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Accessing Lysozyme Nucleation with a Novel Dynamic Light Scattering Detector

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Abstract

Dynamic light scattering was employed to investigate the behaviour of nucleating hen egg-white lysozyme solutions. For these studies a novel fiber-optic based microprobe that suppresses multiple light scattering and contributions from large clusters in the spectra by backscattering detection was employed. The time evolution of small lysozyme clusters was found to obey classical nucleation at the initial stages of the reaction.

1. Introduction

The search for optimal crystallization conditions is an important aspect for obtaining protein crystals suitable for X-ray analysis. The relative lack of kinetic data and non-systematic screening of crystallization attempts has so far prohibited the development of adequate protein crystallization diagnostics. In recent years, hen egg-white lysozyme, a small globular enzyme, has been extensively used for studying nucleation events in the presence of screening electrolytes (Kam *et al.*, 1978; Feher & Kam, 1985; Georgalis *et al.*, 1993, 1994, 1995; Georgalis & Saenger, 1993; Eberstein *et al.*, 1994; Niimura *et al.*, 1995; Muschol & Rosenberger, 1995) employing light and small-angle neutron scattering techniques.†

Dynamic light scattering (DLS) is a well established method that provides information about particle motion, and thus size, by directly measuring the photon autocorrelation function (ACF, Schmitz, 1990). Under ideal conditions (a dilute and monodisperse small particles ensemble) DLS delivers directly the hydrodynamic radius of the particles, R_h (Chu, 1991; Brown; 1993). DLS measurements are however, impossible with very concentrated samples often required in crystallization experiments. Besides difficulties in correctly treating interparticle interactions, major problems arise from multiple light-scattering contributions. These contributions lead to additional spectral broadening and the measured ACF decays more rapidly than that resulting from single scattering events. In practice, one can reduce multiple scattering by using thinner samples or, even better, by employing index matching as in colloidal crystallization studies (Pusey, 1990; Schätzel & Ackerson, 1992, 1993). However, the latter approach can hardly be applied to biological samples since they may denature in other than aqueous solvents.

An alternative way to alleviate the multiple scattering problems is to model photon transport as a diffusive process with samples exhibiting strong multiple scattering (Ishimaru, 1978). Because of the diffusive photon transport involved, the technique is known as diffusing wave spectroscopy (DWS, Weitz & Pine, 1993). Another route is to isolate the singly scattered light from the detected signal by using particular cross-correlation techniques (Drewel *et al.*, 1990; Schätzel, 1991). This type of experiment achieves excellent recovery of the dynamic structure factors (Segré *et al.*, 1995). Very recently, Overbeek *et al.* (1997) and Aberle *et al.* (1997) have realized '3-D' cross-correlation instrumentation for suppressing multiple light scattering in highly turbid solutions.

Other useful approaches directed towards alleviating multiple light scattering in concentrated samples involve microprobes.‡ In comparison to standard DLS, the size of microprobes is considerably reduced by using fiber-optics for launching the beam and detecting the scattered light (MacFadayen & Jennings, 1990). Especially for investigating complex systems like nucleating protein solutions, the use of such miniature instrumentation is highly desirable. There are two important reasons for this: first, proteins are only rarely available in large quantities so that the volumes required by conventional DLS instruments (typically 1 ml) preclude automated high-throughput screening crystallization assays using standard instrumentation. Second, a gradual supersaturation increment implies at some stage turbid solutions, and consequently conventional DLS becomes impossible.

† Aspects concerning protein crystallization have been reviewed by Ducruix & Giegé (1992) and Giegé *et al.* (1995) in a paper with 291 literature citations.

‡ For an historical development on DLS and DWS microprobes see Tanaka & Benedek (1975); Dyott (1975); Auweter & Horn (1985); Ross *et al.* (1987); Dhadwal & Ross (1980); Dhadwal & Chu (1989); Brown *et al.* (1986); Brown & Jackson (1987); Brown & Grant (1987); Brown, Burnett, Mansbridge, Moir *et al.* (1990); Brown, Burnett, Mansbridge & Moir (1990); Wiese & Horn (1991, 1992) and Van Keuren *et al.* (1993).

Fortunately, microprobes may offer simultaneous solutions to both problems. Wilson (1990) and Casay & Wilson (1992) have constructed a device for investigating protein nucleation. It operates in a hanging-drop vapour-diffusion apparatus and uses 90° DLS detection. Besides miniaturization, an attractive feature, is the good performance of this device with only 40 μl hanging drops.

