2009

Probing interactions and phase separations of proteins, colloids and polymers with light scattering

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Probing Interactions and Phase Separations of Proteins, Colloids, and Polymers with Light Scattering

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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Date of Approval:
June 9, 2009

Keywords: Lysozyme, Nucleation, Microrheology, Viscosity, Nanoparticles

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Acknowledgements

I would like to take this opportunity to express my gratitude to all those who helped me during my research work to finish this thesis.

I am deeply indebted to my advisor Dr Martin Muschol, Associate Professor Department of Physics, for his help, invaluable suggestions, encouragement and his patience, during my research and writing of this thesis. He taught me how to ask questions and express my ideas. I will always be grateful to him for his constructive criticisms during our group meeting. His relentless pursuit of perfection has made me a better researcher. I will always be grateful for his kind support even during personal crisis.

I am also thankful to my committee members Dr Ryan Tomy, Dr Garret Matthews, Dr Dennis Killinger and Dr Lo for stimulating discussions and general advice. I am also very grateful to Dr Pritish Mukherjee, Chair of Department of Physics and Dr. Johnson for their help and advice.

I would also like to give special thanks to Shanon Hill, my fellow lab member for her stimulating discussions and support. I would like to thank the rest of my fellow lab members (both past and present), for their cooperation and all the fun we had in the last five years.

I am also thankful to my friends (Anurag, Avis, and Himanshu) for their emotional support, camaraderie and help they provided.

I would also like to thank my parents and family for their constant support and inspiration. Last but not least, I would also give thanks to my wonderful wife “Jayeeta Lahiri” for her constant support and encouragement.
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Probing Interactions and Phase Separations of Proteins,
Colloids and Polymers with Light Scattering

Avanish S. Parmar

ABSTRACT

The broad objective of my research is to investigate the physical characteristics and interactions of macromolecules and nanoparticles, and the corresponding effects on their phase separation behavior using static and dynamic light scattering (SLS & DLS). Light scattering provides a non-invasive technique for monitoring the in-situ behavior of solutes in solution, including solute interactions, sizes, shapes, aggregation kinetics and even rheological properties of condensed phases.

Initially, we investigated lysozyme solutions for the presence of preformed aggregates and clusters that can distort the kinetics of protein crystal nucleation studies in this important model system for protein crystallization. We found that both undersaturated and supersaturated lysozyme solutions contained population of large, pre-existing protein aggregate. Separating these clusters and analyzing their composition with gel chromatography indicated that these clusters represented pre-formed lysozyme aggregates, and not extrinsic protein contamination.

We investigated the effect of chaotropic versus kosmotropic ions (water structure breakers vs. structure makers) on the hydration layer and hydrodynamic interactions of hen egg white lysozyme. Surprisingly, neither chaotropic nor kosmotropic ions affected the protein hydration layer. Salt-effects on direct and hydrodynamic protein interactions were determined as function of the solutions ionic strength and temperature.

Using both static and dynamic light scattering, we investigated the nucleation of gold nanoparticles forming from supersaturated gold sols. We observed that two well separated populations of nuclei formed essentially simultaneously, with sizes of 3nm vs. several tens of nanometer, respectively.
We explore the use of lysozyme as tracer particle for diffusion-base measurements of electrolyte solutions. We showed that the unusual stability of lysozyme and its enhanced colloidal stability enable viscosity measurement of salts solutions at high salt concentration, over a wide range of pH values and temperatures for the common tracer particle polystyrene flocculates.

We applied dynamic light scattering to measure the viscoelastic responses of polystyrene probe particles embedded in solutions and gels of two different polymers: polyacrylamide (PAAm) and poly-N-isopropylacrylamide (poly-NiPAAm).
Chapter 1

Motivation/Introduction

The broad objective of this thesis is to investigate the physical characteristics and interactions of macromolecules and nanoparticles, their effect on phase separation behavior and to characterize the rheological properties of their condensed phases. Using static (SLS) and dynamic (DLS) light scattering provides a uniquely suited set of experimental technique to perform these tasks: light scattering is intrinsically non-invasive and can be used to measure a very wide range of material characteristics, including solute interaction, particle size distributions and aggregation kinetics, and rheological properties of soft condensed phases. My overall research program was divided between two different projects. The first project investigated the interaction effects and nucleation behavior of proteins and gold sol during solid phase formation. The second set of projects focused on optical, non-invasive approaches towards characterizing rheological properties of aqueous solutions and hydrogels. In the following I will motivate the specific research projects we performed within this broader framework.

X-ray diffraction from high-quality protein crystals remains the most reliable approach for obtaining detailed information about the 3-dimensional structure of proteins. This information is critical for understanding how the spatial structure of these ordered polypeptide polymers supports their biological function. Attempts at high-throughput protein structure determinations, however, have been frustrated by the difficulties of establishing suitable solution conditions for promoting the nucleation and subsequent growth of high-quality crystals. Frequently, the main bottleneck is the initial step of crystal nucleation itself. The kinetics of protein crystal nucleation and the morphology of aggregates were among the earliest targets of fundamental studies in protein crystallization. However, fundamental studies of crystal nucleation, using the common model protein small hen egg white lysozyme, produced inconsistent and contradictory
results. Using dynamic light scattering (DLS), we investigated whether intrinsic heterogeneities in commonly used stock materials of lysozyme might contribute to the observed inconsistencies. Chapter 3 summarizes our research efforts in characterizing different commercial sources of lyophilized lysozyme stock and the effect their pre-existing heterogeneities have on protein crystal nucleation and growth.

Another persistent challenge in protein crystallization is to understand how the choice of precipitant affects the subsequent kinetics of protein crystal nucleation and growth. The specific questions we investigated were whether and how different salt ions affect the protein hydration layer and the hydrodynamic interactions of the protein. The hydration layer is commonly considered an important kinetic barrier toward protein aggregation. Similarly, the kinetics of crystal nucleation and crystal growth could also be affected by the effects of hydrodynamic interactions among the protein molecules. Using a combination of static and dynamic light scattering, we investigated the effect of either chaotropic or kosmotropis ions (i.e. ions that either break or reinforce local water structure) on the hydration layer and hydrodynamic interactions of hen egg white lysozyme under conditions supportive of protein crystallization (Chapter 4).

The nucleation and growth mechanisms of colloid gold particles synthesized from solution is of broad interest to the rapidly growing field of nanoparticle chemistry and physics. However, to fully understand the formation of particles at various levels, it is essential to capture and investigate the early stages of nucleation of these nanoparticles, their growth kinetics and the effect of various solution parameters on this process. We applied static and dynamic light scattering to investigate the unusual nucleation and growth kinetics of gold nanoparticles synthesized from the solution phase in the presence of the antibiotic Cephalexin (Chapter 5).

Local measurements of solution viscosity as function of various solution parameters (temperature, pH, solute type and concentration) is often critical for characterizing solute transport in solution. Many commonly used viscosity measurements require bulk samples, can be time consuming, require considerable heating power and thermal equilibration times due to the large thermal capacity of common liquids, and often require independent measurements of solution density in order to obtain viscosity values. As an alternative and nonintrusive method, we used diffusion measurements of
nanoscopic tracer particles to obtain the viscosity of saline solutions as a function of salt type, salt concentration and solution temperature. We compared the performance of two different types of tracer particles to accomplish this task: uniformly sized polystyrene beads \textit{versus} the protein hen egg white lysozyme. The results of this comparison are summarized in chapter 6.

Local measurements of tracer particle diffusivity with optical techniques can be further extended for characterizing the viscoelastic properties of soft materials such as gels and polymer solutions. These materials typically have complex structures spanning multiple length and time scales. The response of these complex materials to shear strain is an important step towards characterizing and understanding their internal structure. Using dynamic light scattering off polystyrene beads embedded in gels, we characterized the viscoelastic behavior of two different types of polymers: cross linked polymer polyacrylamide (PAAm) and poly N-isopropylacrylamide (pNiPAAm). The latter system is particularly intriguing since NiPAAm gels undergo a thermally driven dehydration transition. Results of these experiments are presented in chapter 7.
Chapter 2  
Light Scattering  

2.1 Wave Description of Light  

Light is a minimally invasive probe that can be used to obtained information about the structure and dynamics of molecules. Maxwell’s equation forms the basic of the description of all electromagnetic phenomena. These equations identify the light as a transverse electromagnetic wave with the direction of the oscillating E and B-field is perpendicular to the direction of propagation to each other.

The electric field associated with a plane wave at location $r$ and time $t$ is given by

$$E(r, t) = E_0 \exp(ik.r)\exp(-io\omega t)$$

where $E(r, t)$ is the spatial orientation of the oscillation (polarization) for a field strength of magnitude $E_0$, $\lambda_0$ is the wavelength of light, $k$ is the wavevector ($= \frac{2\pi}{\lambda_0}$), and $\omega$ is angular frequency ($= 2\pi v_0 = \frac{2\pi c}{\lambda_0}$)

2.2 Scattering by an Isolated Polarizable Particle  

The following discussion reproduces in large part the theoretical description of light scattering by Johnson & Gabriel\textsuperscript{2}. In fig.2.1, a plane electromagnetic wave propagates in the $+x$ direction, and the $x$ and $y$ axes define the scattering plane. We assumed that the incident light is linearly polarized along the $z$-direction.

$$E_i = E_{0z} \cos(kx - \omega t)$$

This electric field interacts with electrons in an atom or molecule (located at the origin) to induce an electric dipole moment, which oscillate at an angular frequency $\omega$. The expression for the induced dipole moment is

$$p = \alpha \cdot E_i$$
where, \( p \) is the induced dipole moment, and \( \alpha \) is the polarizability tensor.

For optically isotropic scatterers, \( \alpha \) is a constant and independent of orientation and equation (2.3) becomes

\[
p = p_z = \alpha E_Z = \alpha E_0 z \cos(kx - \omega t)
\]

(2.4)

We know from electromagnetic theory that an accelerating charge generates electromagnetic radiation. Hence, an oscillating dipole produces radiation\(^{19,20}\) and its oscillating electric moments \( p_z \) provides the source of scattered radiation. The solution for the scattered field of an oscillating dipole in the far-field (\( R \gg \lambda_0 \)) becomes\(^{19,20}\)

\[
E_s \propto \frac{d^2p}{dt^2} = -\omega^2 p
\]

(2.5)

So, if we solve the above equations the expression for \( E_s \) at \( R \) resulting from a dipole at the origin in Fig.2.1 is (see Griffith section 11.1.2)\(^{19}\)
\[
E_s = -\frac{\omega^2 p}{(4\pi\varepsilon_0)c^2 R}\sin(\chi) \tag{2.6}
\]

where \(\chi\) is the angle between \(p\) and \(R\).

The intensities corresponding to the electric field \(E\) are given by

\[
I = c\varepsilon_0 E.E^* \tag{2.7}
\]

Thus, the incident radiation \(I_i\)

\[
\langle I_i \rangle = c\varepsilon_0 E_0^2 \left(\frac{\omega}{2\pi}\right) \left[\int_0^{2\pi} \frac{\pi'}{\omega} \cos^2(\omega t) dt \right] = c\varepsilon_0 E_0^2 Z_0 \tag{2.8}
\]

It is more common to use complex variables

\[
E = E_{0z} e^{i(kz-\omega t)} \tag{2.9}
\]

So, \(I_i\) can be written as

\[
\langle I_i \rangle = \frac{c\varepsilon_0}{2} \left|E_i^2\right| \tag{2.10}
\]

From equation (2.7) the intensity of scattered light is given by

\[< I_s > = c\varepsilon_0 E_s^2 \tag{2.11}\]

So, using the above equation (2.11) and equation (2.6) we get

\[
\langle I_s \rangle = c\varepsilon_0 \left[\frac{\omega_0^4 \sin^2 \chi}{(4\pi\varepsilon_0)^2 c^4 R^2}\right] \langle p^2 \rangle \tag{2.12}\]

Where < > denoted the average of the quantity.

Now, using equation (2.4) and equation (2.8), we get

\[
c\varepsilon_0 \langle p^2 \rangle = \alpha^2 \langle I_i \rangle \tag{2.13}\]
Substituting equation (2.13) into equation (2.12), we get

\[
\frac{\langle I_s \rangle}{\langle I_i \rangle} = \frac{\omega_0^4}{(4\pi\varepsilon_0)^2 c^4 R^2} \sin^2 \chi = \frac{16\pi^4 \alpha^2}{(4\pi\varepsilon_0)^2 \lambda_0^6 4R^2} \sin^2 \chi
\]  

(2.14)

Now, I will try to rewrite the equation (2.14) in terms of change in refractive index \(n\) with concentration \(C\) instead of polarizability \(p\) as they are the experimentally determined quantities. Expanding the refractive index \(n\) in a Taylor’s series gives

\[
 n = 1 + \left(\frac{\partial n}{\partial C}\right) C
\]

(2.15)

and then squaring it gives

\[
 n^2 \approx 1 + 2 \left(\frac{\partial n}{\partial C}\right) C
\]

(2.16)

\(n^2\) is also written as \(^2\)

\[
 n^2 = 1 + N \left(\frac{\alpha}{\varepsilon_0}\right)
\]

(2.17)

So, from equation (2.16) and equation (2.17) we get

\[
 \alpha = \frac{2\varepsilon_0}{N} \left(\frac{\partial n}{\partial C}\right) C = 2\varepsilon_0 m \left(\frac{\partial n}{\partial C}\right)
\]

(2.18)

where \(m\) is the scattering mass per particle.

So, inserting equation (2.18) into equation (2.14) and then multiply it by \(N = N_A C/M\), the intensity of scattered light per volume for a gas of particles can be written as

\[
\frac{\langle I_s \rangle}{\langle I_i \rangle} = \frac{4\pi^2 CM}{\lambda_0^6 4R^2 N_A} \left(\frac{\partial n}{\partial C}\right)^2 \sin^2 \chi
\]

(2.19)

where \(N\) is the number of scatterers per unit volume and \(M\) is the molecular weight.
2.3 Scattering by Macromolecules in Solution

The scattering intensity for condensed phases is less than predicted by equation (2.14) and equation (2.19) due to the destructive interference of the scattered light waves. When a monochromatic light is incident onto a dilute macromolecule solution, due to the difference in refractive index of solvent and solute, the incident light is scattered by each illuminated macromolecules into all directions\textsuperscript{7,11}. The scattered light waves from different macromolecules interfere at the detector to produce a net scattering intensity \( I(t) \). If all the macromolecules were stationary then the scattered light intensity would be constant. However, macromolecules in the solution undergo Brownian motion, which constantly changes the optical inhomogeneities and, therefore, the corresponding fluctuations in scattering intensity in the solutions.

In the following, I will relate the intensity of scattered light to these optical inhomogeneities. For this we will consider that the solution of scatterers is composed of \( N \) small volume element \((\delta V)\) with \( \delta V \ll \lambda \). Connection to the scattering theory developed in section 2.2 is made by realizing that fluctuation in concentration or density leads to fluctuation in polarizability. Fluctuation in polarizability by one volume element is defined as

\[
\delta \alpha_v = \alpha_v - \langle \alpha_v \rangle
\]  

(2.20)

where, \( \delta \alpha_v \) is the fluctuations in the polarizability, \( \alpha_v \) is the instantaneous polarizability, and \( \langle \alpha_v \rangle \) is the time average of \( \alpha_v \).

From equation (2.14) we see that the intensity of scattered radiation is proportional to the square of the polarizability. Thus,

\[
\langle (\alpha_v + \delta \alpha_v)^2 \rangle = \langle \alpha_v \rangle^2 + \langle \delta \alpha_v \rangle^2
\]  

(2.21)

On the right hand side of equation, the cross term cancels because the time average of \( \delta \alpha_v \) is zero. Similarly, the contribution of the \( \langle \alpha_v \rangle \) cancels as it is always possible to
pair two scattering volumes such that destructive interference occurs. The net scattering will depend on $\left\langle \alpha_v \right\rangle^2$. Now, using equation (2.21) into equation (2.14) and multiplying by $N=1/\delta V$, we get

$$\frac{I_s}{I_i} = \frac{16\pi^4 \left\langle \delta \alpha_v \right\rangle^2}{(4\pi\epsilon_0)^2 \lambda_0^2 R^2 \delta \alpha}$$

(2.22)

Now, mean square fluctuations in polarization for a given volume element are related to the mean square fluctuations in concentration as

$$(\delta \alpha_v)^2 = \left( \frac{\partial \alpha}{\partial C} \right)_T \left( \frac{\partial C}{\partial V} \right)_T^2$$

(2.23)

Similarly, the refractive index of the solution is related to the polarizability by

$$n^2 - n_0^2 = \frac{\alpha}{(\delta \alpha)\epsilon_0}$$

(2.24)

Where, $n$ is the index of refraction of solute and $n_0$ is the index of refraction of solvent.

