

Detection of Amyloid Fibril Formation in Hen Egg White Lysozyme Using Dynamic Light Scattering

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ABSTRACT Under certain solution conditions, some protein molecules can aggregate to form long fibril structures known as amyloids. In order to study the formation of amyloid in one protein, hen egg white lysozyme (HEWL), we have built a small, dynamic light scattering apparatus. With this apparatus, we have observed the aggregation of HEWL in a 50 mM acetate buffer (pH 5.5), containing moderate concentrations of 2,2,2-trifluoroethanol (TFE). We used a Congo Red assay to determine whether these aggregates actually consisted of amyloid fibrils.

Introduction

Associated with several pathological disorders, most notably Alzheimer's disease and Bovine Spongiform Encephelopathy ("Mad Cow Disease"), is an abnormal type of protein aggregation. The aggregates the proteins form in these cases are known as amyloid fibrils. The amino acid "backbones" of the protein molecules bind to each other to form long strands, ~30-50 Å in width and of widely varying length [1].

The purpose of this project was to determine whether we could induce the formation of fibrils in a protein that is not associated with any amyloid disease, and if so, to study the kinetics of amyloid fibril formation in that protein. We chose hen egg white lysozyme (HEWL), a readily available small protein of 129 amino acids, for this study. There is some evidence that incubating a protein in a buffer solution containing 2,2,2-trifluoroethanol (TFE) can induce the formation of fibrils, as it stabilizes partially folded states of protein [1]. We planned to measure the rate of formation and size of the amyloid fibrils using dynamic light scattering (DLS), a noninvasive technique from

which detailed data regarding the speed of diffusion, and therefore the size, of macromolecules can be obtained.

Although the technique itself is complicated, the principles behind it are fairly simple. DLS measures the rate at which particles diffuse in a solution by analyzing fluctuations in the intensity of scattered light. The rate of diffusion of particles in solution is directly related to the size of those particles. If the speed of the fluctuations in the intensity of the scattered light can be characterized, the size of the particles can be obtained indirectly.

One motivation for studying the kinetics behind amyloid fibril formation is to understand the requirements for the formation of pathogenic fibrils. In this way, we hope to contribute to the ultimate goal of developing pharmaceutical treatments which will prevent the formation of these fibrils, and therefore alleviate the symptoms and suffering of patients suffering from associated disorders.

Experimentation, Materials, and Methods

In order to eventually proceed to test the rate of amyloid fibril formation we constructed a DLS apparatus. A diagram of the experimental setup appears in figure 1. The protein sample scatters the focused beam of a HeNe laser (wavelength = 632.8 nm; power = 5 mW), and an optical system consisting of lenses, irises, and filters collects and focuses this scattered light onto a detector. To convert the scattered light to an electrical signal we used an EG&G Optoelectronics model C30902E silicon avalanche photodiode (APD). This device is a sensitive detector of light at 632.8 nm. When scattered light strikes the light-sensitive surface of the device, it produces a small signal current of approximately 25 nA when biased with approximately 180 V. This current must be

converted to a voltage and amplified prior to measurement, due to the presence of various types of noise in the electronic equipment. To solve this problem we constructed a circuit which would first convert a ~ 10 nA AC current to a 0.1 V AC signal, and then amplify that 0.1 V signal to 3 V. The circuit diagram is shown in figure 2.

We found when testing the DLS apparatus that the exact alignment of every element of the setup was crucial. A misalignment of the laser, or of any mirror or lens resulted in a significant loss of laser light. The test solution was obviously unable to scatter any lost laser light which did not travel through the sample in the test tube. We found that a marginal misalignment of any element in the apparatus by a millimeter or less could easily result in an unobservable signal, even with the amplifying circuitry in place.

The positions of the first two focusing lenses were especially crucial, as these lenses ensure that the laser beam focuses to a well-defined point inside the test tube. We aligned these lenses with the aid of a flip mirror (see fig. 1) that reflected an image of the beam focal region onto a CCD camera, which produced an image of the sample on a monitor.

Stray light was another problem. We controlled this problem by constructing a light tight box of opaque PVC board which enclosed the optical table. Because cables were needed to carry power to the amplifying circuitry, the APD, and the signal from the APD to the oscilloscope, we installed BNC coaxial cable connectors in the box, and drilled small holes for the power cables. We ensured that these resulted in a minimum of stray light entering the box. We utilized a RG 610 Schott Glass Light Filter to absorb any stray light with a wavelength shorter than 610 nm from entering the APD.

