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Heat-Induced Gelation of Globular Proteins. 1. Model for the Effects of Time and Temperature on the Gelation Time of BSA Gels

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ABSTRACT: Globular protein gels have been extensively investigated over many years, although the great majority of studies have used rather crude and/or mixed samples of protein. Moreover, most previous work has concentrated on examining structural and rheological properties of fully cured gels. In the present paper we will discuss heat-induced gelation of the typical globular protein, bovine serum albumin (BSA), not far from critical gel conditions together with aspects of kinetic gelation theory. We will concentrate on a description of the parameters that alter the gelation or gel time, t_c. We will develop a new model to describe both the effect of temperature and polymer concentration on t_c. Gel formation is achieved by isothermal heating. The change in rheological properties during the gelation process is monitored by dynamic shear rheometry as a function of time. The measurements are carried out at different temperatures and protein concentrations to clarify their effects on the gelation. The results are displayed in various diagrams, including a new form of sol/gel state diagram, and discussed in terms of both phase behavior and gelation kinetics.

1. Introduction

Globular protein gels have been extensively investigated over many years, although many of these studies have used rather crude and/or mixed samples of protein. Moreover, most studies concentrate on examining structural and rheological properties of fully cured gels. Consequently there is relatively little work which considers the incipient behavior around the gelation point.[1,2]

In de Gennes' terms,[3] gels are frozen systems and many factors are constrained in the stage of sample preparation. As a result, treatments prior to measurement can strongly affect the properties of the resulting gels. Although similar problems may occur in the incipient gelation behavior, the resultant network is more ideal than that of fully cured gels. Therefore, it is more appropriate to study the gelation process and the effects of the factors upon this rather than on the latter.

Protein gelation is known to be promoted by several kinds of treatments such as acidification, addition of salts, enzyme reaction, and heating.[4,5] While the first three treatments have already been examined by a number of workers, it should be appreciated that gelation induced by temperature, the so-called heat-induced or thermally induced gelation, has received insufficient attention.

Heat-induced gelation of proteins is very important for commercial production such as foods and pharmaceuticals. For example, some foods of milk and soy proteins have been traditionally manufactured by this principle. To control the processes it is essential to understand the fundamental behavior of gelation. Heat-induced gelation is also important in manufacturing lines because it can cause problems such as the fouling of pipes.[6,7] Without sufficient information about the gelation mechanism, it is almost impossible to deal with such difficulties.

Although not a major part of this paper, it is worthwhile reviewing the currently accepted structural features of these globular protein gels. Historically, it was commonly thought that when globular proteins were heated above their denaturation temperature (typically 50−80 °C), the protein unfolded substantially. This is not the case, as has been shown, for example, by SAX-ray scattering experiments. For example, data by Matsumoto and Inoue on ovalbumin sols[8] confirm that the size and shape of the protein macromolecule are only slightly perturbed by heat denaturation. In general it seems that the shape of the original protein changes relatively little (e.g. by < 20%), but internal hydrophobic groups "flip" to the outside of the molecule. The "molten globules" produced then aggregate in order to reduce the exposure of hydrophobic groups to the aqueous environment. The precise mode of aggregation, for example random clumping, more directed rodlike fibrillar, etc., depends upon the pH and external ionic strength. The higher the ionic strength and the closer the pH to the isoelectric point, the more unspecific the aggregation (coagulation), and turbid syneresing gels are formed. Conversely, transparent gels are formed when the pH is far from the isoelectric point, and they are formed at intermediate salt levels. The article by Clark and Lee-Tuffnell[4] summarizes the structural aspects in more detail.

In the present paper, we will discuss heat-induced gelation of the typical globular protein, bovine serum albumin (BSA), and aspects of theory. We will develop a new model to describe both the effect of temperature and polymer concentration on the gelation time. In the second part, we will consider other proteins, β-lactoglobulin (β-Lg), α-lactalbumin (α-La), and mixtures of these with BSA. Here we will also apply the single-component approach outlined in the present paper to β-lactoglobulin gels, and we will also consider the effect of pH and ionic strength on both BSA and β-Lg systems. In both papers we will use the gelation time as our study parameter. More details of the advantage of this approach are given elsewhere.[9]

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In the present work gel formation is achieved by isothermal heating. The change in rheological properties during the gelation process is monitored by the use of dynamic shear rheometry as a function of time. The measurements are carried out at different temperatures and protein concentrations to clarify their effects on the gelation. The results are displayed in various diagrams and discussed in terms of phase behavior and gelation kinetics.

2. The Gelation Time. Theoretical Background

2.1. Analysis of Gelation Kinetics. In the study of incipient behavior of gelation, one of the topics to be examined is the reaction kinetics. A conventional method is to monitor the change in concentration of reactants, intermediate, and final products. Then an appropriate reaction model is constructed to describe the reaction processes, and the reaction rate of each step is determined by analytical or numerical calculation to give the best fit with experimental data.

It is, however, very difficult to apply this kind of procedure to the kinetics of polymer gelation, because polymers have the following characteristics: (1) They have a molecular weight and size distribution. (2) Reactivity of sites is not uniform. (3) Steric hindrance may be significant.

Without the simplifying assumption of uniform reactivity of functional groups,[10] it is difficult to overcome these problems, and many workers now resort to simulation. In the case of biopolymers, the processes may be even more complex, because other reactions, e.g. denaturation and crystallization, are often observed to occur prior to gelation. In order to deal with such complicated processes, we inevitably have to introduce many assumptions.

Several models have been proposed for protein gelation. Some of them seem rather oversimplified because of the difficulties mentioned above. Other models are rigorously constructed but contain a number of variables which cannot be specifically determined by experiments. In this study, we have attempted to propose a new model for protein gelation by considering these problems as carefully as possible. The model was constructed on the basis of gelation time described by a function of two factors, concentration and temperature. Here we explain our model, in light of earlier approaches.

