

## Albumin Aggregates: Hydrodynamic Shape and Physico-Chemical Properties

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### Abstract

Although albumin was known for more than a century, little is known about its tertiary structure. At present two models are known to represent its molecular conformation: the heart-shaped and the prolate ellipsoid. Our results support the prolate ellipsoid model for albumin in slightly alkaline solution. Albumin in solution exists as a monomer and higher aggregates. We isolated the monomer, dimer, tetramer, and hexamer of bovine serum albumin by gradient polyacrylamide gel electrophoresis followed by electroelution. The monomer was stable for 5 months at 8°C, whereas the dimer and tetramer dissociated into smaller forms within few hours after their separation but no higher aggregates were detected. Albumin forms are surrounded by about three layers of water, with Stokes' radii of 3.55, 4.34, 5.18, and 5.85 nm for monomer, dimer, tetramer, and hexamer, respectively. The effective negative charge and density of surface charge ( $- \text{esu.cm}^{-2}$ ), respectively, were 14 and 4244 (monomer), 12.3 and 2495 (dimer), and 10.2 and 1452 (tetramer). The translational diffusion coefficients were 5.88, 4.81, 4.03, and 3.57  $\text{cm}^2.\text{s}^{-1}$  for monomer, dimer, tetramer, and hexamer, respectively. Other physico-chemical properties were calculated such as: frictional coefficient, sedimentation coefficient, electrophoretic mobility, volume of hydrated and unhydrated molecules.

**Keywords:** Albumin monomer; Albumin dimer; Albumin tetramer; Albumin hexamer; Electroelution; Stability; Molecular shape; Hydrodynamic properties.

### Introduction

Albumin has a wide range of applications in various fields including research as a model protein, clinical practice, pharmaceutical preparations, diagnostic reagents, food chemistry, and as standard solution in chemical analysis. The stability of serum albumin and its availability at high purity and low cost, in addition to its solubility and absence of prosthetic groups or bulk carbohydrates encouraged its wide use in various fields. Bovine serum albumin is very close to human serum albumin regarding chemical composition, structure, and physico-chemical properties <sup>[1]</sup>. Human serum albumin (HSA) is a single polypeptide with 585 amino acid residues containing 17 pairs of disulfide bridges and one free cysteine <sup>[2]</sup>. Albumin monomer consists of three

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homologous domains (I, II, III) encompassing the complete sequence, each of which displays specific structural functional characteristics, and each domain is made up of two subdomains <sup>[3]</sup>. Despite various physico-chemical studies for the determination of the tertiary structure and solution conformation of albumin, its three-dimensional molecular structure has remained largely unknown <sup>[4]</sup>. Two models were suggested to represent its molecular conformation: 1) the prolate ellipsoid (cigar-shaped) with dimensions of 140x40 Å and axial ratio of ~3.5 <sup>[5]</sup>; 2) the heart-shaped conformation <sup>[3, 4]</sup>. Both models are supported by different experimental results, and the two disparate views might be reconciled if the tertiary structure of the protein in crystal is different from that in solution <sup>[6]</sup>.

This discrepancy might have resulted from the use of albumin in different studies either as crystalline solid or as solution at different pH values. Ferrer et al. <sup>[6]</sup> demonstrated that the conformation of HSA and bovine serum albumin (BSA) in neutral solution is very similar to the heart-shaped structure of crystalline HSA. However, albumin undergoes several pH-dependent transitions associated with molecular rearrangements that lead to changes in molecular dimensions <sup>[3, 5]</sup>. The normal-to-fast (N-F) transition (between pH 5.0 and 3.5) causes elongation of the molecule, and further decrease in pH below 3.5 causes fast-to-elongated (F-E) transition (acid expansion) associated with an increase in the hydrodynamic axial ratio from 4 to 9 <sup>[7]</sup>. Such transitions are associated with secondary structural changes <sup>[8]</sup>. At slightly alkaline conditions, pH between 7.0 and 9.0, both BSA and HSA undergo conformational change known as normal-to-basic (N-B) transition <sup>[5]</sup>. This isomerization was supposed to be a structural fluctuation, a loosening of the molecule with loss of rigidity, particularly affecting the N-terminal region <sup>[9]</sup>. Dockal et al. <sup>[9]</sup> provided evidence that the loosening of the HSA structure in the N-F transition takes place primarily in domain III, and domain I undergoes a structural rearrangement with only minor changes in secondary structure, whereas domain II transforms to a molten globule-like state as the pH is reduced. Moreover, they showed that in the pH region of the N-B transition domain I and II experience a tertiary structural isomerization that is not observed in domain III. Therefore, N-B transition is associated with increase in axial ratio similar to the N-F transformation.

