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Dielectric relaxation study of aqueous Proteins (Hb & BSA) using time domain reflectometry technique

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ABSTRACT

A dielectric measurement was performed on proteins in an aqueous media using dielectric time domain spectroscopy over a wide range of frequency 10 MHz to 30 GHz. The static dielectric constant (ε_0), relaxation time (τ) and conductivity (σ) have been determined using nonlinear least squares fit method. The hydration numbers (N_{hyd}) were also determined from the static dielectric constant. The hydration number suggests the presence of charges on protein surface which is responsible for maximum interaction of water molecules, whereas it decrease with increase in pH.

***Keywords:** dielectric relaxation, proteins hydration, time domain reflectometry.*

1. INTRODUCTION

Proteins make up fundamental machinery of life. Since evolution, protein adapted to aqueous environment as a result functional processes mediated by proteins, such as binding, recognition and catalysis often involve specific interactions with individual water molecules carried hydration. Therefore, protein hydration is of fundamental interest in biology [1, 2]. The hydration water of proteins is essential to biological activity but its properties are not yet fully understood. The term hydration is commonly used to cover two different phenomena: (a) the total interaction of a solute with its aqueous solvent environment; and (ii) the perturbation of the structure and dynamics of bulk water caused by the interaction with the solute. Here, we use hydration in the latter, more restrictive sense. The water molecules that interact with a protein can, with little ambiguity, is classified as internal or external. Internal water molecules occupy cavities within the protein and are present in most globular proteins [3,4]. They are conserved to the same extent the amino acid sequence and must therefore be essential for function [5]. For most purposes, internal water molecules are best regarded as an integral part of the protein, even though they exchange with external water molecules, typically on time scale of 0.1-1.0 μs[6].

Protein-water interactions are of similar strength as water-water interactions and are therefore not expected to induce extensive structural perturbations. Indeed, magnetic relaxation [6] and computer simulation [7-9] studies indicated that only water molecules in direct contact with the protein surface are significantly perturbed. Moreover, the vast majority of water molecules in this hydration layer are not more perturbed that water molecules in contact with small solute [10]. Nevertheless, these water molecules are often referred to as 'bound'. This term appropriately describe the strongly exothermic adsorption of water molecules on the surface of a dry (vaporized or lyophilized) protein [11], but it is misleading when applied to a protein immersed in an aqueous solvent. Water molecules in the hydration layer of a dissolved protein are not bound in a thermodynamic or kinetic sense. It is therefore not physically meaningful to describe protein hydration in terms of equilibrium between bound and free water as is commonly done [12-14]. In the absence of co solvents, every exposed hydration site is virtually always occupied by a water molecule from the 'bound' to the 'free' state is invariably accompanied by the reverse transition of another water molecule. In other words, we are dealing with a symmetric exchange process for which the equilibrium constant is trivially equal to one. Water simply fills the available space.

In a physiological condition, proteins play their roles mostly in water. The enzymatic activities are also related to their structure change according to the environmental chemical or physical stimulations [15]. Generally, structural change of proteins in water relates to the change of the hydration states [16]. Protein hydration has long been studied by various methods, such as calorimetry [17], infrared spectroscopy [18], osmotic pressure [19], NMR techniques [20], by ^{17}O relaxation measurements [21], or ^{1}H chemical shift analyses [22], and dielectric spectroscopy [23-26].

The dielectric properties of biological systems are very remarkable. They typically display extremely high dielectric permittivity at low frequencies, falling off in more or less distinct steps with increasing frequency. Their frequency dependence permits identification and investigation of a number of completely different underlying mechanisms, and hence, dielectric studies of biomaterials have long been important in biophysics [27]. Dielectric relaxation is a popular method to probe the dynamics of protein solutions. However, several interesting and anomalous phenomena observed in the dielectric spectra have surprisingly eluded molecular explanation despite a number of studies over decades [28, 29].

The molecular understanding of the dielectric functions of an aqueous protein solution is also essential to follow the interaction between a charged species and the protein-solvent system. The study of concentration dependence of dielectric relaxation spectra can also help in understanding the forces responsible for protein association [30].