Once the DLS setup was constructed, we tested particles of known size dissolved in solution. We chose to test latex polystyrene beads [from Sigma-Aldrich], diameter 1.09 μm suspended in solution, at a concentration of approximately 0.25 μL of 10% microsphere solution for every 2 ml of distilled, deionized water. These solutions provided reliable results for only a few hours after mixing. After that period of time, the spheres began to settle out of solution, and come to rest on the bottom of the test tube.

After confirming that the light scattering signal of the polystyrene spheres was consistent with our expectations, we tested HEWL. We worked with protein concentrations varying from 1 mg/mL to 13 mg/mL (70 μM to 909 μM) dissolved in a 50 mM acetate buffer, pH 5.5, containing dissolved TFE in concentrations ranging from 15% to 30%. Initially, dust interfered with our light scattering signal. We determined that our glassware, particularly the disposable borosilicate glass test tubes holding the protein solutions, was significantly contaminated with small particles. We began twice rinsing each piece of glassware with HPLC grade isopropyl alcohol. We used the following rinsing method: we filled each container roughly half full with isopropyl alcohol and agitated the solution for a few seconds. We then inverted each container, so that its opening was facing down, emptying the solution from the container. Without inverting the container, we next rinsed the sides of each container again with isopropyl alcohol using liquid chemical dispensing spray bottles. Each container was then allowed to dry upside down until all the remaining isopropyl alcohol had evaporated. This greatly reduced the small particle contamination.

We prepared each individual protein solution by first preparing a small amount of a 3.5 mM HEWL-buffer solution, and a separate buffer-TFE solution of the desired TFE

concentration. We filtered each solution separately before combining them. We filtered the buffer-TFE solution using Whatman GD/X PVDF hydrophilic membrane 0.2 μm pore size syringe filters, and the HEWL-buffer solution using Whatman Anatotop 10 hydrophilic 0.02 μm pore size syringe filters. We ensured that each solution was not exposed to dust from the air by covering each piece of glassware with parafilm.

We next measured the DLS signal from each solution. We took DLS measurements within thirty minutes of combining the protein and TFE solutions. Each autocorrelation function measured is an average of the autocorrelation from approximately 400 oscilloscope traces, requiring roughly 5 minutes altogether. For each autocorrelation function recorded, we recorded a background autocorrelation function with the laser beam blocked, so that we only observed the portion of the signal not due to the light scattering. When analyzing the data, we then subtracted each background autocorrelation function from the associated autocorrelation function for a protein sample. This allowed us to eliminate the significant 60 Hz and other noise which was still present in our data.

Every photon that strikes the light sensitive-surface of the APD produces a small electric current. We have taken great pains to ensure that only photons originally emitted from the laser and scattered by the solution in question reach the photodiode. So, it is a reasonable assumption that the signal produced by the APD accurately describes the scattered light originating from the test tube at an angle of 90° to the incident laser light. The light is scattered by individual particles in solution. Because there are many particles in solution, and many more photons moving through the solution, a very large number of scattering incidents will occur. Each scattering incident can be viewed as a spherical

light wave emanating from the particle responsible for the scattering. These waves will interfere constructively or destructively to produce a time-varying pattern of light and dark spots of varying intensity at a distance from the test tube. It is this pattern that falls on the light-sensitive surface of the APD. Because all of these particles are diffusing randomly due to Brownian motion, the intensity of the light falling on the APD also changes randomly with time. Large particles diffuse slowly and produce a slowly changing pattern. Small particles diffuse quickly and produce a quickly changing pattern.

Without dwelling too heavily on the mathematics involved, we will here give a brief summary of how each autocorrelation function, $g(\Delta t)$, was obtained from the intensity fluctuations of the interference pattern. There are two portions of the measured intensity of this interference pattern: a time varying intensity fluctuation, $I(t)$, and a constant intensity, I_o . Because we were only interested in the time varying component of the total intensity, we subtracted out I_o so that we were only left with $I(t)$. It was this portion of the signal that we analyzed. If the time varying intensity signal at some time, $I(t)$, is multiplied by the time varying intensity signal at some later time, $I(t+\Delta t)$, the average of this product will approach zero as Δt increases. This is because if enough time has passed for the solution particles to diffuse a significant distance, their scattering pattern becomes uncorrelated. For very small values of Δt , the particles have not had time to move very far; thus, the two signals are not totally different, and when they are averaged together there will be some positive average intensity.