Another important factor that might contribute to the discrepancy in results concerning albumin structure and physico-chemical properties is the presence of albumin in monomeric and oligomeric forms in solution. Commercially available BSA and HSA contain variable proportions of dimer and higher oligomers <sup>[10-12]</sup> where many factors affect albumin aggregation during processing and storage <sup>[13]</sup>. Protein aggregation is not a random process <sup>[14]</sup> but proceeds through specific pathways that often involves an intermediate species termed 'molten globule' which may readily form aggregates <sup>[15, 16]</sup>. Moreover, the native conformation is a dynamic structure, such that at any instant in time there exists an ensemble of species with a distribution of

structural expansion/compaction [17], where it was proposed that aggregates are formed from species within the native state ensemble that are structurally expanded relative to the compact conformations [18, 19].

Albumin oligomers are not often addressed in various studies and little is known about their physico-chemical properties. However, albumin aggregation involves noncovalent and hydrophilic interactions [20]. In this work we studied the heterogeneity of some albumin preparations of both BSA and HSA and addressed their hydrodynamic shape and some of their physico-chemical properties at slightly alkaline conditions (pH 8.3).

## Materials And Methods

Human serum albumin, human serum albumin-fatty acid free (HSA-FAF), bovine serum albumin, bovine serum albumin-fatty acid free (BSA-FAF), and molecular weight markers were purchased from Sigma (USA). BSA monomer was purchased from NenTech (USA). Other BSA were purchased from BDH (UK) and Merck (Germany). Chemicals used were of analytical grade.

*Nondenaturing gradient polyacrylamide gel electrophoresis (gPAGE).* A nondenaturing 4-25% gPAG was used to determine the relative molecular masses ( $M_r$ ) and Stokes' radii ( $R_s$ ) of the oligomers. It was prepared and run as was described previously [21, 22]. In brief, the gPAG was cast and run in the LKB 2001 vertical electrophoresis system (LKB, Sweden). The appropriate amount of albumin (e.g., 50  $\mu$ L of 1 mg/mL fresh solution) was applied to each well of the gradient polyacrylamide gel, molecular weight markers were added in one well, and the gel was run at 20°C in 14 mM Tris and 110 mM glycine buffer of pH 8.3, density ( $\rho$ ) of 1.002 g/mL, and viscosity ( $\eta$ ) of 1.028 g.cm<sup>-1</sup>.s<sup>-1</sup> (Poise) [23]. The voltage gradient applied was 2000 V.m<sup>-1</sup> for 24 h. After the run was complete the gel was fixed, stained with coomassie brilliant blue R-250, and destained.

*Polyacrylamide gel electrophoresis (PAGE):* Homogeneous 7.5% polyacrylamide gel (PAG) was cast in the LKB 2001 vertical electrophoresis system and run at 125 V for 5 h. After the run was complete the gel was used for electroelution of the separated fractions as detailed below.

*Calculation of the relative composition of albumin oligomers.* The relative percentages of the albumin forms were calculated by densitometric scanning of the stained gradient gels.

*Immunological methods.*

*Raising specific antibodies against albumin forms.* Antisera against purified BSA monomer, BSA, BSA-FAF, HSA, HSA-FAF (Sigma, USA), were raised in New Zealand white rabbits. The antigens were individually injected into rabbits at three week intervals, and after three to four injections serum was obtained and tested for specificity.

*Double immunodiffusion.* Agarose gel plates (1.5 mm thick) were prepared from 1% agarose in Tris/glycine buffer, pH 8.3. Holes were made in the gel (1 cm apart). The antibody and antigen (5  $\mu$ L each) were placed in the wells, incubated in a wet chamber at 8°C for 24 – 48 h, then the plates were washed with saline (0.15 M NaCl) to remove the unreacted proteins. The gel plates were dried, stained with coomassie brilliant blue R-250 and de-stained.

