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Note

Effect of Salt Addition on the Fractal Structure of Aggregates Formed by Heating Dilute BSA Solutions

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The fractal dimension, D_f, of aggregates in a dilute BSA system with added salt was evaluated by static light scattering (SLS). A fractal structure was observed for the system with NaCl addition. The values of D_f increased with increasing heating time and ionic strength. The values of D_f were larger than those (D_f=1.8 or 2.1) predicted by the conventional cluster-cluster aggregation model, probably due to a "restructuring" of aggregates during the aggregation process. On the other hand, a fractal structure was not apparent for the system with added $CaCl_2$.

Key words: fractal; aggregate; light scattering; bovine serum albumin; metal salt

On heating a globular protein solution, the protein molecules often aggregate and a gel is formed at a high concentration. Macroscopic physical properties such as the solution viscosity and gel elasticity would be considerably influenced by the structure of the aggregate. A systematic understanding of the behavior of the macroscopic physical properties requires the relationship between the aggregate structure and the physical properties to be investigated. However, the structure of an aggregate can be difficult to characterize due to the disordered shape.

Fractal analysis is very effective for characterizing many kinds of disordered shape.[1] A fractal is a self-similar structure which can be characterized by a non-integer: the fractal dimension, D_f.[1,2] The concept of the fractal has been applied to colloid aggregation in a dilute solution, in which the aggregate structure can be characterized as a kind of fractal: the mass-fractal.[3-5] The fractal structure of an aggregate in a dilute system has been examined by the cluster-cluster aggregation model;[2,6] diffusing particles or aggregates in a certain medium stick to each other with probability P on contact. According to computer simulations, the values of fractal dimension D_f for aggregates are 1.8 at $P=1$ (the diffusion-limited cluster-cluster aggregation, DLCCA) and 2.1 at $P \fallingdotseq 0$ (the reaction-limited cluster-cluster aggregation, RLCCA).[2,6] We have already analyzed the fractal structure of aggregate formed by heating a dilute bovine serum albumin (BSA) solution with the static light scattering (SLS) method;[7] the fractal dimension of the aggregate at pH 7.0 (apart from the isoelectric point of BSA (pH, 4.9)) was about 2.1, this value agreeing with that predicted by the RLCCA model,[2,6] while at pH 5.1, the fractal dimension was about 1.8 which agrees with that predicted by the DLCCA model.[2,5] These results suggest that the average charge level of BSA molecules would influence the fractal structure of an aggregate.

The appearance of protein aggregates was changed by adding metal salts when observed by an electron microscope.[8,9] Salts added to a protein solution would, therefore, affect the fractal dimension. We have reported that the value of D_f for a protein aggregate gel with NaCl or $CaCl_2$ addition was evaluated to be 2.6 to 2.8,[10-12] although the reason for this high value is not yet known due to the lack of a computer simulation for a concentrated system. Fractal analysis of a dilute system with added salt would help to elucidate the mechanism for the development of the fractal structure in a protein aggregate gel with added salt, but the effect of salt on D_f in a dilute system has not been examined. In this study, the effect of adding salt on the fractal structure of the aggregate formed by heating a dilute BSA solution was analyzed by the SLS method.

Bovine serum albumin (BSA) was obtained from Boehringer Mannheim GmbH (Mannheim, Germany; ref. 238040). All other chemicals were of reagent grade.

BSA aggregate suspensions (BSA concentration of 0.1 wt%) were prepared by almost the same method as that described in the previous work.[7] BSA was dissolved in a 50 mM HEPES buffer at pH 7.0, before NaCl was added to the buffer to adjust ionic strength I_s to a specific value; the value of I_s was varied from 0.2 M to 1.0 M. Each solution was kept at 25°C for about 20 min and then heated at 95±0.2°C. The samples were then subjected to SLS measurement. Aggregates formed by heating BSA solutions with $CaCl_2$ were also prepared. BSA was dissolved in a 50 mM HEPES buffer at pH 7, and 5 mM $CaCl_2$ was added; the solution was heated in the same way as that just mentioned and subjected to SLS measurement.