It is to be expected that a fraction of the water molecules finds themselves in a local environment, interact strongly with the protein molecule. The properties of water in biological systems have been studied for well over a century by a wide range of physical techniques [11]. However, none of these techniques provides the spatial and temporal resolution required to directly probe water molecules interacting with the surface of a protein in aqueous solution. The high complexity of proteins and the incomplete understanding of bulk liquid water and of small-molecule hydration, it is perhaps not surprising that progress within the field of protein hydration has been slow and erratic [3].

In this paper, we report static dielectric constant, relaxation time, conductivity and hydration number of hemoglobin (Hb) and bovine serum albumin (BSA) in aqueous solutions at different pH values using. Time domain reflectometry technique in the frequency range of 10 MHz to 30 GHz.

2. EXPERIMENTAL SECTION

2.1. Materials

Hemoglobin powder (Hb, Mw 64500) and Bovine serum albumin (BSA, Mw 66000) were purchased from Himedia Laboratories Pvt. Ltd. and used without further purification. Solutions were prepared using potassium phosphate buffer (pH 7.1, 7.3, 7.4) in deionized water by taking 0.5 mM and 1.0 mM concentrations of monobasic potassium phosphate (KH_2PO_4) and dibasic potassium phosphate (K_2HPO_4) respectively. pH was recorded using Systronics digital (802) pH meter.

Density of solutions was measured by using a picnometer. Density measurements were reproducible without attention to temperature; reproducibility was typically ± 0.001 kg/liter. We assume the density of the solutions is independent of ligation state.

We utilize a time domain reflectometry (TDR) technique for measuring dielectric spectra of liquids up to 30GHz. A Tektronix DSA8200 Digital Serial Analyzer Oscilloscope mainframe along with the sampling module 80E08 has been used. A repetitive fast rising 250mv voltage pulse of 200 KHz with 18ps incident rise time was fed through coaxial line system of impedance 50 ohm. The co-axial cable was copper metal and semi rigid model no. EZ_86/M17 (Huber Suhner Electronics Pvt. Ltd.). The inner diameter of outer conductor is 2.2mm; outer diameter of center conductor is 0.51mm and the diameter of dielectric material is 1.68mm. Sampling oscilloscope monitors changes in step pulse after reflection from the end of line. Reflected pulse without sample $R_1(t)$ and with sample $R_x(t)$ were recorded in time window of 5ns and digitized in 2000 points. A flat ended coaxial line makes contact with the liquid sample, and TDR measure the complex reflection coefficients at frequencies ranging from 10 MHz to 30 GHz. The complex permittivity spectra have been obtained from reflection coefficient spectra by applying the least squares fit method. The details of the apparatus and the procedures of the TDR have been reported previously [31, 33].

3. RESULT AND DISCUSSION

The complex permittivity spectra of 0.5mM & 1.0mM Hemoglobin (Hb) and 0.5mM & 1.0 mM Bovine serum albumin (BSA) are shown in Figure 1 and 2 respectively. It is seen from the Figure 1 & 2 that the real part of permittivity decreases with increase in frequency.