Differentiating equation (2.24) with respect to solute concentration gives

$$2n \left( \frac{\partial n}{\partial C} \right)_T = \frac{1}{(\delta \alpha)\epsilon_0} \left( \frac{\partial \alpha}{\partial C} \right)_T$$

(2.25)

Using equations (2.23) and (2.25), we get

$$\left\langle \delta \alpha_v \right\rangle^2 = \left[ 2n(\delta \alpha)\epsilon_0 \right] \left( \frac{\partial n}{\partial C} \right)_T^2 \left( \frac{\partial C}{\partial V} \right)_T^2$$

(2.26)

Finally, substituting equation (2.26) into equation (2.22), we get the scattering intensity in terms of the mean concentration fluctuation.
\[
\frac{I_s}{I_i} = \frac{4\pi^2 n^2 (\partial V / \partial C)_{T, V}^2 \langle \partial C \rangle^2}{\lambda_0^2 R^2}
\]  

(2.27)

The energy required to produce the concentration fluctuation is the Helmholtz free energy \( F \). Since the fluctuations are small, we can expand \( \delta F \) in terms of Taylor’s series

\[
\delta F = \left( \frac{\partial F}{\partial C} \right)_{T, V} \delta C + \frac{1}{2!} \left( \frac{\partial^2 F}{\partial C^2} \right)_{T, V} (\delta C)^2 + \ldots...
\]  

(2.28)

The first term will be zero because the system is in equilibrium. The probability of concentration fluctuations is equal to \( \exp \left( -\frac{\delta F}{k_B T} \right) \). So, it will be

\[
\exp \left( -\frac{\partial F}{k_B T} \right) = \exp \left[ -\left( \frac{\partial^2 F}{\partial C^2} \right)_{T, V} \frac{(\delta C)^2}{k_B T} \right]
\]  

(2.29)

The ensemble average of \( (\delta C)^2 \) is given by

\[
\langle \delta C \rangle^2 = \frac{\int_0^\infty (\delta C)^2 \exp \left( -\frac{\partial F}{k_B T} \right) d(\delta C)}{\int_0^\infty \exp \left( -\frac{\partial F}{k_B T} \right) d(\delta C)}
\]  

(2.30)

Solving the given integral, we get

\[
\langle \delta C \rangle^2 = \frac{k_B T}{\left( \frac{\partial^2 F}{\partial C^2} \right)_{T, V}}
\]  

(2.31)
Putting equation (2.31) into equation (2.27) we get the concentration dependence of the scattering intensity. However, it is conventional to write the concentration dependence in terms of a virial expansion in powers of particle concentration C. In the following I will use two very important relations whose derivation can be found anywhere else\textsuperscript{2,21}

\begin{equation}
\left( \frac{\partial^2 F}{\partial C^2} \right)_{T, V} = -\frac{8V}{C(V)} \left( \frac{\partial \mu_1}{\partial C} \right)_{T, V}
\end{equation}

\begin{equation}
-\frac{1}{k_BT(V)} \left( \frac{\partial \mu_1}{\partial C} \right)_{T, V} = N_A \left[ \frac{1}{M} + 2B_2 C + 3B_3 C^2 + \ldots \right]
\end{equation}

Using equations (2.31), (2.32), & (2.33) and equation (2.27), we get

\begin{equation}
\frac{I_s}{I_i} = \frac{4\pi^2 n^2 c^2}{\lambda^4 R^2 N_A} \left[ M^{-1} + 2B_2 c + 3B_3 c^2 + \ldots \right]
\end{equation}

In scattering experiments, $I_i$ and $R$ are fixed and we measure $I_s$. These measured quantities can be combined into one quantity called Rayleigh ratio $R_\theta$

\begin{equation}
R_\theta = \frac{I_s}{I_i} R^2
\end{equation}

The advantage is that it is independent of the incident light intensity and the distance to the scattered light detector.

We also define an optical constant $K$ which only depends on the solvent properties, and $\lambda$ but not solute parameters.

\begin{equation}
K = \frac{4\pi n^2}{N_A \lambda^4} \left( \frac{dn}{dC_p} \right)^2
\end{equation}
where \( n_0 \) is the solvent's refractive index, \( N_A \) Avogadro's number, \( \lambda \) the wavelength of incident light, and \( (dn/dC_p)_\lambda \) is the refractive index increment due to the solute. For lysozyme \( (dn/dC_p)_\lambda = 0.185 \) for \( \lambda = 633\text{nm} \). Equation (2.36) is true for incident light polarized in the z-direction. For unpolarized incident light we can make corrections by decomposing the intensity into equal parts of incident light polarized in both the z-direction and the y-direction. Then \( K \) is defined as

\[
K = \frac{2\pi n^2}{N_A \lambda^4} \left( \frac{dn}{dC_p} \right)^2
\]  

(2.37)

So, defining equation (2.34) in terms of \( K \) and \( R_\theta \), we get

\[
\frac{kC_p}{R_\theta} = \left[ \frac{1}{M} + 2B_2 C_p + 3B_3 C_p^2 + \ldots \right]
\]  

(2.38)

In equation (2.38) we have neglected the intraparticle interference effects between the particles. Therefore, this equation applies for small solute particles with major dimension less than \( \lambda/10 \). When the size of the particle is greater than \( \lambda/10 \) the light scattered from two points within the particle will reach the detector at different time which will produce an additional phase difference (due to the path difference for the light scattered from two points) and thus will cause angular dependence of the scattered light intensity.

In practice, \( R_\theta \) is obtained by comparison against a standard of known scattering cross section (in our case, toluene).

\[
R_\theta = \left[ (I_{\text{tot}} - I_{\text{sol}})/I_{\text{tol}} \right] \left[ n/n_{\text{tol}} \right]^2 R_{\theta,\text{tol}}
\]  

(2.39)

where \( I_{\text{tot}}, I_{\text{sol}} \) and \( I_{\text{tol}} \) are the measured scattering intensity of the protein solution, the salt/buffer background and of the toluene standard, respectively. \( R_{\theta,\text{tol}} \) is the Rayleigh ratio for toluene at \( \lambda = 633 \). For our set-up, the manufacturer quotes a Rayleigh ratio of \( R_{\text{tol}} = 13.52 \times 10^{-6} \text{cm}^{-1} \). For interacting particles, this normalized Rayleigh ratio \( R_\theta \) is related to the properties of the protein solution via

\[
KC_p/R_\theta = M^{-1} \left[ 1 + k_s \phi \right] = [M^{-1} + 2B_2 C_p ]
\]  

(2.40)
where $M$ is the molecular weight of the protein, $C_p$ is the protein concentration (in mg/ml), $k_s$ is the direct interaction parameter, and $\phi = \nu C_p$ is the protein's volume fraction. The constant $K$ in equation (2.40) is given by equation (2.37). For our set-up, inverse of the scattering wavenumber $q^{-1} \approx 38$ nm and lysozyme's hydrodynamic radius is $R_h = 1.9$ nm. Since $R_h q << 1$, lysozyme is a Rayleigh scatterer thereby eliminating the need for scattering intensity measurements at multiple angles $\theta$.

### 2.4 Light Scattering Techniques

#### 2.4.1 Static Light Scattering (SLS)

![Fig. 2.2: Overview of Static Light Scattering Measurements](image)

As shown in fig.2.2, during a typical light scattering experiment, incident light $I_i$ impinges on a macromolecular solution, and the scattered light $I_s$ is detected at some angle $\theta$ and distance $R$. As shown on the LHS of fig. 2.2, the signal is noisy due to thermal fluctuation in the local concentration of scatterers. In static light scattering we measure the time-averaged intensity of this scattered light. In general, the scattering intensity of macromolecular solutions is given by

$$
\frac{I_s}{I_i} = \frac{4\pi^2 n^2 C_p M}{\lambda^4 R^2 N_A} \left( \frac{dn}{dC_p} \right)^2 P(q)S(q)
$$

(2.41)
where, \( P(q) \) and \( S(q) \) are the form and static structure factor of the molecules, which account for intraparticle and interparticle interference effects respectively, \( n \) is the solvent refractive index, \( M \) is the molecular weight of the solute (protein), \( dn/dC_p \) the refractive index increment of the solution due to the protein, \( C_p \) is the protein’s mass density, \( N_A \) is Avogadro’s constant, \( R \) is the distance between the origin of the scattering volume and the detector, \( \lambda \) is the wavelength of the incident light., and \( q \) is the scattering wave number given by

\[
q = \frac{4\pi n}{\lambda} \sin\left(\frac{\theta}{2}\right)
\]

Equation (2.41) is a generalized version of equation (2.38) derived above.

Lysozyme is a Rayleigh scatterer as its radius \( R \approx 2\text{nm} \) is much smaller than the wavelength of light \( \lambda = 632.8 \text{ nm} \). Therefore \( P(q) = 1 \). Since, the mean protein spacing \((d) \) is much less than the wavelength of the light, structure factor \( S(q=0) \) can be described by a virial expansion in the solute concentration. The corresponding scattering intensity cab therefore be written as given in equation (2.38). To first order approximation in \( C_p \), equation (2.38) becomes\(^5\)

\[
\frac{kC_p}{R_\theta} = \frac{1}{M} + 2B_2C_p
\]

where we have neglected all higher order terms. A plot of \( KC_p/R_\theta \) vs. protein concentration \( C_p \) varies linearly with protein concentration. The molecular weight of the protein can be derived from the y-intercept at \( C_p=0 \) and values of the second virial coefficients \( B_2 \) is equal to the slope of \( KC_p/R_\theta \). Positive values of \( B_2 \) indicate net repulsion whereas negative values of \( B_2 \) indicate net attraction between proteins\(^6\).

### 2.4.2 Dynamic Light Scattering (DLS)

Unlike static light scattering which measures the time-averaged scattered intensity, dynamic light scattering measures the fluctuation in the scattered intensity with time as shown in fig.2.3. These fluctuations arise from the fact that the particles undergo random
thermal (Brownian) motion. Therefore, the distance between them is constantly varying. The fluctuation in the intensity of scatter light at the detector is due to the constructive and destructive interference of light scattered by the randomly moving particles within the illuminated sample volume. The time dependent changes in intensity contain information about this Brownian motion. DLS measures the temporal correlations of these statistical fluctuations in light scattering intensity.

![Dynamic Light Scattering Analysis](image)

**Fig.2.3: Overview of Dynamic Light Scattering Analysis**

Experimentally, a single photon detector records the number of scattered photons arriving within a short sample time interval ($\approx 10^{-6}$ s). A multichannel digital correlator uses this digitized record of photon counts vs. time to calculate the intensity-intensity autocorrelation function $g_2(\tau)$

\[
g_2(\tau) = \frac{\int I(t)I(t+\tau)dt}{\int I(t)I(t)dt}
\]  

(2.44)

In dynamic light scattering, particle size distributions are derived from the measured intensity autocorrelation function $g_2(\tau)$ in a three step process. First, the intensity correlation function $g_2(\tau)$ is converted into the field correlation function $g_1(\tau)$ via the Siegert relation

\[
g_1(\tau) = \sqrt{g_2(\tau) - 1}.
\]  

(2.45)
An example of the field correlation function is shown in Fig. 2.4. The field correlation function, in turn, is the Laplace transform of the decay rates $\Gamma$ of local concentration fluctuation for the different-sized particles present in the solution\textsuperscript{7-9}

\[
g_1(\tau) = \int_0^\infty G(\Gamma) e^{-\tau \Gamma} d\Gamma,
\]

where $\Gamma$ denotes the decay rate for particles of a given size and $G(\Gamma)$ is the distribution of decay rates derived from the distribution of particle sizes\textsuperscript{7}. Each decay rate $\Gamma$ can be related to the particle's diffusivity and the scattering geometry of the measurements via

\[
\Gamma = D q^2
\]

where $D$ is the particle diffusion constant and $q$ is the scattering vector given by equation (2.42). Finally, the Einstein-Stokes\textsuperscript{17} relation is used to convert diffusion constants into particle sizes

\[
D_0 = k_B T / (6 \pi \eta R_H)
\]

Here $k_B$ represent the Boltzmann constant, $T$ the absolute temperature, $\eta$ the (temperature-dependent) solution viscosity and $R_H$ the hydrodynamic radius of the diffusing particles.

\[\text{Fig.2.4: Field Correlation Function obtained for the polystyrene sphere in water}\]
The interaction effects on mutual protein diffusivity $D_c$ vary both with salt concentration and salt identity \(^{5,14}\). At moderate protein concentrations, contributions from interactions to collective diffusivity increase in direct proportion to the protein concentration. To this approximation, the corresponding collective diffusion coefficient $D_c$ is related to the single particle diffusivity $D_0$ via\(^5\)

$$D_c = D_0 [1 + k_D \phi] = D_0 [1 + (k_S + k_H) \phi]$$

(2.49)

where $k_D = k_S + k_H$ is the sum of the direct and hydrodynamic protein interactions $k_S$ and $k_H$, $\phi$ is the protein volume fraction and $D_0$ is the single-particle diffusivity of the protein given by the Stokes-Einstein relation [see equation (1.48)]. Measuring the protein dependence of the collective diffusion coefficient $D_c$, while simultaneously accounting for the contributions from direct protein interactions $k_S$ and changes in solution viscosity $\eta(C_s,T)$, we can derive values for both the hydrodynamic radius $R_H$ and the hydrodynamic interaction parameter $k_H$ of the protein. Values for the direct protein interaction parameter $k_S$ can be obtained independently from measurements of the static light scattering intensity vs. protein and salt concentration.

### 2.5 Dynamic Light Scattering Analysis for Viscoelastic Measurements

We used Dynamic Light Scattering (DLS) to do microrheological measurements to obtain the viscoelastic properties of the polymer solutions for sol and gel phase. In this dynamics of probe particles are measured by DLS which is embedded in the solution. For a purely viscous medium, the beads embedded in the solution will diffuse through it and will have viscouslike behavior. For an elastic medium the motion of the probe particle will be constrained. Soft materials such as polymers are viscoelastic in nature i.e. they store and dissipate energy. In general the full frequency dependence is given by the generalized Stokes-Einstein equation given by

$$G^*(f) = \frac{k_B T}{\pi \alpha \tau \Delta r^2(\tau)}$$

(2.50)
where, $G^*(f)$ is the frequency dependent complex shear modulus, $\Im\{\Delta r^2(\tau)\}$ is the Fourier transform of the mean square displacement, $a$ is the radius the beads. To get the mean square displacement, the field correlation function $g_1(\tau)$, in turn, is obtained from the experimentally measured intensity correlation function $g_2(\tau)$ via the Siegert relation given by equation (2.45). The field correlation function $g_1(\tau)$ was normalized to get the intercept 1. For DLS, the electric field autocorrelation is given by

$$g_1(\tau) = \exp[-q^2\langle \Delta r^2(\tau) \rangle / 6]$$

(2.51)

where $q$ is the magnitude of scattering vector which is given by equation (2.42). The mean square displacement $\langle \Delta r^2(\tau) \rangle$ is calculated from equation (2.51) as above. Using Mason et. al. method to estimate algebraically the complex shear modulus. In this method we use local power law to describe the mean square displacement of the beads in the complex fluid. Assuming power law form for $\langle \Delta r^2(\tau) \rangle$ leads to elastic $G'(f)$ and viscous $G''(f)$ moduli, which are given by

$$G'(f) = G(f) \cos(\pi a(f)/2)$$

(2.52)

$$G''(f) = G(f) \sin(\pi a(f)/2)$$

(2.53)

where

$$G(f) = \frac{k_B T}{\pi a \langle \Delta r^2\left(\frac{1}{f}\right) \rangle \Gamma[1 + \alpha(f)]}$$

(2.54)

Here,

$a$ is the radius of the bead, $\langle \Delta r^2\left(\frac{1}{f}\right) \rangle$ is the magnitude of $\langle \Delta r^2(\tau) \rangle$ evaluated at $\tau = 1/f$. $\Gamma$ is the gamma function. The local power law $\alpha(f)$ is given by

$$\left[\frac{\partial \ln \langle \Delta r^2(\tau) \rangle}{\partial \ln \tau}\right]_{\tau = 1/f}$$

The relationship between dynamic viscosity $\eta$ and viscous moduli $G''(f)$ is given by
The ratio of elastic and viscous modulus is the loss tangent, which is given by

\[
\eta = \frac{G''(f)}{f} \tag{2.55}
\]

The ratio of elastic and viscous modulus is the loss tangent, which is given by

\[
\tan \delta = \frac{G''(f)}{G'(f)} \tag{2.56}
\]

2.6 Diagram for Dynamic Light Scattering Set-up

**Figure 2.5:** Diagram of Dynamic Light Scattering Set-up from Malvern Instruments. As the laser illuminates the sample, back scattered light is measured by the detector at an angle of \( \theta = 173^\circ \). The digital correlator generates the intensity-intensity correlation function \( g_2(\tau) \). Software algorithms then invert \( g_2(\tau) \) to obtain the size distribution.
2.7 References

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Chapter 3

Effect of Lysozyme Cluster on Nucleation Kinetics of Supersaturated Solution

3.1 Introduction

Knowing the native, 3-dimensional structure of proteins provides important insights into the molecular mechanisms underlying their cellular functions\(^1\), the origins of many debilitating disorders\(^2^4\), and supports the design of new drugs that target those very same disorders\(^5\). X-ray diffraction from protein crystals still remains the primary tool for obtaining high-resolution 3-D protein structures. Attempts at high throughput protein structure determinations, however, have been frustrated by the difficulties of establishing suitable solution conditions to promote the nucleation and subsequent growth of high-quality protein crystals. These difficulties arise, in part, from the large number of adjustable material and solution parameters that affect the nucleation and growth kinetics of protein crystals and, with them, the resulting diffraction resolution. The long-term object is to improve our understanding of the physical chemistry governing protein phase separation in order to develop an approach toward protein crystallization derived from first principles.

Often, the most difficult step in protein crystallization is to induce and control the very first stage of crystal growth: crystal nucleation\(^6\). The kinetics of protein crystal nucleation and the morphology of aggregates leading to crystallization vs. precipitation, therefore, were among the first targets of fundamental studies in protein crystallization\(^7\). A variety of experimental techniques have been used to explore protein crystal nucleation, including neutron\(^8\) and x-ray scattering\(^9\), video microscopy\(^10\), calorimetry\(^11\), static light scattering\(^12^14\) and, most prominently, dynamic light scattering\(^7,15^20\). The results of these and other studies, however, have often remained ambiguous or contradictory. Even for the well characterized and frequently used model protein hen egg-white lysozyme under comparable solution conditions, the existing data don't agree on the induction times for nucleation, the size, the number, or the morphology of the
critical nuclei. An analysis of three recent experiments on nucleation in lysozyme using microscopy 21, microcalorimetry 11 and static light scattering 14 yielded nucleation rates that differed by as much as twenty orders of magnitude 22.

Similar disagreement exists regarding the structure of protein crystal nuclei. Using dynamic light scattering, Georgalis et al. 23-26 reported large populations of amorphous lysozyme clusters in supersaturated solutions. Measurements by other investigators have been unable to detect large cluster populations under comparable solution conditions 9, 27, 28. In addition, the population densities of sub-micron clusters is orders of magnitude above the number of macroscopically observed crystals 10 seen under comparable growth conditions.

Observations of nucleation kinetics in supersaturated lysozyme solution with dynamic light scattering in our laboratory showed significant discrepancies depending on the source of the stock materials. Previous reports have investigated the contamination of lysozyme stocks by protein impurities, and their effects on lysozyme crystal growth and crystal quality 29-31. Lorber et al. 30 correlated protein impurities (ovalbumin, BSA) in lysozyme solutions with changes in the total number and defect density of lysozyme crystals. Protein impurities have been implicated in changes of growth rates on the 101 facet of tetragonal lysozyme crystals 32, the density of optical defects in lysozyme crystals 33 and limitations of X-ray diffraction resolution 34, 35. Even in the absence of contaminating impurities, structural microheterogeneities of lysozyme monomers have been linked to altered crystal habits and crystal quality 36. These report, however, implicitly assumed that the contaminating proteins were either monomeric or small oligomers. In this paper, we report that lyophilized lysozyme stocks contain significant populations of sub-micron clusters. We investigated the composition and physical properties of these clusters, and went on to characterize their impact on the kinetics of lysozyme crystal nucleation in supersaturated solutions.

