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Predicting interactive behavior of cytokines and their receptors by dielectric thermal analysis and thermogravimetry

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Abstract Cytokines and soluble cytokine receptors serve as important protein biomarkers for chronic and infectious disease diagnosis. The development of biosensors capable of detecting cytokines or their soluble receptors in patient bodily fluids is a growing area of research. In an ongoing series of studies to understand the thermal analytical behavior of cytokines and their soluble receptors, dielectric thermal analysis (DETA) and thermogravimetry (TG) were used in investigations to determine if differentiations based on dielectric properties (e.g., conductivity) of the proteins could be identified. Permittivity (ε') and dielectric loss factor (ε'') measurements were performed over a frequency range of 0.1–300,000 Hz. Up to 20 min, water associated with the samples was conductive, interacting with the proteins and affecting the temperature-dependent relaxation spectra of proteins. A trend analysis revealed differences between surface charge at 0.1 Hz and bulk charge at 300,000 Hz. In addition, the greatest change detected among proteins was due to the conductivity (dielectric loss factor). Beyond a 20 min drying time, the observed conductivity was due to intrinsic properties of the proteins with limited dependence on frequency. A 100% water loss was obtained for samples within 20–30 min by TG. Sample drying by TG could serve as a preparatory step in drying protein samples for further DETA and DSC analysis.

Keywords Dielectric thermal analysis · Thermogravimetric analysis · Cytokines · Cytokine receptors · Malaria · Electrical conductivity · *Plasmodium falciparum*

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Introduction

In an ongoing series of studies to understand the thermal analytical behavior of cytokines and their receptors, the cytokines; (IFNγ, TNFα, IL-1α, IL-2, IL-4, IL-6, IL-10), and soluble receptors and antagonists (soluble TNF receptor types I and II, IL-1Ra, and sIL-2 receptor α) were investigated. IL-1α, IL-1β, and IL-1Ra belong to a growing family of interleukin 1 (IL-1) cytokines, that function in immune regulation and inflammatory processes [1]. IL-1Ra inhibits the activity of IL-1 by binding to IL-1 receptors and competitively interfering with IL-1α and IL-1β activity [1]. Soluble cytokine receptors bind to cytokines (ligands) in plasma and the ability to detect ligand-receptor ratios and complexes will aid efforts in diagnosing infection and determining pathological states of infection.

Cytokines and antagonists were re-suspended in various solvents and analyzed by DETA and TG to determine if differentiations based on electrical conductivity and the resulting activation energies of the proteins could be identified. Cytokines and other soluble host factors induced as a result of infection have been implicated in malaria pathogenesis [2–4]. Cytokines are small regulatory proteins secreted mostly by white blood cells in the body [5]. They exert pro-inflammatory and anti-inflammatory effects resulting in modulation of the immune response in chronic

and infectious diseases. Two of the cytokines (TNFα and IFNγ) and soluble receptors (sTNFR1, sTNFR2, sIL-2R, and IL-1Ra) were selected for evaluation in our previous study, and in the current study because of their association with malaria pathogenesis [1]. Malaria remains a major public health disease with over 515 million clinical cases and 1–2 million deaths in children under the age of 5 years. Cytokines and their antagonists (soluble receptors and naturally occurring antagonists) are implicated in the pathogenesis and disease progression of malaria [2]. Accurate diagnosis of malaria at point-of-care remains a challenge. Identification of individual parasite proteins, in particular *P. falciparum* histidine rich protein 2 (PfHRP2) remains the most widely used method in rapid diagnostic tests (RDTs) for malaria. PfHRP2 shows variation in expression among geographic isolates of *P. falciparum* and some *P. falciparum* strains do not express PfHRP2 [6, 7]. As a result, additional blood stage antigens of *P. falciparum* need to be evaluated for use as diagnostic biomarkers along with human biomarkers that can accurately diagnose malaria at point-of-care in malaria endemic countries.