In this work we report first results obtained with a novel microprobe, the ALV-NIBS (non-invasive backscattering) detector, for investigating nucleation events without multiple scattering at a single scattering angle $\simeq 170°$. We focus on an important aspect in protein crystallization, namely resolving the mean size of stable nuclei using the model system lysozyme–NaCl.

2. Materials and methods

The chemicals used in the present work were of analytical grade. Three times crystallized lysozyme was purchased from Sigma (Deisenhofen, Germany) and treated as previously described (Georgalis *et al.*, 1995, 1997*a*,*b*). All experiments were conducted in a buffer containing 0.10 *M* Na acetate pH 4.25, at room temperature, *ca* 298 K. For aggregation experiments, lysozyme and NaCl were prepared as stock solutions at twice the final concentration, they were rapidly mixed in 1:1 ratio and filtered through Minisart sterile filters, 0.2 μm pore size, into standard disposable square cuvettes and monitoring was initiated immediately.

A pictorial description of the NIBS detector is shown in Fig. 1. The instrument is a prototype (patent pending) and it can handle solute contents of up to $\varphi = 0.20$† for proteins (very turbid solutions) minimizing multiple scattering contributions. Such high concentrations, which could previously be studied only with invasive microprobes or using DWS, have been successfully measured with NIBS using water-suspended polystyrene latex particles with radius 125 nm.

Spectra were collected every 30 s using an ALV/SO-SPID double photo-multiplier assembly and the ALV-5000/E digital correlator operating in the pseudo-cross-correlation mode. The optics allow for a very tightly focused beam with mean spot diameter smaller then 100 μm. The scattering angle of NIBS is 170° and corresponds to a scattering vector‡ of 2.65×10^{-2} nm^{-1}. The laser power used for the experiment was 1 mW at the He–Ne laser line (632.8 nm). After the end of the experiments the correlograms were Laplace inverted with a modified version of the program *CONTIN* (Provencher, 1982*a*,*b*) as previously described (Geor-

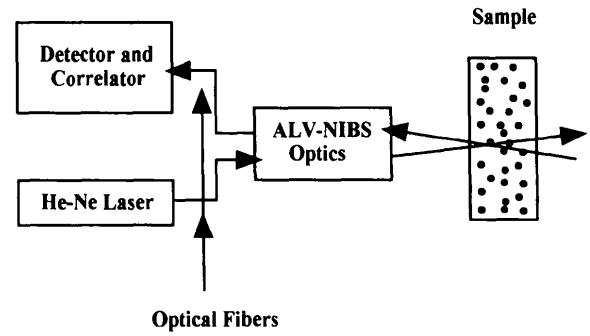

Fig. 1. Pictorial representation of the ALV-NIBS detector.