### 3.2 Materials and Methods

#### 3.2.1 Chemicals

We used three different stock materials of lyophilized lysozyme: 3x recrystallized, dialyzed and lyophilized stock from Seikagaku America (cat# 100910-3, Lot LF 1121) or
Sigma-Aldrich (cat# L-7651, Lot 016K11891), and 2x recrystallized, dialyzed and lyophilized stock from Worthington (cat#2933, Lot 35E8060). All other chemicals were obtained from Fisher Scientific and were reagent grade or better.

### 3.2.2 Preparation of Lysozyme Solutions

Lyophilized lysozyme was dissolved directly into 0.1 M sodium acetate/ acetic acid (NaAc) buffer at pH = 4.5. For crystallization experiments (supersaturated solutions), lysozyme/buffer solutions were mixed 1:1 with salt/buffer stock solution, each at twice the final concentration of protein or salt, respectively. Prior to mixing, both solutions were warmed above the lysozyme solubility temperature applicable to the final solution composition. For the 40 mg/ml lysozyme/ 4% NaCl solutions most frequently used for our nucleation studies, the solubility temperature was determined to be 39 °C following the method of Rosenberger et al. \(^{37}\). Solutions were then transferred to preheated cuvettes and placed into the thermostated holder of our light scattering unit. Supersaturation was induced by quenching the solution temperature to 9°C. Nucleation and growth of clusters in supersaturated solutions was investigated with three different samples: (A) Seikagaku lysozyme containing sub-micron clusters (220 nm syringe filtration), (B) Seikagaku lysozyme after removing sub-micron cluster (20 nm syringe filtration), and (C) Worthington lysozyme which was free of sub-micron clusters (therefore, 220 nm filtration was sufficient). Prior to light scattering measurements all samples were centrifuged in a Fisher accuSpin1R centrifuge at 9,5000 g for 15 min at 25 °C and filtered through either (A) a 220 nm pore size PVDF Fisherbrand or (B) a 20 nm pore size Anotop syringe filter. Actual lysozyme concentrations were determined from UV absorption measured at \(\lambda = 280\) nm (\(\alpha_{280} = 2.64\) ml mg\(^{-1}\) cm\(^{-1}\) \(^{38}\)) with a Thermo Electron Corporation UV1 spectrophotometer.

### 3.2.3 Dynamic Light Scattering (DLS) Measurements

DLS measurements were performed with a Zetasizer Nano S (Malvern Instruments Ltd., UK) with a 3mW He-Ne laser at \(\lambda = 633\) nm. The unit detects the back-scattered light at an angle of \(\theta = 173^\circ\). Sample temperature was controlled by the built-in peltier cooling device. After thermal equilibration of the samples, autocorrelation functions were
collected continuously using acquisition times of 30 s to 60 s per correlation function. Using the "narrow modes" algorithm provided with the Zetasizer Nano software, autocorrelation functions were converted to particle size distributions. Results with alternative inversion algorithms yielded comparable results. A more detailed description of the analysis of DLS data is given in chapter 2.

3.2.4 Thermal Changes in Solution Viscosity

Measurements of solution viscosity as function of temperature are discussed in detail in chapter 5. Briefly, changes in buffer viscosity were derived from measurements of temperature-related changes in apparent lysozyme diffusivity for undersaturated solutions in the range from 5° to 55 °C. Several precautions were taken to assure the observed changes in solution viscosity derived from lysozyme diffusivity were not contaminated by diffusivity changes caused by protein interactions 39 or aggregation.

3.2.5 Separation of Pre-existing Clusters

Pre-existing sub-micron clusters in lysozyme stocks were separated from monomeric lysozyme or small protein aggregates by filtering lysozyme/buffer solutions three times through 100,000 MW cutoff centrifuge filters (NanoSep 100K, Pall Corporation). After each filtration, the cluster fraction on the filter surface was re-dissolved into 0.5 ml NaAc buffer. Successful separation of the non-dissociated clusters from the low molecular weight protein background was confirmed using dynamic light scattering.

3.2.6 SDS Gel Electrophoresis

Aliquots of lysozyme for all three stock materials (Worthington, Seikagaku, Sigma) were analyzed either after 220 nm or 20 nm filtration with SDS PAGE gel electrophoresis. Aliquots of pre-assembled clusters isolated from Seikagaku and Sigma stock were analyzed separately. Using dynamic light scattering, we confirmed that SDS did dissociate pre-assembled clusters into their low molecular weight components. Protein concentrations for the aliquots containing the isolated cluster peak were below the sensitivity of our UV spectrometer (< 5 μg/ml). For SDS gel electrophoresis, 15 μl of
sample was mixed with 15 μl of reducing sample buffer and heated at 95 °C for 4 min, cooled and loaded onto the gel. The gel was a 12% Bis-Tris gel (Invitrogen, Carlsbad, CA) run in 3-(N-morpholino) propanesulfonic acid (MOPS) running buffer according to the manufacturers instructions. To avoid spill over from lanes with high protein concentrations, Seikagaku and Sigma cluster aliquots were loaded onto lanes separated by blanks from their neighbors. The molecular weights of markers used were as indicated in Fig. 3B. Gels were stained using a high-sensitivity silver stain (Silver Snap II, Pierce).

3.2.7 Growth of Macroscopic Crystals

Macroscopic crystals were grown from each of the three types of lysozyme samples (Seikagaku 220 nm; Seikagaku 20 nm; Worthington 220 nm) used in our light scattering studies of cluster formation. To minimize differences in solution conditions, solution volume, and solution-container or solution-air interfaces, macroscopic crystals were grown in the same sample cuvettes used during the DLS measurements. To keep the total number of macroscopic crystals at a reasonable level, though, the temperature-time profile of the samples had to be slightly modified: As in the dynamic light scattering experiments, sample temperatures were initially quenched from 45 °C to 9 °C, but solutions were kept there for only 15 minutes before warming them back up to room temperature (22 °C). Macroscopic crystal were then allowed to grow at room temperature overnight (16 hrs) before their sizes, numbers and quality were assessed.

3.3 Results

3.3.1 Detection and Characterization of Sub-micron Clusters in Undersaturated Solutions.

Fig. 3.1A shows the field correlation functions of light scattered from solutions from each of the three stock materials. Each of the three autocorrelation functions has a rapid decay component, but also a discernable slower tail or "shoulder" extending to longer decay times. These slower decay times indicate the presence of larger aggregates. The corresponding particle size distributions of the unfiltered stock solutions reveal three well-separated peaks (Fig. 3.1B).
Fig. 3.1: Dynamic light scattering from lysozyme solutions prepared with lyophilized stock from different suppliers. All solutions contained $C_{\text{lys}} = 40 \text{ mg/ml} (2.8 \text{ mM})$ of lysozyme dissolved in 100 mM sodium acetate (NaAc) buffer at pH = 4.5, $T = 20 ^\circ \text{C}$ (A) Field correlation function $g_1(\tau)$ vs. delay time $\tau$ for light scattered by (1) Worthington lysozyme; 2x cryst., dialyzed (□) (2) Seikagaku lysozyme; 3x crystallized, dialyzed (○) and (3) Sigma lysozyme; 3x crystallized, dialyzed (▲). (B) Distribution of particle sizes derived from the correlation functions shown in (A). For clarity, the distributions for Seikagaku lysozyme and Sigma lysozyme were offset from the origin. 

The dominant monomer peak is centered at an apparent radius of $r = (1.8 \pm 0.2) \text{ nm}$, consistent with the diffusivity of monomeric lysozyme under these conditions. Seikagaku and Sigma lysozyme solutions also yield significant cluster peaks centered around 60-90 nm. The amplitude of the cluster peak was typically larger for Sigma lysozyme, while its center was located 10-20 nm below the peak position for Seikagaku clusters. The amplitude and position of this second peak, however, varied somewhat with the lot number of the lysozyme stock. No such peak was detected for this batch of Worthington lysozyme. All samples also displayed a small, third peak located around $r = (2.3 \pm 0.2) \mu\text{m}$, which we ascribed to air bubbles induced during sample preparation. Sample filtration through 220 nm syringe filters and/or centrifugation readily removed this third peak.
The subsequent experiments focused on identifying the composition of the clusters forming the second peak in lyophilized lysozyme stock, and on characterizing some of the physical properties of these clusters. We selected the Seikagaku and Worthington stocks for further characterization. To confirm the size distributions of the second peak derived from dynamic light scattering we filtered both the Worthington and Seikagaku solutions through 220 nm syringe filters and measured the resulting autocorrelation functions. As mentioned above, the small population of micron-sized bubbles (third peak in Fig. 3.1B) disappeared from all solutions. The shoulder of the correlation function (Fig. 3.2A) and its associated cluster peak (Fig. 3.2B) found in the Seikagaku solutions, however, were only modestly reduced by this filtration step. Filtering the Seikagaku stock material through a 20 nm syringe filter, instead, eliminated the shoulder in the correlation function (Fig. 3.2A), increasing the average diffusivity of the Seikagaku samples from \( D = 9.5 \, \text{cm}^2/\text{s} \) (unfiltered sample) over \( D = 10.9 \, \text{cm}^2/\text{s} \) (220 nm filter) to \( D = 12.2 \, \text{cm}^2/\text{s} \) (20 nm filter). Concomitantly, the cluster peak associated with the shoulder in the correlation function disappeared after 20 nm filtration (Fig 3.2 B). The effects of sample filtration, therefore, confirmed that the size distributions derived from dynamic light scattering closely matched the physical aggregate sizes in the stock solutions.
3.3.2 Analysis of Cluster Composition

Multiple investigators have reported contamination of lysozyme stock solution by various low molecular weight (< 100 kD) protein impurities \(^{29, 31, 36}\). We set out to determine whether (a) the clusters found in our lysozyme stock were indeed composed of protein and (b) whether these clusters were formed by lysozyme or by one of the miscellaneous impurities previously found in lysozyme stocks. As described in the Materials and Methods sections, we used 100 kD MW cut-off centrifuge filters to separate the cluster peak of Seikagaku and Sigma stock material from the low-molecular weight protein components. As confirmed by dynamic light scattering (Fig. 2.3A), three consecutive filtrations and re-suspensions clearly separated the cluster peak from the low molecular weight background. The size distributions obtained with dynamic light scattering also indicated that the majority of the clusters remained intact after repeated centrifugation and filtration, with only a minor "fragment peak" appearing around 18 nm.

Aliquots containing either lysozyme stock material after filtration through syringe filters or the separated cluster peak were analyzed by SDS PAGE gel electrophoresis, followed by high-sensitivity silver staining (Fig. 3.3B). The left hand side of the SDS gel shows the analysis of Worthington stock material after 220 nm filtration (lane A), Seikagaku lysozyme after 220 nm filtration (lane B) and 20 nm filtration (lane C), and Sigma lysozyme after 220 nm filtration (lane D) and 20 nm filtration (lane E). All lysozyme stocks contain at least three different contaminants with estimated molecular weights of 6, 18 and 29 kD respectively. The 18 and 29 kD impurity bands closely match previous results by Thomas et al \(^{29}\) of an unidentified impurity (18.2 kD) and of an SDS-resistant lysozyme dimer population (28 kD). The smallest molecular weight band (6 kD) has not been reported before and could potentially represent a proteolytic fragment of lysozyme. The analysis of the cluster peaks for Seikagaku and Sigma lysozyme are shown in lane F and G of Fig. 3.3B. Within the resolution limit of the silver staining, the cluster peaks are entirely composed of lysozyme, with no discernable contributions from the protein impurities seen in the stock material. These observations establish that the cluster peak is composed predominately of lysozyme itself. Notice, also, that the SDS resistant lysozyme dimers (29 kD band) do not tend to associate with the lysozyme clusters. Lysozyme stock solutions, therefore, contain at least two different types of
contaminants: protein impurities and non-dissociated lysozyme clusters. Notice that the latter would not be detected by SDS PAGE gel analysis of the stock material.

Fig. 3.3: Isolation and Analysis of Lysozyme Clusters by SDS PAGE Gel Electrophoresis. (A) Confirmation of cluster separation: particle size distribution derived from dynamic light scattering from solution of clusters re-suspended in NaAc buffer after separation (for details, see Material and Methods). (B) SDS PAGE gel electrophoresis of lysozyme stock materials and cluster fractions. Lanes: (A) Worthington lysozyme after 220 nm filtration; Seikagaku lysozyme after (B) 220 nm filtration and (C) 20 nm filtration; Sigma lysozyme after (D) 220 nm filtration and (E) 20 nm filtration. Clusters peak separated from (F) Seikagaku lysozyme and (G) Sigma lysozyme. 15 μl of stock material at 1 mg/ml was loaded. Concentration of isolated cluster peak was below the sensitivity of our UV spectrophotometer (~ 5 μg/ml). Molecular weights of marker lanes are indicated in the margin.

3.3.3 Physical Characterization of Pre-existing Clusters

We investigated whether the observed protein clusters exist in equilibrium with the monomer population or if they are pre-assembled, non-equilibrium structures. Towards that end we determined whether the fraction of protein aggregates in Seikagaku solutions was altered by changes in protein concentration. Seikagaku lysozyme was dissolved in buffer solution without further purification. The protein concentration of a given sample was sequentially reduced from 40 mg/ml down to 5 mg/ml. As shown in Fig 3.4, the ratio of the area under the cluster peak to the area under the monomer peak was essentially independent of lysozyme concentration. Similarly, the location and shape of the protein cluster peak was unaffected by protein concentration.
Fig. 3.4: Dependence of cluster peak amplitude on lysozyme concentration. Ratio of cluster-to-monomer peak area for unfiltered Seikagaku lysozyme in 100 mM NaAc buffer (pH = 4.5) derived from dynamic light scattering data measured at concentrations between 5 to 40 mg/ml.

We had noticed that the location of the cluster peak was sensitive to solution temperature. Charactering the dependence of cluster size on solution temperature using dynamic light scattering requires knowledge of the temperature dependence of the buffer viscosity $\eta(T)$ (see Eqn. 2.49 in chapter 2). To determine $\eta(T)$ we measured the changes in the collective diffusivity $D_c$ of monomeric lysozyme as a function of lysozyme concentration. The $D_c$ vs $C_{lys}$ data were extrapolated to obtain $D_0$, i.e. the diffusivity in the limit of vanishing protein interactions. Using the Einstein Stokes relation (Eqn. 2.49 in chapter 2), values for $D_0$ at different temperatures where then converted into changes of buffer viscosity $\eta(T)$. Details of these experiments and their data analysis will be presented in chapter 6. Values for the viscosity of 100 mM sodium acetate buffer thus determined fell within 3% for those of water at the same temperature and agreed with independent measurements of the dynamic viscosity of sodium acetate buffer at $T = 20^\circ C$. 39.
Fig. 3.5: Thermal collapse of lysozyme clusters. Peak size of lysozyme clusters vs. solution temperature (□) in undersaturated solutions of Seikagaku lysozyme (C_{lys} = 40 mg/ml, 100 mM NaAc, pH = 4.5). As indicated by the width of the error bars (N = 4), sample to sample variations in aggregate size decreased systematically with temperature. Upon cooling (T = 20 °C) lysozyme clusters did not regain their original size prior to heating even during extended measurements over 12 hrs (■).

We next measured the response of the lysozyme cluster peak in undersaturated solutions to increases in solution temperature. After accounting for the temperature-dependence of the viscosity, η(T), the residual change in cluster size distribution was determined in the temperature range of 20°C to 55 °C. As shown in Fig. 3.6, the position of the cluster peak systematically decreased with increasing sample temperature from (93 ± 5) nm at 20 °C down to (72 ± 1) nm at 55 °C, with little change to the overall shape of the size distribution. The error bars in Fig. 3.5 indicate that this behavior was highly reproducible from run to run. When returning the sample temperature from 55 °C down to 20 °C, clusters retained the reduced size and distributions established at 55 °C, even during extended observations for 12 hours.

The lack of concentration dependence in relative aggregate population and the irreversible changes in aggregate size with temperature cycling indicate that these lysozyme clusters are permanent, non-equilibrium structures. This conclusion is further supported by the behavior of the lysozyme clusters after filtration: lysozyme cluster removed after 20 nm filtration did not grow back. Given that these clusters are present in
different concentrations and sizes in most stock materials, we suppose that they represent tightly bound lysozyme clusters formed to different degrees during the supplier-specific purification / lyophilization process.

3.3.4 Effects of Sub-micron Lysozyme Clusters on Crystal Nucleation in Supersaturated Lysozyme Solutions

The characterization of pre-assembled lysozyme clusters detailed so far had been performed in undersaturated conditions, i.e. in solutions without sufficient concentrations of added salt required for lysozyme crystallization. Next we investigated how these pre-assembled lysozyme clusters affected the crystal nucleation process in supersaturated solutions. We dissolved 40 mg/ml of lysozyme directly into 4% NaCl / buffer solutions at a temperature of 45 °C. Using a static light scattering set-up similar to Rosenberger et al. 37, we independently determined the solubility temperature of lysozyme for this combination of solute/solution parameters to be 39 °C. After dissolving the protein, Worthington lysozyme solutions were filtered through 220 nm syringe filters and Seikagaku lysozyme solutions through either 220 nm or 20 nm syringe filters. Distributions of pre-existing clusters were measured at 45 °C, i.e. in undersaturated solution conditions (see Fig. 3.6). Solution temperature was then quenched to 9 °C, which is well below the solubility temperature of 38 °C. The temperature of 9 °C was chosen to accelerate crystal nucleation while keeping the solutions above the liquid-liquid phase separation boundary located around 7 °C (data not shown). Theoretical models 43 and experimental observations 21 have suggested that crystal nucleation rates are enhanced near this phase separation boundary.
Fig. 3.6: Cluster distribution in lysozyme/salt solutions prior to thermal quenching. Solutions containing either Worthington lysozyme after 220 nm filtration (□), or Seikagaku lysozyme after 220 nm filtration (●) or 20 nm filtration (○). C_{lys} = 40 mg/ml in 4% NaCl, 100 mM NaAc at pH = 4.5, T = 45 °C. For clarity, cluster distributions for the different samples have been offset from the origin.