If this calculation is done for many values of Δt , and repeated many times averaging the most recent result with all previous results, we will obtain an autocorrelation function, $g(\Delta t)$. This function can be analyzed to obtain a characteristic

time after which two light-scattering signals are sufficiently different that they average to be zero. This characteristic time changes as the size of the particle changes. A long characteristic time corresponds to very large particles, and a short characteristic time corresponds to very small particles. This process allows the size of macromolecules to be obtained noninvasively.

Mathematically, the autocorrelation function $g(\Delta t)$ is defined

$$g(\Delta t) = \lim_{T \rightarrow \infty} \frac{1}{2T} \int_{-T}^T I^*(t) I(t + \Delta t) dt \quad (1)$$

where $I(t)$ is the time varying intensity signal from the APD observed at time t . For simplicity we will write

$$g(\Delta t) = \langle I^*(t) I(t + \Delta t) \rangle \quad (2)$$

The autocorrelation calculated for $\Delta t = 0$ is therefore simply the mean-squared signal. For $\Delta t > 0$, $g(\Delta t)$ should be a decaying function of Δt , for reasons stated above. We programmed this calculation into our *LabView* data collection software, and used *Matlab* to perform a nonlinear least squares fits of the resulting autocorrelation functions. For a monodisperse (ie. single particle size) solution, we expect the autocorrelation function to obey an exponential function:

$$g_I(\Delta t) = A_0 e^{-\frac{\Delta t}{\tau}} + B_0 \quad (3).$$

Where t is time, and τ is a characteristic time constant associated with the exponential which is defined

$$\frac{1}{\tau} = \frac{32\pi n^2 I_b T}{3\eta(\lambda_o)^2} \cdot \frac{\sin^2(\theta/2)}{d} \quad (4).$$

Here, n is the index of refraction of the medium, I_b is Boltzmann's constant for the medium, η is the medium's viscosity, T is the temperature in Kelvin, λ_o is the wavelength of the incident light in the medium, θ is the angle at which the scattered light is observed, and d is the diameter of the particles scattering the light [2].

This method of analysis works well for the polystyrene spheres, but the proteins aggregate in an imperfect manner; as time passes, particles of all different sizes become present in the solution. This means that the autocorrelation function becomes the sum of a number of decaying exponential functions with different time constants, τ_1, τ_2, \dots . In such cases, an alternative approach is to analyze the autocorrelation function by the method of cumulants [3]. This method involves obtaining the slope of the log of the autocorrelation function in the limit that τ goes to zero, and thus provides an approximate average relaxation time for a non-exponential autocorrelation function. What is observed is actually the intensity of the light scattering, which is the square of the electric field. To account for this fact, we used the square root of the data taken for the cumulant analysis. Following Schmitz's definition for a solution containing many different sizes of particles (Where $g(t)$ is the autocorrelation function):

$$g(t) = \sum_{i=1}^M a_i e^{-\frac{t}{\tau_i}} + b_i \quad (5).$$

Using the expansion approximation for the exponential:

$$\ln(g(t) - b_i) \approx -K_1 t + \frac{1}{2} K_2 t^2 - \frac{1}{6} K_3 t^3 + \dots \quad (6)$$

Taking the derivative of this term, in the limit of $t \rightarrow 0$:

$$\frac{d}{dt} (\ln(g(t) - b_i)) \approx -K_1 \quad (7)$$

where

$$-K_1 = \frac{\tau}{2} \quad (8).$$

We used the Congo Red dyeing technique provided by Wetzel in order to confirm that the HEWL was indeed forming amyloid fibrils instead of a different type of aggregate. This method is one of the most widely used and accepted techniques for confirming the presence of amyloid fibrils. When amyloid fibrils are present, the Congo Red dye exhibits an “apple green” birefringence when the sample is examined between crossed polarizers [4].

Results

The first results we present are those obtained from light scattering by the 1 micron polystyrene spheres. Figure four shows the autocorrelation function of a solution containing 0.25 μL of a 10% microsphere solution dissolved in 2 mL of distilled, deionized water. The data were taken approximately 5 minutes after the two solutions were combined. Using equation (4), we were able to predict the value of τ that an ideal exponential fit would provide for the autocorrelation function of the spheres. The value provided by the nonlinear least squares fit in *Matlab* was 3.89 ms. The predicted value was 3.8 ms. The experimental difference between the characteristic times is approximately 2%.

