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Developmental MRI for NSF

Development of Ultra-Sensitive and Ultra-Low Frequency Dielectric Spectrometers for the Detection, Characterization, and Cataloging of Peptide and Protein Dipolar Sub-Domains and Structural Motifs.

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Proposed Budget $ for 3 Years of Instrument Development

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Project Summary

The intention of the project is to develop ultra-sensitive and ultra-low frequency dielectric spectrometers and chemical techniques to detect, characterize, and categorize the dielectric responses, owing to electric polarization, of the intramolecular secondary structure and intra- and inter-chain structural motifs of peptides and proteins.

The proposed project will develop a set of custom dielectric spectrometers to encompass the frequency range of 10 μHz to 32 MHz with a sensitivity better than 2 fA. Modern electronics and custom amplifiers, combined with real-time instrument control, data acquisition, and computational processing, will modernize current dielectric spectrometers and impedance analyzers to allow detection of the intramolecular electrical phenomena of peptides and proteins. The combination of off-the-shelf and natively developed custom electronics, sample cells, and computer applications will allow the most sensitive and lowest frequency complex dielectric spectra ever performed on biomolecules. The project will design and construct sample cells that allow for controlling and characterizing experimental parameters, such as temperature, conductivity, capacitance, and electrode polarization. This development will involve fabrication of sub-radio frequency circuitry using the latest analog and digital components and techniques. And finally, the project will generate custom computer applications for control of the circuits, instrumentation, sample cell environment, as well as real-time data acquisition, statistical, and graphical treatment of the data.

The project will use the most recent advances in sample cell construction, and chemical and physical techniques to decrease or negate unwanted effects, such as electrode or interfacial polarization, and the temperate Maxwell-Boltzmann distribution of solutions. The chemical techniques will explore electrophoretic gels and trehalose solutions, at different temperatures, to create highly viscous, or soft “frozen”, nano-cells of rotationally and translationally immobilized macromolecules and their respective solvent. Further physical techniques will employ electric or magnetic alignment of the molecular net dipole of the macromolecule in the viscous nano-cells to further enhance intramolecular dielectric responses of intra- and inter-chain structural motifs.

The intellectual merit of the proposed activity encompasses two primary goals. First, it will promote the limits of modern electronics and instrumentation to detect and observe extremely small current and admittance signals in peptides and proteins. Second, the expected dielectric spectra, generated from the first goal, of the peptides and proteins will increase our scientific knowledge and understanding of biological macromolecules. It is apparent to us and other collaborators, that the intramolecular electrical properties of proteins are largely unobserved and uncharacterized.

The broader impact of the project will, first, promote the sensitivity and frequency range of modern electronics and instrumentation, especially for impedance analysis and dielectric spectroscopy. Second, the increased understanding of intramolecular electrical properties and dynamics of peptides and proteins will affect biophysics, biochemistry, proteomics, micro- and molecular cell biology, immunology, endocrinology, pharmacology, bioengineering, and nanotechnology.

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Project Description

a. Research Activities

The proposed research will design, develop, build, and calibrate dielectric spectrometers for the purpose of detecting and categorizing 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.

Generally, the instrumentation will comprise a collection of off-the-shelf and custom preamplifiers, current-to-voltage converters, amplifiers, detectors, and impedance analyzers coupled to custom parallel-plate and cylindrically-symmetric coaxial capacitor-based sample cells and interfaced to PC-based computers for overall control of instrumentation, sample physical parameters, and data acquisition. The Phase I Dielectric Spectrometer (P1DS) is built and generated intriguing results with hemoglobin, which is further discussed under "Current Research and Instrumentation". The proposed project will design and construct two parallel instruments, the Phase II and III Dielectric Spectrometers (P2DS and P3DS), that will calibrate and reproduce dielectric spectra against one another.

Additional emphasis should be made that the current P1DS generated intriguing results with aqueous and dry samples of hemoglobin at ambient temperature. The exciting results were observed at a frequency range of 1 to 20 mHz, a frequency range never previously reported for proteins, in which both the aqueous and dry sample produce similar and corroborative measurements. The discussion of these results are detailed under "Current Research and Instrumentation".

Measurement and Confirmation of Electronic Signals

Theoretically, the measurement of any physical quantity requires the mapping from a source set of an empirical domain onto image elements [1] 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 core, is the basis of the proposed spectrometers. The electronic core will consist of extremely sensitive and reproducible custom and off-the-shelf electronics that have never before been tried for studying the intramolecular dielectric responses of peptides and proteins, herein regarded simply as proteins.

Technically, the proposed electronic core acts as the bridge between the dielectric sample cell, where the actual physical measurement is conducted, and the final reproducible data set. This proposal encompasses two dielectric spectrometers, the P2DS and P3DS. P2DS encompasses an off-the-shelf impedance analyzer and P3DS comprises custom circuitry and digital-signal-processing (DSP) frequency lock-in technology. Although both spectrometers have slightly different specifications, both possess a common frequency range in which parallel runs will confirm dielectric responses of the same samples. The parallel runs from the opposing spectrometers are crucial to confirming the presence of the expected dielectric responses from the sub-domains and intra-chain structural motifs of proteins. The P2DS will possess a broader frequency range than the P3DS, however, the P3DS will possess a greater sensitivity than the P2DS.

