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Dielectric Spectroscopy of Molecular Interactions Based on the Avidin-Biotin Complex

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Abstract— **Dielectric spectroscopy is used to probe the electrical properties of biomolecules dissolved in liquids. A 40 μl cell is constructed out of acrylic with polished, stainless steel electrodes. Experiments are performed on avidin and biotin-labeled BSA, showing characteristics of aggregation. Experiments with avidin and biotin demonstrate shifts in dielectric relaxation of the avidin associated with changes in the dipole moment and size of the molecule due to biotin binding. These shifts are analyzed in the context of biomolecular changes. These experiments demonstrate the utility of impedance spectroscopy to detect changes due to small molecules binding to proteins.**

I. Introduction

Dielectric spectroscopy (DS) has been used for many years to probe the electrical properties of materials [1]. The basic procedure has not changed over the past century; alternating voltages are generally applied to a sample and the magnitude and phase of the current is measured. Current theory is still based on the work of Debye [2] and Oncley [3], who were important contributors to the rich history of DS in applied biophysical measurements.

More recent advances in DS are a result of modern network and impedance analyzers that can cover extremely broad ranges of frequency, usually several orders of magnitude [4]. Data acquisition is automated and time-resolved changes can be observed. Advances in cell construction have increased the stability and accuracy of measurements on small sample volumes.

Binding of antibody-protein pairs as well as β-lactoglobulin (β-Lg) aggregation with hen lysozyme (HENL) have been monitored by DS [5], [6]. These measurements have also been used with Poisson-Boltzmann calculations of protein charges to estimate the internal permittivity of proteins [7].

DS experiments must be performed in solutions of low ionic strength because high conductivity reduces the phase angle between the real and imaginary parts of the impedance. Additionally, in ionic solutions the series polarization impedance of the electrodes becomes significant. Research in the electrode material and surfaces has increased understanding of methods to reduce electrode polarization and other parasitics in measurements [6], [8]. These considerations are employed in the design of the impedance cell built for the experiments in this paper.

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Impedance is attractive for biological measurements because the transduction of electrical signals is straightforward for integrated devices. Thus, it is used in various configurations for monitoring bacteria or counting cells [9], [10]. Notably, impedance spectroscopy is a low-resolution technique when compared to techniques such as NMR or X-ray crystallography. However, its advantage lies in its simplicity, low cost, and speed at which experiments can be performed with the potential for highly-parallelized assays.

The avidin-biotin binding reaction is used heavily in molecular biology because it is well understood and has a high binding affinity ($K_\mathrm{a} \approx 10^{15}$ M^{-1}). Additionally, the three-dimensional structures of the protein, ligand, and complex have been solved [11]. A correct characterization of the avidin-biotin binding event is thus important to demonstrate the capabilities of DS to resolve binding interactions, particularly a large protein with a small molecule. Previously, the interaction between β-Lg and HENL was probed using DS [6]. In that case, an aggregate is formed [12] and the time constant of the resulting complex was outside the measurement frequency range. Here, we choose a model system where the relaxation frequencies of the protein and bound complex are within the frequency bandwidth of the measurement apparatus, allowing extraction of relaxation parameters before and after binding events. We will first demonstrate general protein binding between avidin and biotin-labeled BSA (bBSA) and then avidin with biotin.

II. Theory

In a solution, an applied, alternating electric field applies a torque to most proteins because they possess a large dipole moment. However, the proteins can only respond up to certain frequencies (generally in the MHz range) because of hydrodynamic drag due to interactions with the surrounding water molecules [4]. When the permittivity of a solution containing a protein is plotted, the dielectric relaxation of the tumbling protein mode generally follows a single relaxation Cole-Cole curve as

$$\varepsilon = \varepsilon_\infty + \frac{\Delta\varepsilon}{1 + (j\tau\omega)^{(1-\alpha)}} \tag{1}$$

where ε_∞ is the high frequency permittivity above the tumbling relaxation, $\Delta\varepsilon$ is the change in permittivity, α is the Cole parameter describing the spread of the relaxation [13], τ is the protein rotational relaxation time, and $j = \sqrt{-1}$. The electric dipole moment of the protein is related to $\Delta\varepsilon$ through the Oncley formula

