Korean J. Chem. Eng., **28**(5), 1304-1310 (2011)
DOI: 10.1007/s11814-010-0496-9

Aggregation mechanism of bovine serum albumin based on collision factor by the population balance conservation law

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(*Received 21 September 2010 • accepted 30 November 2010*)

Abstract−This paper discusses a novel approach for exploring the aggregation mechanism of bovine serum albumin using collision factor. The population balance equation consisting of aggregation term was developed and solved by the moment method. Different experiments were implemented to account for effective parameters on protein aggregation and to measure variations in average size of aggregates formed in a time interval. This was done by taking pictures with a CCD camera on a sterio microscope. The pictures were exported to image processing software to analyze average number and size of aggregates. The collision factor appearing in population balance equation was optimized and declared as a global term.

Key words: Protein, Aggregation, Collision Factor, Population Balance

INTRODUCTION

The function of a protein depends greatly on interactions between itself and its ambient. One of the consequences of these interactions is the common phenomenon of aggregation, which is critical to elucidation of protein behavior [1]. Bioactivity may be reduced or completely lost, due to the aggregation. Immunogenicity can be enhanced, and the formulation may be rendered unacceptable based on pharmacopoeial requirements [2]. Therefore, in the case of protein pharmaceuticals one should consider aggregate formation. It is well known that the unique native fold of a protein is crucial to its efficiency even in the complex environment of a living cell. Nevertheless, under some conditions, proteins can fail to fold properly, and this failure results in a wide range of diseases, such as amyloidoses, in which deposition of aggregated proteins in a variety of tissues is involved. Neurodegenerative pathologies, such as Alzheimer's and Parkinson's diseases and transmissible spongiform encephalopathies, belong to this category [3].

In general, the protein aggregation process acts in competition with the normal folding pathway and it takes place from misfolded and partially unfolded states [4]. In particular, a reduced folding stability appears to be a unifying property of amyloidogenic proteins. In other words, the native protein and its aggregates can be seen as originating from a common population of partially unfolded, inter-converting molecules that, due to interactions with solvent and neighbor molecules, proceed towards different pathway.

For this reason, once undertaken the aggregation process in particular conditions of temperature and solvent composition the new macromolecules can coagulate to form different kind of gels. The aggregation process is governed by the balance between attractive and repulsive interactions between denatured protein molecules. Repulsive forces are induced by the surface charge, and the attractive forces originate from the various functional groups exposed by the thermal unfolding of the protein [5].

Bovine serum albumin (BSA), a globular protein within the blood and milk of cows, has often served as a model protein for investigating heat-induced denaturation and aggregation of globular proteins because it is readily available in high purity. It contains three major lobes that are similar to one another and possesses 583 amino acid residues with 17 disulfide bonds and one free cysteine group [6]. To date, heat-induced aggregation and structural changes of BSA have been investigated by using bulk experimental methods [7,8]. It is generally believed that when the temperature is elevated, BSA exposes its thiol group and interior hydrophobic residues, which enables the formation of disulfide bridges, hydrogen bonds and hydrophobic interactions. Such reactions and interactions result in protein aggregation via a non-native and expanded conformational state. During the aggregation process, proteins will inevitably undergo the disruption of their multilevel structures including secondary-, tertiary- and quaternary-structure, and alteration in protein-protein interaction and change in their molecular weight distribution (MWD). It should be noted that such alterations in each level structure and interactions were correlated with the formation of protein aggregation, the reversibility of which depends on the changes in temperature, time of heating, pH and concentration [9].

In particular, when temperature increases, electrostatic interactions are not substantially modified; whereas, hydrophobic interactions are strengthened due to their entropic origin. Moreover, the structures of the aggregates formed depend on pH, which controls the net charge of the protein. Changes in pH lead to titration of groups in the unfolded form, causing destabilization of overall native protein structure and strongly affect the protein aggregation rate. At extreme pH values, far from iso-electric point (*pI*), electrostatic repul-

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sion between like charges in protein increases, resulting in a larger tendency to unfold, and modifies the intermolecular interactions. Also, the ionic strength of the solvent controls the screening of electrostatic interactions and degree of aggregation.

Moreover, at a fixed temperature and pH values, the size of aggregates depends on concentration; in particular, by changing the concentration, it is possible to drive the aggregates formation from oligomers to gel [10].