The electronics of the P2DS will comprise an off-the-shelf Solartron 1260 Frequency Response Analyzer coupled to a 1296 Dielectric Interface. The latter greatly increases the sensitivity of the former to the expected sensitivity range. The combination will allow the sensitivity of the current to be 10 fA, and a frequency range of 10 μHz to 32 MHz.

NovoControl is a competitor and produces nearly comparable analyzers compared to the Solartron impedance analyzers. However, the former is more than twice the expense. The Solartron 1260 and 1296 will be interfaced to an Apple Power Mac personal computer. A custom LabView (National Instruments) software system will be written for instrument control and data acquisition, with the analyzer coupled to custom sample cells.

The electronics of the P3DS will comprise multiple Ametek 7265 and 7280 DSP Lock-in Amplifiers/Detectors running simultaneously with multiple circuits, sample, and reference cells. The combination will allow the minimum sensitivity of the current to be 2 fA, and a frequency range of 1 mHz to 2 MHz.

We have worked with Ametek for a year; their support and lock-in amplifiers are unmatched within the industry. Recent experience with the 7265 proves that the amplifiers should be used in the differential voltage detection mode. The proposed work will develop custom current-to-voltage (I-V) circuits. Using the amplifiers in differential voltage mode and custom I-V converters will increase the sensitivity, greatly decrease noise and settling time, and allow for custom guarding and shielding of the sample cells. These will increase the possibility of measuring the expected dielectric response from proteins, as well as, exponentially decrease the time required to incrementally scan a spectrum. And finally, proper guarding of the sample cell will ensure that stray and parasitic effects, as well as fringe effects, are decreased or fully negated from the measurements.

It is intended to develop the circuits based on the new line of Texas Instruments operational amplifiers. Texas Instruments has recently released new solid-state operational amplifiers that reach far into the GHz range, with instrumental and difference operational amplifiers that offer far greater response time, settling time, and signal amplification. Testing and integration of these operational amplifiers will lie at the heart of the P3DS custom circuitry.

Overall, the P3DS will require three 7265 and three 7280 amplifiers, in which the two sets will run in parallel. In each amplifier set, the first of the three amplifiers will run an admittance response on the short circuit, the second amplifier on a calibrated load impedance or reference cell, and the third amplifier on the sample cell. This technique is similar to those employed by Gross *et. al.* [2], except the three amplifiers will run in parallel and simultaneously calibrate the circuit and reference cell, as well as, measure the complex dielectric response from the sample.

An Agilent Signal Generator, Tektronix Digital Oscilloscope, and Apple Power Mac computer will be used to test components, circuits, and sample materials and cells. This test bench will adequately test from 1 μHz to 80 MHz, as well as, digitally process and record the responses, and permit study of extremely slow and fast phenomena. The test bench will be necessary for characterizing transient and persistent stray and parasitic effects in circuits, cabling, connectors, and sample cells. The test bench will also comprise surface-mount fabrication tools. The ability to create surface-mounted circuit boards will greatly decrease unwanted noise from component effects and connections.

Computer Control and Data Acquisition

Apple Power Mac G5 computers will be used as the primary circuit and instrument control and data acquisition centers. There will be a total of four G5 centers; one for the single P2DS, two for the P3DS, and a fourth for the test bench.

Each computer will be fitted with GPIB (General Purpose Interface Board) interface cards to control instruments and to retrieve data. The latter instruments are the Solartron 1260/1296 Analyzer, the Ametek Amplifiers, the Agilent Signal Generator, and Tektronix Oscilloscope, discussed in the previous subsection. Also, each computer will be fitted with a digital in/out interface card to switch various areas of circuits active or inactive. The latter will allow for further

calibration of the sample and reference cells. LabView Full 7.1 will be used to develop the custom applications to encapsulate the P2DS and P3DS instrumentation into a fully automated spectrometer. LabView contains advanced subroutines for real-time statistical, least-squares regression and fitting, and graphical analysis of streaming data. This capability is vital for the determination of a settled stable measurement, especially at low frequencies, as outlined under the below "Current Research and Instrumentation" section. The raw data will be stored in ASCII text or DDE files for seamless import into Microsoft Excel or OriginLab OriginPro. OriginPro 7.5 will be employed on the raw data for peak and curve fitting of the complex dielectric spectra.

In summary, the P2DS Solartron-based system will require a completely new LabView application to run measurements and create the spectra. The P3DS, however, will port and update the current P1DS application to run multiple lock-in amplifiers and the custom I-V circuitry to create spectra.

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 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, in its most simple design, will consist of a body and two conducting electrodes, separated by a 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 developed since 1897 [3] and five polarization phenomena have been observed: (1) Maxwell-Wagner for heterogeneous phases, (2) electrode polarization, (3) orientational due to permanent dipole moments in polar molecules, and inductional due to (4) atomic and (5) electronic polarizabilities. The latter two occur at extremely rapid rates and are far beyond the expected frequency range for dielectrics of structural motifs, therefore they will not be discussed further.

The expected complex dielectric response from the intra- and inter-chain structural motifs of proteins will be from the permanent dipole moments inherent to those motifs and the electronegative bonding, therein. Therefore, the basic development of the sample cells will be to increase this effect while decreasing all other effects, namely the Maxwell-Wagner (MW) and more importantly, electrode polarization (EP) effects. This will ensure that extraneous electrical effects do not mask the motif dipole moments of proteins.