Autocorrelation functions and cluster distributions prior to supersaturation (see Fig. 3.6) were comparable to those observed in undersaturated solutions without added salt (Fig. 3.5). After 20 nm filtrations, neither the Worthington nor the Seikagaku solutions displayed discernable cluster peaks. The polydispersity of the Seikagaku monomer peak (δ = 0.09), however, was slightly higher than the polydispersity index of the Worthington samples (δ = 0.05). In contrast, Seikagaku solutions after 220 nm filtration displayed a well developed protein cluster peak centered at 80 nm.

The growth of new protein clusters after quenching the protein solutions into the supersaturated region is reflected in the time-dependent changes of the autocorrelation functions shown in Fig. 3.7. Within minutes after thermal quenching, the "shoulder" of the autocorrelation function measured for Seikagaku samples after 220 nm filtration (Fig. 3.7A) and after 20 nm filtration (Fig. 3.7 B) started to grow in amplitude and move towards increasingly longer decay times. Both features are indicators for the growth of significant populations of large (> 50nm) clusters in these solutions. In contrast, within the optical observation volume of our instrument (~ 5 nL), no growth of new protein clusters was discernable in the Worthington samples (Fig 3.7C). After approx. 110
minutes, however, a drop in the zero-intercept of these autocorrelation functions developed. In all samples, such a drop coincided with the appearance of visible protein crystals at the surface of the glass cuvette. This change is consistent with enhanced contributions of static scattering to the autocorrelation function as the laser beam reflects off surface-attached crystals. Particularly intriguing is the persistent difference in the nucleation behavior between Seikagaku samples following 20 nm filtration and the Worthington sample after 220 nm filtration. Neither sample showed a discernable cluster peak prior to supersaturation (Fig 3.6). The larger polydispersity of the Seikagaku samples prior to supersaturation, however, suggests that the pronounced difference in nucleation-related cluster formation of these samples is related to a population of small-diameter aggregates (< 10-20 nm) not resolved as a separate peak by dynamic light scattering.
Fig. 3.7: Dynamic light scattering from supersaturated lysozyme solutions. Temporal evolution of the field correlation function for light scattered from supersaturated lysozyme solutions. Data for the same solutions shown in Fig. 6, but after quenching solution temperature down to 9 °C, i.e. well below the saturation temperature of 38 °C. Seikagaku lysozyme solutions subjected to either (A) 220 nm filtration or (B) 20 nm filtration. (C) Worthington lysozyme solution subjected to 220 nm filtration.
3.3.5 Effects of Pre-assembled Lysozyme Clusters on Macroscopic Lysozyme Crystals

**Fig. 3.8:** Protein crystals grown with lysozyme containing different levels of nondissociated lysozyme clusters. Solutions contained $C_{\text{lys}} = 40\, \text{mg/ml}$ of lysozyme, 4\% NaCl, 100 mM NaAc ($\text{pH} = 4.5$) Lysozyme was dissolved at 45 $^\circ\text{C}$, i.e. above the solubility temperature for lysozyme under these conditions. Solution temperature was then quenched for 15 min to 9 $^\circ\text{C}$. After warming solutions back up, samples were kept at room temperature for 16 hrs. **First column:** Image of the total number of crystals present in glass cuvette containing (A) Seikagaku lysozyme (220 nm filtration), (B) Seikagaku lysozyme (20 nm filtration) and (C) Worthington lysozyme (220 nm filtration). **Second column:** Magnified image of tetragonal lysozyme crystals grown in the three cuvettes above. Notice the changes in the total number and sizes of crystals going from top to bottom. Optical defect densities and the number of twinned crystals decreases in the same order.
Fig. 3.8 show typical images of crystals grown from all three types of solutions (Seikagaku 220 nm, Seikagaku 20 nm and Worthington 20 nm) using a temperature-time profile close to that used during dynamic light scattering measurements (see Materials and Methods). Supersaturated solutions of Seikagaku lysozyme after 220 nm filtration yielded large numbers of relatively small, frequently twinned crystals. Supersaturated solutions of Seikagaku lysozyme after 20 nm filtration generated far fewer macroscopic crystals of larger size (∼1mm), but still with noticeable fractions of twinned and optically defective crystals. Worthington lysozyme produced yet fewer crystals with the highest quality of crystals, as ascertained by visual inspection. Since we grew crystals under the same conditions (temperature profile, containers and sample volume) used in our light scattering measurements, we were unable to numerically quantify the differences in crystal numbers and defect densities between these samples. Nevertheless, the results clearly indicate that the nucleation and cluster growth behavior seen with dynamic light scattering directly corresponds to the outcome of macroscopic growth experiments under the same conditions: Pre-assembled lysozyme clusters dramatically increase the number of submicron and macroscopic protein crystals and enhance crystal defects such as twinning and optical heterogeneities.

3.4 Discussion

Lysozyme is a well characterized protein that is frequently employed in fundamental studies of protein crystal nucleation and growth kinetics. Our analysis of lyophilized lysozyme indicates that commercial sources of this important model protein are consistently contaminated by significant populations of submicron (≤ 200 nm) clusters (Fig. 2.1B). SDS PAGE gel chromatography of the isolated cluster fraction confirms that these clusters are composed of lysozyme. The fraction of lysozyme clusters does not change with protein concentration (Fig. 3.4). Filtration through 20 nm syringe filters permanently removes these clusters from solution (e.g. Fig. 3.2B). Furthermore, clusters sizes shrink irreversibly with increasing sample temperature (Fig. 3.5). In contrast to recent reports of equilibrium lysozyme clusters at high lysozyme and very low ion concentrations \(^45\), the above characteristics identify the clusters described here as non-
dissociated, non-equilibrium lysozyme aggregates already present in the lyophilized stock.

Several laboratories have characterized protein impurities in lyophilized lysozyme stocks, and their impact on subsequent protein crystal growth\textsuperscript{29, 31}. Similarly, the role of structural micro-heterogeneities of lysozyme monomers was raised as possible culprit for changes in crystal growth behavior\textsuperscript{36}. The presence of such sample heterogeneities was typically identified using SDS PAGE gel chromatograph, size exclusion chromatography or affinity chromatography. Hence, it might seem surprising that the significant sample heterogeneity due to non-dissociated lysozyme clusters reported here had not been detected previously. These clusters, however, are likely to evade detection by the above techniques. In SDS gel chromatography, the clusters are dissociated by SDS and make a negligible contribution to the dominant lysozyme monomer band. In column chromatography, in turn, pre-existing clusters will evade standard UV detection due to their low overall concentration. We were unable to obtain UV absorption readings on the aliquots containing isolated lysozyme clusters, even though they yielded a well-defined light scattering peak (Fig. 3.3A) and were readily detected after separation using SDS PAGE gel chromatography with silver staining (Fig. 3.3B).

Our light scattering experiments in supersaturated lysozyme solutions indicate that these non-dissociated lysozyme clusters have a pronounced effect on the crystal nucleation and growth process. The amplitude of submicron cluster populations (Fig. 3.7A) and the number of macroscopic lysozyme crystals (Fig. 3.8A) was significantly enhanced by the presence of pre-existing clusters. In the absence of these clusters (Worthington 220 nm), no submicron lysozyme clusters were detected in supersaturated solutions (Fig. 3.7C) even though macroscopic crystals formed on the container walls (Fig. 3.8C). Non-dissociated lysozyme clusters evidently act as heterogeneous nucleation centers, promoting cluster formation in supersaturated lysozyme solutions. Given the affinity of lysozyme monomers for aggregation with these non-dissociated clusters and given their typical sizes (40-200 nm), non-dissociated clusters should readily incorporate into lysozyme crystals and contribute to defect formation in macroscopic crystal. This expectation is born out by the high densities of optical defects and twin boundaries.
observed in crystals from cluster-contaminated solutions (Fig 3.8A). Both of these macroscopic defect features are bound to degrade X-ray resolution.

Filtration of Seikagaku stock material through 20 nm filters removed the non-equilibrium clusters peak centered around 100 nm completely (Fig. 3.2B). Hence, one might expect that the Worthington samples after 220 nm filtration and the Seikagaku samples after 20 nm filtration should display equivalent nucleation behavior. However, the 20 nm filtered Seikagaku solutions had inductions times for cluster formation closer to 220 nm filtered Seikagaku solutions than Worthington solutions at identical supersaturation (see Fig. 3.7). Dynamic light scattering from undersaturated solutions of Seikagaku lysozyme (20 nm filtration) yielded polydispersities $\delta \approx 0.09$ which were consistently higher than the polydispersity of $\delta \approx 0.05$ measured for Worthington lysozyme (220 nm filtration). Polydispersity implies the presence of small (< 20 nm) unresolved clusters in either solution. The higher polydispersity of "cluster-free" Seikagaku lysozyme over Worthington lysozyme could be related to two factors. SDS PAGE gel chromatography of the two samples (lane A and C in Fig. 3.4) indicates that Seikagaku lysozyme contains slightly higher levels of protein impurities than Worthington lysozyme. Furthermore 20 nm filtration could break up the larger lysozyme clusters in Seikagaku lysozyme into smaller fragments. Cluster fragments generated by filtration through 20nm filters are likely to enhance the polydispersity of the "monomer peak". This latter interpretation is supported by the effects of repeated filtration during isolation of the cluster peak from the monomer background. Dynamic light scattering from isolated lysozyme clusters yielded a small secondary peak centered at 18 nm, indicating the generation of such cluster fragments (first peak, Fig 3.3A). In either case, the enhanced polydispersity is apparently sufficient to lead to a significant acceleration of cluster nucleation and growth kinetics in supersaturated solutions. The apparent lack of discernable cluster formation in supersaturated solutions with Worthington lysozyme is noteworthy. The formation of macroscopic lysozyme crystals from the same solutions clearly indicates that crystals do nucleate and grow under these conditions (Fig. 3.8C). These two observations could be seen to imply that the formation of large (fractal?) protein clusters in lysozyme solutions frequently described by others 24, and seen here in the contaminated Seikagaku samples, is just related to the existence of contaminating
lysozyme clusters. This conclusion, however, would be premature. First of all, the majority of macroscopic crystals obtained from supersaturated Worthington solutions grew on the cuvette walls, apparently via heterogeneous surface nucleation (see Fig. 3.8C). Furthermore, dynamic light scattering only monitors a very small fraction (in our instrument: 5 nL) of the bulk volume of the sample. Therefore, the absence of larger clusters reported by dynamic light scattering might just indicate the dominance of surface over bulk nucleation rates in supersaturated solutions of Worthington lysozyme. In addition, supersaturated lysozyme solutions can form gel phases\textsuperscript{14, 46}. Gelation clearly has to be preceded by the formation of gel clusters. Therefore, pre-assembled lysozyme clusters might just enhance the rates of cluster nucleation and growth in the bulk over heterogeneous nucleation rates at the solution interfaces.

Overall, understanding the mechanisms that control protein crystal nucleation in supersaturated protein solutions and that determine the morphology of nucleation clusters and macroscopic new phases is critical for improving our control over phase separation in macromolecular and colloidal systems. While conceptually straightforward, measurements of crystal nucleation rates for crystals are fraught with experimental obstacles that are difficult to assess and control. Our data on lysozyme nucleation and cluster growth raise yet another experimental concern that, thus far, has received little attention: sample heterogeneity due to pre-assembled protein clusters present in lyophilized stock material. Two specific features of the nucleation behavior in the presence of these non-dissociated clusters are of particular concern. First, filtration through standard 0.22 μm syringe filters or centrifugation up to 15,000 g do little to remove the existing non-equilibrium lysozyme clusters from solution. The presence of such non-dissociated clusters, in turn, dramatically shortens induction times and increases the population densities of sub-micron protein clusters nucleating from supersaturated solutions - two parameters that are frequently assessed for comparison with theoretical models of crystal nucleation. Hence, contamination of lysozyme solutions by non-dissociated, non-equilibrium lysozyme clusters is a likely candidate for explaining some of the large discrepancies in nucleation rates reported in the literature\textsuperscript{22}. 

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3.5 References


Chapter 4

Effect of Chaotropc and Kosmotropic Ions on the Hydration and Hydrodynamic Interaction of Lysozyme

4.1 Introduction

Water molecules bound to the surface and incorporated into the core of protein molecules are considered to play a critical role in regulating the biological functions of proteins and their phase separation behavior \(^{13,14}\). Yet the structure and dynamics of hydration water remain the topic of ongoing experimental and theoretical research efforts \(^{19}\). Neutron scattering and x-ray diffraction from protein crystals indicate that water density near the surface is increased by about 10-15 % beyond the bulk density \(^{36}\), with similar results obtained from molecular dynamics simulations \(^{27}\). NMR, time-resolved fluorescence, and dielectric relaxation spectroscopy have all been used to probe relaxation of water on sub-nanosecond time scales, revealing an overall retardation of the rotational relaxation dynamics of water molecules near protein surfaces \(^{12,24,31}\). Similarly, the ability of salt ions to either disrupt or enhance hydrogen bonding networks is well established \(^{6,9}\). Salt ions are categorized as either water-structure makers (kosmotropic) or breakers (chaotropic). The efficacy of specific salt ions at enhancing or disrupting water structure is similar in many different systems. This rank ordering of salt ions was originally established by Hofmeister's studies of salt-specific effects on protein precipitation \(^{21}\). However, just as the case of water at interfaces itself, no universally accepted model has been put forth to explain the mechanisms mediating the salt-specific effects of the Hofmeister series.

We investigated whether addition of either chaotropic or kosmotropic salt ions at concentrations up to 1 M would alter lysozyme hydration or the hydrodynamic interaction among the lysozyme molecules. Lysozyme is a small globular protein frequently used in studies of protein hydration \(^{6,9}\) and protein diffusion \(^{17,28}\). While salt-specific effects on direct protein-protein interactions have been studied repeatedly \(^{15,17,28}\),
much less is known about salt-specific effects on hydrodynamic interactions and protein hydration. We used five different salts (MgCl₂, NaCl, CsCl, NaI, and NaHPO₄) to investigate ion-specific effects on hydration or hydrodynamic interactions. These salts contained ions varying from strongly kosmotropic (PO₄³⁻, Mg²⁺) to strongly chaotropic (Cs⁺, I⁻) character, and contained at least one negative and positive ion among either group of ions. This allowed us to keep either the co-ion (Na⁺) or counter ion (Cl⁻) to the positively charge lysozyme molecule constant. The overall goal was to gain insights into the effects of chaotropic or kosmotropic ions on the water structure around lysozyme, and on solvent-mediated hydrodynamic interactions among multiple lysozyme molecules. Both questions can be addressed simultaneously by measuring static and dynamic light scattering from lysozyme in salt-water solutions.

4.2 Materials and Methods

4.2.1 Chemicals

Dialyzed, 2× recrystallized and lyophilized lysozyme stock from Worthington Biochemicals (cat#2933) was used for all experiments. As shown in chapter 3 Worthington stock material was least likely to be contaminated by pre-existing sub-micron lysozyme clusters that interfere with light scattering and/or nucleation studies. All other chemicals were obtained from Fisher Scientific and were reagent grade or better.

4.2.2 Preparation of Lysozyme Solutions

Lyophilized lysozyme was dissolved directly into 25 mM sodium acetate/ acetic acid (NaAc) buffer at pH = 4.5. Stock solutions for MgCl₂, NaCl, NaH₂PO₄ and CsCl were prepared by dissolving each into the same 25 mM NaAc buffer at pH = 4.5 at a final salt concentration of 2 M. To avoid complex formation, NaI stock solutions had to be prepared fresh on the day of the experiment and the highest stock concentration used was 0.2M. The pH of all stock solutions was re-adjusted after the addition of salt, if necessary. Lysozyme solutions for light scattering measurements were prepared by 1:1 mixing of lysozyme/buffer with salt/buffer stock solutions, each at twice their final concentrations. Prior to mixing, lysozyme solutions were filtered through 20 nm pore
size Anotop syringe filters while salt solutions were filtered through 220 nm syringe filters. At the higher salt concentrations (≥ 600 mM), lysozyme solutions become supersaturated at room temperature or below and can form crystals. Therefore, after mixing, lysozyme solutions were heated to 45 °C in order to reduce the risk of inducing crystal seeds. Solutions were then transferred to glass cuvettes and placed into the thermostated holder of the light scattering unit. Actual lysozyme concentrations of solutions were determined from uv absorption measured at λ = 280 nm using α_{280} = 2.64 ml / (mg cm)³⁵.

### 4.2.3 Static (SLS) and Dynamic (DLS) Light Scattering Measurements

Both SLS and DLS measurements were performed using a Zetasizer Nano S (Malvern Instruments Ltd., UK) with a 3mW He-Ne laser at λ = 633 nm. The unit collects back-scattered light at an angle of θ = 173°. Sample temperature during measurements was controlled to within ± 0.1 °C by the built-in Peltier element. Correlation functions were determined from the average of 5 measurements, with a typical acquisition time of 60 seconds per correlation function. Scattering intensities for SLS analysis were obtained from the average count rate of the samples and were calibrated against toluene, using the Rayleigh ratio of R_T = 13.52 × 10⁶ cm⁻¹ quoted by the manufacturer³⁷. For DLS measurements, any correlations function with polydispersity values greater than 0.08 was rejected. For the three salts (MgCl₂, NaCl, CsCl) for which temperature-dependent viscosity data were available, light scattering measurements were performed at six different temperatures starting from 40 °C down to 15 °C in steps of 5 °C. After each temperature step, solutions were allowed to equilibrate thermally for 5 min.

### 4.2.4 Dynamic (DLS) and Static (SLS) Light Scattering Analysis

*Dynamic Light Scattering Analysis:* The autocorrelation function of scattered light measured in DLS yield the decay rates Γ of local concentration fluctuations for macromolecules in solution²⁵¹¹. A more detailed description of data analysis of DLS is given in section 2.4.2 of chapter 2.
Collective diffusion coefficient $D_c$ is related to the single particle diffusivity $D_0$ via as given by equation (2.49) in chapter 2.

$$D_c = D_0 \left[ 1 + k_D \phi \right] = D_0 \left[ 1 + (k_S + k_H) \phi \right] \quad (4.1)$$

where $k_D = k_S + k_H$ is the sum of the direct and hydrodynamic protein interactions $k_S$ and $k_H$, $\phi$ is the protein volume fraction.