2.2. Concentration Dependence of Gelation Time. In earlier work[2] we assumed that the gelation proceeded in four steps with different reaction rates.

$$\begin{array}{ll} \mathrm{P} \xrightarrow{k_{\mathrm{ij}}} \mathrm{U} & \text{unfolding of native proteins} \\ \mathrm{U} + \mathrm{U} \xrightarrow{k_{\mathrm{a1}}} \mathrm{A} & \text{aggregation of unfolded proteins} \\ \mathrm{A} + \mathrm{A} \xrightleftharpoons{k_{\mathrm{a2}}} \mathrm{R} & \text{intermolecular reaction of aggregates} \\ \mathrm{R} \xrightleftharpoons{k_{\mathrm{f}}} \mathrm{R'} & \text{intramolecular reaction of aggregates} \end{array} \tag{2-1}$$

Here, as is generally accepted, the protein unfolding step is first order in protein concentration, while the second and the third (intermolecular aggregation steps) are second-order reactions. Following conventional gelation kinetics, the intramolecular reaction step is of one lower order (i.e. first order) compared to the corresponding intermolecular step. Furthermore, Richardson and Ross-Murphy[2] made the assumption, on the basis of the nature of the noncovalent nature of the bonding, that the first two steps were irreversible and the other two could be reversible. This is certainly the simplest kinetic model which reflects all the known aggregation characteristics. They then tried to determine the reaction rate at each step for different concentrations. As a result, although the overall reaction order was estimated between 1 and 2, each reaction order was not determined with enough accuracy.

In this earlier study, the concentrations of initial and final products were used to estimate the approximate values for the four rate constants, although for an accurate estimation of each rate constant, it is necessary to measure the concentrations of the intermediate products in a direct way. In contrast to this complicated reaction model, a simple empirical relationship was proposed by te Nijenhuis.[11] He found the gel time t_c could be written with concentration c as

$$\ln(t_c) = a_1 + a_2 c \tag{2-2}$$

and applied the model to gelatin gelation. The later model of Oakenfull and co-workers[12,13] is of the form

$$t_g = \frac{1}{kc^n} \tag{2-3}$$

Although the latter model can describe experimental data fairly well, it contains some problems as noted by Ross-Murphy.[14] In particular it allows for chemical reactions with reaction orders higher than 4, which are difficult to achieve or accept. It also assumes that a gel can be formed at any finite concentration; i.e. a critical concentration for gel formation is not taken into account. In this earlier work,[14] from a model that was based on the parallels between reciprocal geltime and the gel modulus, another gel time vs concentration equation could also be derived. A detailed comparison of all these approaches is given elsewhere.[15]

Recently Clark[16] proposed a detailed model by combining the branching (cascade) theory with reaction kinetics. The Clark model may be said to be fairly rigorous. However, some points still remain unclear. For example, in his model the functionality f of polymer was used to predict a gelation point, but the results, when expressed in reduced form, i.e. in terms of c/c_0, where c_0 is the critical gelation concentration, were almost independent of f. This is surprising, because f must be largely influential on the gelation rate, as seen from the initial model, but may simply reflect the overall influence of c_0, which is itself a function of f.

2.3. Temperature Dependence of Gelation Time. While several models have been proposed to predict the concentration dependence of gelation time, there are very few models for the temperature dependence of gelation time. This is probably because the effect of temperature is usually assessed in terms of gelation temperature measured by constant rate heating rather than of gelation time by isothermal heating. However, isothermal heating can give more reliable data, and gelation times determined by such a method reflect the process more sensitively. Here we shall explain two empirical models for predicting how gelation time is affected by temperature.

te Nijenhuis[11] measured gelation time t_c of gelatin at different temperatures. By fitting the experimental data, he found that the square root of $1/t_c$ is proportional to temperature T, and wrote the relation as

$$t_c^{-0.5} = a + bT \tag{2-4}$$

where a and b are coefficients. Ross-Murphy,[14] on the other hand, proposed a model by the analogy with the concentration dependence of gelation time, which he had examined earlier. In this model, the critical temperature, T_g, for gelation was introduced in the following way:

$$t_c = \frac{k}{\left(\frac{T}{T_g} - 1\right)^n} \tag{2-5}$$

Here k and n are the prefactor and the scale factor, respectively. He applied this model to te Nijenhuis data of gelatin and obtained an exponent slightly >2 for n.

The model derived in the Appendix combines some features of the above and produces a unified form to describe the gelation time in terms of both the relevant experimental parameters, concentration and time. With the aid of this equation, state diagrams can be constructed, in terms of the critical gel time obtained under different conditions. Below we draw the parallels between this form of kinetic state diagram and equilibrium phase diagrams.

3. Experimental Section

3.1. Materials. BSA samples were pure commercial products from Sigma Chemical Co. (St. Louis, MO) from bovine milk, 1 × crystallized and lyophilized, A-4378, Lot Nos. 13H9338 and 82H9313) These were used without further purification, because the effect of salts contained in them was proved by dialysis to be negligible. The BSA contained 4.8% w/w of water, determined by heating at 104 °C for 24 h. It also contained 0.30% w/w of Na^+ ions (manufacturer's data).

The protein, a white powder, was dissolved in deionized water to give concentrations from 5.0 to 20% w/w. Native pH of the 10% w/w BSA solutions was 6.6 (Lot No. 13H9338) and 7.0 (Lot No. 82H9313). Although the pH of the protein solutions were slightly different depending on the concentrations, such a difference in pH was considered negligibly small in this experiment.

The BSA used here was from different lot numbers, i.e. 13H9338 and 82H9313. According to the manufacturer, the difference in lot sometimes arises because the products are made in different factories. However, it has often been pointed out that such differences in lot number become significant, so we also examined the effect by preparing gels in the range 5.0–20% w/w without pH adjustment. These were prepared by isothermal heating at different temperatures, and gelation points were determined as described below.

3.2. Methods. Rheological measurement was performed with a stress-controlled rheometer, Carri-Med CSL100 (TA Instrument Ltd, Leatherhead, UK), using cone (upper) and flat (lower) plates. The upper plate was made of transparent acrylic polymer, and its geometry was 4 cm in diameter and 2° in cone angle. The frequency and the strain were set at 1 rad/s and 1%, respectively, although strain and frequency sweeps were also carried out to check their effects on the gelation process. After a sample solution was set between the plates, paraffin oil (29436, BDH Laboratory Supplies, Poole, Dorset, U.K.) was placed around the outside to prevent drying. The sample was held at a predetermined temperature and the storage modulus G' and the loss modulus G'' were monitored as a function of time. The measurements were performed at different temperatures.

We also measured gelation temperature by heating at a constant rate, because such a method has very often been used in studies of protein gelation. The samples were heated from 50 to 80 °C using a rate of 1 °C/min. The gap between the plates was adjusted at 65 °C, which is the central value of the temperature range used, to minimize the effect of the temperature change such as increase in sample volume and decrease in gap height.

In the present work, the viscoelasticity of fully cured gels was not examined in detail, because it does not represent sensitively the effect of the relevant experimental factors, as already mentioned earlier. The only measurement carried out for fully cured gels was a frequency sweep at different temperatures. The sample gels were prepared by heating at 70 °C for 6 h. Under this condition, both G' and G'' seemed to reach a plateau. The frequency dependence of the sample was performed at different temperatures from 70 °C down to 20 °C. The strain was set to 5%.