The effect of temperature on multilevel structural nature and interactions of BSA during its aggregation process was investigated by a combination of several spectroscopic and chromatographic methods. The secondary, tertiary and quaternary-level structures of BSA and its aggregates were evaluated upon heating by far-UV CD, near-UV CD, second derivative UV spectroscopy and DLS, respectively [11].

One of the most important properties of many industrial particulate processes is particle properties of the product [1]. In many industrial particulate processes, the dynamic PSD involves the distribution of one or more internal particle properties (e.g., number of radicals, species concentrations, etc.) which can display variations even in particles of identical size [12]. The dynamic evolution of the PSD in particulate processes is commonly obtained by the solution of the population balance equation (PBE) [13]. One dimensional, or univariate, population balance equation models have been employed to describe the evolution of a population of particles, drops, crystals, cells, etc. in dispersed phase systems [14,15]. These models assume that the dynamic evolution of the particle population depends only on a single internal variable (usually particle volume or mass) and that all other particle properties are evenly distributed among the population. However, the need for the employment of a higher dimensional, multivariate PBE model has been noted for a variety of systems such as aerosol systems, granulation processes and cell culture, extraction Columns [15-20].

In practice many engineering problems involving dispersed-phase particulate systems are carried out in one or more regions which can each be considered to be well-mixed. In such cases the chief concern is not the spatial distribution of particles, but rather, the system can be described knowing only the distribution of particles in the internal phase space [21].

In the present work, the aggregation of bovine serum albumin is studied in various pH's, temperatures, protein and salt concentrations. The method of choice is processing of images taken from solution in different time steps to obtain particle size distribution using image processing software. On the other hand, the population balance equation including aggregation term is solved for this case. The collision factor appearing in the population balance equation is obtained using optimization to show the probability of aggregates' collision as a global term.

MATERIALS AND METHODS

Crystallized BSA was obtained from Applichem (A1391) and used without further purification. Samples were prepared in various buffer solutions based on pH. Ionic strength was fixed using different concentrations of NaCl.

Attempts made to consider all conformational isomers of BSA molecule so the pH's selected were 2.5, 3.8, 4.8, 7.58, 9, 11.9. The temperatures studied were 60 °C, below the protein denaturation as well as 65, 70, 80 °C. Solutions in a test vessel were heated with a constant rate of 1 °C/min using a glycerin bath circulating through external wall of the vessel to reach that temperature and kept at a constant temperature for 10 minutes to study the effect of time on average size of aggregated particles. Pictures were taken using a CCD camera COOPLIX 5500 on a sterio microscope. These pictures were exported to image processing 'ANALYSIS' software for particle counting and many other calculations such as mean diameter, sphericity, mean area, and shape factor.

Pictures were taken from different parts of the test vessel with depth of about 5 cm to ensure real statement of particle size distribution. All experiments were done with at least two times of repetition.

To quantify the study on particle collision, population balance equation including aggregation term was expanded based on the work of Hulburt and Katz which agreed well with our system [21].

1. Theory of Particle Agglomeration

The objective of such processes is to build larger particles by "sticking together" two or more smaller ones. The particles which come together are all of measurable size; therefore, the growth of agglomerate takes place in finite steps. Except in cases where precipitation takes place on the surface of agglomerate. The size-distribution change is wholly a function of particle birth and death, and particle inflow to and outflow from the system.

Hulbert and Katz considered the case of a plug flow agglomerator where the size distribution must be considered as a function of axial position as well as time. Hence the number density must be defined as a function of axial position x, mass m and time t. To model the situation we should start with micro population balance, because the number density is a function of position as well as time [21].

A population balance for particles in some fixed subregion of particle phase space can be started as:

Accumulation=Input−Output+Aggregation (Birth-Death)

If we consider the subregion R1 to move convectively with the particle phase-space velocity v, i.e., take the Lagrangian viewpoint, then the population balance for particles in subregion R1 is stated simply as:

$$\frac{d}{dt}\int_{R1} n dR = \int_{R1} (B - D) dR \tag{1}$$

The population balance can be written for Lagrangian region R1 as:

$$\int_{R1}\left[\frac{\partial n}{\partial t} + \nabla\cdot(v_e n) + \nabla\cdot(v_i n) + B - D\right] dR = 0 \tag{2}$$

As the region R1 was arbitrary, the integrand must vanish identically. Thus, the population balance is given as:

$$\frac{\partial n}{\partial t} + \nabla\cdot(vn) + B - D = 0 \tag{3}$$

This equation is a number continuity equation in particle phase space. Hulbert and Katz proposed a mechanistic model for birth and death as follows.