Maxwell-Wagner [4] [5] (MW) effects, or interfacial polarizations, are based on the charge buildup at the boundary of two heterogeneous phases with different impedances. For example, DNA exhibits a huge permittivity, contrary to the absence of permanent dipole moments. The observed permittivity has been attributed to interfacial polarization, and has also been observed in nonpolar latex and colloidal suspensions. This effect exhibits anomalous dispersion with very slow relaxation times that mimic the behavior of Debye relaxation for polar molecules [6]. Although this effect may mask the expected structural motif dielectric response, the effect may prove valuable in studying the dynamics of inter-chain permittivity and hydration layers of proteins.

Electrode polarization is due to the pseudo-ordering of ions and molecules around the electrodes to form an electrical double layer. Since the electrodes carry charge to deliver the applied electric field, mobile ions and polar molecules will orient and order themselves to the electrode charge source, thus forming an ordered electrical shielding layer at the electrode surface. The electronic shielding imposed by electrode polarization is frequency dependent and can impose a significant measurable permittivity, thus potentially overwhelming and masking the measured permittivity of the bulk sample.

The proposed work will investigate a number of techniques to reduce or negate the effect of electrode polarization. The primary thrust is to disrupt the electrical shielding layers around the electrodes. Eight exploratory techniques are proposed herein: (1) four-terminal electrodes, (2) adjusting the ratio of electrode separation versus the electrode area, (3) surface etching and roughening of the electrode, (4) coating the electrode, (5) rotating and vibrating the electrode, (6) pulsing the electrode temperature, (7) pulsed microwave heating of the bulk sample, and finally, (8) DC pulsed or off-frequency AC current through the electrode.

The first four techniques have been previously explored. Klaassen [1] and Takashima [4] discussed the use of the four-terminal technique. The first pair of electrodes stimulate and measure the sample while the second pair are wired into a differential amplifier to measure the potential without interfering electrode polarization. This technique also compensates for wire resistivity and temperature fluctuations in the cabling. This technique is proposed for both the P2DS and the P3DS sample cells. The proposed Solartron equipment for the P2DS is manufacturer ready for four-terminal electrodes. Whereas, the custom I-V converter circuitry of the P3DS will require development of this feature into the custom circuit.

Grosse *et. al.* [7], and historically Schwan *et. al.* [8], determined that a larger electrode separation and area will increase the impedance of the bulk sample and decrease that due to electrode polarization. The limitation to the volume of the sample cell will be determined by the sample availability. The published research also suggested that employing multiple spectra at varying discrete electrode separations will aid in characterizing the electrode and electrode-sample polarization effects. Additionally, the electrodes surface area can be increased by etching or roughening [6] which has also been shown to decrease electrode polarization. These techniques are proposed for both the P2DS and the P3DS sample cells.

Historically, Schwan [9] and Takashima [10], and recently, Tirado and Grosse [11] [2] [7] performed a detailed study of Platinum black coating on Platinum electrodes with colloidal and highly conductive aqueous solutions. It was found that once the electrode separation was maximized that proper application of Platinum black will decrease electrode polarization 2 to 3 orders of magnitude [11]. Platinum black deposition will be employed in both the P2DS and the P3DS sample cells.

The remaining four exploratory techniques have not been published in previous impedance research. These methods are primarily mechanical, thermal, acoustic, or transient electrical pulses to physically disrupt the electrical double layers on the molecular level. It is proposed to study the electrode polarization reduction by each technique within the frequency range of 10 μHz to 10 kHz, where 10 kHz is well above the frequency range of observed electrode polarization effects. It is known that these disruptions must occur at a faster rate than the applied frequency, however, since electrode polarization is only observed at low frequencies, a combination of these and the above published techniques should reduce the effect of electrode polarization substantially.

Parallel plate and cylindrically symmetric sample cells will be designed to apply an electric field to a sample of proteins to measure the dielectric response of intramolecular structural motifs. Numerous novel and previous exploratory techniques will be employed to reduce electrode polarization effects. Temperature control of the sample cells will be employed using circulating

freezers and thermoelectric coolers. All sample cells will be custom designed and machined to meet and balance all specifications and interchangeably interfaced into either the P2DS Solartron Analyzer or the P3DS custom circuitry and Ametek Amplifiers.

Peptide and Protein Samples

It is intended to initially study well-characterized peptides and proteins, especially those with precise 3D structure and well understood secondary structure and structural motifs. In this manner, it will be more straight-forward to develop the instrumentation and correlate the measured signals to dielectric responses from secondary structure and structural motifs, namely α-helices.

Initially, protein samples will be studied in dry powdered and in pure water aqueous forms. Dry powdered protein samples are readily available from Sigma-Aldrich with known dielectric spectra and are simple to run. Powdered solid samples have the great advantage of having constrained rotational movement of the molecules, which will have the effect of decreasing the dielectric response from the molecular net dipole moment. Therefore, allowing the dipole moments of the structural motifs to emanate in the spectra. Dry samples will also produce negligible electrode polarization effects because mobile charge or ion carriers will not exist.

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. It is expected that the molecular net dipole will mask the dielectric responses of the intramolecular motifs. It is conceivable that the rotational inertia will require immobilization to mask the net dipole which will, in turn, allow the observation of dielectric responses of the intramolecular structural motifs.

It is proposed to rotationally immobilize the proteins with three exploratory techniques: (1) single molecular nano-cells, (2) thermal effects, and (3) alignment using electromagnetic manipulation. Essentially, these techniques will increase the viscosity of the medium, thereupon "freezing" the macromolecules rotational orientation.