The purpose of this study was to investigate the thermal behavior and electrical conductivity of human cytokine and soluble cytokine receptors. It is our hypotheses that: (1) intrinsic dielectric signatures of cytokines and soluble cytokine receptors can be differentiated by DETA and other thermal analysis techniques. (2) Charging values for amino acids using the hydroponics index will be related to DETA values, tan delta, conductivity, and activation energy. As a part of our ongoing effort to develop unique "barcodes" for identifying cytokine and cytokine receptor biomarkers, our aim is to identify electrical conductivity patterns unique to cytokines and their receptors to facilitate the development of a predictable electrical model for detecting cytokines and soluble cytokine receptors as well as cytokine receptor interactions in patient samples. In DETA, a sample is subjected to an oscillating sinusoidal electric field. The sample becomes polarized under an applied voltage of 10 V/mm, leading to an ordered array of molecules and alignment of dipoles (Fig. 1). Permittivity or dielectric constant (ε') is the approximate dipole content and is proportional to the capacitance. The dielectric loss factor (ε''), proportional to the conductivity is the energy required to align dipoles and move ions. Ionic conductivity of the samples was measured at a frequency (f) range of 0.1–300,000 Hz. Reproducible signature differences in ionic conductivity and % water loss were obtained for each protein. Tan delta versus log frequency plots were constructed, and activation energies as a function of frequency were calculated from Arrhenius plots of the tan delta ($\varepsilon''/\varepsilon'$, dielectric loss factor/relative permittivity), i.e., log peak frequency versus reciprocal temperature (1/K).

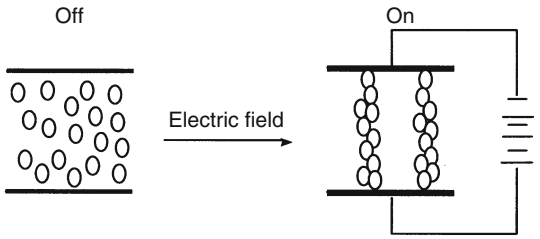

Fig. 1 Protein alignment in an electric field

Materials and methods

Recombinant proteins

Lyophilized recombinant human cytokines were reconstituted to 0.1–1 mg/mL according to manufacturer's instructions in the following solutions: water (IFNγ, TNFα, TNFR2, IL-4, IL-1α, and IL-1Ra), 5 mM Tris pH 8.0 (sTNFR1), 100 mM acetic acid (IL-2), 5 mM acetic acid (IL-6), 5 mM sodium phosphate, pH 7.2 (IL-10), and 1× PBS pH 7.2 (sIL-2Rα). Protein volumes of 10, 20, and 40 μL were used in DETA studies. Human cytokines and soluble cytokine receptors were obtained from Peprotech (Rocky Hill, NJ). Solutions used for cytokine reconstitution were also analyzed using DETA as controls. Amino acid sequences (Peprotech) were analyzed for the frequency of occurrence in cytokines and soluble cytokine receptors using the Protein Information Resources search window (http://pir.georgetown.edu/cgi-bin/comp_mw.pl).

Dielectric thermal analysis

A TAI 2970 DEA was used to measure the electrical conductivity of cytokines and cytokine receptors. 10, 20, and 40 μL of dissolved proteins were placed on a single surface interdigitated gold ceramic electrode and heated to 37 °C. Measurements were taken through a frequency range of 0.1–300,000 Hz. All samples were analyzed using TA Instruments universal analysis software and after sensor calibration all samples were run at 25 °C and isothermal for 60 min. Samples were also ramped at 5 °C per minute to 37 °C in an inert atmosphere with a flow rate of 50 mL in nitrogen. DETA was also performed by placing protein samples on the surface of a single gold ceramic interdigitated electrode in an isolated nitrogen rich dry atmosphere. The samples were ramped at a rate of 10 °C/min from room temperature (25 °C) to 250 °C. Conductivity measurements were recorded at controlled interval frequencies ranging from 0.1 to 300,000 Hz for all temperatures [8]. Dielectric properties were determined as a function of time, temperature, and frequency in order to

obtain the dielectric constant or permittivity (ε'), the dielectric loss factor (ε'') or ionic conductivity, and the loss tangent ($\tan\,\delta = \varepsilon''/\varepsilon'$).