*Static Light Analysis:* A more detailed description of data analysis of SLS is given in chapter 2. For interacting particles, the normalized Rayleigh ratio $R_\theta$ is related to the properties of the protein solution via as given by equation (2.40) in chapter 2

$$\frac{K C_p}{R_\theta} = M^{-1} \left[ 1 + k_s \phi \right] \quad (4.2)$$

where $M$ is the molecular weight of the protein, $C_p$ is the protein concentration (in mg/ml), $k_s$ is the direct interaction parameter, and $\phi = \nu C_p$ is the protein's volume fraction.

### 4.2.5 Growth of Macroscopic Crystals

Macroscopic lysozyme crystals were grown at lysozyme concentrations of 20 mg/ml using all three salts at concentrations of 0.6 M and 1M, respectively. Solutions were placed in sealed crystallization wells and incubated overnight (16 hrs) at 4 °C.

### 4.3 Results

The overall goals of this study were two-fold: to ascertain whether strong chaotropic or kosmotropic ions alter the extent of hydration around individual lysozyme molecules; and to determine whether and how chaotropic or kosmotropic ions selectively alter the water-mediated hydrodynamic interactions among lysozyme molecules. Using measurements of lysozyme diffusion, we tracked changes to the hydrodynamic radius of lysozyme and to its hydrodynamic interactions in the presence of various chaotropic or kosmotropic salt ions.
4.3.1 Chaotropic & Kosmotropic Salts and Water Viscosity

The selection of salts used for this study was driven by several considerations. First, we used salt for which reliable viscosity data vs. salt concentration and, when available, vs. solution temperature. These data are critical both for careful determinations of the hydrodynamic radius of lysozyme (see equation 2.49 in chapter 2) and for quantifying the chaotropic/kosmotropic character of the ions that make up the salts. We chose the following five salts for our study: MgCl₂, NaCl, CsCl, NaH₂PO₄ and NaI. This way we either kept the anion (Cl⁻) or cation (Na⁺) of the salts constant, while selecting corresponding cations/anions ranging from strongly Kosmotropic to strongly chaotropic (see Table 4.2). Na⁺ and Cl⁻ themselves are weakly kosmotropic and chaotropic, respectively. Published values for salt-induced changes to the viscosity of water at 25°C for each salt are summarized in Fig. 4.1.

Since experimental data points are sparse, we used Kaminsky's extension to the empirical Jones-Dole equation 23

\[ \eta(c_s) = \eta_0 (1 + K_1\sqrt{c_s} + K_2 c_s + K_3 c_s^2) \] (4.3)

to derive viscosity values for the specific salt concentrations used in our experiments. Here \( \eta_0(T) \) is the water viscosity at a given solution temperature and \( K_1 \) through \( K_3 \) are empirical fitting coefficients. The resulting fits through the experimental data for \( T = 25 \) °C are displayed as dashed curves in Fig.4.1. Fitting coefficients for each salt, and at all temperatures for which data were available, are summarized in Table 4.1. Values of the linear \( K_2 \)-term or Jones-Dole B coefficient, measured for multiple combinations of ions, can be used to quantify the kosmotropic or chaotropic character of specific ions, and are summarized in Table 4.2. We use these values only to characterize the relative strength of the chaotropic/kosmotropic character for the six ions in our study.
Fig. 4.1: Plot of the viscosity of water/salt solutions at $T = 20$ °C as function of dissolved salt concentration. The slope of the initial increase ($\text{NaH}_2\text{PO}_4$, $\text{MgCl}_2$, $\text{NaCl}$) or decrease ($\text{NaI}$, $\text{CsCl}$) is indicative of the predominant kosmotropic (full symbols) or chaotropic (open symbols) character of the cation/anion combination for a given salt. Symbols represent measured viscosity values for $\text{NaH}_2\text{PO}_4$, $\text{MgCl}_2$, $\text{NaCl}$, $\text{NaI}$ and $\text{CsCl}$, while the dotted lines represent fits through the viscosity data using the Kaminsky equation. Extrapolated viscosity values were used for all salt concentrations for which measured viscosities were unavailable.
Table 4.1: Summary of fitting parameters for viscosity water-salt mixtures at various solution temperatures.

<table>
<thead>
<tr>
<th>Salt</th>
<th>Temperature (°C)</th>
<th>$K_1$ [mM]$^{1/2}$ ($\times 10^{-4}$)</th>
<th>$K_2$ [mM]$^{-1}$ ($\times 10^{-5}$)</th>
<th>$K_3$ [mM]$^{-2}$ ($\times 10^{-8}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>15</td>
<td>-6.20</td>
<td>9.22</td>
<td>-0.27</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>-8.47</td>
<td>10.96</td>
<td>-0.563</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>-7.11</td>
<td>11.39</td>
<td>-0.651</td>
</tr>
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<td></td>
<td>30</td>
<td>-3.54</td>
<td>10.60</td>
<td>-0.266</td>
</tr>
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<td></td>
<td>35</td>
<td>-5.49</td>
<td>11.89</td>
<td>-0.549</td>
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<tr>
<td>MgCl$_2$</td>
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<td>8.82</td>
<td>34.02</td>
<td>6.66</td>
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<tr>
<td></td>
<td>20</td>
<td>8.80</td>
<td>33.96</td>
<td>6.22</td>
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<td></td>
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<td></td>
<td>35</td>
<td>9.07</td>
<td>36.13</td>
<td>5.91</td>
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<td>CsCl</td>
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<td></td>
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<td>34.17</td>
<td>14.62</td>
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<td>NaI</td>
<td>25</td>
<td>1.27</td>
<td>0.86</td>
<td>1.57</td>
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</table>
Table 4.2: Summary of Jones-Dole viscosity B coefficients for the salt ions in this study. Positive values indicate kosmotropic and negative values chaotropic ions. Data adapted from Table 3.1.  

<table>
<thead>
<tr>
<th>Ion</th>
<th>Jones-Dole B-coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>PO$_4$³⁻</td>
<td>0.590</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>0.385</td>
</tr>
<tr>
<td>Na$^+$</td>
<td>0.086</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>-0.007</td>
</tr>
<tr>
<td>Cs$^+$</td>
<td>-0.045</td>
</tr>
<tr>
<td>I⁻</td>
<td>-0.068</td>
</tr>
</tbody>
</table>

4.3.2 Measuring protein hydration and hydrodynamic protein interactions

Combining static and dynamic light scattering, we determined salt-specific effects on lysozyme hydration and on the mutual hydrodynamic interactions among the lysozyme molecules. A detailed analysis of DLS data is given in chapter 2. The diffusive behavior of macromolecules in solution is altered by the presence of direct and solvent mediated hydrodynamic interactions. These interaction effects on mutual protein diffusivities $D_c$ are significant and depend both on salt concentration and salt identity. For moderate protein concentrations, direct and hydrodynamic interaction increase linearly with protein concentration (equation 4.1). Depending on the dominance of net attractive or repulsive interactions, the protein's collective diffusivity $D_c$ can be either higher (net repulsion) or lower (net attraction) than the corresponding single-particle diffusivity (equation 2.49 of chapter 2). By measuring the protein dependence of the collective diffusion coefficient $D_c(C_{LyS})$, while accounting for the contributions from direct protein interactions $k_S$ and changes in solution viscosity $\eta(C_s,T)$, we can derive values for both the single-molecule hydrodynamic radius $R_H$ and the mutual hydrodynamic interaction parameter $k_{H'}$. Values for the direct protein interaction

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parameter $k_s$ are determined independently from the protein-dependence of the static light scattering intensity (equation 4.2).

4.3.2.1 Direct and hydrodynamic interaction of lysozyme in solutions

Figure 4.2 summarizes the changes in light scattering intensity (SLS) with lysozyme concentration $C_{\text{Lys}}$ at $T = 20 \, ^\circ\text{C}$, for a series of increasing salt concentrations and for three (MgCl$_2$, NaCl and CsCl) of the five salts considered in our study. Scattering intensities are displayed as normalized Debye ratios $K_{\text{C,Lys}}/R_\theta$ (equation 2.43 in chapter 2). Debye plots provide a particularly straightforward interpretation of SLS data: The $y$-intercept of the $K_{\text{C,Lys}}/R$ vs. $C_{\text{Lys}}$ data is the inverse of the protein's molecular weight $M_w$, while the sign of their slope indicates whether proteins experience net repulsive (positive slope) or attractive (negative slope) interactions at the given solution conditions $^{15,18}$. The change from positive to negative slopes with increasing salt concentration results from the transition of charge-mediated protein-protein repulsion at low salt concentration to attraction due to short-range protein interactions (van der Waals, hydrophobic, etc). Several previous studies have matched the transition from repulsive to attractive interactions using colloidal DLVO theory $^{17,25,28}$. While successful for any given salt, DLVO theory can not account for the ion-specific differences in protein interactions at the same ionic strengths (i.e. effective charge screening).
Fig. 4.2: Salt-Specific Effects on Debye Ratios $K_{\text{lys}}/R$ and Mutual Diffusivities $D_c$ of Lysozyme: Plot of (Top Row) the Debye ratios $K_{\text{lys}}/R$ and (Bottom Row) mutual diffusivities $D_c$ of lysozyme as function of lysozyme concentration $C_{\text{lys}}$, in the presence of (A) MgCl$_2$, (B) NaCl or (C) CsCl, at increasing salt concentrations (50 mM, 250 mM, 625 mM and 1 M). The y-axis intercepts of the Debye plots yields the inverse of the molecular weight (1/M) of lysozyme, while the sign of the slope indicates whether interactions among the lysozyme molecules are either net repulsive (positive slope) or attractive (negative slope). For the plots of mutual diffusivities, the y-axis intercepts yield the free particle diffusivity $D_0$ while the slope indicates the magnitude and sign of the combined effects of direct and hydrodynamic interactions on lysozyme molecules. All measurements shown were taken at $T = 25^\circ$C.

The bottom row of Fig. 4.2 displays the changes in the coolective diffusion constant $D_c$ of lysozyme under the same conditions used for the SLS measurements in the top row. For all DLS data in Fig. 3.2B the measured size polydispersity $\delta$ was less than 0.08, indicating that changes in $D_c$ are not contaminated by aggregate formation in solution. Any measurements at high salt concentrations suggesting potential aggregate/cluster
formation (high polydispersity, temporal drifts in scattering intensity or $D_c$) were excluded from the analysis. The presence of positive slopes in both DLS and SLS data, together with the strictly linear behavior of both data sets with protein concentration are further indicators that potential contributions due to protein aggregation are negligible 29.

The plots of mutual diffusivity $D_c$ vs. lysozyme concentration are very similar in appearance to the Debye plots in the top row. Mutual lysozyme diffusivities $D_c$ vary linearly with lysozyme concentration, with the slopes changing from positive to negative values as salt concentration increases. As indicated in equation (4.1), the slopes of $D_c$ vs. $C_{Lys}$ measured with DLS are the superposition of both direct and hydrodynamic interactions among the protein molecules. Subtracting the $k_s$ values obtained with SLS, therefore, we determined the magnitude of the hydrodynamic interaction parameter $k_H$ for solution-mediated interactions among the lysozyme molecules. Using this approach enabled us to determine whether the presence of chaotropic vs. kosmotropic ions - similar to the already well-established effects on direct protein interactions- can induce salt-specific changes in either protein hydration or in the solution-mediated hydrodynamic protein interactions.

### 4.3.2.2 Effects of Kosmotropic vs. Chaotropic ions on lysozyme hydrations

Based on the significant influence of salt ions on local water structure, it seems natural to wonder whether chaotropic or kosmotropic ions can alter the extent of the ordered water layer around proteins. Using DLS, we determined whether different salts lead to discernable swelling or contraction in lysozyme's hydration layer. We can obtain the single-particle diffusivity $D_0$ of lysozyme by extrapolating the mutual diffusivity $D_c$ to its y-axis intercept at $C_{lys} = 0$. Using the Stokes-Einstein relation (see equation 1.49 in chapter 1), the radius of hydrated lysozyme can be obtained from the single-particle diffusivity $D_0$ (Fig. 4.2) and values of the solution viscosity $\eta(C_s,T)$. Figure 4.3 displays the resulting values for lysozyme's hydrodynamic radius for each of the five salts. These data are notable in several ways. First of all, when accounting for salt- and temperature dependent solution viscosity and for protein interaction effects on diffusivity, the hydration radii of lysozyme under any conditions are within ± 0.25 Å of one another. These differences are well below the thickness for a single monolayer of water extending
to about 2.6-2.8 Å\textsuperscript{7}. Hence, our experimental resolution permits us to resolve changes down to 1/10 the thickness of a single water layer.

Equally remarkable, while the effects of chaotropic vs. kosmotropic salt ions on the local structure of water are significant, there is no discernable swelling or disruption of the lysozyme hydration layer due to the presence of either kosmotropic or chaotropic ions. This remains true up to salt concentrations of 1M and over the entire range of temperatures in our experiments. This is shown in Fig. 4.3B for the case of MgCl\textsubscript{2}, which is representative for the behavior of all the other salts. These results imply that the overall extent of lysozyme's hydration layer is very stable. The question remained whether the net charge of the protein itself might determine whether chaotropic/kosmotropic ions can disrupt the protein's hydration layer. It has been shown before that the Hofmeister series for the solubility of lysozyme was inverted\textsuperscript{33}, presumably due to the net positive charge of lysozyme at pH=4.5\textsuperscript{34}. According to Debye-Hückel theory, the concentration of cations near the positively protein surface will be reduced from their bulk concentrations\textsuperscript{22}. To investigate this possibility, we included NaH\textsubscript{2}PO\textsubscript{4} and NaI in our measurements, salts with either a highly chaotropic (I\textsuperscript{-}) or kosmotropic (PO\textsubscript{4}\textsuperscript{3-}) co-ion. Yet, neither of these two negative ions altered the hydrodynamic radius of lysozyme (Fig. 4A).
Fig. 4.3: Effects of Chaotropic and Kosmotropic Salt Ions on Lysozyme Hydration. (A) Mean hydrodynamic radius $R_h$ of lysozyme in the presence of various salts with predominately chaotropic or kosmotropic salt ions and for salt concentrations varying from 50 mM to 1 M. $R_h$ values for different concentrations of the same salt were averaged since they displayed no discernable systematic variations (see 4.3B). (B) Hydrodynamic radius $R_h$ of lysozyme in the presence of MgCl$_2$ at different solution temperatures $T$, and for MgCl$_2$ concentrations ranging from 50 mM to 1 M.

It is well known that water becomes progressively disordered with increasing temperature $^{14}$. We therefore determined whether there were temperature-dependent variations in the hydrodynamic radius of lysozyme in the presence of chaotropic vs. kosmotropic ions. Fig. 4.3B shows the results of a typical measurement with MgCl$_2$ over the temperature range of 15-40 °C. The range of temperature values was limited due to problems with bubble formation (high $T$) and the onset of phase separation (low $T$). Within these limitations, there are, again, no indications for any salt-specific effects on
protein hydration with solution temperature. The lack of any discernable effects on the hydrodynamic radius of lysozyme with salt concentration and salt type simultaneously indicates that there is also no salt-induced swelling of the protein itself (which might be otherwise difficult to discriminate from changes to the protein's hydration layer.

4.3.2.3 Salt specific effects on direct and hydrodynamic protein-protein interaction

To convert the slopes of our static and dynamic light scattering data (Fig. 4.2A and B) into direct and hydrodynamic interaction parameters (defined in equation (4.1) and (4.2)), we use the value $v = 0.703 \text{ ml/g}$ for the specific volume of lysozyme $^{35}$. Figure 4.4 displays the resulting values for the direct and hydrodynamic interaction parameters $k_s$ and $k_h$, as function of solution temperature and salt concentration. The systematic variations become more apparent when displayed against solution temperature (shown here for MgCl$_2$, NaCl and CsCl, and for increasing salt concentrations). At the lowest salt concentrations (50 mM), the direct protein interactions parameter $k_s$ remains positive at all temperatures. For the same salt concentration, repulsive protein interactions are more prominent in the 1:1 salt solutions (NaCl, CsCl) than the 2:1 MgCl$_2$ solutions. Both observations are consistent with the Debye theory of diffusive charge screening. At low salt concentrations, protein interactions will be dominated by protein-protein charge repulsion, with the 2:1 salt MgCl$_2$ more effective than NaCl and CsCl in screening out this charge repulsion $^{22}$.

With increasing salt concentration charge repulsion progressively diminished and net protein repulsion (positive $k_s$) turns into net attraction (negative $k_s$). While the salt-induced decrease in net repulsion, at least qualitatively, follows the logic expected for salt screening of protein charges, salt specific effects rapidly emerge even at moderate salt concentrations. In particular, NaCl at or above 250 mM is significantly more effective in promoting attractive lysozyme interactions than either MgCl$_2$ or CsCl. The dashed horrizonatl lines in Fig. 4.4A indicate the range of interaction parameters $k_S$ (or, equivalently, second virial coefficients $B_{22}$) considered favorable for protein crystal growth $^{18}$. As shown in Fig. 4.5, we were able to obtain lysozyme crystals with all three salts when incubating solutions at low temperature and at sufficiently high salt concentrations to reach the "crystallization band" in Fig. 4.4A. Lysozyme solutions
incubated with 1M NaCl yielded larger numbers of smaller crystals, consistent with the enhanced attraction among lysozyme monomers and, therefore, the increased supersaturation of the solutions under otherwise identical growth conditions.