Electrophoretic gels can be prepared to control the pore size within the gel matrix. The size of the pores is determined by the concentration of the gel within the total volume of the solution. The proposed project will require experimentation on different electrophoretic gels that provide the lowest impedance. Electrophoretic gels are the most obvious choice for the proposed protein study because the gels are aqueous in nature and imitate *in vivo* environments. Other semi-solid and highly viscous chemical matrices will also be investigated, such as ionic or quaternary ammonium liquids.

Once a set of suitable macromolecular chemical matrices is found, dielectric studies can be performed at various temperatures, namely –50 to 20°C. It is expected to reduce the temperature below the freezing point of water to immobilize the molecular rotation. The use of trehalose will allow the matrix and proteins to maintain their structural integrity. Trehalose is a sugar and commonly known as "nature's antifreeze". The combination of chemical matrices and thermal soft-freezing, or highly viscous solution, will greatly constrain the protein while greatly reducing the full-width-at-half-maximum of the normally broad Maxwell-Boltzmann distribution, thus increasing the long-term alignment and coherence of the proteins. The latter feature, due to this powerful combination of chemical nano-cells and temperature, will greatly reduce the response of the molecular net dipole moment and enhance the dielectric responses of the intramolecular structural motifs.

The confirmation of dielectric responses of structural motifs can be confirmed by chemical manipulation of the samples, such as pH, electrolytic, and solvent variations, as well as, partial or full denaturation by heating or selective enzymatic breakdown.

The dielectric responses of the above chemical and thermal combination can additionally be enhanced by electrically or magnetically aligning the molecular net dipole moment while the sample

matrix is setting into a higher viscous or nearly “frozen” state. Since alternating electric fields at the proposed frequency ranges are transverse waves, rigidly orienting the macromolecules prior to a spectral run will allow polarization studies to be performed during a run. The polarization studies should reveal critical angles where dielectric responses are maximized owing to the dipole moments of secondary structure and structural motifs.

Task Analysis and Time-Line

It is approximated that the first twelve to eighteen (12 – 18) months, Phase I, will be devoted to the design and development of the sample cells, custom circuitry, and software integration. The sample cells will require integration of the liquid and thermoelectric cooling systems and temperature controllers, metallurgy and Platinum black coating, as well as, proper guarding and shielding, with all sample cell design being custom machined to design specifications. The empty air-filled sample cells will be tested first on the P2DS because of its off-the-shelf convenience, but its software application must be developed from scratch. The circuitry development will require component tests of the Texas Instruments high-performance difference and instrumental amplifiers and integration of the four-terminal electrode technique and guarding which will couple to the lock-in amplifiers. The current P1DS software application will also be ported to accommodate the multiple lock-in amplifiers and custom circuitry of the P3DS.

It is approximated that the next three (3) months, Phase 2, will be devoted to the P2DS and P3DS calibration, stability, reproducibility, and cross-tests on known dielectric responses of standard organic solvents, water, carbohydrates, peptides, and proteins. An indication of electrode polarization will also be forthwith during this calibration period.

And finally, the last twelve to eighteen (12 – 18) months, Phase III, will be devoted to detecting, and confirming such observations, of intramolecular dielectric responses of secondary structural dipole moments and structural motifs. Accumulated data on varying protein samples will lead to categorizing intra- and inter-chain dielectric responses of secondary structure and structural motifs.

Therefore, Phase I will be devoted to the design and development of the instrumentation, both P2DS and P3DS. Phase II will comprise the calibration and reproducibility of both spectrometers. And, Phase III will attempt to detect and confirm dielectric responses of intramolecular structural motifs. It is intended, therefore, that the entire project should span approximately three (3) years. Specific personnel roles are detailed under the section, “Project and Management Plans.”

b. Description of Research Instrumentation and Need

The website "http://qdynamics.org:97/research" contains in depth theoretical, expected dielectric response from peptides and proteins, and current and proposed instrumentation.

Rationale of Technical Feasibility and Developmental Foundations

The classic work "Polar Molecules" in 1929 by Peter Debye [12] formalized the complex dielectric spectra, and anomalous dispersion and absorption, of polar molecules. 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" [13] [14]. Essentially, the change in magnitude and 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 [15], or

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

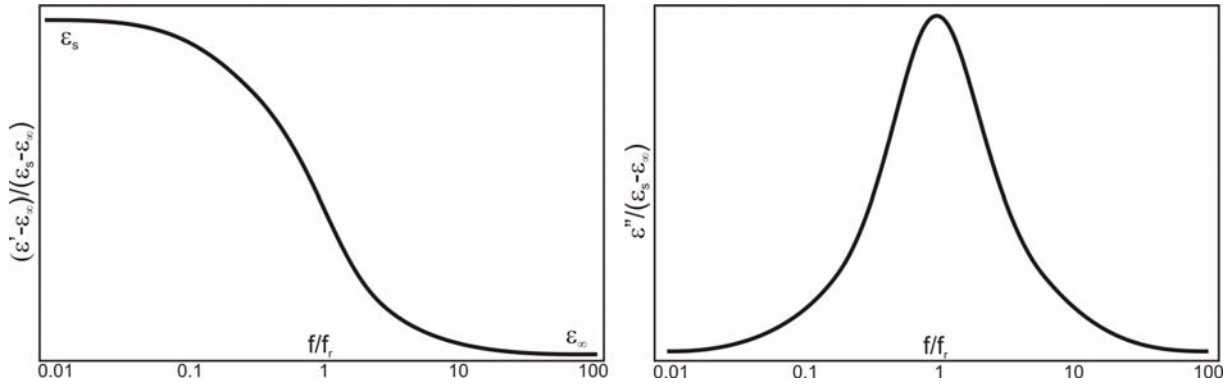
(Eq. 1)

where τ is the relaxation time, ω is the incident angular frequency or $\omega=2\pi f$, ε_s is the static permittivity, ε_∞ is the permittivity at optical frequencies, ε_0 is the permittivity of free space. Comparing Equation 1 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