*Immunoblotting of albumin forms.* Albumin was separated on gPAGE then the albumin forms were electro-transferred onto agarose gel layer containing the corresponding specific antibody, as detailed elsewhere [22].

*Electroelution of albumin forms.* After the electrophoresis run in the homogeneous 7.5% polyacrylamide gel was complete, the gel was cut longitudinally and one lane was stained with coomassie brilliant blue R-250 for a short time and de-stained and the rest of the lanes were compared with the stained one in order to locate the position of individual albumin forms. The gel portion that contains the required albumin form was cut and placed in the electroelution apparatus, developed by Atmeh [24], and electro-eluted as described elsewhere [25]. The eluted albumin fraction was tested against the appropriate specific anti-albumin by the double immunodiffusion technique.

*Purity of the isolated albumin forms.* Each of the isolated albumin forms was run on homogeneous 7.5% polyacrylamide gel immediately after the elution. The gels were fixed, stained, and de-stained.

*Calculation of the translational diffusion coefficient ( $D_t$ ).* The translational diffusion coefficient was determined from the Stokes'-Einstein equation:

$$D_t = k_B T / 6\pi\eta R_s \dots\dots\dots(1)$$

where  $k_B$  is Boltzmann constant, T absolute temperature,  $\eta$  is the dynamic viscosity of the medium, and  $R_s$  is the Stokes' radius.

*Calculation of the lengths of the axes of prolate ellipsoid.* The values of the long axis (a) and the short axis (b) as well as the axial ratio (a/b) of ellipsoidal albumin forms were predicted by the Software UltraScan (Borries Demeler, Ph.D., University of Texas

Health Science Center at San Antonio , UTHSCSA, USA). The software utilizes the linear Lamm model <sup>[26]</sup> for the analysis of ultracentrifugation and other molecular parameters <sup>[27]</sup>.

*Calculation of other properties.* The above mentioned UltraScan software was used to calculate the following parameters, depending on the proper equation, as follows. Calculation of the translational coefficient of friction (*f*) of albumin forms utilizes the Stokes' equation:

$$f = 6\pi\eta R_s \dots\dots\dots (2)$$

Calculation of the radius of unhydrated spherical particles (*R<sub>o</sub>*) having the same mass as the albumin form utilized the equation:

$$R_o = \{3M_r v(\text{bar}) / 4\pi N\}^{1/3} \dots\dots\dots (3)$$

which can be derived as follows. The volume of one mole of a spherical particle equals the product of its molecular mass by its partial specific volume [*v*(bar)], and the volume of one molecule is obtained by dividing the product by Avogadro's number (*N*), or calculated from its radius (*R*), i.e.,

$$\text{volume of one molecule} = M_r v(\text{bar}) / N = 4\pi R^3 / 3 \dots\dots\dots (4)$$

thus the value of *R<sub>o</sub>* can be obtained. The partial specific volume [*v*(bar)] of albumin oligomers is considered as that of the monomer, i.e., 0.733 cm<sup>3</sup>.g<sup>-1</sup>.

Calculation of the coefficient of friction of spherical unhydrated particle (*f<sub>o</sub>*) and its frictional ratio (*ff<sub>o</sub><sup>-1</sup>*) depends on the Stokes' equation (2) using the value of radius of unhydrated sphere (*R<sub>o</sub>*):

$$f_o = 6\pi\eta R_o \dots\dots\dots (5)$$

The frictional ratio or the shape factor (*ff<sub>o</sub><sup>-1</sup>*) can be calculated from equations 2 and 5 and equals to the ratio *R<sub>s</sub>* / *R<sub>o</sub>*. Calculation of the sedimentation coefficient (*s*) depends on the following form of Svedberg equation:

$$s = M_r [1 - v(\text{bar})\rho] / Nf \dots\dots\dots (6)$$

*Calculation of the relative migration ratio and relative mobility of albumin oligomers.* The distance traveled by the albumin monomer in each gel (7.5% PAG) was measured and considered as 1 unit of migration. The distances traveled by the corresponding albumin oligomers were measured and their relative migration ratios were calculated as fractions of the migration distance of the monomer. The relative mobility (*U<sub>r</sub>*) of albumin oligomers equals the ratio between the free electrophoretic mobility (*U*) of the oligomer and that of the monomer since they migrate on the same gel and experience the same electric field strength. Thus we can derive the following equation for the relative migration ratio of the oligomer <sup>[28]</sup>

$$U_{r \text{ oligomer}} = U_{\text{oligomer}} / U_{\text{monomer}} \dots\dots\dots (7)$$

Therefore, electrophoretic mobility of albumin oligomers can be calculated from that of the monomer.