Since the BSA contains a small amount of Na^+ ions, we checked the effect of the residue by dialysis treatment with a cellulose membrane (mol wt cut-off: 12 kDa, D-9777, Lot 82H0674, Sigma Chemical Co.). The BSA solution was put into the membrane and immersed in 1 L deionized water. It was stored at 5 °C for 3 days, and the deionized water was altered frequently. The concentration of BSA solution after dialysis was determined by UV measurement at 280 nm. The gelation of the dialyzed BSA solution was measured by the isothermal heating method as described earlier.

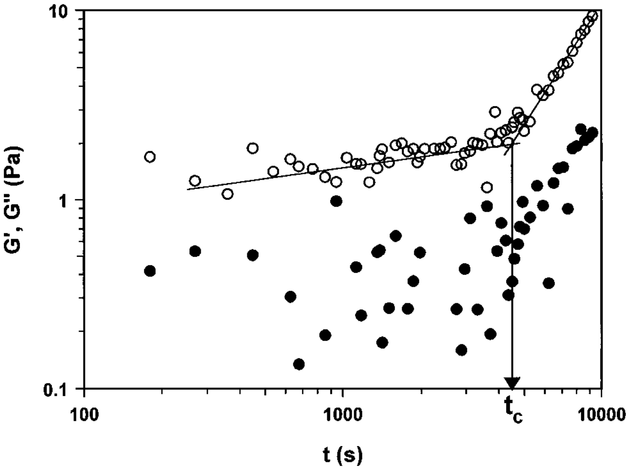

Figure 1. Experimental determination of gelation time for heat set globular gels, using the t_{c2} criterion. Data are for 10% w/w BSA in water at 58 °C: G', open circles; G'', filled circles.

4. Results and Discussion

4.1. Gelation Process. A typical trace of G' and G'' against time t is shown in Figure 1. The sample was 10 w/w % BSA solution (Lot No. 13H9338) at 58 °C. Both G' and G'' increase slightly with time in the early stages. Although both G' and G'' showed rather large deviations due to low torque signals, G' seemed higher than G''. After a certain time they both increase much more rapidly and then seemed to approach a plateau. No crossover of the G' and G'' was found during the gelation process.

This figure reveals, rather unexpectedly, that G' is already higher than G'', even from the beginning of the measurement. This tendency was recognised for other protein concentrations and temperatures and also at different frequencies and strains. The sample was still in the sol state, but the typical feature of solutions, i.e. that G'' is higher than G' in a wide range of frequency with slopes 2 and 1, respectively, was not observed. However, in terms of concentration the protein solution, $[\eta] \sim 0.033$ dL/g,[17] was certainly in the dilute regime ($c^* \sim 1/[\eta] \sim 30\%$ w/w).

One possibility would be that the sample was already highly structured well before the gelation point in a way analogous to a charged colloid system. There is other work which supports this explanation. First, there is the common observation of dimer (and higher) molecular weights found by light scattering for globular proteins. Recently Gimel and co-workers[18] reported the aggregation of β-lactoglobulin in dilute solution after heat treatment. They used both dynamic light scattering and small angle neutron scattering to calculate the fractal dimensions of aggregates. Their results suggested that the globular proteins form packed aggregates by heating even at very low concentrations where gelation cannot occur, although after heating the aggregates will be far less reversible.

Several methods have been used to determine a gelation point. Among these methods, Winter and Chambon's criteria[19] seem the most rigorous. They proposed that the shear frequency dependence of G' and G'' can be described as a power law, the powers of which become equal at the gelation point. It is known, however, that in kinetic gelation experiments, where gelation takes place from the solution state, this method is often very difficult to apply.[20] This is because the viscoelastic moduli near the gelation point are too low to give enough signal for conventional instruments. Furthermore, some samples clearly do not behave as a sol even well before a gelation point.[21]

Careful work by Matsumoto and Inoue[22,23] have confirmed this behavior for a number of globular proteins, including ovalbumin and BSA. They have investigated the behavior of solutions of the protein (in phosphate buffer) at different ionic strengths, and analyzed the behavior in terms of classical colloid science. The repulsive Coulombic interaction between the proteins could be fitted by a size-modified Yukawa potential. For measurements at 20 °C, they were able to construct a state diagram showing the transition from Newtonian flow to "yield stress" behavior for different protein and buffer concentrations.

Since the Winter/Chambon method could not be applied to our samples for the reasons mentioned above, we attempted to determine gelation points from a point at which the G' increased rapidly. On a log−log scale this point can be easily recognised as an apparent discontinuity (Figure 1). This method was found in most cases. However, when gel formation was very fast or the gel formed was very "weak", gelation points were not clearly detected in this way. We, therefore, used another method, i.e. the time at which the G' rose above a threshold value, ~2−3 Pa. Such procedures for determination of gelation points have already been adopted and discussed in detail elsewhere.[9] Here we denote this gelation time, t_c, to represent a gelation point determined as a function of time. In later sections the gelation point is also expressed as a function of temperature, which we will call gelation temperature, T_g. In these experiments, temperatures for preparing gels ranged from 40 to 90 °C.

At temperatures higher than ca. 70 °C, numerous bubbles were observed to appear in the sample, especially near the edge. This phenomenon became more significant at higher temperatures and for longer measuring times. The formation of bubbles was due mainly to evaporation of water contained in the sample. The water evaporation was believed to cause partial concentration of protein, and as a result apparent t_cs may become shorter. In order to reduce this problem, we used paraffin oil as a protectant. Preliminary experiments had showed this was more effective than silicone oils, even though the latter were more viscous at the measurement temperature.

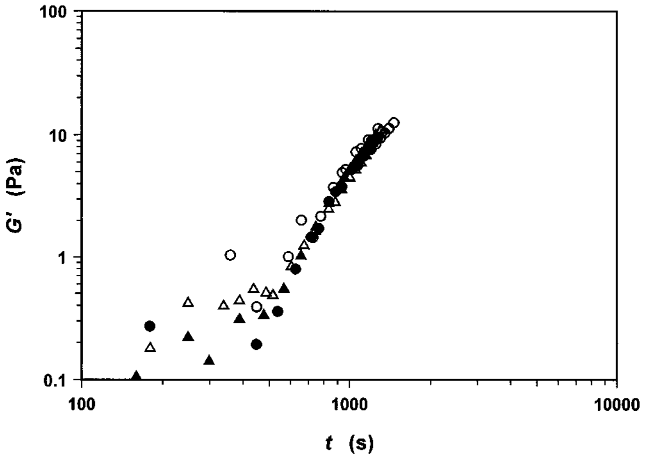

Figure 2. Trace of G' vs time at different percentage strains: BSA 10% w/w at 62 °C; frequency, 1 rad/s. Symbols indicate strain: 0.5% (open circle); 1% (filled circle); 5% (open triangle); 10% (filled triangle).