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

(Eq. 2)

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. 1)

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, $\omega\tau$, is unity at the center of the relaxation transition. Debye [12] determined the relaxation time to be related to

$$\tau_{\text{int rinsic}} = \frac{4\pi a^3 \eta}{kT} \qquad \text{(Eq. 3)}$$

or the relaxation time is related to the volume of the molecule a^3, the viscosity of the solvent η, and the temperature T of the solution. Hence, a frequency scan will reveal the relaxation time, leading to an understanding of the effective molecular size of the responding dipole and the viscosity of the surrounding environment.

The electric permittivity, or frequency-dependent dielectric constant, nullifies the sample cell geometry by the relation

$$\varepsilon^*(\omega) = \frac{C^*}{C_0^*} = \frac{Y^*}{Y_0^*} = \frac{Z_0^*}{Z^*} = \varepsilon' - i\varepsilon'' \qquad \text{(Eq. 4)}$$

or the complex dielectric permittivity ε^* is the complex capacitance C^* of the sample divided by that of the same *in vacuo* reference cell $C_0{}^*$, or similarly for the admittance Y^*, or its inverted impedance Z^*. Admittance Y^* is the measured complex current I^* over the applied voltage V and impedance Z^* is the inverse of admittance. This is the foundation of the classic and well-proven techniques of admittance, impedance, modulus, and dielectric spectroscopy.

The driving forces for the formation of secondary structure in peptides and proteins, herein known simply as proteins, are steric hindrance and 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 = 3.33564×10^{-30} C m) [16],[17]. The moments of water is 1.85 D [18] 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 of a protein possesses the greatest potential energy. The reduction of the potential energy due to the amide bond dipoles will dominate the folding of the secondary structures.

The secondary structure of proteins naturally form structural motifs which alleviate the high-energy dipolar interactions created by the electrical nature of bonded amino acids, the amide or peptide bonds. The most prevalent motifs are hairpin turns, random coils, α-helices, and β-sheets, in which the latter two possess the most structure and the greatest potential energy reduction due to the peptide bond dipole moments. Generally, α-helices and β-sheets reduce the dipolar potential energy with the formation of hydrogen bonds.

The α-helical structural motif will form a hydrogen bond between a carbonyl oxygen and the amino hydrogen of every 4^{th} residue. This imposes a periodicity of 3.6 residues for the α-helix. Although the α-helix greatly reduces the net dipolar potential, it still possesses an overall permanent dipole moment in the direction of the helical axis. This is because the α-helix has the amide bonds pointing nearly parallel to the helical axis, thus causing the formation of a net dipole moment along the axis of the helix. Therefore, a strong permanent dipole moment exists for α-helices that exhibit a strong dielectric response [19], in which 1 – 3 kD are reported for poly-γ-benzyl-L-glutamate. The other structural motifs, although not possessing as strong permanent dipole moments, will exhibit detectable dielectric response via induced polarizability, such as in β-sheets, or via randomly oriented dipoles, such as in random coils. The latter may additionally lead to valuable statistical and environmental data for understanding intra-protein structure and dynamics.

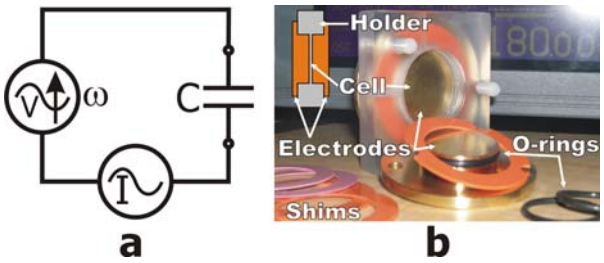
Dielectric studies by Oncley *et. al.* [20] on native freely-suspended proteins in solution reveal relaxation times ranging from 16 ps to 2.5 μs. These relaxation times translate to frequencies on the order of tens to hundreds of kHz. Application of the Debye relation, Equation 3, reveals that the high viscosity of the molten-core of globular proteins will greatly lower the observed frequency of intra-protein inter-chain 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 sub kHz range for the inter-chain motifs.

Current Research and Instrumentation

The Principle Investigator (PI) has actively pursued the current research endeavor for two years, and prior to that, passively for three 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 Phase I Dielectric Spectrometer (P1DS). In addition, to the PI's personal investment into the pure research endeavor, three international corporations, Ametek International (formerly Perkin-Elmer), National Instruments, and Mettler-Toledo, have contributed equipment and technical assistance to the development of the P1DS. Millipore has also pledged equipment support. And finally, Stephan Isied, a top protein electrochemist from Rutgers University, NJ, is actively collaborating on the project.

The P1DS is based on a highly sensitive lock-in amplifier, the Ametek 7265. This is a detector that tightly focuses its detection range on the single applied electric field frequency. The technology inherently filters noise and extraneous signals. The 7265 has a frequency range of 1 mHz to 250 kHz and a current sensitivity of 2 fA. This is more sensitive than any commercially available impedance analyzer.