The first protein solution we analyzed was 15% TFE, with 7 mg/ml HEWL at pH 5.5. The proteins in this solution did not aggregate at all and provided no measurable fluctuations in the light scattering signal. No autocorrelation functions could be obtained from this solution. Even slightly higher TFE concentrations, however, yielded surprisingly different results.

Figure 5 shows a typical autocorrelation function, from light scattered by a solution containing 7 mg/ml (489 μ M) HEWL with a TFE concentration of 20%. The data was taken 30 minutes after the protein was combined with the TFE/buffer solution. Like the autocorrelation function of the spheres, this function closely matches an exponential function, and is easily fitted to equation (3). The exponential fit provided by *Matlab* is graphed over the data. In this case the fit provided by Matlab gives a value for τ of 3.4 ms. The value for τ provided by the cumulant method of analysis for this example is 5.6 ms. This curve is an example of an autocorrelation function that closely fits an exponential curve: we can summarize that the particles are all still close to the same size.

Figure 6 shows the autocorrelation function of the same solution, taken 10 hours after the mixing of the HEWL and the TFE/buffer solution. This curve was not described well by a single exponential, presumably because aggregates of many different sizes were present in the solution. Because of this wide distribution of particle sizes, the exponential fit provided by the least squares method in Matlab was poor. This can be clearly seen by the exponential fit plotted over the data. The characteristic time provided by the cumulant analysis method in this case is $\tau = 17.6$ ms. Similar results were obtained for data taken on protein solutions with a HEWL concentration of 7 mg/ml, and TFE concentrations of 18% and 20%.

Figure 7 summarizes the characteristic time, τ , as a function of the total aggregation time for several solutions of identical protein concentrations but differing TFE concentrations. The graph clearly indicates that the rate of aggregation of HEWL is dependent upon the concentration of TFE in solution.

Discussion

Despite the early problems with dust and the alignment of the laser beam, towards the end of the program we began to see very good results. The Congo Red dyeing results were inconclusive, though this may have been because the general Congo Red dyeing methods available were designed for dyeing tissue samples containing amyloid fibrils, and our protein aggregates were in solution. The next step would be to use a fluorescence dye test with the dye Thioflavin T. In the presence of amyloid fibrils the emission spectra of the dye is red shifted 120 nm [1]. Although at the moment we have not yet confirmed that the aggregation we have observed is due to the formation of amyloid fibrils, on several occasions we recorded protein aggregation in solution on a time scale of hours. These preliminary results are very encouraging. If we had more time to continue this project, we would simply continue to test solutions at various levels of HEWL and TFE concentrations. At the moment we have only extensively studied a solution with a HEWL concentration of 7 mg/ml (489 μ M) at TFE concentrations of 24%, 20%, 18%, and 15%. We have shown that somewhere between 18% and 15% there is a TFE concentration below which HEWL will not form any type of aggregate.

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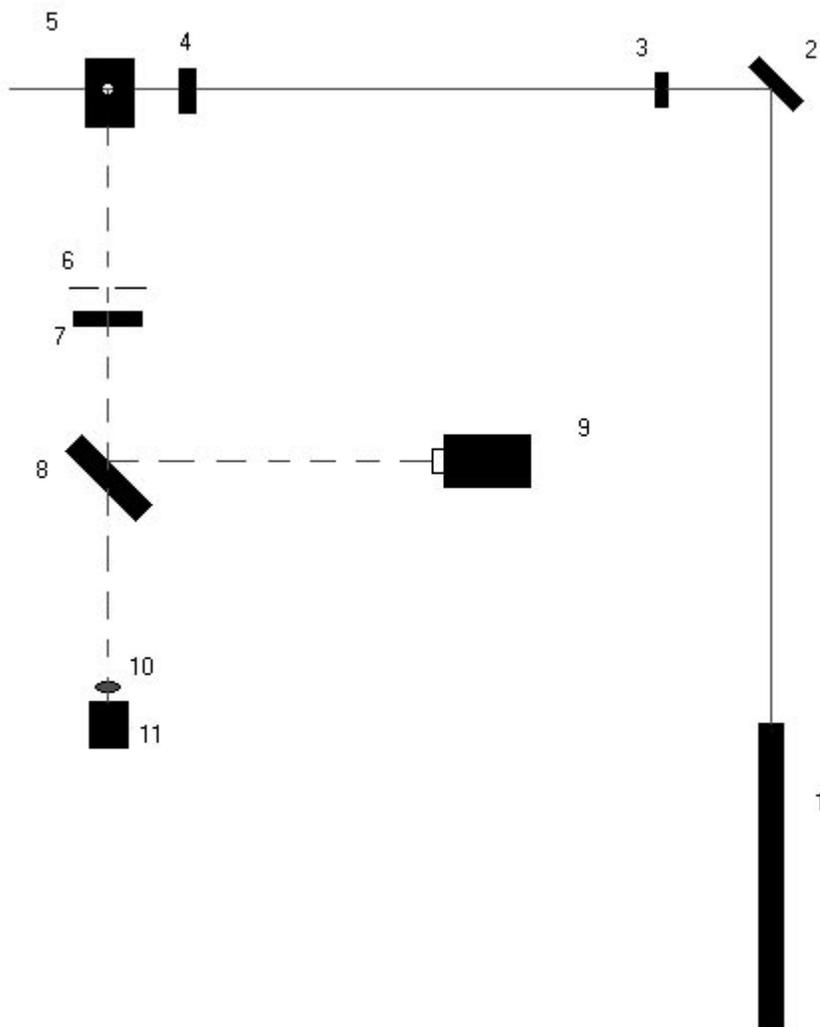