Particle nucleation under the chemical environment is ignored. Only particle birth due to collision agglomerator is considered. At time t and position x, we let the rate of agglomeration of particles

of mass m1, an m2, be proportional to the product of the number densities n(x, m_1, t) and m(x, m_2, t). The proportionality factor for collision a(x, t) depends only on the environment, not on the particle size or shape. Thus,

$$B(m)=\frac{1}{2}a(x,t)\int_0^{\infty}n(x,m-\zeta,t)n(x,\zeta,t)d\zeta \quad (4)$$

Where ζ is the mass of one of the colliding particles and $m-\zeta$ is the mass of the other. The factor 1/2 ensures that collisions are not counted twice. Particles of mass m will be lost by agglomeration at the rate:

$$D(m)=a(x,t)n(x,m,t)\int_0^{\infty}n(x,\zeta,t)d\zeta \quad (5)$$

Incorporating the two functions into population balance gives:

$$\frac{\partial n}{\partial t}+u_x\left(\frac{\partial n}{\partial x}\right)=a\left[\frac{1}{2}\int_0^{\infty}n(x,m-\zeta,t)n(x,\zeta,t)d\zeta - n(x,m,t)\int_0^{\infty}n(x,\zeta,t)d\zeta\right] \quad (6)$$

The method chosen to solve the population balance equation was the method of moment. The moment of the distribution is defined as:

$$m_j=\int_0^{\infty}p^j n(x,p,t)dp \quad (7)$$

where p is a dummy of integration. This moment transformation can be used to reduce the Eq. (6) to:

$$\frac{\partial m_0}{\partial t}+u_x\left(\frac{\partial m_0}{\partial x}\right)=-\frac{1}{2}am_0^2 \quad (8)$$

The solution of the first moment of population balance equation gives the number of particles, the second and third moments of population balance equation can also be solved to calculate the size and area of agglomerated particles.

Second and third moments:

Table 1. Conditions of experiments

No. of exp.	pH	Temperature (°C)	Salt conc. (M)	Protein conc. (g/*l*)
1	2.5	70	0.1	2.5
2	4.8	70	0.1	2.5
3	7.6	70	0.1	2.5
4	9	70	0.1	2.5
5	11.9	70	0.1	2.5
6	4.8	60	0.1	2.5
7	4.8	65	0.1	2.5
8	4.8	80	0.1	2.5
9	4.8	70	0	2.5
10	4.8	70	0.2	2.5
11	4.8	70	0.1	0.5
12	4.8	70	0.1	5
13	4.8	70	0.1	7.5
14	4.8	70	0.1	1.5

$$\frac{\partial m_1}{\partial t}+u_x\left(\frac{\partial m_1}{\partial x}\right)=-\frac{1}{2}am_0m_1 \quad (9)$$

$$\frac{\partial m_2}{\partial t}+u_x\left(\frac{\partial m_2}{\partial x}\right)=-\frac{1}{2}am_1m_2 \quad (10)$$

The parameter 'a' depends on chemical and thermal properties of the environment. To optimize this parameter as explained before, different experiments were done. The variables studied were pH, ionic strength of the solution and also temperature. The conditions of experiments done are shown in Table 1.

RESULTS AND DISCUSSION

1. Effect of pH

The more compact and folded nature of BSA molecules at higher pH causes more charged functional groups not in contact with buffer solution. These groups are exposed to charge screening when the BSA molecule begins to unfold with temperature increase. On the other hand, at low pH, because charged functional groups have already been exposed to the solution, increasing temperature does not significantly affect surface charge [22].

As it is clear at low pH due to the extended structure of proteins, the initial size is larger but as the charged functional groups have already been exposed to the aqueous solution and screening effect of NaCl, the average size of aggregates does not significantly increase. At higher pH values gradually exposing the functional groups to saline solution,causes the repulsive force to reduce and more aggregates form. This effect is shown in Fig. 1.

2. Effect of Temperature

The experiments were done close to protein iso-electric point with concentration of 2.5 g/*l*. When serum albumin is heat-treated, it goes through two structural stages. The first stage is reversible while the second stage is irreversible but does not necessarily result in a complete destruction of the ordered structure. Heating to 65 °C can be regarded as the first stage, with subsequent heating above that as the second stage.