The circuit of the P1DS is extremely simple, Figure 1a. It employs the radio-frequency current-to-voltage (I-V) mode of impedance spectroscopy, in which a voltage is applied across a capacitor-based sample cell using a sine-wave function generator. The lock-in amplifier then detects the current. The sample cell is a brass pair of capacitive electrodes encased by a Plexiglas body with the distance between the electrodes controlled by shims.

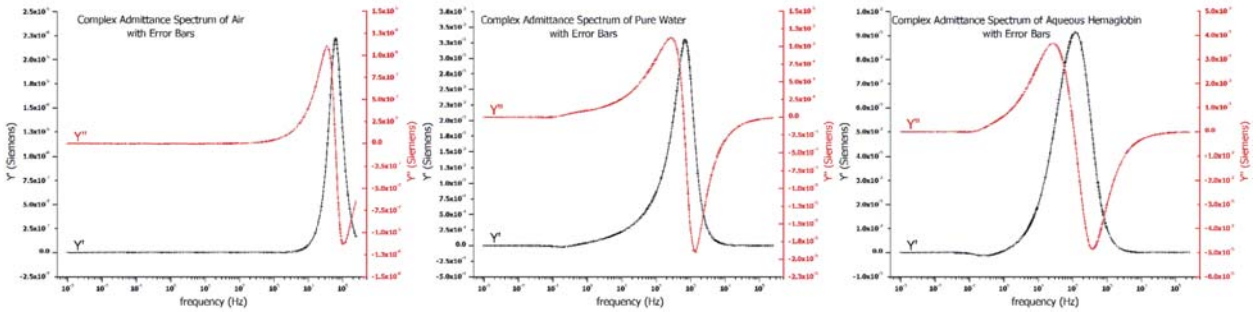


(Fig. 1)

The entire circuit, generator, and detector are computer controlled and monitored using GPIB (General Purpose Interface Board) and digital input-output interface boards. Software is written in National Instruments' LabView Full v7.0, which integrates the entire system into a single user-friendly computer application. The application allows for a single frequency study or a sweep through any range and resolution of frequencies. The application sets the frequency, determines a settled and stable measured current, and then averages any number of values. The application will acquire the data and display real-time statistical and graphical reports on the data. The application will perform the latter for each resolved frequency within the specified range and generate a real-time complex impedance or admittance spectrum. The application then exports the data to ASCII text

files for import into Microsoft Excel or OriginLab OriginPro, for further processing and graphical or statistical analysis.

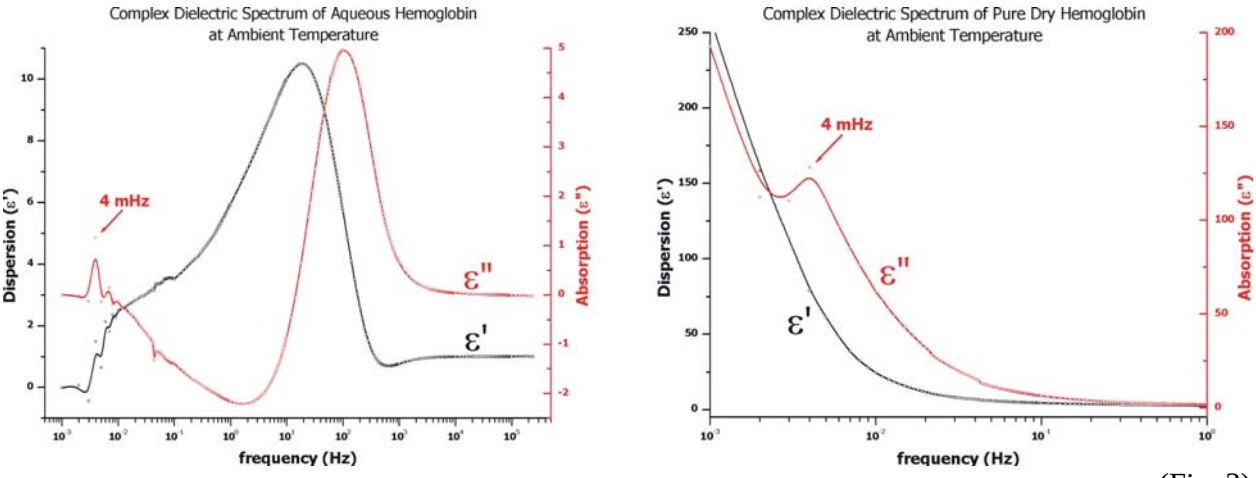
The most difficult aspect of the application is a complicated subroutine that determines the time for the measured current to settle to a constant value. This is especially important at frequencies less than 50 Hz. For example, the settling time at 1 mHz can range from three to eight hours, in which a spectrum from 1 mHz to 50 mHz, at a resolution of 1 mHz, can require up to four days. The settling subroutine uses differential calculus and least-square regressions on multiple real-time functions culminating to eight functional parameters to determine a constant current. Although this subroutine required months of analyzing, programming, and testing, this subroutine, along with the primary application, allows for full 1 mHz to 250 kHz frequency range spectra producing extremely smooth and well-formed impedance or admittance spectra with extremely small random errors. The complex admittance spectra for the P1DS for Air, Water, and 10% Aqueous Bovine Hemoglobin, respectively, at ambient temperature are shown below in Figure 2.