Figure 1. 1: 50 mW HeNe 632.8 nm laser. 2: mirror. 3: silica lens, diameter = 0.5 in., focal length = 16 mm. 4: Focusing lens, diameter = 0.5 in., focal length = 19 mm. 5: Aluminum block designed to hold the test tubes which in turn held the solutions. 6: Adjustable iris. 7: focusing lens, diameter = 1 inch, focal length = 10 cm. 8: Flip mirror. 9: CCD (charge coupled device) camera. 10: RG 610 Schott Glass Light Filter. 11: Avalanche Photodiode. This setup was constructed on an optical table. We employed fixed and adjustable optical mounts to secure every element of the apparatus. The APD was placed in an adjustable mount, which allowed us to center its light sensitive surface on the focused image.

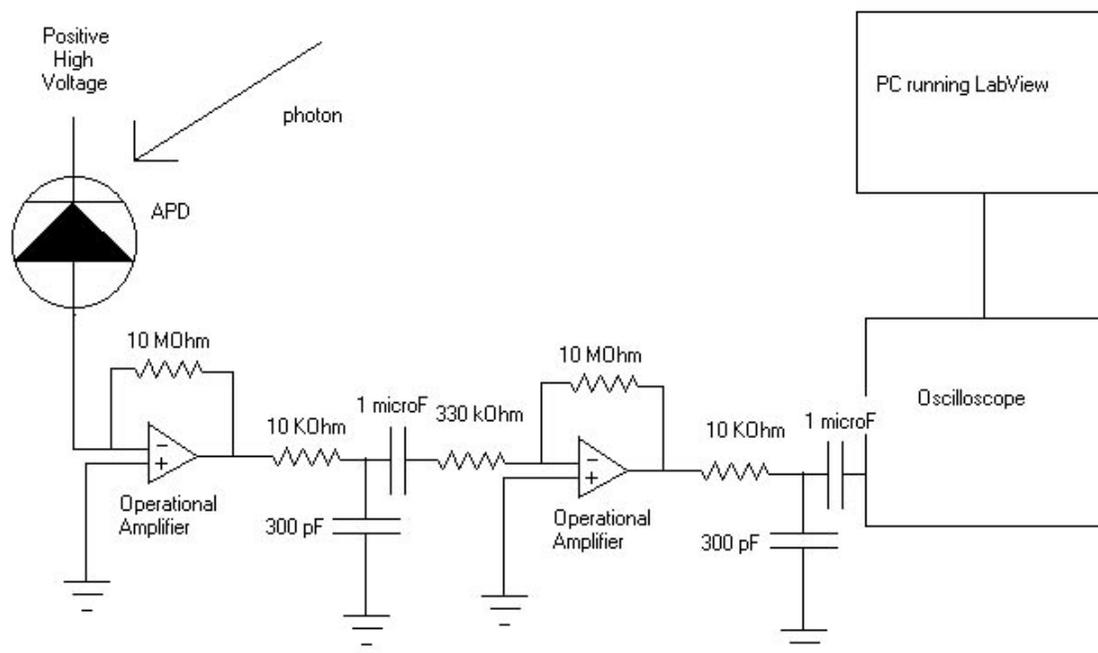


Figure 2. The circuit diagram of the amplifying circuitry shown above is fairly self-explanatory. Shown below is the pin connection diagram of the operational amplifier. We used the Motorola MC1741C semiconductor Op. Amp