Thermogravimetric analysis

A TAI 2950 TG was used to measure the percent (%) mass loss from the proteins when heated to temperatures below the melting point, but above the boiling point of water [7]. Samples were loaded into platinum pans and heated in a nitrogen environment to 150 °C. Isothermal conditions were then maintained to ensure reaction completion at 150 °C. The instrument was calibrated with calcium oxalate hydrate (water loss 12.2 ± 0.1%). TG curves were collected from 25 to 37 °C at a heating rate of 5 °C/min then held isothermally at 37 °C for 60 min. The stability of water in an aqueous solution of 5 μL of protein was determined as a function of time. TAI Universal Software was used to calculate the mass loss as a function of time. Differential thermal gravimetric (DTG) analysis curves were taken as the first derivative of the TG curves and aided in the interpretation of water loss at body temperature.

Results and discussion

Log ionic conductivity versus time and trend analysis of conductivity

Plots of log ionic conductivity versus time are shown in Fig. 2. Conductivity was concentration dependent with high conductivities of 10^5–10^8 observed at low frequencies at 25–50 min. High conductivity levels were followed by a sharp decrease in measured conductivity for cytokines and soluble receptors with low conductivities between 10^0 and 10^4 pS/cm observed for IFNγ and TNFα. Overall, higher conductivity was observed in receptors compared to cytokines. Conductivities for TNFα and soluble TNF receptor 1 (sTNFR1) shown in Fig. 2a and b are representative of the plots obtained. A trend analysis was performed to determine if changes in conductivity observed were consistent at low (10^{-1} Hz), medium (10^2 Hz), and high (3×10^5 Hz) frequencies, at a conductivity range of 10^4–10^8 pS/cm. Results of the analysis for cytokines and soluble receptors are summarized in Table 1. With a 10^6 change in frequency an approximately 90% change in conductivity was observed. The ratio of average log conductivity at high frequency