*Estimation of the net charge on albumin oligomers.* The net number of unit charges of a non-spherical macromolecule is related to its free electrophoretic mobility ( $U$ ) by Debye-Hückel equation:

$$U = (Z\varepsilon / 6\pi\eta R_s) \times F \dots\dots\dots(8)$$

where  $Z$ , is the number of unit charges;  $\varepsilon$ , the unit charge and equals  $1.602 \times 10^{-19}$  Coulomb;  $F$ , a retardation factor which results from the electrophoretic effect where its value depends on  $R_s$  and the composition and strength of the small ions of the buffer solution<sup>[29]</sup>. The value of  $F$  can be calculated from the equation:

$$F = \{X(\kappa R_s) / 1 + (\kappa R_s)\} \dots\dots\dots (9)$$

where  $\kappa$  is related to the thickness of the ion atmosphere around the macromolecule and equals the reciprocal of the radius of the ion cloud;  $X(\kappa R_s)$  is the Henry's function<sup>[28]</sup>.

The net number of unit charges for albumin oligomer can be calculated by comparison with that of the monomer by using equations 7 and 8, thus we can derive the following equation:

$$U_{\text{oligomer}} / U_{\text{monomer}} = (Z_{\text{oligomer}} / Z_{\text{monomer}}) \times (R_{s \text{ monomer}} / R_{s \text{ oligomer}}) \times (F_{\text{oligomer}} / F_{\text{monomer}}) \dots(10)$$

The factors  $F_{\text{monomer}}$  and  $F_{\text{oligomer}}$  can be calculated from equation 9, and the Stoke's radius of the oligomer was determined experimentally. On rearrangement, equation 10 gives:

$$Z_{\text{oligomer}} = (U_{\text{oligomer}} / U_{\text{monomer}}) \times (R_{s \text{ oligomer}} / R_{s \text{ monomer}}) \times (F_{\text{monomer}} / F_{\text{oligomer}}) \times Z_{\text{monomer}} \dots (11)$$

*Calculation of the density of surface charge ( $C_d$ ).* The density of surface charge in electrostatic units was calculated from the following relation<sup>[30]</sup>:

$$C_d = 3.82 \times 10^{-11} Z / R_s^2 \dots\dots\dots (12)$$

**Results**

*Albumin oligomers in commercial products.*

*BSA monomer.* A commercial preparation of BSA monomer that is stated by the manufacturer to contain 98% pure monomer was run on non-denaturing 4-25% gPAGE and found to contain several bands of variable proportions corresponding to monomer, dimer, tetramer, and hexamer, with a monomer percentage of 38.4% (Table 1). All the bands reacted with anti-bovine albumin and anti-bovine albumin monomer on immunoblotting against agarose gel containing the specific antibody, indicating that the bands are albumin aggregates and not impurities.

**Table 1.** Distribution of albumin polymeric forms in human and bovine serum albumin from different commercial sources. Each albumin preparation was run on non-denaturing 4-25% gPAGE, pH 8.3, for 24 h at 15°C. Values are expressed as mean±SD, n = 4.

Albumin type	Monomer %	Dimer %	Tetramer %	Hexamer %	Higher aggregates %
BSA monomer	38.4±2.3	33.6±2.8	18.7±2.8	9.4±1.2	--
BSA (BDH)	33.7±0.3	28.6±0.7	16.8±0.3	12.4±0.6	8.4±0.1
BSA (Merk)	38.4±0.9	28.6±0.3	18.4±0.4	14.7±0.5	--
BSA (Sigma)	38.7±1.0	30.1±1.4	21.6±1.1	9.6±0.6	--
BSA-FAF (Sigma)	36.3±0.4	27.8±0.5	25.3±0.5	10.7±0.3	--
HSA (Sigma)	53.1±3.6	30.1±2.4	13.0±1.0	3.9±0.9	--
HSA-FAF (Sigma)	51.2±2.7	29.2±2.0	13.7±0.9	5.9±0.6	--

**BSA.** Three different commercial preparations of BSA and one BSA-FAF were tested for the presence of aggregates and found to contain monomer, dimer, tetramer, hexamer, and one preparation showed higher aggregates (Figure 1), and the monomer represented about one third of the mass (Table 1). All the bands reacted with anti-bovine albumin and anti-bovine albumin monomer on immunoblotting against agarose gel containing the specific antibody.