(Fig. 2)

where black represents the real component of the complex dielectric spectrum, and red the imaginary.

Similarly, the current P1DS produced complex dielectric spectra of aqueous and dry bovine hemoglobin, as shown below in Figure 3.



(Fig. 3)

where the left data represents 10% (w/w) aqueous bovine hemoglobin for the full frequency range of 1 mHz to 250 kHz. The large broad dispersion around 10 to 100 Hz is probably due to electrode or interfacial polarization. However, the data at less than 20 mHz has never been observed before and may represent the expected dielectric response from intramolecular hemoglobin. To confirm the aqueous results, the right dielectric spectrum was derived from pure dry hemoglobin. An obvious

and strong primary peak at 4 mHz arises in the absorption spectrum, with additional harmonic or overtone peaks around 8, 12, and 16 mHz as observed in aqueous solution. Additionally, the real dispersion of the aqueous sample increases with frequency, indicative of normal dispersion, and may represent resonant, as opposed to relaxation, processes.

The less than 20 mHz complex dielectric response of dry and aqueous hemoglobin reveals normal dispersion as displayed by the real permittivity and strong absorption peaks as revealed by the imaginary permittivity. Both the real and imaginary results confirm the primary and harmonic responses of each of the aqueous samples. Further investigation of this phenomena is required, however, the dielectric data has not been previously reported and represents a possible resonance process due to normal dispersion, as opposed to relaxation processes as traditionally observed by anomalous dispersion. A resonant dielectric response would indicate a forced harmonic oscillator in the classic sense, or a quantum mechanical process in the modern perspective.

The harmonic responses are only obvious in aqueous solution, and not in the dry sample. Is it possible that intramolecular hydration layers exist, thus further enhancing or mediating the resonant processes of an intramolecular vibration beyond the primary response? This may lead credence because the dry sample exhibits anomalous dispersion, implying relaxation, but the aqueous exhibits normal dispersion, implying resonance. Does the energetic absorption of the aqueous hemoglobin in this 20 mHz range affect its conformational structure or enzymatic activity? Will a similar response be seen in myoglobin or other species of hemoglobin? Will shifting of the frequency response occur in the presence of oxygen or carbon dioxide, or with pH or salt changes? And finally, are there other dielectric responses, either at a lower frequency or hidden in the electrode polarization response? The proposed P2DS and P3DS will allow for greater frequency resolution and range, and detection sensitivity, as well as chemical and physical control of the samples, to further the study of intramolecular dielectric responses of peptides and proteins.

The proposed P2DS would allow for greater frequency resolution in this range, thus confirming and clarifying the primary and harmonic responses. The P3DS would provided greater sensitivity to reproduce and study the above results. Such studies will also be performed on an array of peptide and proteins, in varying physical and chemical environments.

Justification of Proposed Versus Current Instrumentation

Our research of published dielectric instrumentation reveals that most groups employ off-the-shelf impedance analyzers. In this arena, three manufacturers produce such equipment; Agilent Technologies (formerly Hewlett-Packard (HP)), NovoControl Technologies, and Solartron Analytical. Generally, Agilent provides high frequency ([20,110G] Hz) analyzers with poor sensitivity (>20 μA), NovoControl provides ultra-low to medium frequency ([3μ,20M] Hz) analyzers with excellent sensitivity (>10fA), and Solartron provides ultra-low to medium frequency ([10μ,32M] Hz) analyzers with excellent sensitivity (>10fA). Agilent analyzers have a frequency range much higher than the expected frequency response of this proposed research. NovoControl and Solartron provide excellent analyzers for the expected response, however, NovoControl's price is more than twice that of Solartron. Of these manufacturers, research groups have employed HP and Solartron for study of biomolecules.

Grosse *et. al.* [2] [7], Facer *et. al.* [21], Bordi *et. al.* [22], and Weingartner *et. al.* [23] collectively use HP Models 4191A, 4192A, 4284A, 4294A, 8510C, and 8720C impedance and network analyzers. Of these six models, four are obsolete and unavailable and only two are currently available; the 4284A and 4294A. The currently available Agilent impedance and network analyzers will collectively measure within the frequency ranges of 20 Hz to 110 GHz with a maximum sensitivity of 20 μA. Although Agilent produces excellent products, these analyzers, with

their frequency and sensitivity ranges, are targeted towards manufacturers of electronic components, such as resistors and capacitors. Recent discussions with Agilent also shows that they do not produce accessory test fixtures for their analyzers to increase the sensitivity. Our current research proves that the minimum current sensitivity required to measure the dielectric responses of internal structural motifs and intra-chain dynamics is within the femtoamp (fA) to picoamp (pA) range. Therefore, any Agilent products, or respective accessory equipment, are either greater than the frequency range or require greater sensitivity for the expected response from proteins. Therefore, Agilent products will not be required in this proposed instrument development.

Two other groups, Smith *et. al.* [24] and Bruni *et. al.* [25], use the Solartron Models 1250 and 1255 Impedance Analyzers with the test accessory 1296 Dielectric Interface. The former impedance analyzers have a collective frequency range of 10 μHz to 10 MHz in which the latter dielectric interface acts as an attached potentiostat to greatly increase the current sensitivity of the analyzers. Solartron currently produces the Model 1260 Impedance Analyzer that possesses a frequency range of 10 μHz to 32 MHz and with the attached 1296 Dielectric Interface a current sensitivity of 10 fA.