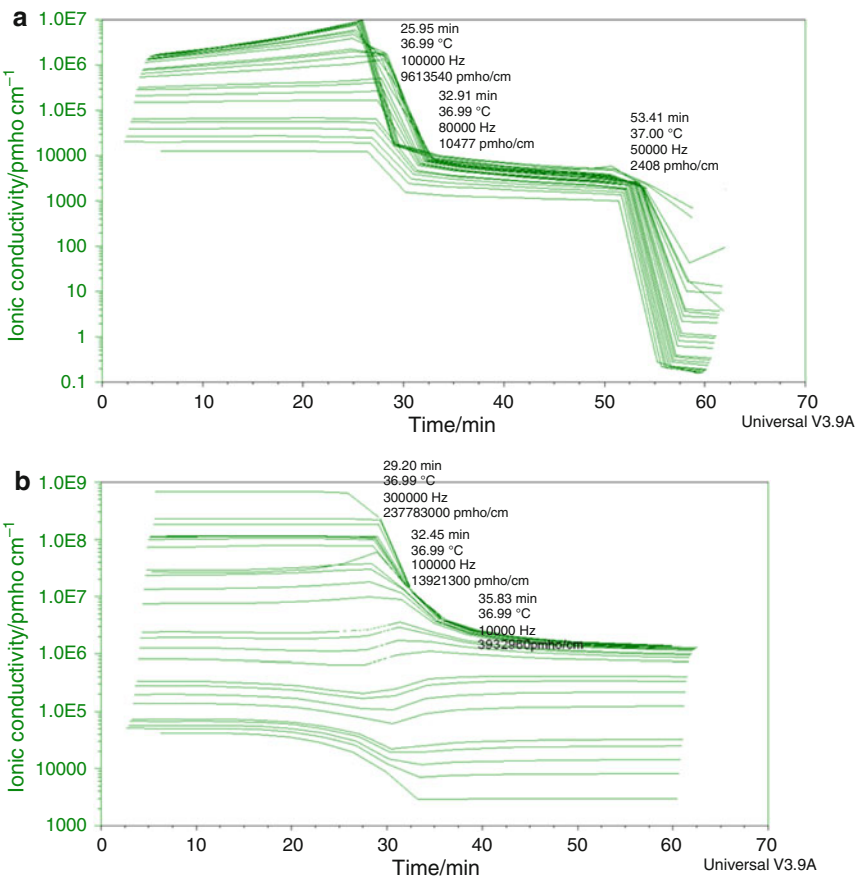

Fig. 2 Log ionic conductivity versus time, frequency range of 0.1–300,000 Hz, 20 μL, 37 °C. **a** TNFα; **b** sTNFR1

Table 1 Trend analysis of conductivity at low, medium, and high frequency

	Proteins	Conductivity/pS/cm			Log conductivity/pS/cm			
		Frequencies/Hz			Frequencies/Hz			
		3.00E+05	1.00E+02	1.00E−01	3.00E+05	1.00E+02	1.00E−01	
1	TNF alpha	3.30E+07	6.60E+06	3.33E+04	7.52	6.82	4.52	
2	IL-10	8.27E+07	4.78E+05	1.71E+03	7.92	5.68	3.23	
3	IL-6	6.22E+07	4.47E+06	2.73E+04	7.79	6.65	4.44	
4	IL-4	1.16E+08	6.41E+05	4.56E+03	8.06	5.81	3.66	
5	IL-2	2.78E 08	7.61E+06	3.02E+04	8.44	6.88	4.48	
6	IL-1 alpha	1.44E+08	3.79E+06	1.76E+04	8.16	6.58	4.25	
7	IFN gamma	5.00E+08	5.00E+06	1.49E+04	8.70	6.70	4.17	
AVE					8.09	6.45	4.11	1.96837
SDV					0.40	0.49	0.48	
% Relative error = SDV/AVE × 100 = %RE					4.91	7.62	11.80	
8	sTNFR1	6.51E+08	1.39E+07	1.49E+05	8.81	7.14	5.17	
9	sTNFR2	2.07E+08	1.00E+06	1.73E+04	8.32	6.00	4.24	
10	sIL-2 R	6.83E+08	6.43E+06	2.65E+05	8.83	6.81	5.42	
11	IL-1-Ra	4.07E+08	1.19E+06	6.61E+04	8.61	6.08	4.82	
AVE					8.64	6.51	4.91	1.759674
SDV					0.24	0.56	0.51	
%RE					2.78	8.60	10.46	

Table 2 Summary of cytokines and soluble cytokine receptors: DETA, time, temperature, and peak frequency data from tan delta versus temperature plots at 10 °C/min

Cytokine	Receptor	Time/min	Temperature/°C	Peak frequency/Hz	Tan delta	Comment	
					$\varepsilon''/\varepsilon'$	With water	Without water
IFN gamma		2.8	47	91,000	8.5	x	
		15	168	0.31	12		x
		21	225	1.1	11		x
TNF alpha		3.1	50	3,000	14	x	
		3.9	58	30,000	33	x	
		14	159	51	28		x
		15	161	55	24		x
	sTNF receptor 1	19	190	0.34	212		x
		20	207	5,000	63		x
		19	208	100,000	35		x
	sTNF receptor 2	3.7	56	300,000	10	x	
		6.6	86	10,000	13	x	
		14	162	5,200	16		x
		15	165	9,900	17		x
	sIL-2 receptor	4.1	330	10,000	300		x
	IL-1 Ra	4.2	56	28,000	18	x	
		6.1	75	9.9	11	x	
		10	114	5.3	8.7		x
		16	178	0.31	9.5		x