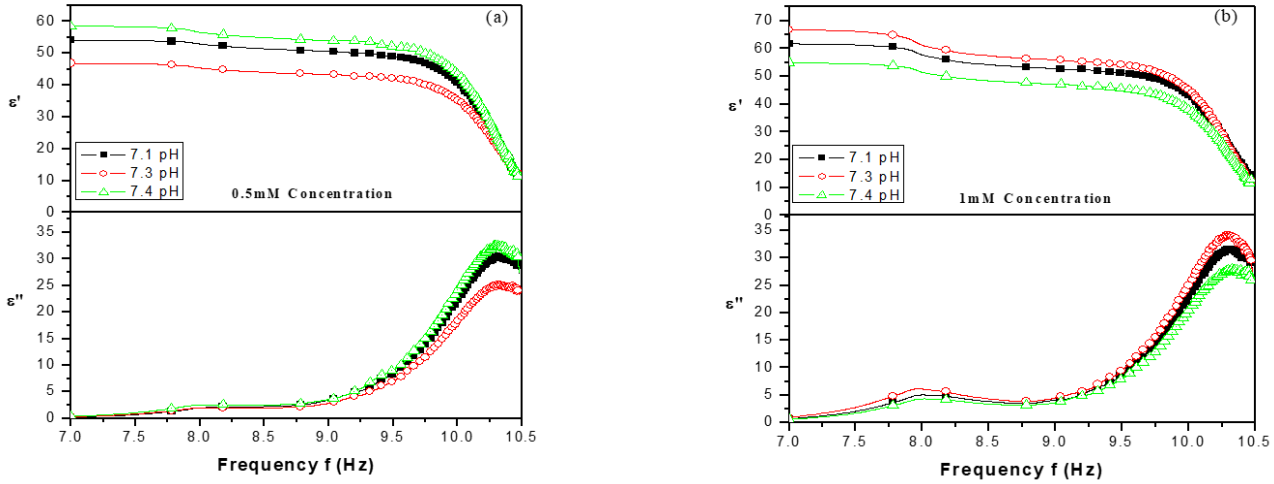


Fig. 1: Complex permittivity spectra vs. Frequency (Hz) of Hemoglobin (Hb) in various pH
(a) 0.5mM Concentration and (b) 1mM Concentration at 25^{0}C.

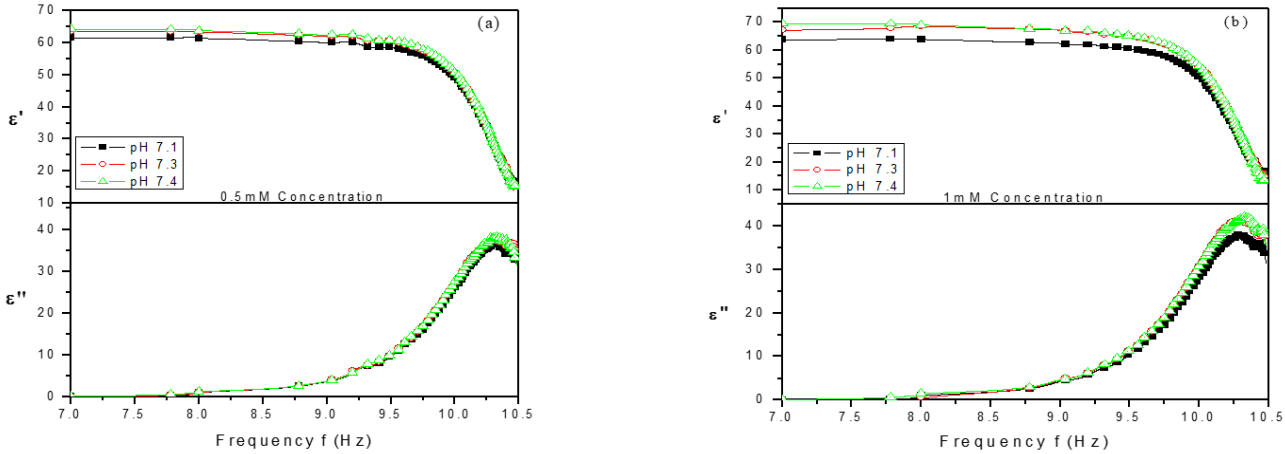


Fig. 2**:** Complex permittivity spectra vs. Frequency (Hz) of Bovine serum albumin (BSA) in various pH
(a) 0.5mM Concentration and (b) 1mM Concentration at 25^{0}C.

The complex permittivity spectra were fitted to Havriliak-Negami equation using least square fit method [34].