It is proposed to use the Solartron 1260 Impedance Analyzer with the 1296 Dielectric Interface as the electronic core of Phase II Dielectric Spectrometer (P2DS). The study of Smith *et. al.* [24] on hydration layers of ovalbumin prove that this off-the-shelf electronic core will provide excellent sensitivity and reproducibility for the proposed P2DS and expected dielectric response. We intend to develop custom parallel-plate and cylindrical/coaxial sample cells and personal computer-based control and acquisition software to interface with this Solartron electronic core.

Lock-in amplifier and detector technology has not been used in the published research of biomolecules. As with the current Phase I Dielectric Spectrometer (P1DS), we will employ two sets of lock-in amplifiers from Ametek International, formerly Perkin-Elmer, for the Phase III Dielectric Spectrometer (P3DS). Model 7265 ranges in frequency from 1 mHz to 250 kHz with a current sensitivity of 2 fA, and Model 7280 ranges in frequency from 0.5 Hz to 2 MHz with a current sensitivity of 10 fA. In the P3DS, both amplifier models will be used in tandem and interfaced to computers to act as a single spectrometer.

Although both Ametek lock-in models possess an internal current-to-voltage (I-V) converter in current mode, the P3DS will employ the newest Texas Instrument (TI) broadband operational amplifiers to develop a custom I-V converter for two important reasons. First, the custom I-V converter will allow the use of the differential voltage mode of the amplifiers, thereby allowing far superior guarding, shielding, and preamplification within the custom circuit itself. This will substantially decrease the stray and parasitic effects, and thus decrease overall noise and increase sensitivity and reproducibility of the instrument. Second, the modern Texas Instruments operational amplifiers will greatly decrease the settling time at the low frequencies, which will greatly increase the sweep or scan speed of a spectrum. Employing the custom I-V converter along with custom parallel-plate and cylindrical/coaxial sample cells will make the P3DS the most sensitive and reproducible instrument available between a frequency range of [1m,2M] Hz and a greater than 2fA sensitivity.

In summary, the proposed dielectric spectrometers, P2DS and P3DS, will exhibit far greater frequency range and sensitivity than has ever been attempted in the study of dielectrics of proteins, or biomolecules. The superior sensitivity and ultra-low frequency range should detect and quantitatively and reproducibly measure the complex dielectric response of intra-chain structural motifs within peptides and proteins. The above, never-before observed, hemoglobin response will be an early candidate for testing with the proposed instrumentation.

c. Impact of Infrastructure Projects

infinite quanta, inc. is a nonprofit 501(c)(3) organization formed for the purpose of performing pure scientific research. Specifically, its purpose is, "to perform pure scientific research and develop innovative and applied scientific instrumentation for the general purpose of acquiring constructive scientific knowledge and the distribution of such pure knowledge and applied innovation through various forms of information transmission, i.e., scientific journals, video, internet, etc." The Articles of Incorporation, the I.R.S. tax-exempt application and ruling, as well as, the corporate bylaws are located at http://qdynamics.org:97/organization/corporate.

Essentially, the purpose of the corporation is to develop scientific instrumentation for the purpose of providing such tools to the overall research community. Our product is public dissemination of scientific knowledge. Our organization will grow to encompass personnel of an innovative, intellectual, and creative spirit. This includes all people regardless of age, gender, ethnicity, creed, sexual orientation, or religion.

We have built, and will continue to build, a collaborative network with for-profit industry and nonprofit academic institutions. The collaborations are based on developing scientific instrumentation and its corresponding results, as well as, teaching and promoting the education of scientifically interested and creative people. Such academic collaborations will undoubtedly lead to advanced degrees in the sciences and engineering.

As we continue, successes in our research endeavors are expected to draw scientific and technical expertise from the local region of Northeast Florida. As the organization grows, it will inspire those outside the area to move to the area for employment, or inside the area for employment, contribution, and collaboration.

Our current and future academic-based collaborations will inevitably lead to graduate students working within our research facility and under our guidance. Fresh and open perspectives from the next generation of scientists will be highly valued and received in our organization. The graduate students will fulfill the graduate requirements and courses of the department and university they attend while performing research duties and training at infinite quanta. Jonathan Rose is currently on our development team and intends to receive his Ph.D. through this effort.

Our corporate bylaws will inspire our organization to incorporate a scientific mentoring program with local primary and college students. In fact, funding of this proposal will allow us to mentor two or three students. These students will be active in the laboratory, attend group meetings, and learn the tools of research. We, at infinite quanta, have a working relationship with Florida Community College at Jacksonville (FCCJ), which is a Florida State accredited college. Through this relationship, the students may attain independent research credit from FCCJ, that is equally transferable to any nationally accredited academic institution.

d. Project and Management Plans

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 most of 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. This will include testing and standardizing any off-the-shelf electronic components, custom circuits, instrumentation, and temperature control equipment. They will fabricate specially designed low-noise circuits and integrate those circuits with off-the-shelf amplifiers and instrumentation to create the overall P2DS and P3DS dielectric spectrometers.

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.

Stephan Isied will closely collaborate with Lukacs to plan and interpret the chemical and biological experiments. The choice and synthesis of peptides and proteins, as well as the solvents, media, and characteristics, will be determined by this cross-discipline effort.

Once the Phase II and III Dielectric 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 maintain the computer applications, prepare samples, and examine the spectra of the peptide and protein samples. Lukacs, Rose, and Isied will process, analyze, and interpret the data and develop new samples, or sample characteristics, for further studies.

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