing aid within a customer's ear in less than two hours using common dental materials.
1968 - 1970	Bendix. Testing and developing of procedures for microwave weather systems used in commercial airplanes.
1970 - 1995	Sensormatic Electronics. Developed test strategies in the production area for printed circuit boards, test methods for silicone wafers, wrote computer programs for microchip testing, and developed equipment for testing magnetic and optical devices.
2000 - 2007	Consultant on electronics used in the study of molecular properties of peptides and proteins.

Walter A. Petersen
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EDUCATION 1956	Bachelor of Science degree in Metallurgical Engineering from the Polytechnic University of New York
1963	Master of Science degree in Metallurgical Engineering from the Polytechnic University of New York
EXPERIENCE 1956 -1960	Grumman Aircraft Engineering, Bethpage, NY. Failure analysis and materials specification for airframe components.
1960 – 1984	International Nickel Company, Sterling Forest, NY. Research Metallurgist specializing in welding research; Patent Agent for Corporate Patents & Licensing Group; Principal Metallurgist Processing Research; Senior Project Manager Product Development & Marketing. Devised welding consumables, filed and prosecuted patent applications; invented weldable, low melting point stainless and nickel base alloys and coinage alloys; marketed oxide dispersion strengthened nickel base alloys for gas turbines.
1984-1987	General Electric Aircraft Engines, Cincinnati, Ohio. Manager Advanced Quality and Metallic Materials Marketing. Marketing and preparation of military proposals.
1987 – 1996	Polymet, Cincinnati, Ohio. Director R&D and Quality Assurance. Created process for weld repairing single crystal gas turbine blades. Designed continuous wire grinding machine. Prepared ISO quality assurance system.
1996 - 2007	Self employed Consultant. Welding wire processing, quality assurance, machine design, chemical industry security, proposal preparation.
PUBLICATIONS & PATENTS	12 U.S. patents, 18 publications.

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EDUCATION 1984 – 1991	Bachelor of Science degree in Computer Science, Mathematics and Physics, Rutgers University, New Brunswick, NJ.
EXPERIENCE 2000 – 2007	Farious Net Solutions, Sussex, NJ. Owner/President. Provide custom hardware and software solutions for large-scale server installations, high speed network and data storage technologies.
1992 – 2001	Rutgers University, New Brunswick, NJ. Co-Adjunct Professor. Taught classes in computer architecture design and development of microprocessors and Internet/networking technologies.
1999 – 2001	Diamond Machine. Prototype Designer. Computer aided design of parts for computerized milling machines.
PATENT	U. S. Patent #6,904,364, Navcell Pier-to-Pier GPS

Budget Justification

The budget includes all projected equipment, materials, supplies, personnel, consultants, and administration required for the 36 month development for the proposed Dielectric Spectrometers 4 through 6, DS4 – DS6, and environmental and chemical techniques, as discussed in the "a. Project Description". All costs have been aggressively minimized while maintaining the specifications of the equipment and level of expertise in order to achieve success of the proposed project.

The bulk of the equipment expenses are for the electronics from Agilent, Keithley, and Tektronix, the Apple workstations, and the freezers and heaters from Julabo. The signal generators from Agilent and Tektronix, totaling $55.6k, will be used to apply the proper pulse, noise, monochromatic, or polychromatic electric field to the sample channel and cell. The combination of the generators range in frequency from 1μHz to 3GHz and apply the voltage waveforms necessary for the implementation of the proposed DS4 – DS6. The voltmeters and electrometers from Keithley as required for DS4, totaling $64.4k, will allow for a three sample channel instrument with the proposed four-wire capacitive sample cell configuration. The digital oscilloscopes from Tektronix as required for the DS5 and DS6, totaling $187.6k, will allow for a minimum of three sample channels per instrument. A minimum of two scopes for each DS5 or DS6 are required to implement the tandem data acquisition technique for obtaining long continuous waveforms at high sampling rates. The technical details of the latter are under "a.1. Measurement of Dielectric Signals".