**HSA.** One commercial preparation of HSA and one HSA-FAF were tested for the presence of aggregates and found to contain monomer, dimer, tetramer, and hexamer; and the monomer represented about one half of the mass (Table 1). All the bands reacted with anti-human albumin on immunoblotting against agarose gel containing the antibody.

**Effect of storage on the distribution of albumin forms.** When solutions of each of BSA, BSA-FAF, HSA, and HSA-FAF were stored at 8°C for 4 months and separated on non-denaturing 4-25% gPAGE, the tetramer and hexamer disappeared with no higher aggregates, while the dimer percentage decreased (Table 2).

**Table 2.** Distribution of albumin polymeric forms in human and bovine serum albumin (Sigma, USA) after incubation for 4 months at 8°C. Values are expressed as mean±SD, n = 4.

Albumin type	Monomer %	Dimer %
BSA	82.2±0.6	17.8±0.6
BSA-FAF	83.3±0.9	16.7±0.9
HSA	76.9±1.2	23.1±1.2
HSA-FAF	81.3±0.5	18.7±0.5

*Isolation of albumin forms.* Solutions of BSA, BSA-FAF, HSA, and HSA-FAF were run separately on 7.5% PAGE and the bands of each albumin corresponding to the monomer, dimer, and tetramer, were cut and electro-eluted. The eluted fractions were immediately run on another 7.5% polyacrylamide gel to check their purity; all the fractions gave one band (Figure 1).

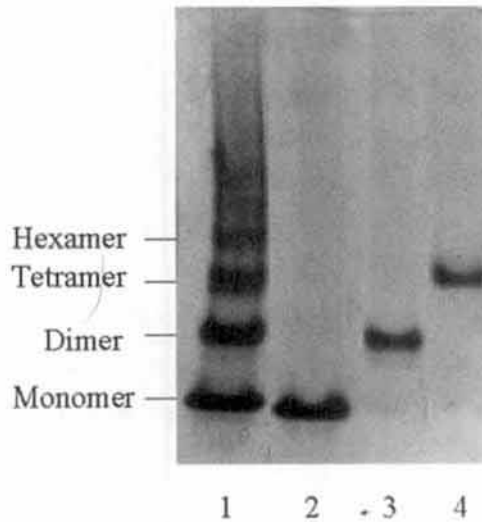


Figure 1. Bovine serum albumin aggregation forms. Lane 1, total albumin; lanes 2-4, pure monomer, dimer, tetramer, respectively.

*Immunological properties of albumin forms.* The immunological properties were tested by the double immunodiffusion method. 1) Each of the antibodies raised against BSA monomer, BSA, and BSA-FAF reacted with each of BSA, BSA-FAF, BSA forms (monomer, dimer, and tetramer) and bovine plasma. 2) Each of the antibodies raised against HSA and HSA-FAF reacted with each of HSA, HSA-FAF, HSA forms (monomer, dimer, and tetramer) and human plasma.

### **Physico-chemical properties of albumin forms.**

*Relative Molecular mass and Stokes' radius.* The measured relative molecular masses of BSA oligomers were  $134,000 \pm 1,100$  (mean  $\pm$  SD,  $n = 6$ ) for the dimer,  $264,000 \pm 2,000$  for the tetramer, and  $396,000 \pm 3,800$  for the hexamer. For the subsequent calculations of the physico-chemical properties of the oligomers their molecular mass values were used as multiples of that of the monomer (molecular mass 66,500). Stokes' radii of the oligomers are tabulated in Table 1. Since BSA is very similar to HSA in terms of structure and physico-chemical properties<sup>[31]</sup> we used BSA-FAF for detailed calculations of the physico-chemical properties of albumin forms.



Translational diffusion coefficient ( $D_t$ ), frictional coefficient ( $f f_o^{-1}$ ), hydrated volume, unhydrated radius ( $R_o$ ), sedimentation coefficient (s). The values of these parameters are tabulated in Table 3.