Fig. 4.4: Dependence of Direct and Hydrodynamic Interaction Parameters on Salt Type, Salt Concentration and Solution Temperature. Plot of the net strength of (top row) direct lysozyme interactions $K_S$, (bottom row) corresponding hydrodynamic interactions $K_H = K_D - K_S$ as a function of solution temperature $T$, and for four different salt concentration $C_s$. Data are shown for (left column) MgCl$_2$, (middle column) NaCl and (right column) CsCl. $K_S$ and $K_D$ are derived from the slopes of the SLS and DLS data respectively. The band of negative $K_S$ values indicated by the two horizontal dashed lines in the top row is considered favorable for protein crystallization growth.
**Fig. 4.5:** Protein crystals grown from lysozyme solutions in the presence of chaotropic vs. kosmotropic cations. Microscope images of tetragonal lysozyme crystals grown with (left column) 0.6 M or (right column) 1M of (top row) MgCl₂, (middle row) NaCl or (bottom row) CsCl. All solutions contained 20 mg/ml of lysozyme in 25 mM NaAc buffer (pH = 4.5) and were incubated overnight (16 hrs) at 4 °C. The lysozyme crystals grown at [NaCl] = 1 M show a mixture of tetragonal crystals and (sea urchinlike) spheres of needle crystals. The latter are most likely orthorhombic crystals.
4.4 Discussion

Lysozyme's hydrodynamic radius of $(1.89 \pm 0.025)$ nm remained unaltered by the presence of salts containing either strong chaotropic or kosmotropic ions. This remained true up to salt concentrations of 1 M (NaH$_2$PO$_4$, MgCl$_2$, NaCl, CsCl) or up to the onset of lysozyme precipitation (NaI). Previous measurements had noted the lack of changes in lysozyme hydration in the presence of NaCl up to 0.4 M or sodium acetate up to 2.5 M$^{28}$ and MgCl$_2$ up to 1 M$^{17}$. Our measurements extend these observations to a series of salts with either predominately chaotropic or kosmotropic character and put a much tighter limit (0.25 Å or less than 1/10th of a monolayer of water) on residual changes that might evade detection. The data also indicate that it did not matter whether the chaotropic or kosmotropic ion carried the same (Mg$^{2+}$, Cs$^+$, Na$^+$) or opposite charge (PO$_4^{3-}$, Cl$^-$, I$^-$) as the net charge of lysozyme. Hence, the elevation (negative ions) or depression (positive ions) of local salt concentrations beyond their bulk concentrations near the positively charge lysozyme surface did not alter these results. Variations in solution temperature did not produce any discernable changes in lysozyme hydration in the presence of various salts, either.

The lack of any discernable changes in lysozyme hydration by either chaotropic or kosmotropic salts seem surprising given the pronounced salt-specific effects on viscous dissipation in bulk water (see Fig. 4.1). Apparently, neither chaotropic nor kosmotropic ions are able to alter the extent of the hydration layer around lysozyme. This could imply that the protein surface residues and surface structure is much more effective at ordering water than either chaotropic or kosmotropic ions. Alternatively, ion-specific effects onto surface water might only change the fast relaxation dynamics of water occurring at or below picoseconds, much faster than the microsecond relaxation times probed in translational diffusion of lysozyme. This later viewpoint seems somewhat difficult to reconcile with the obvious salt-specific effects on bulk water viscosity which do need to be accounted for. Hence, specific effects on water relaxation even at a much faster time scale should translate into increased viscosity near the protein's surface$^{20}$.

We prefer the interpretation that neither chaotropic nor kosmotropic ions will perturb the structure and dynamics of surface water, but that ion-specific effects are
mediated via direct interactions with the protein. This is supported by the clear ion-specific effects on direct protein-protein interactions obtained with static light scattering (Fig. 4.2A). However even there, the ordering of specific ion effects on attractive lysozyme interactions ($\text{Na}^+ > \text{Mg}^{2+} > \text{Cs}^+$) is at odds with considerations of either charge screening ($\text{MgCl}_2 > \text{NaCl}, \text{CsCl}$) or the typical order of these cations within the Hofmeister series ($\text{Mg}^{2+} > \text{Na}^+ > \text{Cs}^+$). It is hard to image that the two-fold higher bulk concentrations of (weakly) chaotropic Cl$^-$ ions in MgCl$_2$ vs. NaCl solutions should be able to compensate for the strong kosmotropic character of Mg$^{2+}$ compared to the moderately kosmotropic Na$^+$ ions. This implies that there are other ion-specific effects on protein interactions beyond the scope of the Hofmeister series.

As with protein hydration, there are no indications that hydrodynamic protein interactions are directly modified by ion-specific effects. However, hydrodynamic interactions are strongly anti-correlated to direct protein interactions thereby coupling them indirectly to salt-specific effects on direct protein interactions. With increasing salt concentration, hydrodynamic interactions transition from net attraction to repulsion while direct protein interactions move in the opposite direction (Fig. 4.4). We have previously noted that trend in lysozyme solutions at fixed temperature for both NaCl and sodium acetate. This anti-correlation is not dependent on any specific salt ion and persists as a function of temperature. Experiments on hydrodynamic interactions with pairs of colloidal spheres can provide guidance in the interpretation of this observed coupling. Specifically, direct attractive interactions are likely to bias diffusion in favor of co-linear motion towards one another. Hydrodynamic momentum transfer will oppose such motion, resulting in enhanced hydrodynamic repulsion. Similarly, with proteins experiencing net repulsion, the direct interaction will tend to push other proteins out of the way, thereby decreasing solution-mediated momentum transfer when compared to non-interacting particles. Hence, enhanced attraction or repulsion among the lysozyme molecules would be accompanied by corresponding increases or decreases in hydrodynamic interactions, as observed in our experiments.
4.5 References

37. Malvern Instruments Technical Support Library, UK
Chapter 5

Nucleation and Growth of Gold Nanoparticles

5.1. Introduction

The properties of colloidal gold, silver and other such similar metal colloids have been of interest for centuries with an extensive scientific research going back to Michael Faraday in 1857 \(^1\). In 1908, Mie presented a solution to Maxwell’s equations that describes the extinction (absorption and scattering) of spherical particles of arbitrary sizes \(^2\). Ever since, various models and approximations have been developed to study nanoparticles systems \(^3,4\). Over the years, it has been realized that the morphology and the growth-rate of these nanostructures in the solution phase can be controlled and designed by tuning the reaction parameters. The wet chemical synthesis of nanomaterials has advanced to the level where it is possible to tailor make particle shapes, sizes and their distributions by manipulating various parameters during the growth process \(^5,6\). However, to achieve control over the synthesis, it is important to understand the process of nucleation and growth of crystallites from the cluster level upward, includes the specific roles played by various physical and chemical parameters such as temperature, concentration, pH, stirring, osmotic potential, incubation time etc. The mechanisms involved in the growth of nanoparticles follow different rules than those applicable to bulk materials. Over the last several decades, the mechanism of nucleation and growth processes of colloidal particles synthesized by various methods has been researched in detail. The initial swell in nucleation studies began predominantly with condensation \(^7,8\) and crystallization \(^9,10\) studies during the early twentieth century. However, mechanistic studies of colloid and cluster formation began when LaMer and Dinegar \(^11\) synthesized sulfur hydrosols nucleating from supersaturated solutions. Uniform particle size was achieved by short nucleation and relatively long growth periods. Studies on kinetics and mechanisms of particle formation showed incompatibility with Lamer’s supersaturation theory \(^12\). Models and statistical theories began to be developed for understanding the
formation of the critical nucleus and spontaneous growth which gives rise to particular sizes\textsuperscript{13,14}. Overbeek\textsuperscript{15} did extensive studies on the particle growth rate and the particle size distribution citing the possible rate-determining steps. Analysis of the activation barrier in the nucleation process, studies on the parameters relevant for kinetic or thermodynamic control, and factors controlling the growth process have improved our understanding of the overall process. However, the nucleation event itself is quite complicated and difficult to study experimentally. It depends on numerous factors like nucleation rates, cluster mobility, maximum cluster density, spatial and size distribution of clusters, and modes of growth. To fully understand the formation of particles at various levels, it is essential to capture and investigate the early stages of nucleation of the nanoparticles, their growth kinetics and the effect of various parameters. Henceforth, the study of mechanisms of crystal growth is currently attracting increasing interest and recent advancements in the instrumental techniques have made it feasible for \textit{in-situ} experimental investigation of the process with higher resolution and precision. Among the experimental techniques used to study and understand the kinetic and thermodynamic nature of nanocrystal nucleation and growth are small angle X-ray scattering (SAXS)\textsuperscript{16}, UV-visible spectroscopy, X-ray absorption spectroscopy\textsuperscript{17}, time dependent TEM\textsuperscript{18}, and DLS\textsuperscript{19}. Though these techniques are highly efficient for the \textit{in-situ} measurements of particle size and shape determination, the main problem with all methods is that they obtain information about larger clusters (around $>1-2$nm). The limitation on the time scale of the measurements is yet another issue since nucleation and growth of the nanoparticles during laboratory syntheses proceeds quite fast. The nucleation events, in particular, are difficult to resolve since they represent a transient, metastable state. Additionally, complications due to the reaction set-up (multi step synthesis processes, high temperature/pressure etc.) prevent combination of light scattering with X-ray scattering for simultaneous \textit{in-situ} measurements. Therefore, a careful investigation of nucleation and growth of nanocrystallites in the solution phase demands a synthesis protocol that is (1) single step (2) can be coupled with standard light/X-ray scattering set-ups (3) and has a slow-enough reaction rate to capture the growth process.

Recently, Ramya Jagannathan et al reported a novel synthesis route where they used the antibiotic cephallexin to reduce chloroauric acid\textsuperscript{20}. In this method colloidal gold
capped by antibiotic in aqueous solution is readily prepared by a facile one-step protocol. Their NMR and X-ray photoelectron spectroscopy (XPS) results have shown that the sulphur moiety present in the beta lactam is responsible for the dual role of reducing and capping (stabilizing) the gold nanoparticles\textsuperscript{20}. Interestingly, in this method, they could control the morphology of the gold nanoparticles from quasi-spherical to flat triangular flakes and finally to truncated triangles and hexagons by increasing the concentration of gold ions correspondingly. Their transmission electron micrograph also showed the presence of a large number of smaller 1-3 nm particles.

This one-step synthesis-route is a promising model system for studying the growth of the gold nanoparticles. The rationale behind choosing this particular synthesis method over several other established methods was due to the following reasons: a) the reaction is sufficiently slow (approx. 1.5 hour at 28 deg C), b) establishes the mechanism of colloidal gold synthesis by a biomolecule, specifically an antibiotic c) scattering and absorption studies can be performed using a simple system with no auxiliary chemicals or processes needed.

Here we report our results on \textit{in situ} dynamic light scattering studies at various incubation temperatures to understand the nucleation and growth mechanism.

5.2 Materials and Methods

5.2.1 Synthesis of Gold Nanoparticles

We followed with slight modification the one-step synthesis protocol developed by Jagannathan et al\textsuperscript{20} to synthesize the antibiotic functionalized gold nanoparticles. In short, $10^{-4}$ M chloroaauric acid was reduced by $10^{-5}$ M of the antibiotic- cephalaxin. Both the antibiotic and chloroaauric acid were first diluted to twice their final concentrations into the distilled water before mixing them to induce the formation of nanoparticles. For the DLS studies, we passed both stock solutions through 0.22 μm syringe filters to filter-out any performed aggregates. Using DLS, both the stock solutions were checked for the presence of such pre-existing particle clusters (or dust particles) that might interfere with subsequent nucleation studies\textsuperscript{21}. The 2x stock solutions were cooled to 5 °C, mixed in equal proportion to their final concentration and then placed into a quartz cuvette for light scattering measurements. The pH of the solution was monitored by using a digital pH
meter. The pH was stable around ~ 3.7. Following the particle synthesis, the resulting colloidal gold nanoparticle suspensions remained stable without aggregation or precipitation.

5.2.2 Dynamic light scattering (DLS) measurement

Dynamic light scattering (DLS) measurements were performed using a Zetasizer Nano S (Malvern Instruments Ltd., UK) with a 3 mW He-Ne laser operating at a $\lambda = 633\text{nm}$. For details see chapter 1. Glass cuvettes containing the mixed chloroauric acid/cephalexin solutions were placed inside the thermostated sample holder of the DLS unit and were allowed to equilibrate to their set temperature (15 °C, 25 °C or 35 °C) for five minutes. Intensity autocorrelation functions of scattered light were collected continuously using acquisition times of 60 seconds per correlation function. Throughout the experiment, the total intensity of scattered light changed dramatically due to the incessant nucleation and growth of strongly scattering gold colloids. Therefore, the measurement software protocol was set up to first measure the total scattering intensity and to adjust a variable neutral density filter in the detection arm accordingly, in order to keep the avalanche photodiode count well below saturation. Relative scattering intensities were corrected for this variable attenuation.

The complete analysis of Dynamic Light Scattering (DLS) measurements for particle size distribution is described in Chapter 2. For our system parameters, $q = 2.64 \text{nm}^{-1}$ hence, particles with hydrodynamic radii $r_h$ close to or below $q^{-1} \approx 38 \text{nm}$ could be treated simply as isotropic Rayleigh scatterers. Finally, the distribution of diffusion coefficients can be converted into particle size distributions using the Stokes-Einstein as given by equation (2.49) in chapter 2.

During the nucleation studies, the amplitude of the autocorrelation functions steadily increased as nucleation and aggregation of the gold sol progressed. Correlation functions with intercepts at $t \rightarrow 0$ smaller than 0.2 were excluded from our analysis due to their intrinsic noisiness. Otherwise, correlation functions were converted into particle size distributions using the "general purpose" inversion algorithm provided with the Zetasizer Nano S software. Particle size distributions obtained from alternative inversion algorithms yielded comparable results.
5.3 Results and Discussion

In Figure 5.1, we show the temporal evolution of the intensity correlation function of light scattered from the solution undergoing the synthesis of gold colloids at 15 °C. Due to the reduced synthesis rate, the early stages of the nucleation and growth of the colloidal gold particles are more readily resolved at T = 15 °C. Initially, no correlations are detected since the concentration fluctuations of the gold solution alone are too fast to be resolved by DLS. Fig. 5.2 displays the temporal evolution of the intercept of the intensity correlation function $g_2(\tau)$ vs. the incubation time of the sample. There is a significant latency period of approx. 30 min before the onset of nucleation and growth of gold particles as detected by DLS. This latency period decreases significantly as the solution temperature is raised to 25 °C or to 35 °C. After a period of rapid increase, the $g_2 (\tau)$ intercept eventually levels off around 0.78, below the theoretical limit of 1. The lower plateau value of 0.78 arises from contributions to the dynamic signal from purely static scattering off the various interfaces (air/glass/solution).

Fig: 5.1. Normalized temporal correlations of the intensity of scattered light, $g_2(\tau)$-1, vs. delay time $\tau$ obtained at different time points (see label on curve) during the synthesis of colloidal gold particles from chloroauric acid solutions ($10^{-4}$ M) in the presence of the antibiotic cephalexin ($10^{-5}$ M), incubated at 15 °C. With increasing incubation period, the correlations of the scattered light arising from the gold colloids nucleating and diffusing in the aqueous suspension increases significantly.

Together with the zero intercepts of the intensity correlation functions, Fig. 5.2 also shows the total intensity of scattered light during the synthesis of the gold colloids.
Obviously, the rapid increase in the temporal correlations of scattered light (~ 30 min) significantly precedes the upswing in overall scattering intensity (~ 80 min), both of which are associated with the nucleation and growth of the gold colloid particles. This is an intriguing observation since increases in static scattering intensity are frequently used as indicators for the onset of nucleation events in supersaturated solutions. Our observations suggest that the correlation amplitude of dynamically scattered light is a much more sensitive and reliable indicator for nucleation events than "kinks" in static light scattering data. Nevertheless, DLS is unlikely to capture the actual nucleation event due to at least two complicating factors. First, the dynamic signal during the very early phases of nucleation is contaminated by contributions from residual dust and air inclusions. In addition, the shot noise of the photon detector limits resolution of very small populations of particles.

Fig. 5.2 Intercepts of the intensity correlation function of scattered light (○) and the overall intensity of scattered light (△) as vs. the incubation time of the sample. The solid squares highlight the time points for most of the correlation functions displayed in Fig. 5.1, and their corresponding particle size distributions shown in Fig. 5.4. A fit through the intercepts of $g_2(\tau) \cdot -1$ vs. incubation time with a simple sigmoidal functions faithfully reproduces the experimentally observed behavior, as expected for an "activated process" such as nucleation. Notice also the significant lag of the total scattering intensity compared to the upswing in the amplitude of the correlation function. This implies that dynamic light scattering is a much more sensitive indicator of the nucleation event than static light scattering.

Figure 5.3 displays the particle size distributions obtained from the autocorrelation functions during the early stages of the nucleation and aggregation
process (see also open squares in Fig. 5.2). Noticeably, the larger particle peak around 20-30 nm emerges ahead of the smaller aggregates near 1-2 nm. However, caution is required when interpreting this result. First, as indicated by the Stokes-Einstein relation (equation 2.49 in chapter 2) and equation 2.42 in chapter 2, the autocorrelation of light scattered by small 1-2 nm aggregates decays at rates of only few microseconds. Unfortunately, the correlation functions \( g_2(\tau) - 1 \) remain rather noisy, particularly at these short delay times, until the amplitude of the zero-intercept is well above 0.5. In addition, the scattering intensity of the particles increases approximately quadratic with particle volume. As a result, a single particle of radius 25 nm will scatter one million times more light than a 2 nm particle. As is apparent from Fig. 5.2, the overall scattering intensity from the solutions remains rather weak prior to approx. 85 minutes into the experiment. In addition, the contribution to the scattering intensity from the small particles never exceeds 20% of the total scattering intensity (see Fig. 5.6). All these factors might collude to minimize the contributions of smaller particles to the dynamic light scattering signal during the very early stages of nucleation.

Fig. 5.3 Examples of particle size distributions obtained during the early phases of the synthesis of gold colloids in the presence of cephalixin during incubation at 15 °C. Fig. 5.2 indicates at what point in the nucleation process these particle size distributions where obtained. The particle distributions at all temperatures eventually showed two well-resolved particle peaks centered around 25 nm and 1 nm, respectively. At higher temperatures, the apparent delay between the emergence of the larger and smaller peak was much less pronounced.
Figure 5.4 summarizes the temporal evolution of the two peaks in the particle size distribution vs. the incubation time for samples at 15 °C, 25 °C and 35 °C, respectively. Most strikingly, the particle distribution is bimodal with two narrow peaks located around $r_h \approx 25$ nm and 0.5-1.5 nm. Following a brief latency period, the two well-separated populations of gold nanoparticles emerge from the supersaturated solutions nearly simultaneously, with the larger particles slightly preceding the smaller particles particularly at the lowest reaction temperature of 15 °C. As discussed above, it is not obvious whether this apparent difference in latency of nucleation is just a consequence of the limited detection sensitivity for the smaller aggregates. We are therefore, cautiously, concluding that both populations of gold nanoparticles nucleate essentially simultaneously. The observations of two different particle populations of distinct mean size are consistent with earlier observations made by Jagannathan et. al \cite{20} in a separate study (as discussion above) where the TEM micrographs showed the presence of larger particles surrounded by a large number of smaller particles\cite{20}.