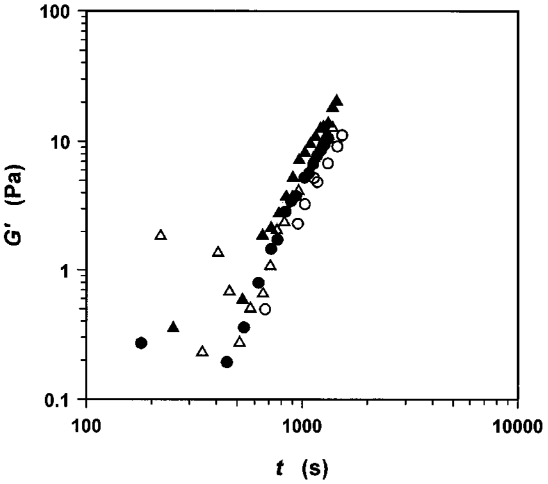

Figure 3. Trace of G' at different frequencies: BSA 10% w/w at 62 °C; strain, 1%: Symbols show frequencies: 0.5 rad/s (open circle); 1 rad/s (filled circle); 5 rad/s (open triangle); 10 rad/s (filled triangle).

4.2. Effects of Strain and Frequency. Figure 2 shows G' of BSA at different strains. The sample at lowest strain (0.5%) showed rather large deviations because of low torque signals, but there were no significant differences in t_c. It seems reasonable to conclude that the strain in this range did not affect t_c. This means that, within the range, e.g., 0.1−10%, the strain can be set arbitrarily to obtain enough torque signal. We chose a strain of 1%, because it is still possible that larger strains may affect the apparent gelation time.

Figure 3 shows the corresponding trace of G' at different frequencies. Here t_c seems to shift slightly to shorter times with the increase in the frequency. Since this difference is much smaller than the experimental errors between each measurement, we concluded that the effects of frequency on t_c were not significant for the range employed. However, the reliable range of frequency was found to be very limited because of experimental problems at the higher and lower ranges, e.g., <0.1 or >10 rad/s, so we chose 1 rad/s for the remaining experiments.

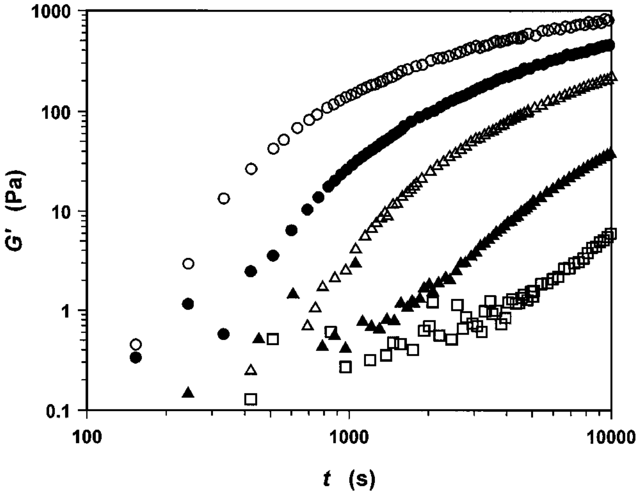

Figure 4. *G*′ vs time at different temperatures: BSA 10% w/w; strain, 1%; frequency, 1 rad/s. Key: 66 °C (open circle); 64 °C (filled circle); 62 °C (open triangle); 60 °C (filled triangle); 58 °C (open square).

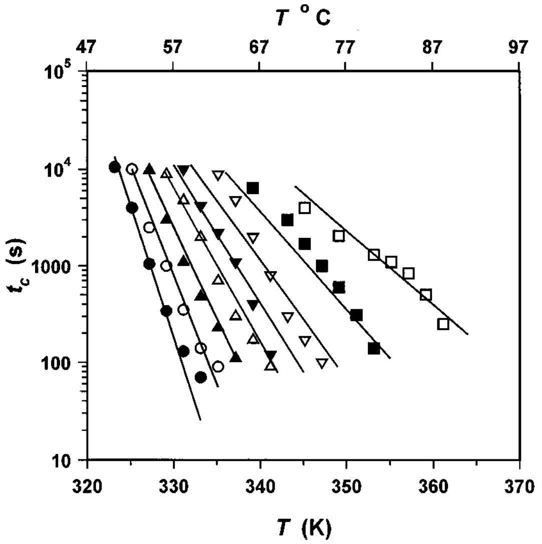

Figure 5. Gelation time vs absolute temperature for different protein concentrations. Solid lines were obtained from eq A-13. Protein concentrations are, respectively, 6% (open square), 7% (filled square), 8% (open triangle down), 9% (filled triangle down), 10% (open triangle up), 12% (filled triangle up), 15% (open circle), and 20% (filled circle). Here, and in Figure 6, the size of the symbols reflects the expected error in the middle of the experimental range, i.e. when 3000 s ≳ t_c ≳ 300 s. Outside this range the errors become larger, because determination of the gelation time becomes more difficult. This is reflected in the increased scatter.

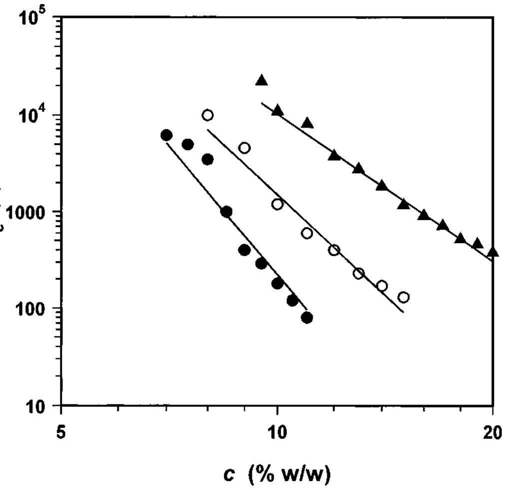

Figure 6. Gelation time vs BSA concentration at different temperatures. Solid lines were obtained from eq A-13. Symbols correspond to different temperatures: 66 °C (filled circle); 61 °C (open circle); 56 °C (filled triangle).

4.3. Effects of Temperature and Concentration. Figure 4 shows traces of *G*′ plotted against time *t* at different temperatures. The BSA concentration was 10% w/w. It was found that the change in *G*′ slowed as the temperature decreased, but except for the lowest temperature data when $t/t_c \lesssim 3$ and the data are truncated, these appear to approach a plateau value.