Thus, it may be concluded that in the reversible structural stage, some of the alpha-helices are transformed to random coils. If the side chains of neighboring residues of two peptide strands point in opposite directions and are perpendicular to a plane so that hydrogen bonds can form between the strands, then IR, CD, and Raman spectroscopy will detect them as beta-sheets. This means that aggre-

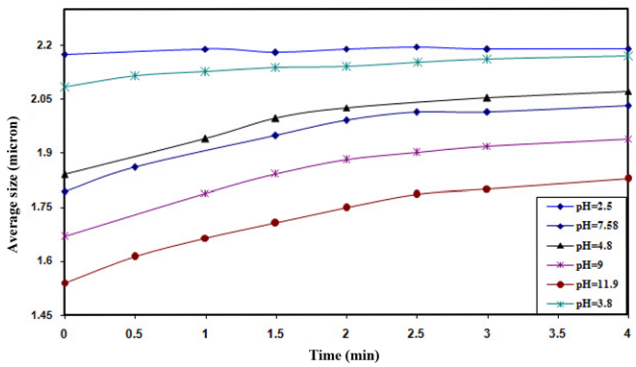

Fig. 1. Effect of pH on aggregates size of BSA, NaCl=0.1 M, T= 70 °C, Cp=2.5 g/*l*.

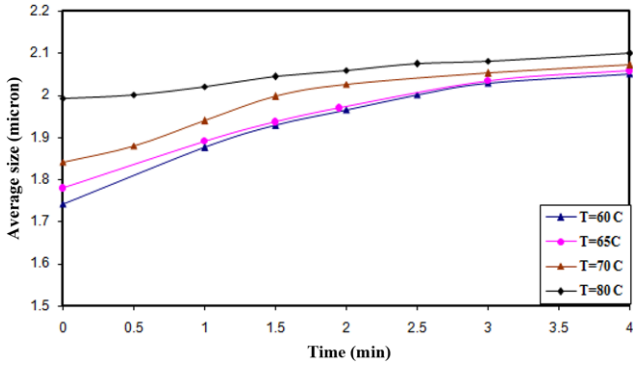

Fig. 2. Effect of temperature on aggregates size of BSA, NaCl=0.1 M, pH=4.8, Cp=2.5 g/*l*.

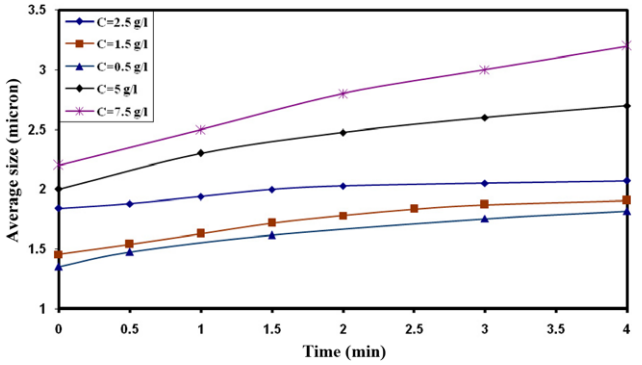

Fig. 4. Effect of protein concentration on aggregates size of BSA, pH=4.8, T=70 °C, NaCl=0.1 M.

gates are formed through the hydrogen bonding of beta-sheets between monomers. The beta-sheets formed are most likely to be antiparallel, since they are bound to be on the surface of the monomers. Parallel sheets are less twisted than antiparallel and are always buried. Antiparallel sheets can withstand greater distortions and greater exposure to solvent. As the temperature is increased past the reversible stage, unfolding of the pocket exposing Cys-34 takes place, giving easy access to the formation of disulfide bridges. Since disulfide bridges are covalent bonds, this stage is irreversible [3].

The effect of temperature on aggregation is shown in Fig. 2. Increasing the temperature causes higher molecular weight aggregates to form. It is predicted that the variation of aggregates' size with time is reduced at higher temperatures because most of the functional groups have been exposed to the solution and formed aggregates reaching that temperature.

3. Effect of Ionic Strength

The shielding effect is enhanced by increase in counter ions and ionic strength reduces the electrostatic repulsion between BSA molecules, which expedites the aggregation of BSA molecules [22]. When the ionic strength was increased, the increased charge-shielding reduced the repulsive force between functional groups, resulting in a slightly larger molecule. This effect is shown in Fig. 3.