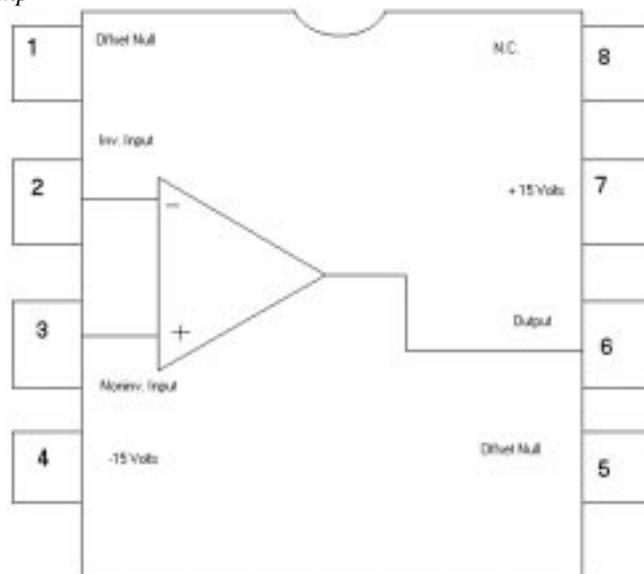


Figure 3
(Top View)

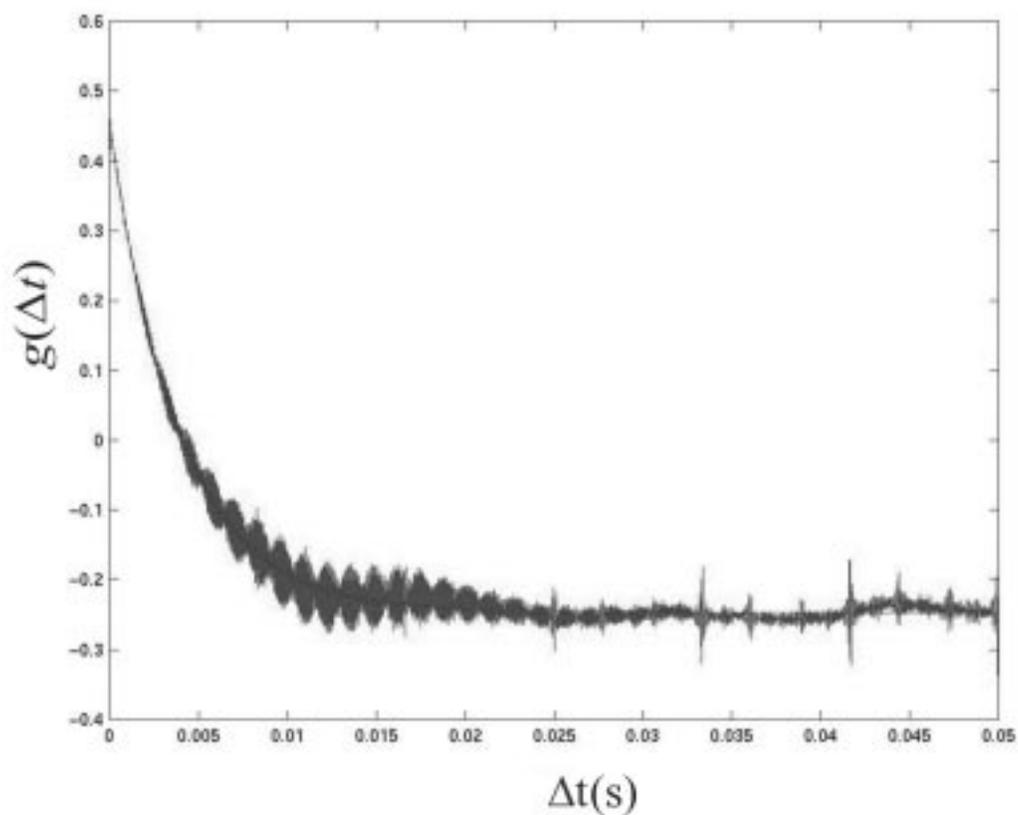


Figure 4. Shown is the autocorrelation function, $g(\Delta t)$, for $1.09 \mu\text{m}$ polystyrene spheres in water, scattering HeNe laser light (632.8 nm) at an angle of 90 degrees, at room temperature. Values of τ in seconds are plotted on the x-axis.