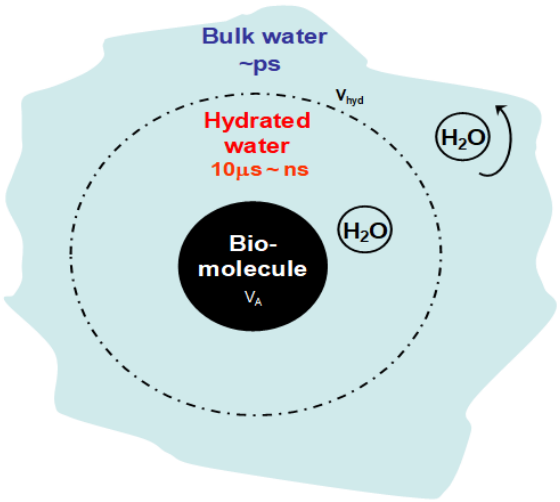
$$\varepsilon^*(\omega) = \varepsilon_\infty + \frac{(\varepsilon_0 - \varepsilon_\infty)}{[(1+(j\omega\tau)^{1-\alpha}]^\beta} - j\frac{\sigma}{\omega \in_v} \qquad (1)$$

where ε_0 is the static dielectric constant, ε_∞ is the permittivity at high frequency, τ is the relaxation time, σ is the dc conductivity of the solution and $\in_v$ = 8.85 X 10^{-12} *F/m* is the permittivity of vacuum. With ε_0, ε_∞, τ, α, β and σ as fitting parameters in equation 1. The Havriliak Negami equation includes three relaxation models as limiting forms: the Cole-Cole (β=1, 0≤α≤1), Davidson-Cole (α=0) and 0<β<1 and Debye (α=0, β=1) relaxation models [35, 36]. For the systems studied here, the values of α and β are found to be 0 and 1 respectively within experimental error. The resulting values of dielectric relaxation parameters for aqueous protein solution at different pH are listed in Table 1.

Table – 1: Dielectric relaxation parameters for Hemoglobin (Hb) and Bovine serum albumin (BSA) in aqueous solutions at 25^{0}C. The number in bracket denotes uncertainties in the last significant digits obtained the least squares fit method e. g. 49.53 (3) means 49.53 ± 0.03.

C (mM)	pH	ε_0	τ(ps)	σ (mho/m)	β
		Hemoglobin (Hb)			
0.5	7.1	49.53(3)	8.64(3)	0.1534(1)	1.00(04)
0.5	7.3	51.35(3)	8.69(3)	0.1780(1)	1.00(05)
0.5	7.4	53.18(3)	8.96(3)	0.1874(1)	1.00(04)
1.0	7.1	53.01(5)	8.83(5)	0.3072(2)	0.99(07)
1.0	7.3	55.57(7)	9.21(6)	0.3209(2)	1.00(07)
1.0	7.4	57.49(5)	9.72(5)	0.3418(1)	0.99(07)
		Bovine Serum Albumin (BSA)			
0.5	7.1	60.51(2)	8.58(2)	0.06287(7)	0.98(02)
0.5	7.3	61.82(2)	8.60(1)	0.06362(7)	0.99(02)
0.5	7.4	62.63(2)	8.65(2)	0.06442(7)	0.99(03)
1.0	7.1	62.97(2)	9.09(2)	0.09203(9)	0.99(03)
1.0	7.3	66.99(2)	9.25(1)	0.09224(9)	1.00(02)
1.0	7.4	68.62(3)	9.54(2)	0.09749(9)	1.00(03)

We assumed that the protein in solution is spherical and has spherical hydration shells and solvent outside of hydrated protein has the same dielectric properties as that of water as shown in Figure 3.



$$V_D = V_{hyd} + V_A$$

Fig. 3: Illustration of the model for the dielectric excluded volume (V_D), the effective displacement volume (V_A) of the protein, and the volume of the hydrated water (V_{hyd})

Therefore, the microwave dielectric excluded volume V_D is assumed to have a dielectric constant [40] of $\varepsilon_{ex} = \varepsilon_{\omega\infty} = 5.0$. The effective permittivity of voids in bulk water may be modeled by electrostatic theory. We define the dielectric excluded volume fraction R_V as the total volume of the hydrated protein molecule (V_D) per liter of the solution. The permittivity of the solution can be described by the Maxwell-Wagner formula [32].