In Figure 5, t_c values for different concentrations *c* were plotted against temperature *T*. This strongly suggests that t_c increases with the decrease in both *T* and *c*. The sample of 5% w/w did not form a gel even at 90 °C for 1000 s, and therefore under the conditions employed here, a minimum concentration for gelation, C_0, appears to exist between 5 and 6% w/w. Here, and in Figure 6, the size of the symbols reflects the expected error in the middle of the experimental range, i.e. when 3000 s ≳ t_c ≳ 300 s. Outside this range the errors become larger, because determination of the gelation time becomes more difficult. This is reflected in the increased data scatter.

It seems that the series for high concentrations were explained by exponential functions of temperature. Although this applied well to the higher concentration range, the deviations became larger as the concentration lowered. This was probably due to the evaporation effects, which made t_c shorter. The formation of disulfide bonds, which is often observed for proteins at high temperatures, may also be involved[24] although this has not yet proved important for our system. Evidence suggests that for BSA few disulfide bonds are formed below ~80−85 °C.[4]

The lines drawn are calculated from the model discussed in the Appendix, given as eq A-13. Elsewhere[15] we have tested several models, including all those discussed in Section 2 to predict the effects of concentration *c* and temperature *T* on gelation time t_c. Each model can explain the effect of one factor alone, *c* or *T*, but cannot evaluate the cross-term effect of the two factors, which is not negligibly small. *Over this restricted range* of t_c the data appear to follow a series of single power laws. Nevertheless there must be a critical temperature for each concentration and *vice versa*, a point clarified later.

On the other hand, the effects of both *c* and *T* including their cross-term are properly assessed in our model. Here we examined the model by comparison with the experimental data of BSA. In the second paper, we will extend the model to other (including mixed) systems. The calculation was carried out by multiple regression with eq A-13, because experimental errors of ln t_c were assumed to follow the normal distribution. The data used for the fitting were BSA samples which were measured at various levels of concentration and temperature. The software package used was JMP 2.0 for the Macintosh (SAS Institute Inc, Cary, NC). At this point it should be re-emphasized that the model is essentially empirical, and we do not attach real significance to the values of the parameters, not the least because of the relatively restricted range over which reliable data can be collected.

The appropriate fitting curves are also displayed in Figures 5 and 6. The concentration dependence of t_c

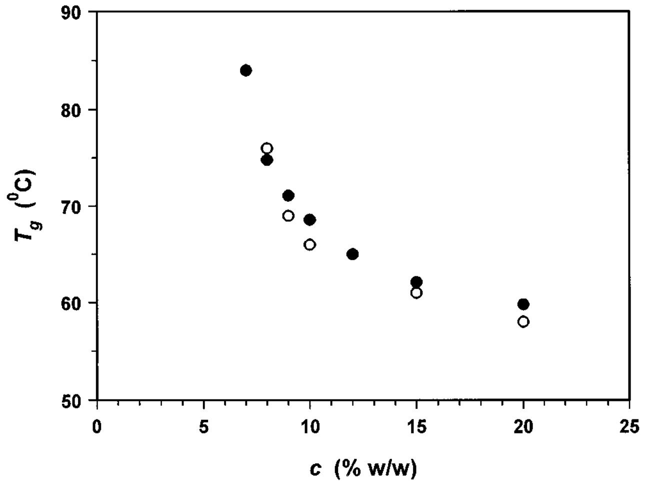

Figure 7. Gelation temperature vs BSA concentration. Key: open circle, constant rate heating (1 °C/min); filled circle, isothermal heating with time threshold of 1 min.

for BSA seems to be explained by the model with satisfactory accuracy. Although the slight curvature of the data at 61 and 56 °C was not reproduced well, the slope of each level is estimated successfully. For these BSA gels, the temperature dependence is also described quite satisfactorily by the fitting curves. Although slight deviations appear in the low concentration range, there seems no systematic error at any temperature. This confirms that the regression was carried out properly.

Figure 6 shows t_c at different temperatures T plotted against concentration c. This indicates that t_c became longer with the decrease in c, and in this case t_c can be seen to follow a power law in c. It is suggested that the concentration dependence of t_c became much stronger with the increase in T, as indeed it must as the critical gel temperature is approached. Also included are the theoretical lines calculated using eq A-13.

From the theory viewpoint, the interaction between c and T is well described by the additional term, although we have not found a clear explanation of its physical meaning. In practice, determination of coefficients need to be carried out in the logarithmic form (eq A-13), but not in the nonlinear form (eq A-14), because we assume that errors in $\ln(t_c)$ follow a normal distribution.

4.4. Gelation Processes Under a Constant Heating Rate. In Figure 7, T_gs are shown for different concentrations c. This indicates that the temperature T_g increased with the decrease in c. In this figure, T_g determined by isothermal heating is plotted. Figure 7 also shows the results for the 10% w/w BSA sample heated at a constant rate (1 °C/min). The changes in dynamic moduli were very similar to those seen by isothermal heating (Figure 1), and we can determine gelation temperature T_g in the same way; i.e., a rapid increase in G' is regarded as the gelation point. In this case, the time threshold was 1 min. The results shown in Figure 7 suggest that the experiment of heating at a constant low rate of 1 °C/min gave nearly the same value of gelation temperature as that by isothermal heating. This means that constant rate heating *can* be used to determine the gelation point. However, this method may cause other problems, because the temperature increase during the measurement may change experimental conditions, e.g. sample volume and plate gap. These changes cause artifacts, in particular, when the temperature range is large. It can also create other problems in analyzing data. As the reaction kinetics is usually strongly dependent on temperature, it is necessary to consider the thermal history at different temperatures. Such an analysis would not be easy to make. Consequently, we conclude that gelation points are best determined by isothermal heating.

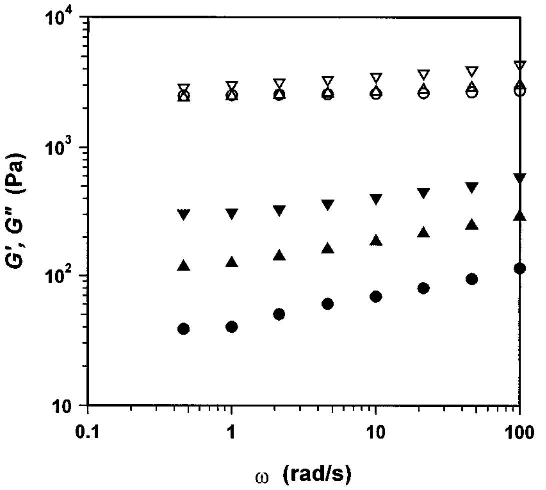

Figure 8. G' and G'' of fully cured gel vs frequency. BSA concentration: 10% w/w, heated at 70 °C for 6 h. Strain: 5%. Key: G', open symbols; G'', closed symbols. Measurement temperatures are as follows: 70 °C, circles; 50 °C, triangle up; 30 °C, triangle down.