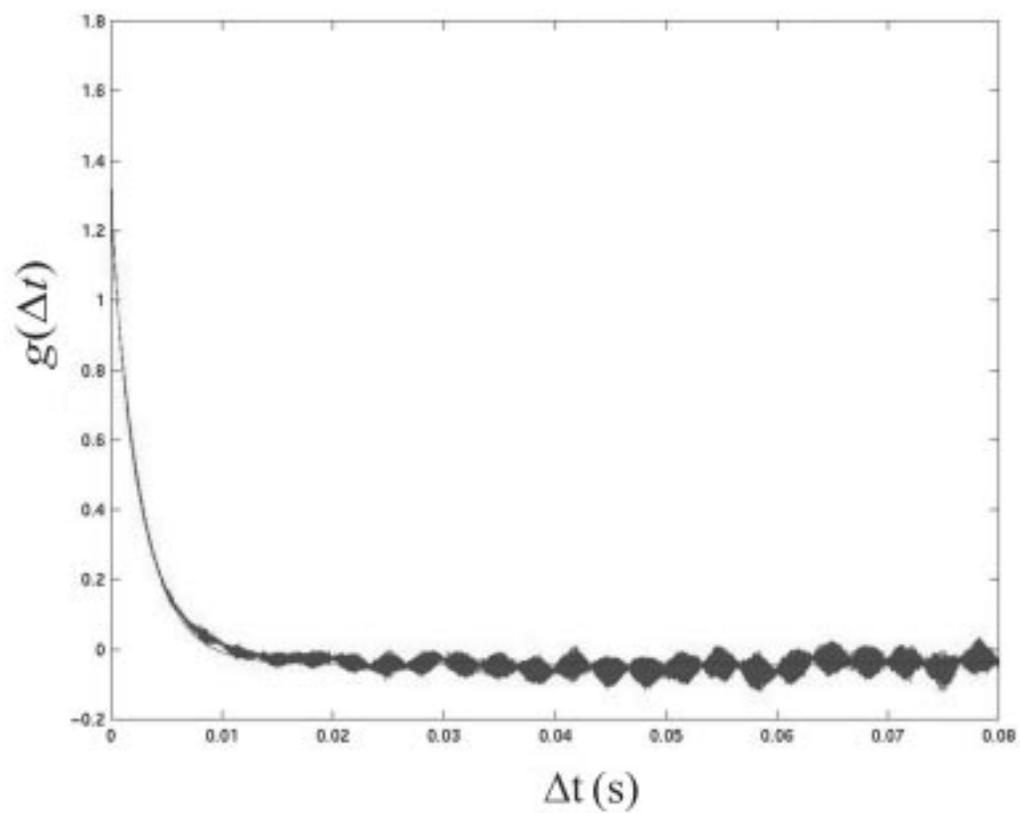


Figure 5. Autocorrelation function $g(\Delta t)$ versus Δt for HEWL in 20% TFE pH 5.5, 7 mg/ml, 30 minutes after the initiation of protein aggregation.