4. Effect of Protein Concentration

The last effect studied was the concentration of protein. At fixed temperature and pH, the size of aggregates depends on concentration [23]. It is predictable that higher concentration of protein causes more effective interactions and due to the shielding effect of solution, the repulsive force is reduced, which causes more aggregates to form, thus the probability of aggregation and its size increases.

5. Collision Factor Estimation and its Functionality with Effective Parameters by Application of Population Balance Equation

As we mentioned before, the solution for the first moment of population balance equation gives the number of particles per unit volume, the second and third moments of population balance equation can also be solved to calculate the size and area of agglomerated particles.

Previous sets of equations were solved numerically using FORTRAN programming. To optimize the collision parameter 'a', the optimization software 'AIMMS' was used.

To fit parameter 'a' with this software, we introduced a number of sets, parameters, variables and constraints, and also an objective function which here is minimization of the difference between number of particles obtained by solving the first moment of population balance equation and the output of image processing software for particle counting up to an acceptable error range.

Applying parameter optimization algorithm for all experiments gives a specific 'a' for each case, where the results are shown below. In the following curves, the effect of each operational parameter on average number of aggregates is shown, and model outputs and experimental results have been compared. The dependency of colli-

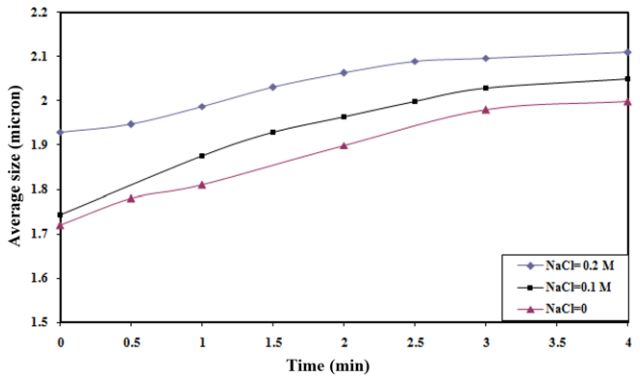

Fig. 3. Effect of ionic strength on aggregates size of BSA, pH=4.8, T=70 °C, Cp=2.5 g/*l*.

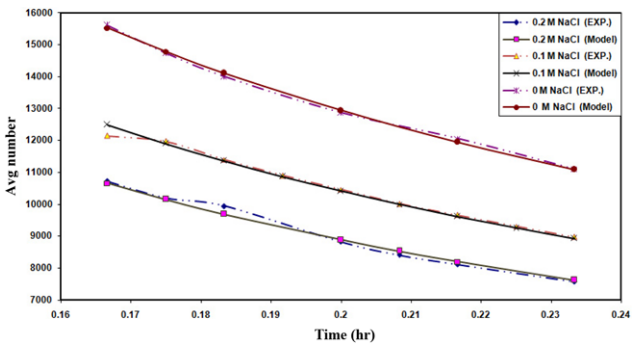

Fig. 5. Effect of ionic strength on average number of BSA aggregates, pH=4.8, T=70 °C, Cp=2.5 g/*l*.

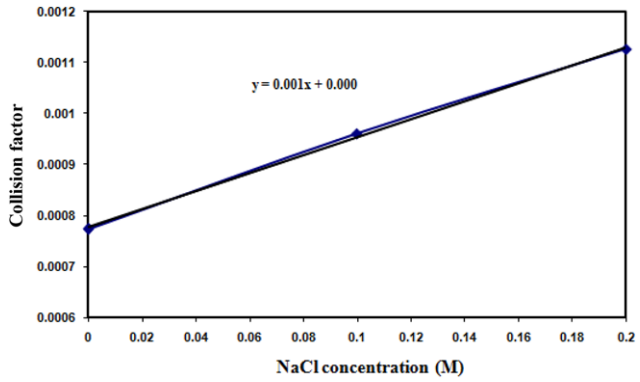

Fig. 6. Effect of ionic strength on collision factor, pH=4.8, T=70 °C, Cp=2.5 g/*l*.