**Fig. 5.4** Changes in the mean particle size for both the small and large gold colloids as function of incubation period and solution temperature. Two well separated populations of gold colloids with surprisingly tight limits on their particle distributions emerged at all incubation temperatures. The radius for the peak of either population of gold colloids remained essentially unchanged throughout the entire observation period of several hours.
Figure 5.5 summarizes the temporal evolution of the relative scattering intensity from the solutions at $T = 15\,^\circ\mathrm{C}$ and $25\,^\circ\mathrm{C}$, respectively. Following the initial lag-time for nucleation, the overall scattering intensity from these solutions rapidly increases with time, closely following a power law with exponents around 1.7.

![Graph showing the changes in total scattering intensity for T = 15°C and T = 25°C over incubation time.](image)

**Fig. 5.5** Changes in the total intensity of scattered light during the synthesis of colloidal gold particles at $T = 15\,^\circ\mathrm{C}$ and $25\,^\circ\mathrm{C}$. In contrast to the relative distribution of gold colloids (Fig. 5.5) the total number of colloidal gold particles rapidly increases throughout the incubation period. In addition, the synthesis clearly proceeds significantly faster at $T = 25\,^\circ\mathrm{C}$ than at $T = 15\,^\circ\mathrm{C}$. Intensity data shown here have been corrected to account for neutral density filters inserted in front of the detector in order to prevent saturation.

Figure 5.6 shows the corresponding changes in relative scattering intensity for the small vs. the large colloidal particles over the same time period. In stark contrast to the rapid increase in total scattering intensity, the relative contributions to the scattering intensity from either particle population remain nearly fixed at a ratio of approximately 20% for the small colloids vs. 80% for the larger colloids. Again, this suggests the remarkable feature that both populations are nucleating and growing at identical rates throughout the synthesis process. The chemical origin of the co-existence of two different size ranges with tight control over particle size, nucleation and growth rates is not obvious to us, but does suggest that all three components of the synthesis are somehow
tightly coupled to one another. We believe that this is the first report of simultaneous nucleation and growth of two size ranges. It seems that various functional groups on the antibiotic molecule (cephalaxin) might be playing a significant role in this process.

![Graph](image)

**Fig. 5.6** Percentage of total light scattered by either population of colloidal gold particles during synthesis at \( T = 15 \, ^\circ\text{C} \). Similar to the overall sizes of the two colloidal gold particles, the relative populations for either peak does not appear to change throughout the nucleation and growth period shown in our data. The results at \( T = 25 \, ^\circ\text{C} \) and \( 35 \, ^\circ\text{C} \) are comparable but have been omitted here for clarity.

Our DLS data exhibit, a highly unusual and surprising nucleation and growth process for gold nanoparticles mediated by the presence of cephalaxin. In addition, these two nanoparticle populations reach their respective final sizes very rapidly and then cease growth altogether. At the same time, the total number of gold colloid continues to grow rapidly, and their rate of formation is a sensitive function of incubation temperature. It is intriguing to note that, at all temperatures, we observe bimodal distribution of particles in a homogenous system. These observations raise important fundamental questions relating to the nucleation and growth mechanisms resulting in the observed behavior. What causes the apparent *simultaneous* nucleation of two distinct gold nanoparticles from an essentially homogenous solution? What distinguishes these two particle populations? What causes the rapid cessation of growth not just for one but both of these particles, and why does it occur at such different sizes? Why do these two populations not "compete"
for nutrient but continue to nucleate and increase in numbers essentially in lock-step with one another?

5.4 References


Chapter 6

Lysozyme as Tracer for Measuring Viscosity of Aqueous Solutions

6.1 Introduction

Solution viscosity is a fundamental parameter controlling power dissipation over many length scales, ranging from flow of macroscopic objects down to the diffusive motion of nanoparticles. A wide variety of methods is in use for measuring the bulk viscosity of fluids, including capillary viscometers, falling ball viscometers, vibrational viscometers, rotating disk viscometers 1-3 and, more recently, piezoelectric or magnetostrictive resonators 4, 5. However, due to the thermal capacity of common liquids, viscosity measurements can be time consuming and often require independent measurements of solution density to convert kinematic into dynamic viscosity values. In addition, bulk measurements are not applicable for mapping out spatial variations in viscosity in such diverse systems as biological blood flow 6 or during phase transitions in glassy systems 7. Monitoring diffusive motion of sub-micron tracer particles provides a convenient way to obtain spatially resolved data on solution viscosity, with good temporal resolution, and no need for additional density measurements when changing solution conditions (e.g. solution temperature or solute concentration). Tracer diffusion also permits remote-sensing of viscosity changes for solutions at extremes of temperature or pressure 2, 3.

Viscosity data are critical for evaluating dynamic light scattering measurements on how solution conditions affect colloidal diffusivity. Changes in colloidal diffusivity with solution conditions typically contain contributions from both altered solution viscosity and from solution-specific changes in colloidal interactions and/or aggregation behavior, and their corresponding effects on solute diffusivity 8-11. Ideally, one would like to measure solution viscosity and solution specific effects on diffusive solute transport independently, and without the need for reverting to time-consuming bulk viscosity measurements. Using a suitable tracer particle, dynamic light scattering can be
used to perform both tasks. In practice, however, saline solutions readily induce a loss of colloidal stability and subsequent aggregation of the most commonly used tracer particle: uniform populations of polystyrene beads. Even surface coatings can extend the range of stability of polystyrene beads only moderately. Here we report that the small protein hen-egg white lysozyme provides an attractive alternative as tracer particle for dynamic light scattering measurements of the viscosity of saline solutions.

6.2 Materials and Methods

6.2.1 Chemicals

As tracer particles we used either monodisperse polystyrene nanobeads (Polysciences Inc., cat # 64006) or two times recrystallized, dialyzed and lyophilized lysozyme (Worthington Enzyme, cat # 2932, Lot: X6J8946). Using DLS, we obtained hydrodynamic radii of $R_h = (32.0 \pm 0.6)$ nm for the microbeads and $R_h = (1.89 \pm 0.03)$ nm for lysozyme. All chemicals were obtained from Fisher Scientific and were reagent grade or better.

6.2.2 Lysozyme Stock Solutions

Lyophilized lysozyme was dissolved directly into 25 mM sodium acetate/acetic acid (NaAc) buffer at pH = 4.5. Stock solutions of MgCl₂, NaCl, and CsCl were prepared by dissolving each salt directly into 25mM NaAc buffer at pH = 4.5 to a stock concentration of 2M. The pH of all stock solutions was readjusted after addition of the salt, if necessary. Prior to mixing lysozyme stock solutions were filtered through 20 nm Anotop syringe filters and salt /buffer stock solutions were filtered through 220 nm syringe filter to remove any particulate impurities. After mixing the protein and salt stock solutions at a ratio of 1:1, the mixtures were incubated at 45°C for 5 min. This reduced the risk of inducing crystal seeds at high salt concentrations. Solutions were transferred to glass cuvettes and placed into the thermostated cuvette holder of a dynamic light scattering unit (Zetasizer Nano, Malvern Instruments Ltd., UK). Actual lysozyme concentrations of all solutions were determined from uv absorption measured at $\lambda = 280$nm using $\alpha_{280} = 2.64$ ml / (mg cm)$^{12}$. 
6.2.3 Dynamic Light Scattering (DLS)

All dynamic light scattering (DLS) measurements were performed with a Zetasizer Nano S (Malvern Instruments Ltd., UK) with a 3mW He-Ne laser at $\lambda = 633$nm. For details see chapter 1.

6.2.4 Tracer Particle Measurements

For measurements with polystyrene beads, 300 $\mu$l of the polystyrene standard (1% w/v) was dissolved either into 12 ml of water, with an added 10 mM of NaCl, or into 100 mM NaAc solution. Both solutions were filtered through a 0.22 $\mu$m pore size PVDF syringe filter. For water or NaAc solutions, the temperature-dependence of their viscosity was measured from 50 °C down to 5°C in 5°C steps, allowing 10 minutes of thermal equilibration after each temperature change. For the three saline solutions in this study (MgCl$_2$, NaCl, and CsCl), DLS measurements were performed at six different temperatures between 40 °C and 15 °C, again in steps of 5°C, and at four different salt concentrations (50 mM, 250 mM, 625 mM and 1M). Analysis of correlation data used the average of three (for polystyrene) or five (for lysozyme) correlation functions, with a typical acquisition time of 180 and 60 seconds, respectively.

6.2.5 Analysis of Tracer Diffusivity

Tracer diffusivities were derived from the decay rates of measured intensity autocorrelation functions $g_2(\tau)$. A detailed description of the analysis is given in chapter 2.

6.3 Results and Discussion

6.3.1 Viscosity Measurement Using Polystyrene Nanobeads

DLS-based measurement of solution viscosity is essentially a two-step process: (a) determine the hydrodynamic radius of the tracer particle in a solution of known viscosity (e.g. water at 20 °C); (b) convert changes in tracer diffusivity under different solution conditions (temperature, composition, pH, etc) back into changes in solution viscosity using the Stokes-Einstein relation [given by equation (2.49) in chapter 2]. This approach
imposes two constraints: First, the hydrodynamic radius of the tracer particles remains constant (e.g. no swelling or aggregation). Second, effects of interactions among the tracer particles on the diffusive relaxation dynamics are properly accounted for [see equation (2.49) in chapter 2].

![Figure 6.1: Viscosity of Water](image)

**Fig.6.1: Viscosity of Water:** (A) Plot of the viscosity of water as a function of temperature measured by Dynamic Light Scattering (DLS) using polystyrene latex ($R_H = 31.9$ nm) as a probe. Connecting lines are added as visual guides only.(B) Plot of relative viscosity of measured water viscosity using lysozyme to the tabulated value in the literature. The dashed line represents ±2.5% error.

Using dynamic light scattering, we measured the diffusivity $D_0$ of polystyrene nanobeads as function of solution temperature. To convert tracer diffusivities into solution viscosity using the Einstein Stokes relation [equation (2.49) in chapter 2], we need to determine the hydrodynamic radius of the tracer particles under known solution conditions. For polystyrene beads in water solutions at $20^\circ$C ($\eta = 1.002$ mPa s) we obtained $D_0 = 4.20 \times 10^{-12}$ m$^2$/s. This yields a hydrodynamic radius for the polystyrene
beads of $R_h = 32.0$ nm, which compares favorably with the manufacturers quoted dry diameter of 64.8 nm. Using this value for $R_h$, we determine polystyrene diffusivity in water as function of temperature between $5^\circ$ and $50^\circ$ C and derived the underlying changes in water viscosity $\eta(T)$. As shown in Fig. 6.1, water viscosities obtain using polystyrene tracer diffusivity were within ± 2.5% of tabulated values for water viscosity $\eta(T)$. We repeated temperature-dependent viscosity measurements using 100 mM and 250 mM NaAc buffer at pH = 4.5. While measurements at 100 mM NaAc buffer provided reliable data (see Fig.4.2A), at 250 mM NaAc concentration polystyrene beads had lost their colloidal stability and flocculated.

![Fig.6.2: Viscosity of 100mM NaAc using different probes:](image)

(A) Plot of viscosity of 100mM NaAc measured by Dynamic Light Scattering using lysozyme (open circle), and polystyrene latex (dark circle). Measured viscosity values compared with tabulated$^8$ (dark square) viscosity values at $T = 20^\circ$C. (B) Relative viscosity of 100mM NaAc using lysozyme as tracer particle to the polystyrene latex as a tracer particle. The dashed line represents ±2.5% difference in values using two different probes.
6.3.2 Viscosity Measurements Using Lysozyme

To address the problem of polystyrene flocculation even at very modest ionic strengths we explored the use of the protein lysozyme as alternative tracer particle. Hen-egg white lysozyme is a small (14.3 kD) enzyme whose 3-dimensional structure has been carefully characterized. Depending on salt identity, at moderate concentrations lysozyme will remain soluble for salt concentrations up to 1 M or more. Due to the four disulfide bonds in its native structure, lysozyme does not unfold up to 74 °C. More specifically, we have shown (see Fig. 6.3) that the hydrodynamic radius of lysozyme is essentially unchanged over a wide range of solution conditions, and irrespective of the chaotropic or kosmotropic character of salt ions added to the solution.

**Fig.6.3: Dependence of pH and temperature on hydrodynamic radius:** (A) Plot of ratio of mutual diffusion coefficient ($D_c$) and free particle diffusivity ($D_0$) i.e. ($D_c/D_0$) as function of lysozyme concentration $C_{\text{Lys}}$ for pH = 3 at $T = 20^\circ C$ & $50^\circ C$ and for pH = 4.5 for $T = 20^\circ C$. (B) Mean hydrodynamic radius $R_H$ of lysozyme for part (A) derived from the measured free particle diffusivity $D_0$ and corrected for the salt and temperature-dependent changes in water viscosity.
One additional concern requiring attention is the effects of particle interactions on diffusion measurements. Dynamic light scattering measures the diffusive relaxation kinetics of thermally induced fluctuations in local tracer concentrations\textsuperscript{13,21}. The Stokes-Einstein relation [equation (2.49) in chapter 2] only applies to thermally agitated diffusion, without contributions from (direct or hydrodynamic) particle interactions. Polystyrene beads can be used at sufficiently high dilution to fulfill this requirement. Protein interactions in solution, however, significantly alter the diffusivity measured at the finite protein concentrations needed for sufficient scattering intensities\textsuperscript{8,22}. These changes in $D_c$ are due to the potential of net force between lysozyme at different salt concentrations. For low salt concentrations residual charge repulsion among lysozyme molecules is only partially screened, causing a net increase in the relaxation rate and, therefore, mutual diffusivity (see e.g. Fig. 6.4A). At higher salt concentrations, short-ranged attractive forces dominate which slow down mutual diffusion. Over the range of lysozyme concentrations used in our study lysozyme's mutual diffusivity $D_c$ changes linearly with concentration (see Fig. 6.4). Extrapolating $D_c (C_{\text{Lys}})$ to its infinite dilution limit ($C_{\text{Lys}} = 0$) yields the corresponding free particle diffusivity $D_0$ appearing in Eqn. [(2.49) in chapter 2]. Extrapolation of the diffusion data to infinite dilution also guards against the potential effects of (concentration-dependent) protein oligomerization on measured diffusivities. As additional precaution against contamination of diffusivity data from temperature-induced oligomerization or precipitation we rejected any autocorrelation functions whose polydispersity exceeded 0.08.
Fig. 6.4: Effect of temperature and salt concentration on Diffusion coefficient $D_c$ of lysozyme: Plot of diffusion coefficients $D_c$ as a function of lysozyme concentration $C_{\text{lys}}$ (A) for 50mM CsCl as a function of increasing temperature for the temperature range of 15°C - 40°C. (B) for CsCl at increasing salt concentrations (50mM, 250mM, 625mM and 1M). The sign of slopes indicate whether interactions among the lysozyme molecules are either net repulsive (positive) or net attractive (negative). The y-axis intercepts at $C_{\text{lys}} = 0$ yields the free particle diffusivity $D_0$.

To compare the performance of polystyrene with lysozyme, we repeated the viscosity measurements vs. temperature in 100 mM NaAc buffer. From the extrapolated free-particle diffusivity of $D_0 = 11.02 \times 10^{-11}$ m²/s and a viscosity value of $\eta = 1.029$ mPa s for NaAc buffer at 20 °C, we obtained $R_H = 1.89$ nm for the hydrated lysozyme monomer. Mutual diffusivities $D_c$ of lysozyme vs. temperature were then repeated for the same series of NaAc solution temperature. The extrapolated free-particle diffusivity values $D_0$ were converted into corresponding changes in buffer viscosity $\eta(T)$ using the
Stokes-Einstein relation [equation (2.49) in chapter 2]. NaAc buffer viscosity values obtained with either polystyrene beads or lysozyme as tracer particles fall within 2% of previous measurements using a Cannon-Ubbelohde viscometer.

We extended lysozyme-based viscosity measurements to an extended range of salt concentrations (50 mM to 1 M) and to three salts with ranging from kosmotropic (MgCl$_2$) to weakly kosmotropic (NaCl) to predominately chaotropic (CsCl). Fig.6A displays the changes in the mutual diffusivity $D_c$ of lysozyme in 50mM CsCl/ 25 mM NaAc buffer solutions at pH = 4.5 as function of solution temperature. Fig. 6.4B shows the corresponding changes in mutual diffusivity $D_m$ for a fixed temperature of T= 25°C at four different concentrations of CsCl. As discussed above, changes in the potential of net force from repulsion (low salt concentration) to attraction (high salt concentration) causes the slope of the mutual diffusivity $D_c$ vs. lysozyme concentration $C_{lys}$ to change from positive to negative values (Fig. 6.4B).

Fig. 6.5 summarizes the temperature- and concentration dependent viscosity changes for CsCl, NaCl and MgCl$_2$ solutions. The dashed lines in Fig. 6.5A represent fits through the viscosity values assuming that the temperature dependence arises solely from an Arrhenius-type activation process, i.e. $\eta = A \exp(\Delta G/RT)$. Over the limited range of temperatures, the corresponding fits show reasonable agreement, but systematic deviations are apparent. We did not explore these deviations further since they are not the focus of the current work. Fig. 5.5B displays the dependence of solution viscosity on salt concentrations for a fixed temperature (T = 20 °C). For the case of MgCl$_2$ and NaCl, solution viscosity increases with increasing salt concentration, consistent with the dominant kosmotropic character of its cations (Mg$^{2+}$, Na$^+$. Similarly, the viscosity of CsCl solutions decreases since both its constituent ions are chaotropic. The dashed lines are fits through the data with the Kaminsky equation

$$\eta(C_s) = \eta_0 (1 + K_1 C_s^{1/2} + K_2 C_s + K_3 C_s^2) \quad (6.1)$$

This equation is an extension to the more commonly used Dole-Jones equation, with the $C^{1/2}$ term accounting for ionic screening effects (Debye-Hückel theory) and the higher-order terms representing empirical extensions to match experimental data at intermediate (linear) and higher (quadratic) salt concentrations.
Fig. 6.5: Measured viscosity of CsCl, NaCl, and MgCl₂ as function of temperature and concentration using lysozyme as a probe for DLS measurement: Plot of viscosity vs. temperature for CsCl, NaCl, and MgCl₂ at various concentrations. Dashed lines are added as visual guides only. For MgCl₂ measured values are compared with tabulated values using Kaminsky equation 17 (dark lines values). (B) Plot of the relative viscosity of measured viscosity using lysozyme as tracer particle to the tabulated value 17. The dashed line represents ±3% error.
Table 6.1: Temperature and concentration dependence of the viscosity for NaCl, MgCl₂, and CsCl solutions in 25mM sodium acetate buffer at pH = 4.5 obtained from measurements of lysozyme diffusivity.