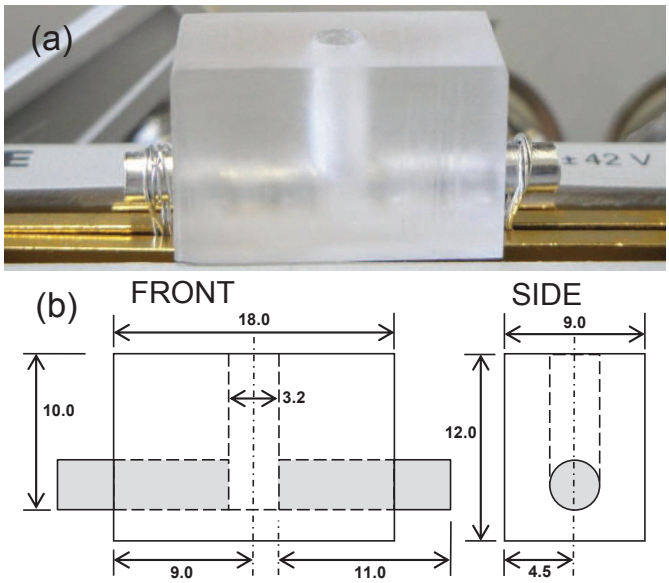

Fig. 1. (a) Photograph and (b) schematic of dielectric cell (dimensions in mm). The interrogated volume is 40 μl. Liquids are added and removed using microcapillary tubes.

$$\mu = \sqrt{\frac{2Mk_bT\varepsilon_0\delta}{Ng}} \tag{2}$$

where μ is the dipole moment, M is the protein molecular weight in kilodaltons, k_b is the Boltzmann constant, T is the temperature in Kelvin, ε_0 is the permittivity of free space, N is Avogadro's number, g is the correlation parameter assumed to be 1 for dilute protein solutions [14], and $\delta = \lim_{c\to 0} \Delta\varepsilon/c$ is the dielectric increment where c is the molar concentration of the protein.

Assuming the protein is roughly spherical, the effective hydrodynamic radius of the protein, a, is estimated from the relaxation time taken from the fitted permittivity spectrum,

$$\tau = \frac{4\pi\eta a^3}{k_bT} \tag{3}$$

where η is the solution viscosity. Many additional factors can be employed to describe the actual measured permittivity of the solution containing proteins, but these relations are a standard interpretation of the observed primary relaxation.

III. Materials and Methods

A. Dielectric Cell

Because the proteins to be investigated are the most expensive component for these tests, it was desired that the interrogated volume of the cell be small. Starting with our most recent design [6], the cell size was reduced considerably by machining the cavity in a single piece of acrylic, with dimensions of 1.8 cm X 1.2 cm X 0.9 cm. A schematic and photograph of the cell attached to the impedance analyzer are found in Fig. 1.

1/8 inch round stainless steel (Grade 304) was used for the electrodes. Each were cut with edges slightly tapered on an end mill to ease the insertion into the acrylic. A polishing process was then employed to give a consistent, smooth surface and to reduce electrode polarization. The electrode faces were ground with 600, 800, and 1200 grit silicon carbide abrasive discs in a Spectrum System 2000 (LECO Corp.). The electrodes were then press-fit into the acrylic.

The electrodes were connected via wires to the terminals of a 16047E test fixture mounted on an Agilent 4294A Precision Impedance Analyzer. The Analyzer was controlled through a GPIB connection to an attached computer running National Instruments LabVIEW for communication, automation, and data logging. The acquired spectra were then post-processed in MATLAB.