Apple Mac Pro computers are the only workstations available to handle the proposed equipment connectivity, instrument control, data acquisition, and most importantly, post-processing of the raw data into final noise-rejected dielectric spectra. The proposed pseudo-random noise for TDDS and the polychromatic spectroscopy under FDDS, both detailed in section a.1., will require memory and computational resources beyond conventional single processor personal computers. Other manufacturers, such as Dell or HP, can not provide workstations with the required specifications for the proposed software and project. Three Apple workstations and three APC backup power supplies are required and total $30.5k.

The liquid freezers and heaters from Julabo comprise another $53.5k. Thermoelectric coolers and heating coils were seriously considered for this project because of their low cost. However, these will introduce electrical interference into the dielectric measurements and will not maintain the temperature stability required for the proposed goals. The Julabo temperature equipment provides a stability of 0.01°C or 0.02°C, depending on the model, and the very low dielectric nature of silicone oil will ensure that no electrical interference is introduced into the sample cells due to temperature control. No other known manufacturer or method can provide the thermal stability or electrical immunity required to achieve the thermal goals of this project. Additionally, each freezer or heater can manage multiple sample cells and thus will allow for simultaneous studies without the extra cost of a single heater/cooler per sample cell.

The only commercially-available dielectric spectrometers with capabilities close to the proposed spectrometers are provided by NovoControl Technologies. Each of the three proposed spectrometers, DS4 – DS6, can handle a minimum of three simultaneous sample channels, therefore a minimum of nine simultaneous sample channels are proposed for this project. Simultaneous sample runs are necessary for dielectric spectroscopy because each spectrum acquisition may require long periods of time, in some cases up to 30 hours for a single spectrum. The ability to run multiple samples simultaneously is crucial for the success of this project. Therefore, we calculated the "equivalent" hardware from NovoControl for four simultaneous sample channels. Four NovoControl Concept 80 systems would cost $787.6k, based on NovoControl's 2006 pricelist. Four separate Concept systems are required from NovoControl because each system can only handle a single sample channel. The development of the proposed advanced spectrometers is well within the

acquisition cost of NovoControl equipment. In summary, each of the three proposed spectrometers can handle multiple simultaneous sample channels while performing far more advanced capabilities, i.e., both TDDS and FDDS modes, four-wire sample cells, polychromatic excitation and responses, etc., as well as provide commercial availability of such proposed capabilities back to the Test and Measurement industry, as detailed under section "a. Project Description".

Computer-controlled switching hardware is required to construct automated reference capacitor decade boxes for calibration of the proposed sample channels. It is proposed that each of the three spectrometers will require a single switch box. The radio and microwave switch boxes are provided by National Instruments. National Instruments also provides LabView which is the critical software development platform to create the proposed software. The proposed software is the vital link to the success of the proposed goals, instrument control, data acquisition, and dielectric spectrometers. Mathworks MatLab will also be used in conjunction with LabView for advanced real-time data and graphical analysis and reporting. Both software development platforms are industry standards for research-level instrument control, data acquisition, and data analysis. The total cost from these two manufacturers is $20.0k.

Additional required software will be for circuit analysis and electronic simulations from Cadence, sample cell design from Nemetschek, and data analysis and linear and nonlinear regression from OriginLab, all totaling $12.5k. Circuit analysis software is required to construct the ultra-low noise printed circuit boards for the proposed isolated and amplified sample channels, as well as the reference capacitance boxes, as discussed under "a.1. Measurement of Dielectric Signals". The Nemetschek VectorWorks software is computer-aided and design software and will be used to design and construct the sample cells, as discussed under "a.3. Sample Cells". Linear and nonlinear regressions would be in the form of the Arrhenius fit and Havriliak-Negami phenomenological dielectric relations, as discussed in "a.5. Expected Dielectric Responses, Interpretation, and Publication".

And finally, some wet-chemistry equipment is required for peptide and protein sample preparation from Eppendorf, Fisher Scientific, and Millipore, totaling $21.9k. This equipment includes volumetric pipettes for liquid handling and measurement, a centrifuge for protein washing, a pH and conductivity meter for sample preparation and calibration, and filters for the already owned water purifiers.

It is difficult to provide approximations or near estimates for sample cell fabrication, preamplifier PCB fabrication, and protein and chemical supplies, however a value around $120k has been factored in for these necessities. The total equipment, hardware, and material expenses are $565.9k for the full three years of development. Official manufacturer quotes are included in the "Supplementary Documents" of this proposal.