$$\varepsilon_s(\omega) = \varepsilon_w(\omega)\frac{2(1-R_V)\varepsilon_w(\omega) + (1+2R_V)\varepsilon_{ex}}{(2+R_V)\varepsilon_w(\omega) + (1-R_V)\varepsilon_{ex}} \qquad (2)$$

where, R_V is the Volume fraction of protein and is as follows:

$$R_V = \frac{(\varepsilon_{w0} - \varepsilon_{s0})(2\varepsilon_{w0} + \varepsilon_{w\infty})}{(2\varepsilon_{w0} + \varepsilon_{s0})(\varepsilon_{w0} - \varepsilon_{w\infty})} \qquad (3)$$

$R_V = 0$ if the dielectric constant of the voids is $\varepsilon_{w0}(\varepsilon_{s0} \equiv \varepsilon_{w0})$ and $R_V=1$ when $\varepsilon_{s0} = \varepsilon_{w\infty}$. The excluded volume of proteins is determined as follows [36].

and
$$V_D = \frac{R_V}{cN_0} \tag{4}$$

where, ε_{w0} =78.3 and $\varepsilon_{w\infty}$ = 5.0 are taken for the static and high-frequency dielectric constants of pure water at 25^0C, c is the molar protein concentration, and N_0 is the Avogadro's constant.
The mass (in Kilograms) of free solvent per liter can be calculated as follows.
$$W_{fw} = (1 - R_V)d_0 \tag{5}$$
where, d_0 is the density of the solvent (for bulk water at 25^0C, d_0 = 0.99707 Kg/L). Therefore, the total mass of water in 1 L of the solution can be found as
$$W_w = d - 10^{-3}Mc \tag{6}$$
where, M is the molecular weight of protein, d is the solution density and c is the protein concentration. The molecular weights of hemoglobin and bovine serum albumin are 64,500 and 66,000, respectively. The difference between the total mass of water and that of the free water is the mass of hydrated water per liter of the solution. Therefore, the number of hydrated water molecules for each protein molecules is given by
$$N_{hyd} = (W_w - W_{fw})/(0.018c) \tag{7}$$
The N_{hyd} is determined by the measured quantities ε_{s0}, d and c. Therefore the errors in the measured quantities can be determined as follows [39].
$$\Delta N_{hyd} = \left\{\left[\frac{d_0\delta R_v}{0.018c}\right]^2 + \left[\left(N_{hyd} + \frac{M}{18}\right)\frac{\delta c}{c}\right]^2 + \left[\frac{\delta d}{0.018c}\right]^2\right\}^{1/2} = \left(\delta N_1^2 + \delta N_2^2 + \delta N_3^2\right)^{1/2} \tag{8}$$
According to equation (3),
$$\delta R_v = \left|\frac{3(2\varepsilon_{w0} + \varepsilon_{w\infty})}{\varepsilon_{w0}(\varepsilon_{w0} - \varepsilon_{w\infty})(2 + \varepsilon_{s0}/\varepsilon_{w0})^2}\right|\delta\varepsilon_{s0} \tag{9}$$
where, $\delta\varepsilon_{s0}$ is the root mean square deviation. For a typical hemoglobin solution with c=0.5 mM and pH = 7.1 at 25^0C, we have $\delta c/c \approx 2\%$, $\delta d \approx 0.001$ Kg/L, $\delta\varepsilon_{s0} \approx 0.3$, $\varepsilon_{s0} \approx 49.53$, $N_{hyd} = 27820.27$, $\Delta N_{hyd} = 697.20$. For hemoglobin and bovine serum albumin solution concentration (c), density (d), dielectric constant (ε_0), dielectric excluded volume (V_D) are listed in Table 1 and 2 respectively.