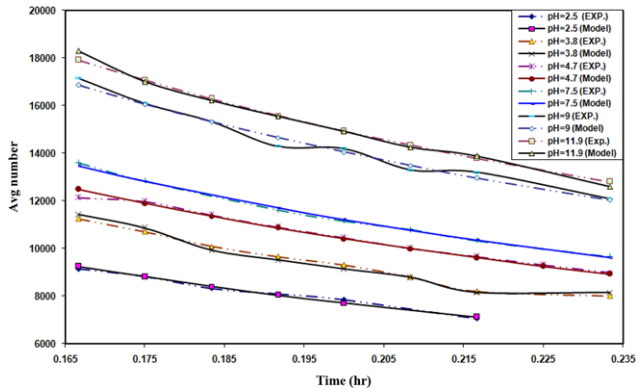

Fig. 7. Effect of pH on average number of particles, NaCl=0.1 M, T=70 °C, Cp=2.5 g/*l*.

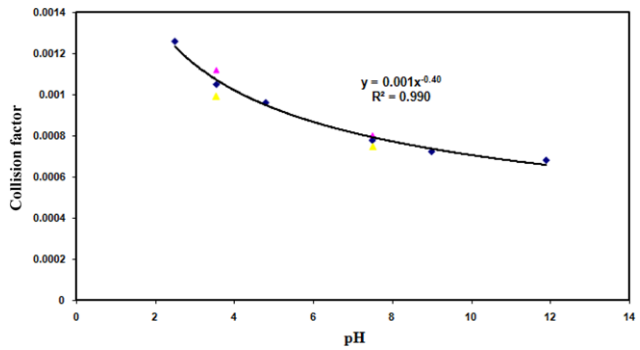

Fig. 8. Effect of pH on collision factor, pH=4.8, T=70 °C, Cp=2.5 g/*l*.

sion factor on each of the operating conditions is introduced as a function.

The effect of ionic strength on average number of aggregates is shown in Fig. 5. Increasing the number of ions causes the screening effect of charge to increase and the probability of particles' collision augment which is shown in Fig. 6.

Fig. 7 shows the effect of pH on collision factor. Due to the expanded structure of proteins at low pH, the probability of collision and aggregation increases. This effect is shown in Fig. 8.

The relation between protein concentration and collision factor

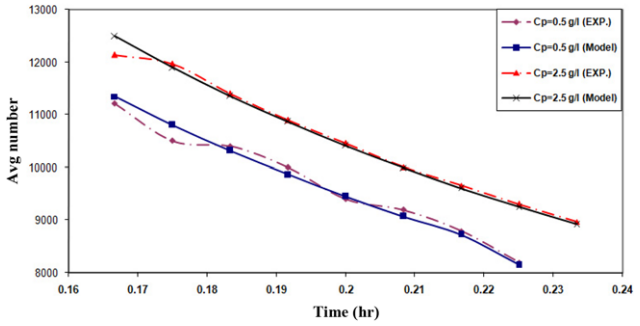

Fig. 9. Effect of protein concentration on average number of BSA particles, NaCl=0.1 M, T=70 °C, pH=4.8.

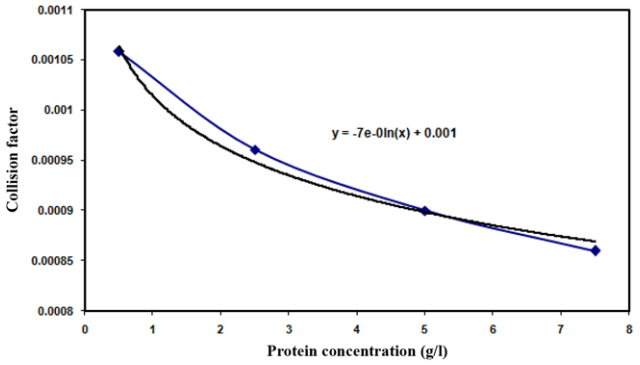

Fig. 10. Effect of protein concentration on collision factor, NaCl= 0.1 M, T=70 °C, pH=4.8.

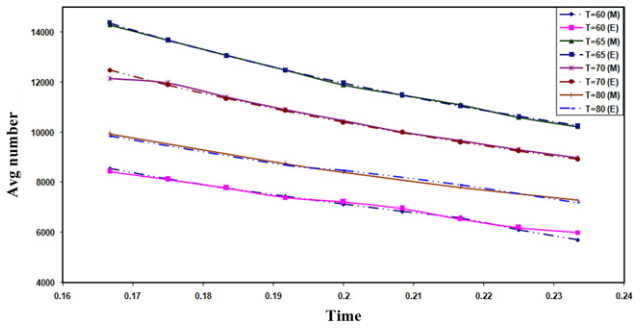

Fig. 11. Effect of temperature on average number of particles, NaCl =0.1 M, Cp=2.5 g/*l*, pH=4.8.

is shown in Fig. 9. Increasing the protein concentration causes larger aggregates to form. It is predicted that due to the neglected effect of velocity in solving population balance equations, the probability of collision decreases and aggregation mostly occurs through electrostatic interactions. The effect of protein concentration on collision factor is shown in Fig. 10.