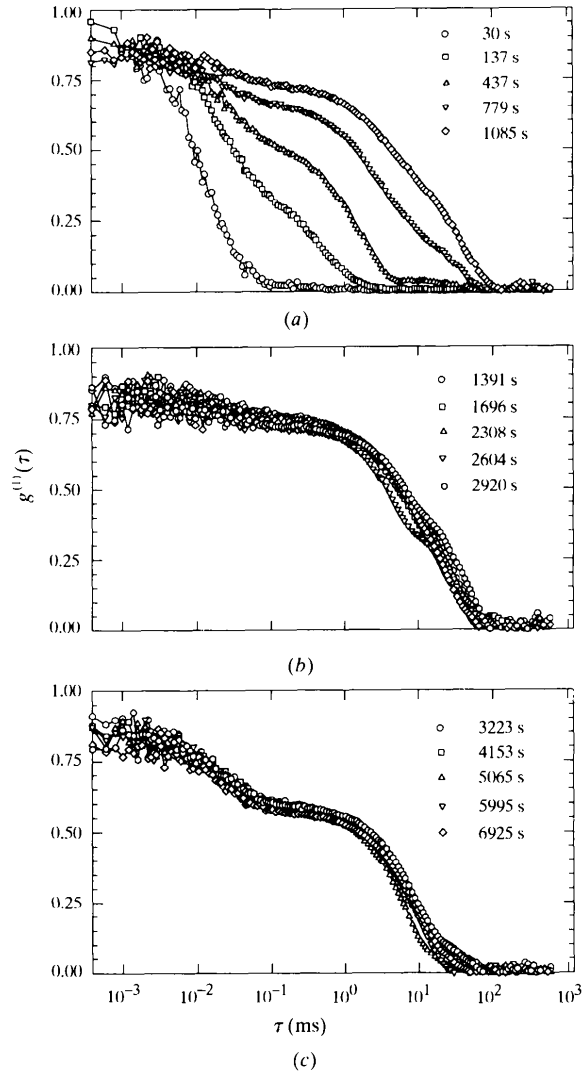

Fig. 2. Field ACF $g^{(1)}(\tau)$, plotted as a function of delay time τ for the 5.60 m*M* lysozyme sample. For clarity, only every tenth spectrum is displayed in (*a*) and (*b*) and every 20th in (*c*). Note the changes in the behaviour of the ACF, *i.e.* growth of the intermediate components for between 30 and 1085 s in (*a*) and the collapse of the spectra at longer times, between 1391 and 2920 s (*b*) and 3233 and 6952 s in (*c*).

† $\varphi = (4\pi/3)\alpha^3 C_n$, denotes the volume fraction of a solute with a radius α and number concentration C_n.

‡ The scattering vector **q** defines the spatial resolution of the experiment. Its magnitude is $\mathbf{q} = (4\pi n/\lambda)\sin(\theta/2)$ where n is the refractive index of the solution, λ the wavelength of the incident light and θ the scattering angle.

galis *et al.*, 1993, 1995). Solution viscosities for correcting the data for NaCl were obtained from standard tables (*CRC Handbook of Chemistry and Physics*, 1984–1985).

3. Results and discussion

In this report we focus our attention on an especially clear example involving 1.40 and 5.60 m*M* lysozyme in 0.5 *M* NaCl, 0.10 *M* Na acetate pH 4.25. The conditions chosen for the first sample are lie below the optimal lysozyme crystallization conditions. In the second sample we have used a lysozyme concentration higher than usually employed in previous work. The chosen electrolyte is at an intermediate final concentration that triggers nucleation, and is successful in both cases. Rapid nucleation and concomitant crystallization is expected only with the sample containing 5.60 m*M* lysozyme, which could not be examined so far by DLS at forward angles due to rapid development of multiple light scattering.

In forward angles, scattering from fractals dominates the spectra and intermediate components cannot be retrieved with confidence upon Laplace inverting the spectra. In the present work we pay special attention to these populations, believed to be stable lysozyme nuclei.

The set of correlation functions corresponding to the 5.60 m*M* sample displayed in Fig. 2 shows the ACF's are not monomodal. The smallest components that are of interest are captured with precision by the NIBS detector. The results are satisfactory but the low laser power employed may induce some uncertainty at small lag times. In Fig. 3 we have plotted the time evolution of the mean hydrodynamic radii, corresponding to lysozyme concentrations of 1.40 and 5.60 m*M*. For both solutions, radii pass through a maximum, indicating approach to a critical size, at around 5.0 and 8.0 nm respectively, and relax after some time to constant values of 3.2 and 6.5 nm, after several hours. Crystals were observed within 3 h in the latter sample, but after two days with the 1.40 m*M* lysozyme sample.

The observed events may be dictated by a cluster restructuring mechanism (Meakin, 1988). Clusters may undergo several internal rotations until they reach an energetically more favourable, compact conformation. Such ideas, well known in colloidal aggregation, have been used by us, without formal proof or theoretical support, to interpret events observed in supersaturated lysozyme solutions. They have been recently disputed by some groups (Muschol & Rosenberger, 1996) and verified by others who conducted the right experiments (Tanaka *et al.*, 1996). An exact verification of the underlying events will require theoretical computations of cluster–cluster aggregation, using suitable potentials. Unfortunately, the solvent-mediated interactions, which drive these process are only approximately tractable