From equation (4), the error in the volume of a hydrated protein molecule can be estimated as
$$\Delta V_D = \left\{\left[\frac{\delta R_v}{cN_0}\right]^2 + \left[V_D\frac{\delta c}{c}\right]^2\right\}^{1/2} = \left\{\left[\frac{0.018\delta N_1}{d_0 N_0}\right]^2 + \left[V_D\frac{\delta c}{c}\right]^2\right\}^{1/2} \tag{10}$$
The weight ratio of bound water mass to protein mass is
$$W_{hyd} = 18N_{hyd}/M \tag{11}$$
The hydration number is determined by the charge density at the protein surface, which progressively decreases. As a consequence the protein-water interaction is progressively weaker, with fewer water molecules bound by Coulomb forces in the protein solution. The estimated dielectric excluded volume per protein molecule, number of hydration water molecules, and derived weight ratio are listed in Table 2. The average number of hydration water molecules per protein molecule is shown in Figure 4 which can be referred as bound water molecules.

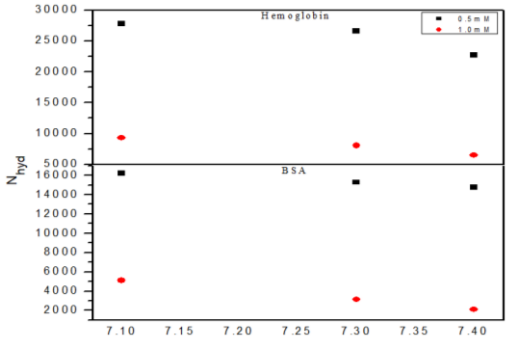


Fig. 4: Hydration number with concentration (mM) for proteins.

Table – 2: Protein hydration information extracted from the dielectric measurements at 25^{0}C.

C (mM)	pH	Density (gm/c^3)	VD (nm^3)	W_{hyd}	N_{hyd}
			Hemoglobin (Hb)		
0.5	7.1	0.97288	1022 ± 24	77	27790 ± 712
0.5	7.3	0.98447	949 ± 22	74	26642 ± 690
0.5	7.4	0.97088	877 ± 21	63	22726 ± 625
1.0	7.1	0.96465	442 ± 11	26	9322 ± 290
1.0	7.3	0.97168	392 ± 10	22	8061 ± 269
1.0	7.4	0.96529	356 ± 09	18	6494 ± 246
			Bovine Serum Albumin (BSA)		
0.5	7.1	0.99607	600 ± 16	44	16235 ± 514
0.5	7.3	1.0019	552 ± 15	42	15298 ± 498
0.5	7.4	1.0059	523 ± 15	40	14772 ± 490
1.0	7.1	1.0015	256 ± 07	14	5106 ± 224
1.0	7.3	1.0084	185 ± 06	09	3140 ± 199
1.0	7.4	1.0064	157 ± 06	06	2100 ± 189

The experimental quantity that is directly obtained from the analysis of the measurements is the microwave dielectric excluded volume V_D per protein molecule as given in Table 2. The important is that, for native proteins, this volume is larger than the bare molecular volume in the crystal, V_{HB}~1022 nm^3 and V_{BSA}~600 nm^3 [37]. The excess is attributed to the hydration shell, and knowing V_D, the weight ration W_{hyd} and Hydration number N_{hyd} are easily obtained. Approximately 0.3-0.7 gm of protein-associated water per gm of dry protein [38]. The extent of association of this water to the protein molecule is such that it ca be considered a shell that contributes to the proteins effective radius of rotation. Using spheres with r_{HB} = 62.48 A^0 and r_{BSA} = 52.31 A^0 to approximate hemoglobin and bovine serum albumin molecules at pH 7.1 and 0.5mM concentration, the average molecular size of bulk water molecules, with density d_w = 1, is 3.1 A^0.

The weight ratio values obtained ranges from 18 – 77 for hemoglobin and 06 – 44 for bovine serum albumin seem to be varying with concentration as it showed decreasing trend with increasing concentration of solution. The average value for N_{hyd} of hemoglobin found to be 16839 that of 442 for bovine serum albumin. It is inferred that N_{hyd} for hemoglobin is 1.7 times greater than N_{hyd} for bovine serum albumin. These values obtained supported the theory that number of bound water, generally decreases as the size of protein increases (BSA having high molecular weight).