**Table 3.** Calculated physico-chemical properties of BSA forms.

Parameter	Monomer	Dimer	Tetramer	Hexamer
$R_s \times 10^9$ (m)	$3.55 \pm 0.01^a$	$4.34 \pm 0.01$	$5.18 \pm 0.02$	$5.85 \pm 0.03$
$R_o \times 10^9$ (m)	2.68	3.28	4.26	4.87
$D_t \times 10^7$ (cm <sup>2</sup> .s <sup>-1</sup> )	$5.88 \pm 0.03$	$4.81 \pm 0.03$	$4.03 \pm 0.04$	$3.57 \pm 0.03$
$f \times 10^9$ (g.s <sup>-1</sup> )	$6.88 \pm 0.02$	$8.41 \pm 0.02$	$10.04 \pm 0.02$	$11.34 \pm 0.02$
$f_o \times 10^9$ (g.s <sup>-1</sup> ) <sup>b</sup>	5.2	6.55	8.25	9.45
$f f_o^{-1}$	1.32	1.28	1.22	1.22
Unhydrated Volume (nm <sup>3</sup> )	80.9	161.9	323.8	485.7
Hydrated Volume (nm <sup>3</sup> )	187.4	342.4	582.2	838.6
$Z_{eff}$ (-e)	14.0	12.3	10.2	nd
$Z_{eff} \times 10^{10}$ (Coulomb)	22.4	19.7	16.3	nd
$C_d$ (-esu.cm <sup>-2</sup> )	4244	2495	1452	nd
$U \times 10^9$ (m <sup>2</sup> .V <sup>-1</sup> .s <sup>-1</sup> )	-7.42	-4.75	-2.90	nd
Sedimentation coefficient (s) $\times 10^{13}$ s	4.26	6.97	11.68	15.52

<sup>a</sup> mean  $\pm$  SD, n = 4; <sup>b</sup>  $f_o = 6\pi\eta R_o$

*Molecular shape of albumin forms.* The length of the axes of albumin molecules, a and b, were predicted by the UltraScan software from the values of  $D_t$  and  $M_r$  of each albumin form as well as the density and viscosity of the buffer used at 20°C. The values are shown in Table 4.

**Table 4.** Molecular dimensions of BSA forms as prolate ellipsoids predicted by the UltraScan Software (UTHSCSA).

Parameter	Monomer	Dimer	Tetramer	Hexamer
a (long axis) (nm)	9.01	10.57	11.61	12.8
b (short axis) (nm)	1.46	1.91	2.58	3.01
a/b ratio	6.15	5.53	4.50	4.25
% increase in a axis relative to monomer	--	17.3	28.9	42.1
% increase in a axis relative to dimer	--	--	9.8	21.1
% increase in a axis relative to hexamer	--	--	--	10.2
% increase in b axis relative to monomer	--	30.8	76.7	106.2
% increase in b axis relative to dimer	--	--	35.1	57.6
% increase in b axis relative to tetramer	--	--	--	16.7

*Hydration properties.* The calculated thickness of hydration shells surrounding albumin forms (monomer, dimer, tetramer, and hexamer) were in the range of 0.87 – 0.98 nm, equivalent to 3.0- 3.5 layers of water, which in turn is equivalent to 3,510 – 11,700 water molecules per particle (Table 5).

**Table 5.** Calculated hydration characteristics of albumin forms.

Parameter	Monomer	Dimer	Tetramer	Hexamer
Volume (hydrated) (nm <sup>3</sup> )	187.4	342.4	582.2	838.6
Thickness of water of hydration <sup>a</sup> (nm)	0.87	0.96	0.92	0.98
Number of hydration layers	3.1	3.4	3.3	3.5
Number of water molecules bound to one albumin molecule	3500	6000	8700	11700
Hydration factor ( $\delta$ ) (g water/g albumin)	0.95	0.82	0.59	0.53

$$^a = R_s - R_o$$

*Charge characteristics.* The effective charge of BSA monomer at pH 8.3 was taken as -14 charge units from the work of Böhme and Scheler<sup>[32]</sup> who reported the effective charge as a function of pH. This value was used to calculate  $Z_{eff}$  for the dimer and tetramer using equation 11. We found that  $Z_{eff}$  of the dimer was about two units less than that of the monomer, and that of the tetramer was about two units less than that of the dimer (Table 3). Surface charge was calculated according to equation 12, and the values of the dimer and tetramer were about two thirds and one third, respectively, that of the monomer (Table 3). Equation 8 was used to calculate the free electrophoretic mobility of albumin forms where the mobilities of the dimer and tetramer were about two thirds and one third, respectively, that of the monomer; values are shown in Table 3.