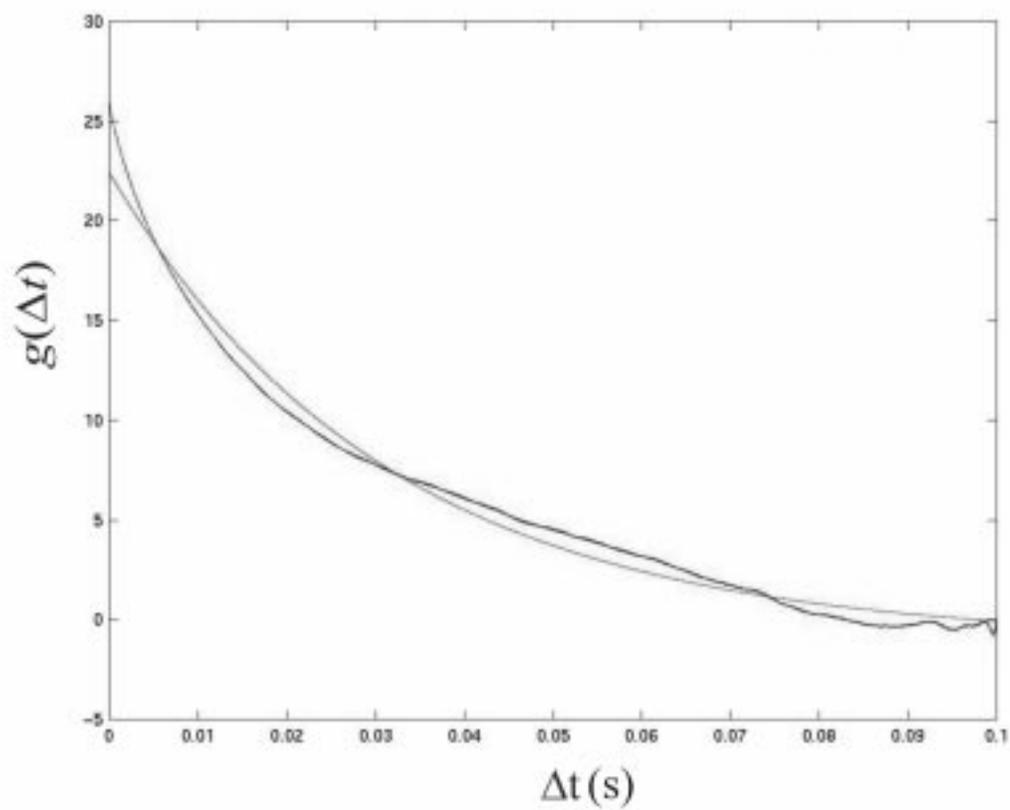


Figure 6. Autocorrelation function $g(\Delta t)$ versus Δt for HEWL in 20% TFE pH 5.5, 7 mg/ml, taken 5 hours after the initiation of aggregation.

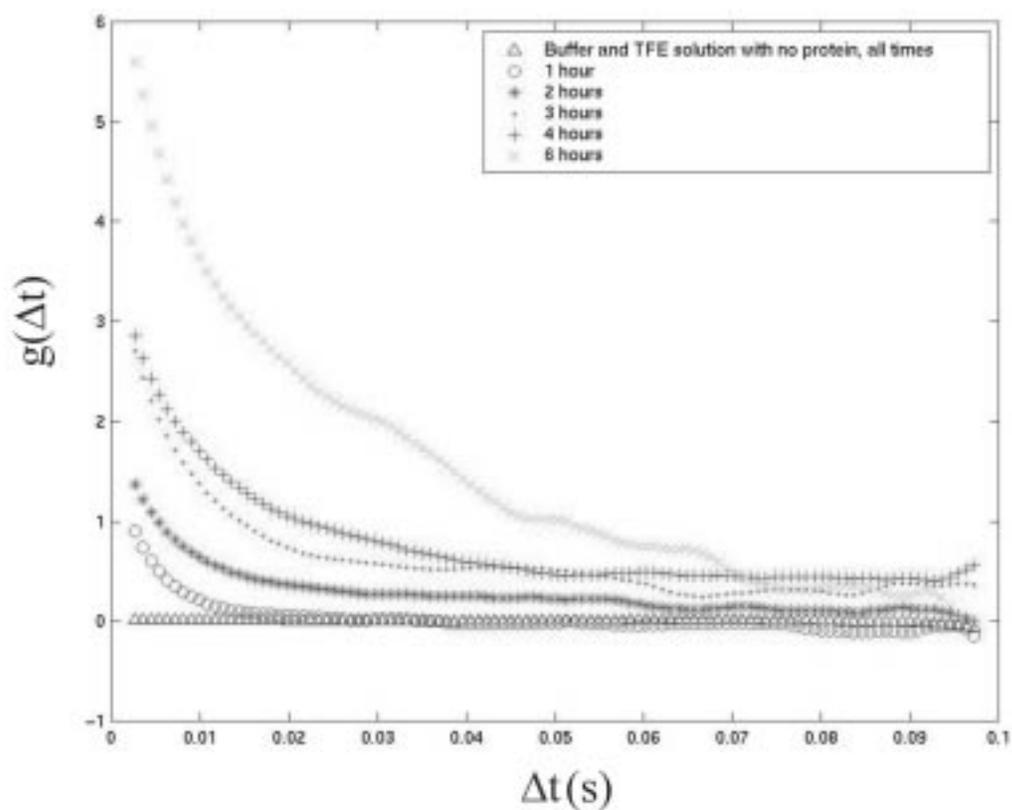


Figure 7. Evolution of the autocorrelation function $g(\tau)$ [24% TFE 7 mg/ml HEWL solution] after initiation of aggregation . The lengthening of the exponential curves indicates that the size of the particles in solution is increasing as a function of time. Similar results were obtained for data taken on protein solutions with a HEWL concentration of 7 mg/ml, and TFE concentrations of 18% and 20%.