From calculations it can be concluded that hydration number N_{hyd} derived from direct dielectric measurements is greater. The above estimate suggests that the hydration shell corresponds roughly to a single atomic layer of water surrounding protein molecules i.e. 'bound water' that is more tightly packed than bulk water.

Increased N_{hyd} form dielectric calculations may contributed by free water. From previous studies [36, 38], it is clear that buffer ions will also bind water molecules, therefore deionized pure water was used in study with buffer having very low molarity sufficient for imparting charges on surface of protein.

As stated earlier hydration number showed inverse trend with concentration as well as pH of solution likewise relaxation time showed correlation with pH of solution at different frequencies. Samples were prepared with low molarity buffers with varying pH. Biological buffers with ranging pH plays important role of stabilizing proteins confirmation. α- dispersion is a function of pH of sample. Present study was undertaken to study dynamic changes of proteins confirmation and its interaction with ligands with respect to charge and hydration on it.

Therefore wide range of pH was selected sufficient for imparting different charges on protein surface and are biologically important. pH 7.1, 7.3 and 7.4 are ranges of pH found in biological systems. Experimental measurements on hemoglobin binding are often performed at pH 7.4. Changes in surface charge of protein as a function of pH affects binding of polar water molecules to it resulting into changes of interactions of proteins with its ligands. Present study was carried using two different proteins Hemoglobin and Bovine Serum Albumin. Analysis of structural composition of Hemoglobin showed presence of four polypeptide chains having maximum percentile of non polar Alanine, Leucine and Valine amino acids along with polar Lysine, Asparagine, Arginine and Threonine amino acids in minimum percentile.

Terminal amino and carboxylic groups from amino acids undergo deprotonation and protonation respectively with change in pH. Ionisable side groups from polar amino acids also contribute to protonation or deprotonation of solution. Water molecules are either hydrogen bonded to proton donors and acceptors of proteins or associated with ionizable acidic and basic dielectric properties of protein.

In case of hemoglobin pH ranges from 7.1 to 7.4 carboxyl terminals of non polar amino acids stated from all four polypeptides undergoes protonation imparting net negative charge. Also ionizable acidic side groups from polar amino acids contribute to negative imparting positive charge. Amphipathic dipolar water molecules interact with oppositely charged amino acids through hydrogen bonds. Therefore hemoglobin molecule and water around it form a strongly coupled system. Two mechanisms appear to provide a qualitative understanding of this, namely mechanical damping of the protein motion by adsorbed water and a dynamic electrical coupling between the fluctuating electric dipoles of the adsorbed water and the polar side groups of the protein molecule. Displacement of water molecules occurs against displacement of the hydrogen bonded protein ligand i.e. “Oxygen”. According to Bohrs effect, consequent rise in pH increases affinity of oxygen towards Hemoglobin, resulting displacement of more number of hydrogen bonded water molecules by oxygen ultimately responsible for decrease of hydration number with rise in pH of protein as shown in Table 2. Protons contributed by ionizable groups from Hemoglobin in sample contribute to hydrogen bond, proton relay network that link ligand oxygen to ‘Fe’ atom of Hemoglobin responsible for enhancement of activity of protein. Similar concept is considered for decrease in hydration number for BSA along with rise in pH. Study of relaxation time of protein may prove significant for concern study. At pH 7.1, 7.3 & 7.4 for different molarity of buffers, imparts varying charges on surface of hemoglobin and bovine serum albumin.

4. CONCLUSION

The dielectric dispersion of aqueous protein has been studied at various pHs using time domain reflectometry technique. The static dielectric constant, relaxation time, d.c. conductivity, hydration number has been determined. The dielectric data reveal the formation of a solvation sheath of bound water molecules around the proteins. The present study helps to determine interaction of hemoglobin with its ligand at different pH using dielectric property and hydration number study along with BSA.

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