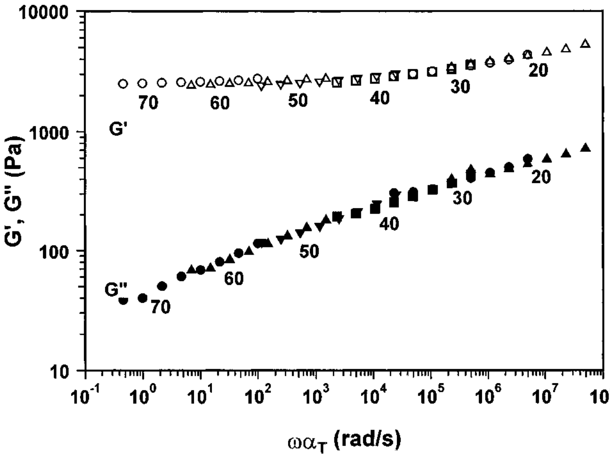

Figure 9. Time–temperature superposition master curve of BSA gel from Figure 8. Reduced temperature: 70 °C. Key: open symbols, G'; closed symbols, G''. Measurement temperatures are as follows: 70 °C, circles; 60 °C, triangle up; 50 °C, triangle down; 40 °C, squares; 30 °C, circles; 20 °C, triangle up.

4.5. Viscoelasticity of Fully Cured Gels. The frequency (ω) dependence of G' and G'' for a BSA gel fully cured at 70 °C was shown in Figure 8. The different symbols represent various temperatures at which the sample was maintained. The figure shows that both G' and G'' increased as the temperature was lowered. The change was more significant in G″ than in G'. From this a time–temperature superposition master curve (Figure 9) was calculated. The result seems satisfactory, and the shape of the curve is typical for polymer gels. It does not show any real evidence of a high frequency glass transition, and any (creep) flow must be restricted to very low frequencies. It can be concluded that in the temperature range from 20 to 70 °C the gel structure did not change significantly.

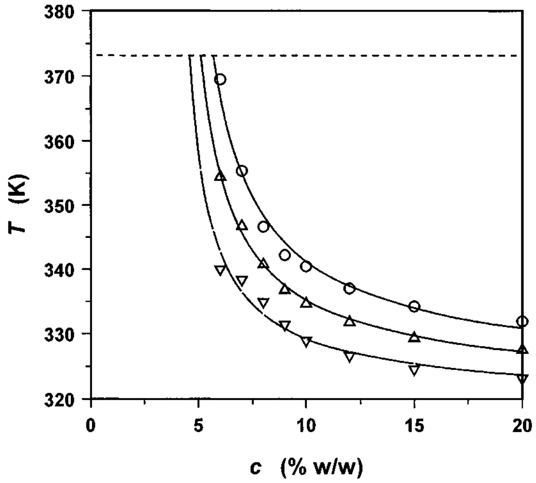

Figure 10. Temperature vs concentration state diagram for BSA illustrating the sol/gel boundary. Symbols represent threshold times for gelation (circles, 100 s; triangle up, 1000 s; triangle down, 10000 s); curves were obtained from eq A-13. The dashed line indicates the boiling point of the aqueous solvent.

As mentioned above, dialysis treatment did not affect t_c significantly. As will be mentioned in the following paper, the t_c of BSA is very sensitive to the presence of salt such as sodium chloride, in particular, when the protein concentration is lower than 10% w/w. If the salt had been removed by dialysis treatment, t_c would change significantly. However, this experiment, illustrated in Figure 7, showed that there was no difference in t_c between the two samples, and we can conclude that the salt residue in BSA was not removed by the dialysis treatment. This may be because BSA has a number of ionic groups in its peptide chain. If the salt is firmly bound to the polymer, it would not be removed by ordinary desalination methods such as dialysis. As far as t_c is concerned, it can be said that the effect of the remaining salts is negligibly small. This helps to confirm our strategy of using commercial products without further purification.

4.6. State Diagrams. We have demonstrated that gelation time t_c can be estimated accurately by our model. Although t_c can be described as a function of concentration c and temperature T, it is also possible to determine a form of a state diagram by the model. Here we shall demonstrate the procedure and compare the results with other theoretically predicted diagrams.

The relationship between gelation time and the two factors, c and T, was already shown in Figures 5 and 6. Consequently, if we examine these curves after a certain gelation time, e.g., 100 s, we can calculate points furnishing different c and T factors, to give this threshold time. In other words, the gel is formed under the given condition of c and T if the sample is maintained for the threshold time of 100 s.

Plotting T against c gives a diagram as shown in Figure 10. Here the threshold times were chosen as 100, 1000, and 10000 s. In the diagram the upper region represents the sample in the gel state and the lower the sol state. The boundary between the sol and the gel regions corresponds to the so-called gelation curve.

With this diagram we can predict the possibility of gel formation for any given concentration and temperature. For example, if a 10% w/w BSA solution is heated at a temperature higher than 63 °C, which is shown in the gel region at 1000 s in the diagram, it is expected to form gels after 1000 s. The critical parameters, i.e., concentration and temperature, can also be estimated by giving time and one of the two parameters. In this way, the state of a sample, sol or gel, can be determined fairly accurately with this diagram.

Experimental studies on this type of state diagram were carried out by several workers, e.g. by Kawanishi et al.[25] and Tan et al.[26] for synthetic polymers, and by Tanaka et al. for biopolymers.[27] In the latter studies the gelation curves, or sol/gel boundaries, were determined by use of rheological techniques such as rheometry and viscometry. Some of them showed coexistence, binodal, or spinodal lines, which were determined by means of optical methods. In most of these diagrams, the two-phase region, as well as the gel region, was located on the lower temperature side; i.e., a two-phase region exists at lower temperatures, giving the upper critical solution temperature (UCST) diagram. In contrast, when the two-phase region is located on the higher temperature side, we have a lower critical solution temperature (LCST) phase diagram.