Temperature effect on average number of particles is shown in Fig. 11. The effect of temperature on collision factor is different before and after denaturation. Before denaturation the probability of collisions due to the solid structure of protein is higher than that in denatured case. At denaturation temperature its multilevel structure goes through changes, which causes the collisions to occur at lower rate. After denaturation the effect of temperature on molecu-

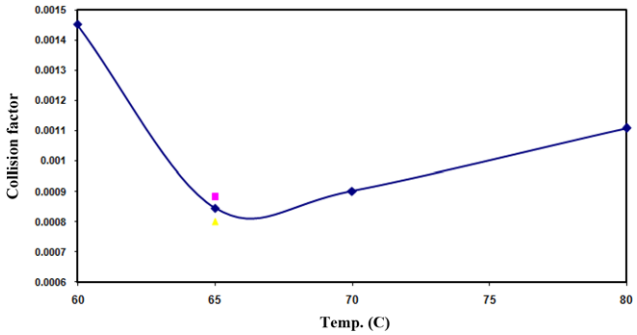

Fig. 12. Effect of temperature on collision factor, NaCl=0.1 M, T= 70 °C, pH=4.8.

Table 2. Obtained collision factor in each case

a	NaCl	pH	Cp	Temp.
0.0007729	0	4.8	2.5	70
0.000960798	0.1	4.8	2.5	70
0.001126626	0.2	4.8	2.5	70
0.00126	0.1	2.5	2.5	70
0.00105	0.1	3.55	2.5	70
0.000777	0.1	7.5	2.5	70
0.000721854	0.1	9	2.5	70
0.0006797	0.1	11.9	2.5	70
0.00145169	0.1	4.8	2.5	60
0.000844963	0.1	4.8	2.5	65
0.001107332	0.1	4.8	2.5	80
0.001058717	0.1	4.8	0.5	70
0.000960798	0.1	4.8	2.5	70

lar pulses as well as ion screening effect causes the collision factor to increase which is shown in Fig. 12.

Now we have obtained parameter 'a' for each case that is shown in Table 2. To introduce the collision factor as a global variable we defined it as a function of temperature, pH, protein and salt concentration. Excluding some of the results, the collision factor was introduced in two global collision models using parameter estimation of MATLAB software:

$$a(pH, T, C, Cp) = -0.0015 + 0.013C - 0.0395C^2 - 3.81\ln(Cp) - 0.0004\ln(pH) + 0.00163\exp(0.00163T) \quad (11)$$

$$a = 5.8E-7 \times pH^{-0.3066} Cp^{-0.01339} T^{1.9507} C^{0.4007} \quad (12)$$

Some of the collision factors excluded in regression were obtained using the above correlations with substituting the sample conditions, which all of them were in an error range less than 7-8%. The second model is more compact and gives the results in lower error range, so it is preferred.

CONCLUSIONS

The effect of different parameters on BSA aggregation was discussed. Results showed that at low pH due to the extended structure of proteins, the initial size is larger, but as the charged functional groups had already been exposed to the aqueous solution the average size of aggregates did not significantly increase. The higher heating temperature and the longer heating time will result in both higher amount and larger molecular weight of BSA aggregates. As the result of experiments shows, when the ionic strength was increased, the increased charge-shielding reduced the attractive force between functional groups, which resulted in a slightly larger aggregate formation.

The significance of this study rests on representing correlation models as a function of temperature, pH, protein and salt concentration for collision factor for the first time based on population balance equation, which expresses the probability of collisions which leads to aggregation with an error estimation of at most 7-8%.

NOMENCLATURE

$a(s^{-1})$: collision factor
B(L) : birth of particles with size L
D(L) : death of particle with size L
R : region in particle phase space
m_j : Jth moment of population distribution
ζ(kg) : is the mass of one of the colliding particles
u (m/s) : particle velocity
n : population density with size L

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