Calibration was performed using air and water. The cell constant, k, and the parasitic capacitance of the cell, C_p, were found through the formula

$$C = k\varepsilon + C_p \tag{4}$$

using the permittivities of air ($\varepsilon = 1$) and water ($\varepsilon = 78.4$ at 25 °C [15]). The measured cell constant was found to be 0.0337 pF and the parasitic capacitance was 0.194 pF at 1 MHz. Sweeps were made over 601 logarithmically-spaced points from 10 kHz to 110 MHz with settings of a bandwidth of 4 and an oscillator strength of 500 mV. The cell constant was then used to extract the permittivity of the measured solutions. The temperature of the room did vary minimally, so a normalized permittivity approach was used, where the permittivity at 110 MHz was the normalization point.

B. Protein Solutions

Avidin (A9275), bBSA (A8549), and biotin (B4501) lyophilized powders were obtained from Sigma and used without further purification. The proteins were reconstituted in 0.1 mM HCl and stored in microcentrifuge tubes at concentrations of 20 mg/ml, 20 mg/ml, and 5 mg/ml, respectively. They were then refrigerated until used.

C. Experimental Protocal

The first experiments were reactions of avidin and bBSA. The cell was initially filled with 60 μl of 0.1 mM HCl buffer. Frequency sweeps were taken every 30 seconds as 3 μl were withdrawn from the cell and replaced with 3 μl of the avidin solution. This was repeated 2 times. Next, the second protein was added by withdrawing 2 μl from the cell and replacing with 2 μl of the bBSA solution. This was repeated 3 times. After the cell was rinsed with ethanol and DI water and dried, the procedure was repeated starting with bBSA, followed by avidin.

The second experiments examined the dielectric properties of avidin before and after binding with biotin. Because the changes in $\Delta\varepsilon$ and τ were expected to be small, the inital concentration of avidin was raised to 3 mg/ml. While continuously recording sweeps, two additions of 2 μl of the biotin solution were made spaced 10 minutes apart. The concentration of biotin was such that after the first addition