For BSA, the gel region was on the higher temperature side. Although a two-phase region has not been observed in this study, globular proteins are believed to have an LCST phase diagram. Indeed early work by one of the present authors included state diagrams for the BSA system (although, of course, for a different batch of samples) as a function of pH and ionic strength, which were determined using both optical methods and small-angle X-ray scattering and published as long ago as 1981.[28] More recent work by San Biagio and coworkers suggests that complex LCST-type phase behavior (here attributed to spinodal demixing) is seen for the unfolded BSA, and does not appear to be present with the native protein.[29] As Tanaka and collaborators[27] showed with the gelatin in water/methanol system, two-phase and spinodal regions might appear if the quality of the solvent is changed. This can be achieved in practice by changing pH, adding salt, or adding organic solvents. Such phase separation behavior may give us useful information about the inhomogeneous structure of protein gels, often observed by microscopy.[4,30]

There are very few experimental studies on LCST phase diagrams of gel systems. Eliassaf and Silberberg[31] examined the phase behavior of poly(methacrylic acid) and determined a diagram from shear modulus and turbidity. With the same polymer, Nakamura and co-workers[32] tried determining the phase diagram from the change in the shear modulus. Although the position of the gelation and coexistence curves in the diagram seemed slightly different from those of previous workers, qualitatively they look the same. These diagrams also appear similar to those of BSA obtained in the present study, i.e. the gelation curve slopes upward with the decrease in concentration.

These earlier experimental works on phase diagrams were performed without considering time effects, or kinetics, in the measurement of gelation and phase separation. It may be said that the results merely describe the state at a certain time. Since polymer systems are known to need a very long time to reach equilibrium (usually on the order of days), such measurement conditions cannot always be achieved in practice. Therefore it is reasonable to regard most of the diagrams experimentally obtained as representing merely a pseudoequilibrium state.

In contrast the theoretical models described earlier, and in the Appendix, were constructed on the assumption that the system has reached an equilibrium. Therefore it is not possible to regard the experimental results, or curves derived from them, as representing a true phase diagram. Nevertheless we find that this representation of the behavior of our protein samples does coincide with those derived from thermodynamic theory, at least qualitatively.

The kinetics of phase behavior has became more important in recent years. In polymer morphology, an understanding of the kinetics of phase transformation, such as spinodal decomposition and nucleation, is now thought to be essential.[33] For such systems, the reaction is much more complex; the gelation and the phase separation occur cooperatively, and a number of problems concerning phase behavior and its kinetics remain to be solved.

As can been seen above, however, the gelation temperature can be described successfully by a function of time and concentration and very acceptable fits obtained. By giving appropriate parameters, we determined the sol/gel boundaries as shown with the lines in Figure 10 for BSA. Overall it seems that experimental results are reproduced fairly well, although the deviations became larger near the critical regions in which gel cannot be formed.

4.7. Significance of the New Model. Although the model was derived by a rather simple procedure, it can explain experimental data quite satisfactorily. It should be re-emphasised that the model can successfully estimate the gelation time in terms of both the protein concentration c and temperature T; this is something which could not be achieved by any previous model.

We are aware, of course, that there are several problems not addressed by the model. They are, for example, as follows.

1. Critical conditions which are predicted by gelation theories have to exist, such as the critical concentration C_0 and the critical temperature T_g. In our model, N_{b0} is introduced as a critical parameter but does not appear in the final equation.

2. The protein concentration c and the temperature T have to satisfy physical requirements which depend entirely on the system. In our protein/water systems, they can be written as follows: $0 < c < 1$ (w/w), $t_c = 0$ at $c = 1$; $t_c = \infty$ at $c = C_0$. $273.15 < T < 373.15$ (K); $t = 0$ at $T = 373.15$; $t_c = \infty$ at $T = T_g$. These conditions are not treated in our model.

3. The gelation process may proceed in several steps such as denaturation and aggregation. The model does not describe the details of such reactions.

Problems 1 and 2 may be solved by introducing into the model more parameters which describe critical points, such as T_g and C_0. However, not only does this procedure increase the number of variables but it also makes calculation more complicated. Determination of these variables requires more experimental data, in particular, close enough to the critical point. This is very difficult to achieve in practice. Even if such parameters are determined from experimental data which are obtained relatively far away from the critical conditions, they do not have a physically important meaning. Consequently, we must conclude that as the experimental data which can be used for comparison have not yet been obtained, it is reasonable not to introduce any parameters relevant to critical conditions. Nevertheless, it should be noted that the model can evaluate the parameters near critical conditions.

Problem 3 is also very difficult to deal with at this stage. It certainly is possible to construct detailed reaction models which can describe each step, e.g. denaturation and aggregation. In order to achieve this, however, the model needs to have more variables to be determined by other experiments. This is a problem because in this study gelation time is the only representative parameter which can be measured. Obviously estimation of the rate constants at each process requires more detailed data such as the concentration of intermediate products. As such data have not yet been obtained so far, we decided not to discuss in our model the detailed steps of the gelation process.

Because of the neglect of the above problems, the model obviously fails to give reasonable results under some critical conditions. For instance, the values of t_c at zero and infinity should be predicted by the model. The former corresponds to the system in which gel formation occurs immediately, and therefore its kinetics is determined by the diffusion of polymer particles. In the latter case, on the other hand, the situation is that a gel is formed very slowly and its process is dependent on the rate of junction formation. These may be understood in terms of fractal models as the diffusion-limited aggregation and reaction-limited aggregation, respectively. These phenomena have already been observed for a number of colloidal systems and also explained theoretically. Similar behavior is expected to occur for our globular protein systems. However, the model proposed here cannot predict the process at which t_c becomes zero or infinity, even at rather moderate conditions. At this stage, the problem has to be left unanswered. Consequently, the model for gelation kinetics proposed here is simply a qualitative description of the phenomena, and is not one constructed from formal principles. Here the recent approach of Tanaka and co-workers,[34-37] and specifically the recent extension to f-functional systems[38] may prove invaluable.

5. Conclusions

In this paper we have investigated the heat-induced gelation behavior of a single component globular protein, BSA, using a dynamic oscillatory rheometer. The effects of two important factors, concentration and temperature, on gelation time were clarified. We also found several points to which more attention should be paid, such as the effect of heating rate. Gelation kinetics were evaluated from the gelation time. The effects of temperature and concentration were examined and the results were shown as a diagram. The diagram suggested that BSA may have an LCST phase diagram.

The time effects on phase behavior have not been examined in previous studies. The present study showed that the phase behavior can be described more accurately by considering reaction kinetics. This approach is expected to apply to other systems: not only biopolymers but also synthetics. Although the precise gelation mechanism has not yet been clarified in this study, it will be considered in future work.

Appendix

A1. Construction of a New Combined Model for the Concentration and Temperature Dependence of Gelation Time. The different equations introduced in Section 2 treat gelation time as an individual function of each factor, i.e. concentration and temperature.