## Discussion

The aim of this work was to study the molecular shape and oligomeric states of albumin in slightly alkaline solution, and to calculate some of the physico-chemical properties of the oligomers. Albumin molecule has high conformational flexibility, which is crucial to perform a wide range of functions including ligand transport in the body, where a breathing mechanism was suggested to explain how one protein could bind so many ligands<sup>[33]</sup>. In the light of such flexibility and variable roles of albumin, it may not be reasonable to confine albumin structure to a rigid conformation such as the heart-shaped model at neutral pH. Randolph et al.<sup>[17]</sup> considered the native conformation of proteins as a dynamic structure involves a distribution of ensemble of species. However, albumin undergoes several transitions according to the pH of the environment, thereby, attains variable conformations during its interaction with ligands<sup>[35]</sup> and body cell membranes such as hepatocytes<sup>[34, 35]</sup>. The N-B transition at slightly alkaline pH involves a tertiary structural rearrangements<sup>[9, 36]</sup> and is more related to the physiological role of albumin as ligand transporter to the liver, and the N-B equilibrium is likely to alter in tissues and organs where pH changes occur, therefore, ligands are released and transported across membranes<sup>[35]</sup>. It has been reported that many ligands and drugs bind to albumin depending on the state of N-B equilibrium<sup>[35]</sup>. In this work we choose to study albumin at a slightly basic conditions close to those present *in vivo* or used in various ligand binding studies. The high values obtained for translational diffusion coefficient, negative charge, and hydration of albumin molecules

(Tables 3, 5) are in conformity with the biological role of albumin as ligand transporter, where it approaches the cell membrane, penetrate the unstirred water layer surrounding the cell to deliver the ligand to the cell. This is in accordance with the reported results that domain I of albumin is less flexible and more hydrated than domains II and III<sup>[37]</sup>, and the suggestion that domain I is the site of interaction with cell membranes<sup>[35]</sup>.

The presence of oligomers in the freshly prepared solutions of different commercial BSA and HSA albumin preparations can be attributed to the preparation procedures including lyophilization<sup>[13]</sup>. When the solutions of both BSA and HSA were stored for 4 months at 8°C, oligomers dissociated into dimer (17 – 23%) and monomer (77 – 83%) with no higher aggregates (Table 2). We also observed this phenomenon for the isolated pure oligomers. The monomer solution was stable for five months when stored at 8°C and no oligomers were detected. On the other hand, isolated pure dimer and tetramer underwent dissociation into monomer, and monomer and dimer, respectively, within few hours of isolation, but no higher aggregates were detected. In a previous study<sup>[22]</sup>, highly diluted pure HSA solutions (25 µg/mL and less) did not contain any oligomers as was detected by the sensitive immunoblotting method against anti-human albumin included in an agarose gel matrix. These observations can be explained by non-covalent binding between the monomers in the oligomers<sup>[38, 39]</sup>. This is in agreement with the work of Boersen et al.<sup>[40]</sup> who reported that aggregation of ovalbumin is primarily driven by physical interactions, and the work of Levi and Flecha<sup>[39]</sup> who reported the presence of equilibrium between HSA monomer and dimer in solution. Therefore, the presence or absence of oligomers must be stated when albumin is used in different studies especially those that address the physico-chemical properties, otherwise less accurate and contradictory results may be obtained. Many of such recent studies overlook the presence of oligomers<sup>[6, 8, 9, 37]</sup>.