(3×10^5 Hz) to average log conductivity at low frequency (10^{-1} Hz) for cytokines shows a 97% difference in conductivity. There is a difference of 76% conductivity for the receptors for the same ratio for the cytokine receptors (Table 1). A change of 10^6 (10^{-1} to 3×10^5) in frequency (Hz) is six orders of magnitude variation or

about 90% change. At low frequency the changes observed are associated with surface charge while changes observed at high frequency are associated with bulk charge. The standard deviation obtained from the data at high frequency or ±0.24 is lower than that observed at low and medium frequencies, ±0.51 and ±0.56, respectively. The percent relative error (% RE) is at a range of 3–10% for average log conductivity values for low, medium, and high frequencies (Table 1). The variations observed in the conductivity values are appropriate. Furthermore, the log conductivity values at high frequency show clearer distinctions between cytokines and receptors. The major difference observed among proteins is due to the conductivity or loss factor (ε''). Frequency peaks for the six proteins investigated previously were selected at low frequency (0.1 Hz) and high frequency (100,000 Hz) to highlight the conductivity differences observed for cytokines and cytokine receptors (Table 2). Differences observed between cytokines and cytokine receptors in general reflect fundamental properties of the proteins and are due to intrinsic dipoles of the amino acid side chains as well as the hydrophobicity and hydrophilicity of the individual proteins.

Tan delta versus log frequency

The curves for tan delta versus log frequency (Debye plots) showed a strong dielectric loss spectra for IFNγ, TNFα, sTNFR1, sTNFR2, and IL-1Ra with two major peak areas at low (0.1–100 Hz) and high (1,000–100,000 Hz) frequency ranges in different scan cycles at different times and temperatures. Dielectric loss spectra for TNFα and sTNFR1 are shown in Fig. 3a and b. The spectra are representative of the plots obtained. In contrast to sTNFR1 and sTNFR2, soluble IL-2Rα revealed a weak spectrum with a single peak at 10,000 Hz. Further analysis of the spectra revealed two signatures for cytokines associated with and without water (Table 2). Increased frequencies were associated with short cycle times and low temperatures and decreased frequencies were associated with longer cycle times, higher temperatures, and drying of the proteins. For example, IFNγ had peak frequencies and peak times of 14–15 min; at short time cycle (<15 min), with water association at high frequency (of 91,000 Hz), 2.8 min at 47 °C. A second signature representing low frequency (0.3 Hz), 15 min at 168 °C was observed. Similarly, TNFα had a high frequency peak (5000 Hz), 3 min at 51 °C and a second signature at a low