Fractal dimension D_f of the aggregate was evaluated from the SLS measurements by using the following equation:[7]

$$I(q) \propto q^{-D_f} \tag{1}$$

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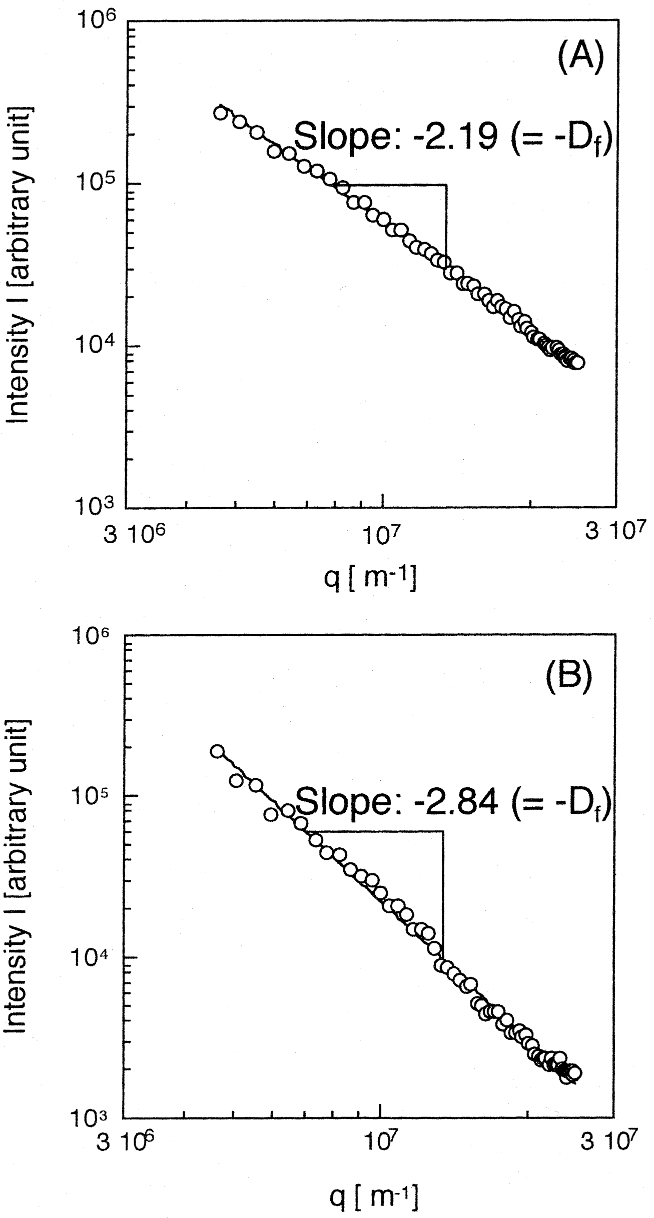

Fig. 1. Dependence on q of Scattered Light Intensity I for Heated BSA Solutions.

(A) Solvent: 50 mM HEPES buffer (pH 7.0, ionic strength=0.2M). Heating time was 90 min. (B) Solvent: 50 mM HEPES buffer (pH 7.0, ionic strength=0.6M). Heating time was 13 min.

where $I(q)$ is the total scattered light intensity and q is the scattering vector defined by

$$q=(4\pi n_s/\lambda)\sin(\theta/2) \tag{2}$$

where n_s, λ, and θ are the refractive index of the solvent, the wavelength of the light source in a vacuum, and the scattering angle (the angle between the incident and scattered beams), respectively. SLS measurement was done similarly to that described in the previous work[7] by a System 4700 instrument (Malvern); the light source was a 30 mW helium-neon laser with a wavelength of 632.8 nm (NEC Co. Ltd., Japan). The value of q was varied from 5×10^6 to $3\times10^7\,\mathrm{m}^{-1}$ (scale of 2.1×10^{-7} to 1.3×10^{-6} m). D_f was evaluated from Eq. (2) by the slope of the double logarithmic plot of $I(q)$ vs. q.

Table 1. Fractal Dimension D_f of BSA Aggregates Containing NaCl

Ionic strength I_s [M]	NaCl concentration [M]	Heating time [min]	Fractal dimension D_f
0.1	0.0868	90	2.11[7]
0.2	0.186	40	2.08
0.2	0.186	90	2.19
0.2	0.186	120	2.27
0.3	0.286	17	2.08
0.3	0.286	25	2.39
0.4	0.386	20	2.53
0.5	0.486	16	2.50
0.6	0.586	13	2.84
1.0	0.986	10	2.12
1.0	0.986	11	2.71

Figure 1 shows the double logarithmic plots of $I(q)$ vs. q for the samples prepared from a BSA solution containing NaCl. Both plots were linear, indicating that the aggregates prepared from the BSA solutions containing NaCl were fractal. The values of D_f for A and B were 2.19 and 2.84, respectively, from the slope of each plot.