The length of albumin monomer axes,  $2a = 18.02$  nm and  $2b = 2.92$  nm, and frictional ratio of more than one ( $f/f_0^{-1} = 1.32$ ) indicate that the molecule in solution at pH of 8.3 is an elongated prolate ellipsoid. This in accordance with the reported length of albumin at basic conditions to be longer than at neutral pH<sup>[32]</sup>. Moreover, it has been reported that hydration of albumin induces a degree of unfolding and denaturation and change in conformation<sup>[37, 41]</sup>. This is expected from the flexibility of albumin molecule where it changes molecular shape under various conditions due to the relative motions of its domain structures<sup>[4]</sup>. Molecular shape changes are also observed *in vivo*, such as the changes that occur in plasma albumin of patients with myocardial ischemia<sup>[42]</sup> and acute stroke<sup>[43]</sup> where albumin attains a conformation called ischemia-modified albumin. This form of albumin differs from the native one in its cobalt binding characteristics<sup>[44]</sup> which led the Food and Drug Administration (USA) to approve a test known as Albumin-Cobalt Binding (ACB) test to aid in better diagnosis of heart attacks. The ellipsoidal structure is in contrast with the heart-shaped conformation that was

suggested in crystalline albumin <sup>[3, 4]</sup> and at neutral pH <sup>[6]</sup>. Our results and similar reports about the ellipsoidal shape at non-neutral conditions contribute to the reconciliation of the two disparate views of albumin conformation <sup>[6]</sup>.

Regarding the molecular shape of the oligomers, the increase in the length of a and b axes of the dimer was not double that of the monomer; and the same applies for the tetramer, while the b axis of the hexamer is double that of the monomer (Table 4). Moreover, the effective charge, the density of surface charge, and frictional coefficient decreased gradually from monomer to dimer to tetramer (Table 3).

From the above mentioned observations we can propose the following points about the molecular shape of albumin forms and the mode of their intramolecular interactions. 1) Due to the small increase in a and b axes of the dimer (less than two folds) than that of the monomer, the monomers bind laterally to form dimer, and we assume that the monomer shape is a "flattened ellipsoid" where axis b is not equal to axis c. Therefore, the calculated b value may be an average of b and c values. This conclusion is supported by the heart-shaped model where the thickness of the molecule is less than the other dimensions <sup>[6, 45]</sup>. 2) The small increase in a axis of the dimer can be explained by small unfolding with some displacement between domains I and III. In support of that, Dockal et al. <sup>[9]</sup> attributed the origin of molecular rearrangement during N-B transition to domain I. 3) Due to the more negative domain I and more positive domain III, and the very small net charge on domain II <sup>[5]</sup>, we expect that the arrangement of the monomers in the dimer to be "antiparallel", that is domain I of the first monomer is electrostatically attracted to domain III of the second one. This may explain the decrease in the effective charge of the dimer. 4) The same reasoning can be applied to the tetramer and hexamer. 5) The presence of ~3 water layers around albumin forms is in agreement with the work of van Oss and Good <sup>[46]</sup> who reported the presence of three hydration layers around albumin. Other workers reported less and variable amounts of water of hydration <sup>[47, 48]</sup>, which may be due to the higher albumin concentrations (up to 20 folds) they have used.

The reaction of BSA, BSA-FAF, and BSA monomer, dimer, and tetramer with each of the antibodies raised against BSA monomer, BSA, and BSA-FAF; and the similar results with HSA, may be explained by the presence of a degree of cross-reactivity between albumin domains <sup>[49]</sup> and hence the presence of fatty acids or aggregation may not shield all the epitopes of albumin molecule. This looks reasonable in the light of the wide antigenic area on albumin molecule where about one third of the surface of an albumin molecule is antigenic <sup>[5]</sup>.

## Conclusion

Commercially available BSA and HSA preparations are heterogeneous and contain variable proportions of aggregates. Albumin monomer and oligomers have high degree of hydration, high effective negative charge, and high translational diffusion coefficient, which are consistent with the physiological role of albumin. Albumin aggregation involves weak intermolecular interactions and not covalent bonding. All albumin forms show immunological cross-reactivity. The estimated molecular shape of albumin in alkaline solution as prolate ellipsoid reconciles the two opposing models of albumin tertiary structure.

## Abbreviations

BSA: bovine serum albumin

BSA-FAF: bovine serum albumin-fatty acid free

HSA: human serum albumin

HSA-FAF: human serum albumin- fatty acid free

F-E transition: fast-to-expanded transition

N-F transition: normal-to-fast transition

N-B Transition: normal-to-basic transition

gPAGE: gradient polyacrylamide gel electrophoresis

PAG: polyacrylamide gel

PAGE: polyacrylamide gel electrophoresis

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