Industrial contributions to our nonprofit pure research endeavor have reduced this budget by over $110k in hardware and equipment. These contributions made the DS1 through DS3 spectrometers a reality and the results are outlined under "b.2. Current Research and Instrumentation". I, Stephen Lukacs, have worked without charge for the last four years, and will donate the space for, this project.

Three employees will work for the full three years of research and development, Lukacs, Rose, and Macias. They will perform the majority of the instrument construction, routine experiments, and ensure the instrumentation is in proper working order. Lukacs will receive an annual salary of $90k for a full 40 hours per week. This is the average annual salary for a Ph.D. as reported by *Chemical & Engineering News*, **84**(45), November 6, 2006. Rose will receive an annual salary of $40k for 40 hours per week and will assist Lukacs with the design and fabrication of the sample cells, software development, and routine experiments. Macias will receive an annual salary of $15k for 15 hours per week. Macias' primary function will be construction, assembly,

maintenance, and calibration of the electronics, signal generators, and detectors. The latter functions will not require a full 40 hours per week.

Consultants will be employed for the development thrust during the first 18 months. Carter and Petersen will develop the electronics, fabricate the sample cells, and provide metallurgical input, respectively. The consultant fees will total $47.5. Therefore, the total employee salary and consultant fees for the entire three years of development is $482.5k.

And finally, Lukacs owns the 600 ft^2 space slated for this project, therefore, rent will not be required or charged to the project. The temperature controllers will probably use quite a bit of electricity, especially since the three spectrometers will be automatically running experiments 24 hours a day, 7 days a week, utilities have been factored into the budget. Also, some small legal and accounting fees for the nonprofit requirement of infinite quanta are included. These will total $21.6k for the entire three years of development. Publication subscriptions have been included for a total of $1.5k and project-related travel at $6k.

Although the total budget is for $1,077.6k, it is far less expensive when compared to the limited capabilities of acquiring commercially-available dielectric spectrometers. It is hoped that NSF realizes that the potential data, science, and understanding derived from the proposed project will far outweighs its cost.

Facilities, Equipment, and Other Resources

The Principal Investigator, Stephen Lukacs, owns a new 600 ft^2 space which will be used, without charge, for the proposed project. It is laboratory space with full electrical and water utilities. This will provide enough room to house the equipment and hardware, perform the necessary development, and run the routine experiments for the proposed project. The space will include an office.

Major technical and scientific corporations have donated over $90k in equipment and hardware for the advancement of this project. Mettler-Toledo donated the AT261 analytical balance for sample preparation. Millipore Corporation donated the Elix5 and Milli-Q water purification systems to provide the purest water possible for aqueous solutions of peptide and protein samples. This contribution has made a marked difference in reducing conductivity of the samples and thus decreasing the undesired electrode polarization effect during the dielectric studies of peptides and proteins.

Fluke Corporation donated a 397 Arbitrary Signal Generator and Tektronix donated the TDS5104 4-Channel 1-GHz Digital Phosphor Oscilloscope. The generator and oscilloscope have been used to create the Dielectric Spectrometer 3 (DS3) Frequency-Domain Dielectric Spectrometer (FDDS). DS3 acts as the primary analog-to-digital waveform converter for dynamic-signal analysis and sine-swept mode experiments and has led to a publication submission to the *Review of Scientific Instruments*. National Instruments has donated GPIB interface hardware and cables, as well as, LabView v8.2 software. This made the DS1 and DS2 possible and provided the dielectric results on gelatin, myoglobin, and hemoglobin, as outlined in "b.2. Current Research and Instrumentation". This interface equipment and software will be used with the donated Fluke RCL meter for initial electrode, sample cell, and reference calibrations.

The Principal Investigator has donated three computers to the project for the purpose of administration, financial tracking, proposal and publication preparation, web site development, data analysis and presentation, and theoretical calculations. The data analysis will include least-squares regression and peak-fitting of dielectric data. The theoretical calculations can include potential dielectric simulations of peptides and proteins with the AMBER or GROMACS software packages.

The proposed project will not require any clinical or animal facilities. The proposed project is not concerned with organisms of any kind, simply peptides and proteins.