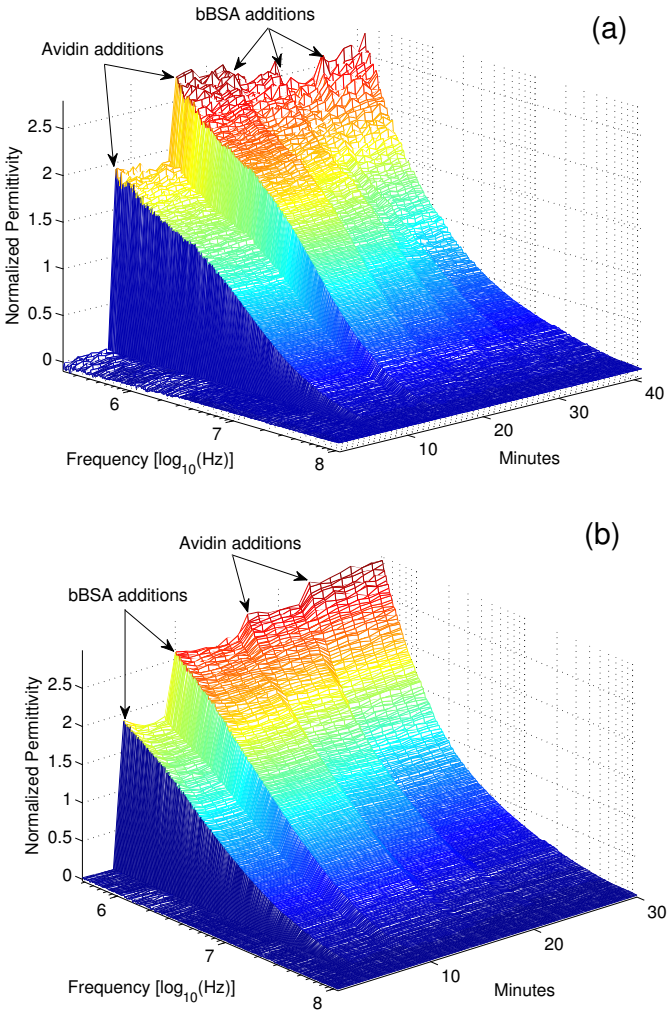

Fig. 2. Normalized permittivity of (a) avidin + bBSA and (b) bBSA + avidin interactions. When the second protein is added, the proteins bind and the original relaxation falls off considerably.

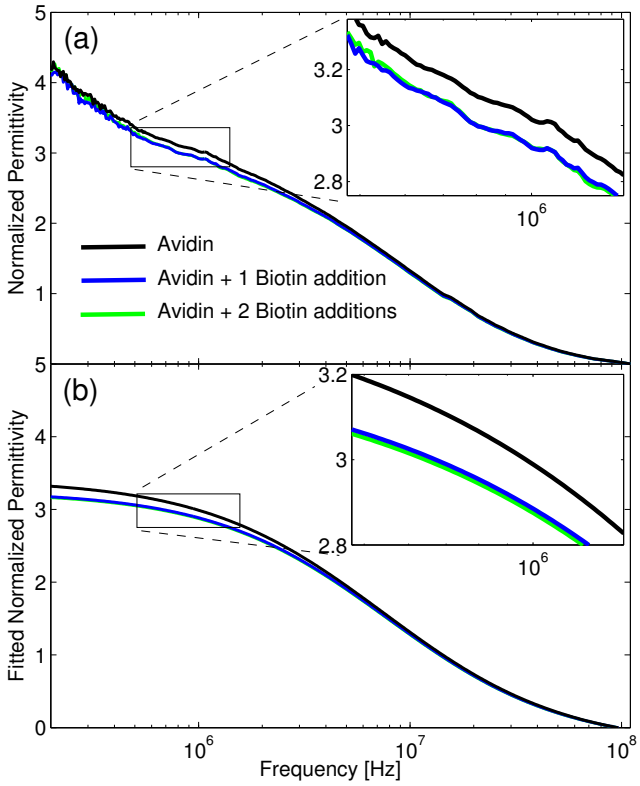

Fig. 3. (a) Raw and (b) fitted normalized permittivity of avidin before and after binding with biotin. After first biotin addition, binding sites of avidin become saturated (blue line), causing second biotin addition to unaffect the relaxation curve (green line, barely visible behind blue). To avoid electrode polarization, the lower frequency bound on the fitting function was set to 1 MHz.

the number of biotin molecules exceeded the number of available avidin binding sites.

IV. Results and Discussion

A. Avidin-bBSA: Protein-Protein Interaction

Fig. 2 shows the results of the avidin-bBSA binding experiments run first with avidin, followed by bBSA (Fig. 2a), then bBSA followed by avidin (Fig. 2b). This shows how the aggregation of the protein is clearly visible. The first protein has a distinct dielectric relaxation that increases proportional to concentration. When the second protein is added, this relaxation falls off and eventually is overtaken by a larger relaxation at a lower frequency. Because the reaction is so quick, it is not easily resolved in time because the diffusion of the molecules is slow, even with mixing.

The data was parameterized using a least-squares fitting of Eq. 1. Because this equation assumes a single solute molecule, a bimodal or trimodal should be used when the complement protein is added. However we used the single relaxation model to capture the trend of the largest relaxation. In both experiments, the relaxation time τ increased until moving outside the available frequency window of our apparatus. Thus the general size of the avidin-bBSA aggregate is unaccessible, except that it must be at least 2 or 3 times larger than either individual protein.