**NaCl**

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**MgCl₂**

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CsCl

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6.4 Conclusion

We have compared polystyrene beads vs. the small protein lysozyme for use as tracer particles for light-scattering based viscosity measurements of aqueous saline solutions. Compared to traditional techniques, dynamic light scattering measurements of tracer diffusivity requires minimal solution volumes (< 100 μl), can be used to measure spatial or temporal variations in viscosity, determine the effects of extreme solution conditions (high pressure, high temperature) and can be applied to confined geometries (microfluidics). One of the main drawbacks of commonly used polystyrene beads as tracer particles, however, is their limited colloidal stability, which renders them prone to flocculation at or below physiological concentrations (~ 150 mM) of electrolytes.

As indicated in the above results section, lysozyme provides a stable and robust alternative to polystyrene beads, remaining soluble for many different salts up to concentrations of 1M or more. There are several reasons why lysozyme is a good choice as a tracer particle for viscosity measurements in saline solutions. First of all lysozyme is a small, globular protein with a well-defined molecular weight. Therefore, variations in tracer size arising from particle synthesis are negligible. As globular protein,
lysozyme's shape is sufficiently compact to be considered a uniform sphere for the purpose of translational diffusion measurements. Furthermore, lyophilized lysozyme stock with low levels of impurity and contamination of disordered aggregates is readily available. Therefore, with some care to avoid contamination from non-specific lysozyme clusters \(^1^4\), lysozyme solutions with a very low degree of polydispersity can be readily prepared.

Lysozyme is also a protein with an unusually high degree of structural stability. Due to the presence of four disulfide bridges in its structure, lysozyme is resists thermal unfolding up to temperatures of 74 °C \(^2^4\). Furthermore, we have shown that lysozyme retains its hydrodynamic radius of 1.9 nm within very tight limits over a wide range of pH values (pH 2-8) and temperatures (5-50 °C). In addition neither strongly kosmotropic nor chaotropic salt ions, at concentrations at or below 1M, were able to disrupt lysozyme's hydration layer \(^2^0\). Despite the common use of lysozyme in light-scattering studies of protein phase separation and crystallization \(^2^5-^2^8\), lysozyme actually tends to remain soluble over a wide range of solution conditions, as well, particularly when compared to polystyrene beads. This stability is closely related to the combination of a large net charge of lysozyme, particularly at acidic pH values \(^2^9\), with its overall small radius and modest short-range attraction \(^8\).

These advantages of lysozyme are tempered by the salt-specific effects on its solubility \(^1^7\) and the loss of net charge upon approaching of its isoelectric point around pH = 11 \(^2^9\). The net charge repulsion, which promotes lysozyme's colloidal stability at intermediate salt concentrations, does affect the diffusive relaxation dynamics of the concentration fluctuations measured in dynamic light scattering. This necessitates the use of dilution curves (see Fig. 5A) in order to extract the single-particle diffusivity appearing in the Einstein-Stokes relation in equation (1.49) in chapter 1. However, this latter complication is specific to DLS measurements which require sufficient protein concentrations in order to resolve the dynamic concentration fluctuations against the static scattering background. The latter limitation can be readily addressed and the use of lysozyme as tracer particle further extended by using fluorescence correlation spectroscopy in conjunction with low concentrations of fluorescently labeled lysozyme.
In short, lysozyme provides an attractive and readily feasible choice as tracker particle to monitor the viscosities of ionic solutions over a wide range of parameters and with many advantages compared to typical viscosity measurement in bulk samples.

6.5 References

Chapter 7

Probing the Viscoelastic Behavior of poly-N-isopropylacrylamide (poly-NiPAAm) During Thermally Induced Gel Collapse

7.1 Introduction

Soft materials such as polymers, gels, and many biomaterials are viscoelastic in nature \(^1,2\) i.e. they store as well as dissipate energy when an external stress is applied. Solids are elastic in nature and can store energy upon application of shear strain. Liquids are viscous in nature and dissipate energy. Soft materials exhibit both of these properties and are viscoelastic in nature. Generally, the viscoelasticity of soft materials i.e. elastic and viscous modulus is measured using rheometer. Rheometer measures the stress response upon application of a well defined strain \(^3\). The viscoelastic behavior of soft materials is typically condensed into the complex shear modulus \(G^*(f)\) where the real part measures the in-phase elastic response of the medium \(G'(f)\) and the imaginary part measures the out-of-phase viscous response \(G''(f)\). More recently a complimentary technique called microrheology \(^4-9\) has been developed to measure such viscoelastic behavior of soft materials. In microrheology a microscopic probe particle is embedded in the soft medium and its local displacement as function of an external force is measured to determine its viscoelastic properties. There are many different techniques used for measuring the displacement of probe particles, including particle tracking measurements \(^10\), Diffusion wave spectroscopy (DWS) \(^11\) and quasielastic light scattering (QELS) \(^12\).

In our experiments, the external force is random thermal motion of the probe particles. This motion can be very different from those in purely viscous fluids. It can be either subdiffusive motion or can be locally bound. Hence, we need to establish a relationship between the average microscopic motion of a colloidal probe particles to the macroscopic viscoelastic response of the complex medium. There are many advantages of this technique over conventional rheometer. First, of all only a very small amount of sample is required around 100μl compared to conventional rheometers which require
millilitres of sample. This is important for biological materials which are not available in large quantity or are intrinsically small (single cells). Secondly, no external stress is applied as probe particles are thermally driven at all frequencies. Again, this is advantageous for many biological relevant materials as larger external stress can restructure then irreversibly. Thirdly, since these probe particles are very small, their inertia can be neglected and the viscoelastic properties of materials can be measured at higher frequencies.

In this chapter, we will use the dynamic light scattering (DLS) to measure the viscoelastic properties of various soft materials in the sol as well as in the gel regime, and also probed a thermally induced gel collapse near and far from the phase transition temperature.

7.2 Materials and Methods

7.2.1 Preparation of Polyacrylamide (PAAm) Sample

The polyacrylamide (PAAm) solution is prepared in distilled water with 2.2 wt % of acrylamide monomers are mixed with 0.1 wt % of tetramethylenediamine, which acts as catalyst. For the formation of a sol phase, 0.03 wt % of the cross linker Methylenebisacrylamide are added. Adding 0.2 wt % of Methylenebisacrylamide leads to the formation of a gel phase, instead. Ammonium persulfate 0.5 wt % is added as initiator to the solution. Finally, 0.005 wt % of polystyrene beads (RH = 450 nm) is added to the solution. Fig. 6.1 shows the absorption of a PAAm solution undergoing gelation at 260nm as a function of time. The plateau in absorption around 50 minutes indicates that the polymerization reaction is completed. To achieve the formation of a gel phase, the solution is kept under N2 for 40 minutes.
7.2.2 Preparation of Poly-N-isopropylacrylamide (poly-NIPAAm) Sample

The poly-N-isopropylacrylamide (poly-NIPAAm) gels are prepared in distilled water with 4.86 wt % NiPAAm mixed with 0.019 wt % ammonium persulfate which is the initiator, 0.49 wt % TEMED as the activator and 0.113 wt % of BisAAm as cross-linker. For microrheological measurements 0.005 wt % of polystyrene beads ($R_H = 450$nm) are used are added in the solutions as probe particles. To achieve gel formation, the solution is exposed to UV irradiation at $\lambda = 360$nm for 40 minutes.

7.2.3 Dynamic Light Scattering Measurements

For DLS studies, we filtered the solution without beads through 0.02 $\mu$m syringe filters to filter out any pre-assembled clusters. DLS measurements performed with beads in solution yielded bead sizes of $454 \pm 5$ nm, confirming that the beads do not aggregate in solution. The 0.005 wt % beads concentration was chosen to ensure that the scattering signal dominated by scattering from beads dominates (vs. the polymer in solution). However, the concentration also has to remain small enough to prevent the multiple scattering. Glass cuvettes containing the gel with beads as probe particles were placed inside the thermostated sample holder of the DLS unit and were allowed to equilibrate to their set temperatures (20°C for PAAm sol and gel and 5, 31, or 33°C for poly-NiPAAm gel) for 5 minutes. Autocorrelation functions were obtained from averages.
of 5 measurements at each temperature. A detailed analysis of DLS data is given in chapter 2.

7.3 Results

7.3.1 Viscosity of Water using Polystyrene Beads

As first test we used the generalized Stokes-Einstein equation (equation 2.50 in chapter 2)\(^6\) to measure the viscosity of water at \(T = 20^\circ C\). From the field correlation function \(g_1(\tau)\) we derived mean square displacement \(<\Delta r^2(\tau)\>\) [(equation (2.51)]. Using the analysis as given in chapter 2 we calculated the full frequency dependence of the viscous modulus of polystyrene beads diffusing in water as shown in Fig. 7.2. As the motion of the beads in water is purely diffusive, the viscous modulus varies linearly with frequency (Fig.7.2). Using equation (2.55) we calculated the viscosity of water as function of frequency as shown in Fig.7.2. As expected, the viscosity of water at all frequency was found be 1.00cp, in agreement with tabulated values\(^16\). We also calculated the viscosity of water using the Stokes-Einstein equation given by equation (2.49) yield the same value. We therefore had confirmed the reliability of our analysis for a purely viscous medium.

![Viscous modulus G’’(f) and viscosity η(f) of water at T =20°C as a function of frequency derived by microrheological measurement using polystyrene beads. Viscosity of water was constant and match tabulated values.](image)

Fig.7.2: Viscous modulus G’’(f) and viscosity η(f) of water at T =20°C as a function of frequency derived by microrheological measurement using polystyrene beads. Viscosity of water was constant and match tabulated values.\(^16\)
7.3.2 Microrheological Measurement for Polyacrylamide (PAAm)

We used dynamic light scattering measurements to measure the viscoelastic properties of cross-linked polyacrylamide (PAAm) using polystyrene as probe particle in the sol and gel regimes. The aim was to reproduce existing result in order to confirm that we have command at this technique.13

Fig. 7.3: (A) Correlation functions for the sol (open square) and gel (dark square) phase for the polyacrylamide (PAAm) sample with embedded polystyrene beads ($R_{H} = 450$nm) at 20°C, (B) Plot of the mean square displacement of probe particle for PAAm sample for sol (open square) and gel phase (dark square), (C) local slope of sol (open square) and gel phase (dark square) (D) Elastic modulus $G'(f)$ (dark circle) and viscous modulus $G''(f)$ (open circle) and corresponding viscosity $\eta(f)$ of sol phase as a function of frequency. The horizontal line shows the corresponding water viscosity at that temperature, and (E) same as in (D), but for gel phase.

Fig. 7.3A shows the field correlation function for polystyrene beads dispersed in either the sol or gel phases. We can see that a bead in the sol phase displays a much faster
decay than in the gel phase. The correlation functions in Fig. 7.3A are converted into mean square displacement [see equation (2.51)], plotted in Fig. 7.3B. The slope of the mean square displacement for sol phase is around 0.8 to 0.9, for all time scales. A slope closer to 1 shows the dominance of viscous behavior. As expected for the sol phase, the viscous modulus \( G''(f) \) is greater than the elastic modulus \( G'(f) \) for the entire frequency range shown in Fig. 7.3C. For gel phase, the mean square displacement has a slope near 0.9 at short time scales, indicating predominately viscous behavior. At longer time scales it approaches 0.3 which shows the elastic behavior expected for gel samples (Fig. 7.3B). In this case the elastic modulus dominates over the viscous modulus in the low frequency range and become comparable to each other at high frequency. Our result reproduces previous work by Dasgupta et al. 13. We conclude therefore, that we have control over this method. In the following, we are going to use this method to study the thermal dehydration transition in poly-\( N \)-isopropylacrylamide (poly-NiPAAm).

7.3.3 Poly-N-Isopropylacrylamide (poly-NIPAAm) System

7.3.3.1 Gel Phase Transitions

Poly-NiPAAm undergoes a thermally controlled volume phase transition. During this transition the number and placement of crosslinks doesn’t change but the gel conformation and density changes. This is essentially a first-order phase transition with a sharp temperature onset (see Fig. 7.4) When the phase transition occurs, the polymer network collapses, the chains become more densely packed and the solvent water is expelled from the network. It was first predicted by Dusek and Patterson in 1968 17. Tanaka was the first one to describe it for ionize acrylamide gel 18. There are many factors which can trigger this phase transitions including pH, temperature, high pressure, uv light, etc. In this section we are going to discuss the temperature induced phase transition of poly-N-isopropylacrylamide (poly-NIPAAm). A more detailed discussion of polymer gels and phase transitions of gels can be found elsewhere 19,20.

Light scattering experiments are performed on the poly-NIPAAm gel with beads embedded inside the gel network. Scattering intensity data is shown in Fig.7.4. The sudden jump in the scattering intensity indicates that the phase transition occurs at 34°C.
At this point the gel becomes turbid (white) and it is not possible to use DLS to analyze bead motion.

**Fig. 7.4:** Changes in the total scattering intensity of poly-N-isopropylacrylamide (poly-NIPAAm) as a function of temperature. Intensity data shown here have been corrected to account for neutral density filters inserted in front of the detector in order to prevent detector saturation.

### 7.3.3.2 Microrheological Measurement with Poly-N-isopropylacrylamide (poly-NIPAAm)

As DLS measurement of microrheological behavior can not been performed for turbid sample, we limited our DLS measurements to the temperature range of 5°C (far from the transition temperature), to 33°C (very near to phase transition temperature). We made sure that the solution remains transparent during the measurement. Fig. 7.5A shows the correlation functions for bead movement at 5 and 33°C. It shows that at 33°C the decay rate is faster than at 5°C. We also notice a double decay mode at 33°C. The mean square displacement was derived from the correlation functions shown in Fig. 7.5B. [A detailed analysis of DLS data for measuring the viscoelastic behavior of polymer gels is given in Chapter 2]. At both temperatures the elastic modulus dominates over the viscous modulus at low frequency range and they become comparable at high frequency range (Fig. 7.5C and D).
Fig. 7.5: (A) Correlation functions for 450 nm polystyrene beads embedded in *poly-N*-isopropylacrylamide (poly-NIPAAm) gel at 5°C (open square) and 33°C (dark square), (B) Plot of the mean square displacement of the probe particle for poly-NIPAAm gel sample at 5°C (open square) and 33°C (dark square) (C) local slope of gel at 5°C (open square) and 33°C (dark square) (D) Elastic modulus $G'(f)$ (dark circle), and viscous modulus $G''(f)$ (open circle) and corresponding gel viscosity $\eta(f)$ as a function of frequency at 5°C. The dark line shows the corresponding water viscosity at that temperature, and (E) same as (D) for gel at 33°C.

Fig. 7.6 shows the microrheological measurements of the polymer gels at three different temperatures (5, 31, and 33°C). It can be seen that the elastic modulus values at the lower frequency are comparable for all three temperatures. At higher frequencies the elastic modulus decreases as the temperature increases. The viscous modulus is constant at lower frequency range and increases linearly at high frequencies. Like the elastic modulus, the viscous modulus also decreases as the temperature increases. At all temperatures the elastic modulus dominates over the viscous modulus at lower temperatures and frequencies, and is comparable or dominated by the viscous modulus at high frequencies. Viscosity values decreases as the frequency increases for all temperatures and level off towards water viscosity. It should be noted that at 5°C the viscosity value reaches the water viscosity for frequency around 8kHz, whereas for 31°
and 33°C it level off to water viscosity value around 5kHz and 2kHz respectively as shown in fig.7.6.

**Fig.7.6:** Comparison of elastic modulus $G'(f)$, viscous modulus $G''(f)$, and viscosity $\eta(f)$ as function of frequency at three (5, 31, and 33°C) different temperatures. The dark line shows the corresponding water viscosity at that temperature.

Note the dip of $\eta(f)$ below the water viscosity at $T = 33°C$. We are not yet certain whether this is due to an artifact in our data analysis or the presence of yet another relaxation process not properly accounted for in our data analysis. This work is in progress and will try to finish it before leaving.
7.4 References

Chapter 8

Conclusion

Using static and dynamic light scattering (SLS & DLS) we investigated a series of fundamental problems concerning phase separation kinetics of macromolecules and polymers, and the resulting rheological properties of their condensed phase.

We have characterized the interactions of proteins in solution and their effects on their aggregation behavior under conditions that promote protein crystallization. We have shown that it is necessary for nucleation and growth studies in protein crystallization to carefully characterize whether the starting materials is a homogenous collection of monomers or contains substantial populations of pre-formed large aggregates. The clusters present in the stock materials of hen-egg white lysozyme, a frequently used protein in crystallization studies, will distort the nucleation kinetics and increase crystal defect formation. These clusters might well be the reason for the persistent contradiction in existing nucleation data on the size of crystal nuclei, their induction time or the total number of nuclei generated under comparable conditions.

Using DLS and SLS, we have also determined the (non-)effects of chaotropic (water structure breaking) versus kosmotropic (water structure making) ions on the hydration layer and the hydrodynamic interactions of lysozyme. Our results show that neither protein hydration nor solvent-mediated hydrodynamic interactions displays any obvious salt-specific effects, while salt-specific effects on direct protein interaction were prominent.

DLS has been used to monitor the simultaneous nucleation and growth of gold nanoparticles synthesized from solution in the presence of the antibiotic cephalexin. Their nucleation kinetics were measured at three different incubation temperatures (15, 25, and 35°C). It seems that two populations of nuclei with distinctly different sizes formed simultaneously. Equally intriguing, the sizes of these two nuclei populations remained
essentially fixed, only their numbers increased over time. Decreasing the temperature only slowed down the induction period prior to nucleation.

We also used DLS to measure the viscosity of aqueous solution with lysozyme as a tracer particle. We find that lysozyme provides an advantageous tracer particle for viscosity measurement of saline solution up to 1 M where other probe particles like polystyrene beads flocculate. Due to its inherently high structural and colloidal stability, lysozyme provides a useful tracer particle for high salt concentration and a wide pH and temperature range, which are relevant for biological solutions and sample processing in aqueous environments.

Finally we extended tracer particle measurements with DLS to characterize the microrheological properties of polymers in their gel or sol phase. The viscoelasticity of the medium i.e. the elastic as well as viscous modulus, were determined over a range of "force frequencies" spanning five decades. We also succeeded in performing microrheological measurements for PAAm in the sol and gel regime. For poly- NiPAAm we measured the viscoelastic behavior near and far from the phase transition temperature in the gel regime.
About the Author

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