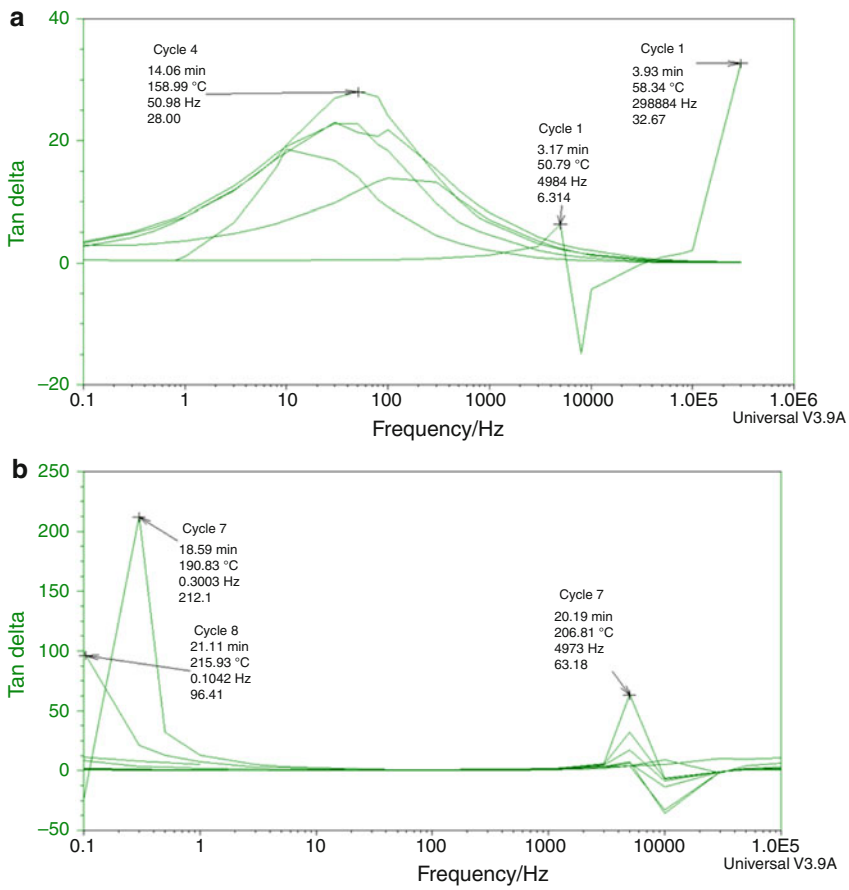

Fig. 3 Debye plot of tan delta versus log frequency for temperature ramp ups, 20 µL, 250 °C. **a** TNFα; **b** sTNFR1

frequency peak (51 Hz), 14 min at 159 °C (Table 2). IL-1Ra and the soluble cytokine receptors varied in their spectra probably due to different levels of water types (hydrophilic vs. hydrophobic) associated with amino acid residues and differences in protein conformation. Hydration levels serve as a marker for differentiating the proteins [9–11]. A temperature range from ambient to 180 °C was observed for IL-1Ra, with frequency peaks ranging from 0.3 to 280,000 Hz. It appears water is lost early at 5 Hz, 10 min, and 114 °C, which is close to the boiling point of water. The two receptors for TNFα: sTNFR1 and sTNFR2 showed varying time, temperature, and frequency dependent responses. Soluble TNFR1 showed modest cycle times of 19–21 min at high (5000 Hz) and low (0.3 Hz) frequencies while sTNFR2 had a broad distribution in time and temperature frequency dependent responses. At 21 min, 44 Hz, and 227 °C, sTNFR2 may be completely denatured (Fig. 3). Both sIL-2Rα and sTNFR1 had very high tan delta values; 300 and 212, respectively, compared to the other proteins at a tan delta range of 8.5–63, indicating increased charge mobility and ionic conductivity of amino acid residues at the frequency ranges of the scans (Table 2) in sTNFR1 and sIL-2Rα.

Arrhenius plots of log peak frequency versus reciprocal temperature, activation energies, and thermogravimetry

Activation energies (E_a) associated with the electrical charging of cytokines and cytokine receptors were calculated from the slopes of Arrhenius plots of log peak frequency versus reciprocal temperature, 1/K. E_a is the energy required to initiate charge mobility. The energy thresholds for mobility of charges in the amino acid residues of cytokines and cytokine receptors can be identified by E_a. The lower the E_a, the higher the charge mobility, and the more reactive (as in amorphous solids) the sample. Activation energies are frequency and temperature-dependent. Linear curves were obtained from the calculations of the Arrhenius equation, with most R^2 values close to 1.0. Frequency dependent decrease in E_a was observed for IFNγ, IL-1Ra, and sTNFR1 with E_a decreasing (increasing mobile charges) from low to high frequency (Table 3). Frequency dependent increase in E_a was observed for TNFα (19, 20, 23 J/mol) as well as for sIL-2R (14, 17, 35 J/mol) (Table 3). E_a for sTNFR2 was frequency independent. At medium frequency sTNFR2 had increasingly mobile charged species (9.2 J/mol) compared to 33 J/mol at high frequency. Positive slope E_a curves were observed for IFNγ, TNFα, and sTNFR2 at low, medium, and high frequencies. IL-1Ra had a negative slope at low and medium frequencies, sTNFR1 had a negative slope at high frequency and sIL-2Rα had negative slopes at medium and high frequency (Table 3). Correlation coefficients (R^2) were high with values close to 1.0. The negative activation enthalpy is associated with the protein-folding process and "transient" of water molecules coordinated around proteins undergoing denaturation [12]. In a previous study TG and DETA studies were described and a TG/DETA overlay plotted [13]. Employing TG and DETA in tandem of the cytokines and cytokine receptors was very instructive. Mass loss of water was clearly identified in <20 min and <120 °C, where we observed increased conductivity in cytokines which gave us a basis of associating water as markers. TG aided the separation of data to distinguish water wet samples from dried samples for better interpretation. Clearly, protein samples were dry based on TG and contained reduced frequency DETA response at >20 min.