B. Avidin-Biotin: Protein-Ligand Interaction

Fig. 3 shows the results of the avidin-biotin binding experiment. The three curves denote the solution permittivity with (1) avidin only, (2) avidin + 1 biotin addition, and (3) avidin + 2 biotin additions. When biotin was first added, the change in permittivity $\Delta\varepsilon$ of the dominant relaxation decreased, and the relaxation time τ remained roughly the same. This reflects the change in dipole moment before and after each avidin molecule binds two biotin. The binding is internal (avidin has two β-barrels), thus the hydrodynamic radius and consequently τ would not be expected to change substantially. When the second biotin addition is made, the permittivity spectrum remains constant. This indicates the number of bound molecules remains the same; only the number of free biotin increases. The high binding affinity between the two molecules ensures that after the first biotin addition a very high percentage of binding sites are occupied.

TABLE I

MEASURED AND CALCULATED DIPOLE MOMENT μ AND HYDRODYNAMIC RADIUS a OF AVIDIN AND BIOTIN-BOUND AVIDIN.

	Avidin	Avidin + Biotin
μ_{meas} [D]	599 ± 7	591 ± 11
μ_{calc} [D]	632^a	630^b
a_{meas} [Å]	19.4 ± 0.1	19.0 ± 0.2
a_{calc} [Å]	24.6^a	24.6^b

Measurement error ranges represent 95% confidence intervals. [a]Calculated using PDB 2avi with biotin removed at pH 7. [b]Calculated using PDB 2avi with biotin included at pH 7.

Because the relaxation of biotin occurs above 110 MHz, any permittivity contributions from biotin in the bulk solvent would be absorbed by normalized permittivity.

The observed shift in dielectric properties of bound and unbound avidin is remarkable considering the small physical changes the molecule undergoes. Avidin is dimeric at low concentrations in solution [16] where the binding of each avidin dimer ($M \approx 30$ kDa) to two biotin molecules ($M \approx 0.5$ kDa) is accompanied by an increase in mass of less than 2%. Because the signal of interest, in this case $\Delta\varepsilon$, is proportional to the square of the dipole moment μ^2 (Eq. 2), the protein to protein-ligand transition is resolvable. The measured dipole moment of avidin alone was ~ 599 Debye (D). This result was pleasantly unexpected as it is larger than most proteins and no published measured value for avidin currently exists. The dipole moment of biotin-bound avidin was somewhat smaller, ~ 591 D. Two possible mechanisms for this decrease are dipole vector cancellation of the added biotin molecules, and structural adjustments of the dimer that accompany binding. We introduced a calculation method to perhaps elicidate these mechanisms.

Theoretical approaches have been developed to calculate parameters μ and a based on deposited three-dimensional structures. We implemented the approach described in [7] for μ, and used a least-squares sphere-fitting algorithm to estimate a. The results of these computations are displayed in Table 1 alongside corresponding DS measurements. The agreement is quite good, and discrepancies of a probably reflect errors due to the non-spherical shape of avidin. Dimeric molecules also typically rotate about the shorter axis of rotation [7], thus, a_{calc} is more indicative of the longer axis of avidin, and a_{meas} the shorter. When the two biotin molecules were included in the calculations for μ_{calc} and a_{calc}, they remained nearly unchanged. It is plausible that avidin flexes and tightens around biotin [17], which if true, would resolve the paradox of why μ_{meas} decreases by an amount larger than the predicted amount of 2 D. This would also be consistent with the small observed decrease in measured hydrodynamic radius with the addition of biotin.

V. CONCLUSION

These experiments clearly show that dielectric spectroscopy can be used in experiments of binding between proteins, as demonstrated for the case of avidin and bBSA. Notably, the reaction of small molecules with a much larger protein is also demonstrated. The avidin-biotin experiment shows the potential of dielectric spectroscopy to resolve small structural and chemical changes in molecules, which could have important implications for creating more sensitive biosensors for medical applications. These methods could also play a vital role for new drug discovery and protein signaling techniques.

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