The Table summarizes the values of D_f for aggregates prepared from BSA solutions containing NaCl at various I_s values, including the result from the previous work (I_s=0.1 M).[7] The values of D_f were larger for longer heating time at an identical value of I_s and tended to increase with increasing I_s. The values of D_f are larger than the maximum of 2.1 predicted by the conventional "cluster-cluster aggregation model" mentioned before.[2,5,6]

Meakin *et al.*[15] have simulated an aggregate structure by a modified cluster-cluster aggregation model that allows for "restructuring" of the aggregate as follows:

① Restructuring is allowed to take place immediately after two clusters have contacted each other, but no subsequent restructuring is allowed.

② Restructuring takes place in three distinct stages and the process can be stopped after each stage.

They reported that the fractal dimension was increased from about 1.8 to about 2.2 by the simulation. The peptide chain of a protein molecule does not have a fixed structure like a crystalline material but a flexible fluctuating structure;[16-18] so restructuring of the aggregate can take place during the aggregation process. The increase in D_f in the Table would have been caused by such restructuring of the aggregate. However, the maximum value of D_f (2.84) in the Table is larger than that calculated by the model of Meakin *et al.* The foregoing assumptions in their model are thus too simple. Restructuring during the aggregation of protein molecules would probably be more complicated, so the value of D_f for the BSA aggregate might have been substantially increased by NaCl addition.

In Fig. 2, I vs. q plots for the aggregates containing $CaCl_2$ are shown. No linear relationship is apparent, implying that the aggregates in these samples were not fractal. However, the aggregates in these samples would probably have been fractal, considering that the aggregates in BSA gels containing $CaCl_2$ were fractal in the

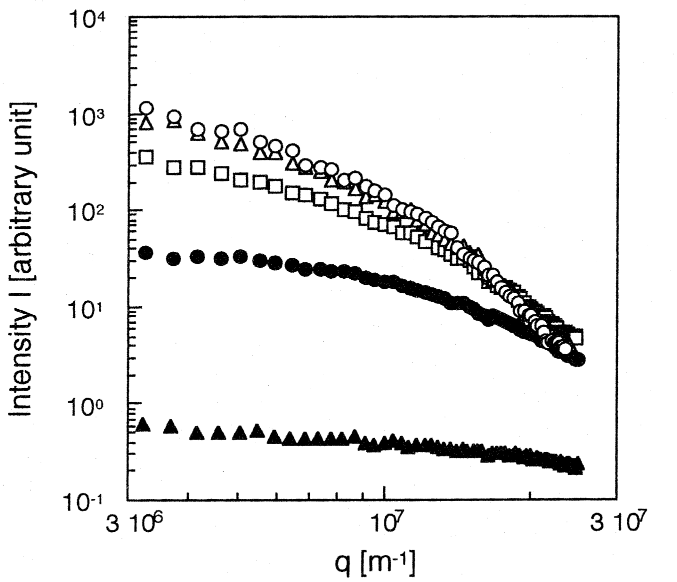

Fig. 2. Dependence on q of Scattered Light Intensity I for BSA Solutions Heated for Various Times.

Solvent: 50 mM HEPES buffer (pH 7.0, $CaCl_2$ concentration was 5 mM). Heating time: ○, 45 min; △, 30 min; □, 10 min; ●, 5 min; ▲, 2 min.

range of one to several decades micrometers from an image analysis.[10-12] An experimental method other than SLS used in this study should be developed to confirm that an aggregate formed in the presence of $CaCl_2$ is fractal.

In the previous works, the values of D_f for BSA and β-lactoglobulin gels with added salt were approximately 2.6–2.8,[10-12] while those of D_f for the gels prepared without adding salt were about 2. The larger values of the fractal dimension for the gels would have been caused by restructuring. Salt addition would restructure a protein aggregate and increase the value of D_f.

We concluded from the previous works[10-12] that the elastic behavior of a protein aggregate gel was a reflection of the fractal structure of the aggregate in the gel. In principle, the elastic behavior of a gel, therefore, could be controlled by varying D_f. Further investigation on the effect of aggregation conditions on D_f is necessary.

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