Table 3 Activation energies of cytokines and cytokine receptors at low to high frequencies (log peak frequency vs. 1/temperature (K) × 1000)

Cytokine	Receptor	E_a/J/mol			Slope	R^2
		Low frequency	Medium	High		
IFN gamma		45			+	0.998
			27		+	0.998
				10	+	0.981
TNF alpha		19			+	0.99
			20		+	0.988
				23	+	0.998
Average		32	23	17		
	IL-1Ra	111			–	0.999
			88		–	0.927
				2.8	+	0.998
	sTNFR1	103			+	0.995
			14		+	0.977
				5.8	–	0.985
	sTNFR2	47			+	0.999
			9.2		+	0.918
				33	+	0.999
	sIL-2R	14			+	0.977
			17		–	0.867
				35	–	0.935
Average		69	34	19		

Key: Low frequency 0.1–1 Hz; medium 100–500 Hz; high 100,000–300,000 Hz

Conclusions

A wide range of effects of applied frequency in an AC electric field shows transition properties as a function of frequency over a range of 0.1–300,000 Hz. A trend analysis of changes in conductivity among the proteins revealed a change reflecting fourteen orders of magnitude.

Limited frequency and dielectric loss factor dependence were observed in the conductivity of the proteins. Conductivity and activation energy were dependent on the intrinsic properties of the proteins reflecting the differences in the amino acid composition of the proteins. However, water associated with the proteins represents a useful marker for differentiating the dielectric response of the proteins and can be used to predict the behavior of the protein. Electric properties are the only significant variables that help define the thermal behavior of cytokine and cytokine receptors. Different solvents used to reconstitute lyophilized recombinant proteins were subjected to DETA analysis in order to determine the level of conductivity of the solvents. Although the solvents were conductive, dielectric signatures of the individual proteins could be differentiated from each of the solvents. In addition to the dielectric properties of proteins investigated in the current study, thermal analysis by differential scanning calorimetry (DSC) will be performed. Serum proteins other than cytokines such as human albumin, immunoglobulin (Ig) M and IgG are currently under investigation to determine their distinct dielectric signatures. Reports by Michnick et al. [14] show human albumin with a high thermal stability compared to bovine serum albumin. In protein mixtures such as in human serum, it will be important to predict the interaction of proteins and determine their responses under thermal processing conditions for the development of diagnostic platforms, protein based pharmaceutical agents and other solid state studies of macromolecules [15, 16]. Future investigations will include DETA and DSC of varying concentrations of proteins and establishing the influence of the fluid environment surrounding the proteins on the dielectric spectra obtained. A larger sampling of cytokines and cytokine receptors will need to be investigated to determine if the dielectric properties identified are consistent for cytokines, cytokine receptors, and antagonists. The external environment and the interaction of the fluid environment on the interface of the protein and fluid can influence the relaxation of the charged amino acid residues [17, 18]. The data obtained supports our hypothesis. Differentiation of cytokines will benefit efforts to identify and differentiate cytokines associated with the different pathogenesis of malaria.

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