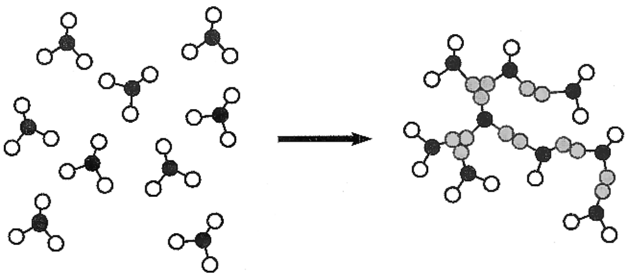

Figure 11. Illustration of gelation model. Open circles represent unreacted sites, while shaded circles represent reacted ones. In this case two or three reacted sites form one junction.

However, it was found, for the first time in the experimental part of this study, that the cross-term effect, or interaction, of these two factors is not negligible. Therefore, a new model is required that can take account of such a cross-term effect.

Here we derive such a new model. The derivation of equations is made by scaling where applicable, and therefore coefficients are often neglected. We do not consider each step in the reaction but discuss the overall process. This means that detailed mechanistic reactions such as denaturation and aggregation are not explained.

First we assume that gelation is described as an *n*th order irreversible reaction of junction formation as shown in Figure 11. One junction is formed by *n*-fold sites on polymer particles. The process can be explained by two parameters: N_a, the number of active sites of polymers per unit volume, and N_b, the number of junctions composed of *n* reacted sites. As the active sites are assumed to be already reactive enough from the beginning of the process, N_a is thought to decrease simply with time from the initial number of active sites N_{a0} to zero. The initial image of gelation process made here seems similar to that proposed by Tanaka and Stockmayer,[35] although its treatment is quite different.

The gelation process can be written as follows:

$$-\frac{dN_a}{dt} = kN_a^{\,n};\quad N_a = N_{a0} \text{ at } t = 0 \qquad \text{(A-1)}$$

Here *k* is the reaction rate constant, which is later expanded as a function of temperature. The solution of eq A-1 is given as

$$t = \frac{1}{k(n-1)}\left(\frac{1}{N_a^{\,n-1}} - \frac{1}{N_{a0}^{\,n-1}}\right);\quad n \neq 1 \qquad \text{(A-2)}$$

$$= \frac{1}{k}\ln\left(\frac{N_{a0}}{N_a}\right);\quad n = 1 \qquad \text{(A-3)}$$

Here only eq A-2 is used for model construction, because *n* is considered to be higher than unity, as shown later. These equations automatically satisfy the final condition, i.e. $N_a = 0$ at $t = \infty$. The number of junctions N_b is given as

$$nN_b = N_{a0} - N_a \qquad \text{(A-4)}$$

and therefore eq A-2 becomes

$$t = \frac{1}{k(n-1)}\left(\frac{1}{(N_{a0} - nN_b)^{n-1}} - \frac{1}{N_{a0}^{\,n-1}}\right) \qquad \text{(A-5)}$$

Next the process of cluster formation described above needs to be related to gelation time t_c. To achieve this, we here introduce an important assumption that gelation occurs, or an infinitely large cluster is formed, when N_b exceeds a critical value N_{b0}. In other words, a network is assumed to percolate throughout the system, if the junction density becomes higher than a percolation threshold.

In this model, whether the gelation mechanism is described by tree statistics[39] or lattice percolation theory[40] is thought not to affect the gelation kinetics significantly, because such a difference would only change N_{b0} slightly. Then, t_c can be written in terms of N_b as

$$t_c = \frac{1}{k(n-1)}\left(\frac{1}{(N_{a0} - nN_{b0})^{n-1}} - \frac{1}{N_{a0}^{\,n-1}}\right) \qquad \text{(A-6)}$$

If $N_{b0} \gg N_{a0}$ is satisfied, eq A-6 can be shown to give

$$t_c \approx \frac{nN_{b0}}{kN_{a0}^{\,n}} \qquad \text{(A-7)}$$

Here only the first terms of both numerator and denominator on the right hand side are left, because other higher order terms are negligibly small if $N_{b0} \gg N_{a0}$. It is reasonable to assume that N_{a0} is proportional to the protein concentration *c*. By generalizing this equation, we obtain

$$t_c \sim \frac{1}{N_{a0}^{\,n}} \sim \frac{1}{c^n} \qquad \text{(A-8)}$$

Here the notation ∼ is used to express that the two terms can be equated by scaling but not by absolute values, and therefore the coefficients are neglected. This form of the equation is identical to te Nijenhuis' and Oakenfull's models, although we would argue this derivation is more rigorous. It is also clear that eq A-8 is only a limit case, and higher order terms could be retained and exploited, although not without the disadvantage of generating even more coefficients. In particular eq A-8 will *not* apply when N_{b0} is *not* $\gg N_{a0}$, a condition which holds, close to the gel point, for low concentration systems.

The effect of temperature *T* (K) can also be treated by eq A-7. In this equation, t_c is proportional to the reciprocal of the rate constant *k*. We introduce the Arrhenius model to connect *k* with *T* such that

$$k = A\exp\left(\frac{-E_a}{RT}\right) \qquad \text{(A-9)}$$

where E_a is an apparent activation energy and *R* is the gas constant. Therefore, substituting into eq A-7, we obtain

$$t_c \sim \exp\left(\frac{E_a}{RT}\right) \qquad \text{(A-10)}$$

A general formula can be given by the combination of eq A-8 and eq A-10 as follows:

$$t_c \sim \frac{1}{c^n}\exp\left(\frac{E_a}{RT}\right) \qquad \text{(A-11)}$$

For BSA gels experimental errors of t_c do not seem to follow the Gaussian distribution, but those of ln t_c do.

Therefore, by taking natural logarithms and introducing coefficients a_1, a_2, and a_3, eq A-11 gives

$$\ln(t_c) = a_1 + a_2 \ln(c) + a_3 T \tag{A-12}$$

It has been shown above that this equation can explain the temperature dependence and the concentration dependence of gelation time with satisfactory accuracy. However, as mentioned earlier, the model needs to be able to handle the effect of the cross term of ln c and T. In order to solve this problem, we introduce the simplest form for the cross term as shown in

$$\ln(t_c) = a_1 + a_2 \ln(c) + a_3 T + a_4 T \ln(\mathrm{c}) \tag{A-13}$$

where a_4 is a coefficient. By inverting the logarithm, we obtain

$$t_c = a_1 c^{(a_2 + a_3 T)} \exp(a_4 T) \tag{A-14}$$

Here the coefficients are changed accordingly.

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