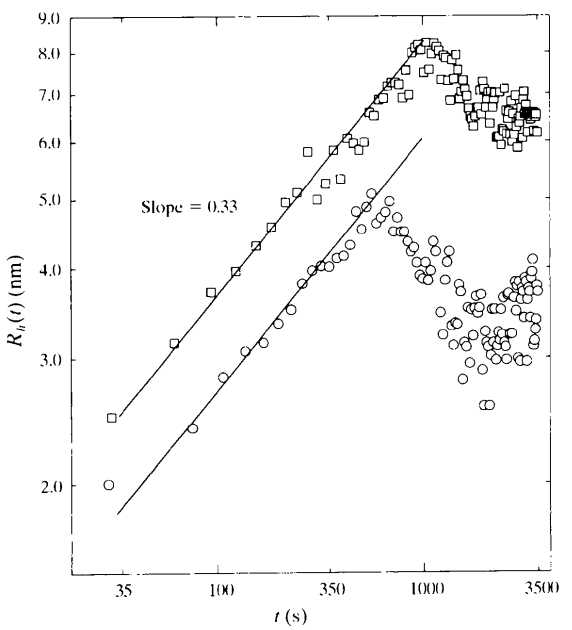

Fig. 3. Time evolution (only the first hour of the reaction) of the mean cluster hydrodynamic radius $R_h(t)$ of the smallest component for the samples containing 1.40 (○) and 5.60 m*M* (□) lysozyme. After passing the maxima at around 5.0 and 8.4 nm, radii relax to values around 3.2 and 6.5 nm, respectively. The solid lines correspond to a power-law growth with an exponent of 0.33 for both samples.

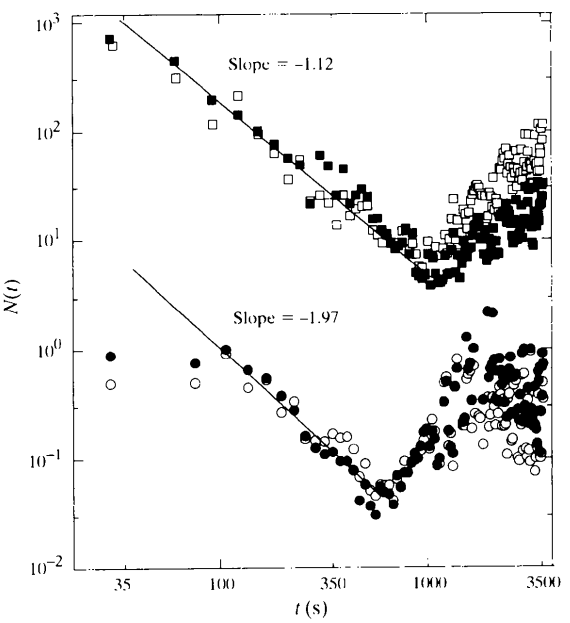

Fig. 4. Time evolution (only during the first hour of the reaction) of the relative number of nuclei in solution $N(t)$, for the samples containing 1.40 (○) and 5.60 m*M* (□) lysozyme. The solid line corresponds to a power-law growth with an exponent of −1.97 for the 1.40 m*M* and −1.12 for the 5.60 m*M* lysozyme sample. For comparison $N(t)$ has been determined from the total scattered intensities (*i.e.* involving contributions from monomers and fractals) and plotted with closed symbols. The exponents deduced from either plot are nearly identical.

even for monomer lysozyme–NaCl system (Soumpasis & Georgalis, 1997). Needless to say, that unless the monomeric state is understood, attempts to investigate interactions between clusters will be unsuccessful.

If the nuclei are assumed to be compact, they should include 15–75 monomers, respectively, at the maxima. For the initial growth stages *i.e.* less than the first 500–1000 s, there is a clear power-law kinetics relationship describing cluster ripening (Landau & Slyojov, 1965) as $R_h(t) \simeq t^{0.33}$ (Schätzel, 1996). This behaviour is qualitatively similar to the observations made by Casay & Wilson (1992) who found nucleation in hanging drops after 100 min associated with lower speed of equilibration compared with batch crystallization setup. Our estimates of the hydrodynamic radii of the nuclei are also within the range determined by these authors and agree with more recent works using X-ray (Georgalis *et al.*, 1995) or neutron small-angle scattering (Niimura *et al.*, 1995). In our previous studies, however, growth of nuclei could not be quantitatively studied due to contributions from larger fractal clusters which dominated the spectra at forward scattering angles.

We have also determined roughly the amplitudes derived from *CONTIN* analyses, to obtain estimates of the relative number of nuclei, $N(t)$, as a function of time, Fig. 4.† From the amplitudes we can obtain approximate estimates of $N(t) \propto I(t)/R_h^6(t)$. We find clear power-law kinetics, $N(t) \simeq t^{1.97}$ for the 1.40 mM lysozyme sample, and $N(t) \simeq t^{1.12}$ for the sample containing 5.60 mM lysozyme. The agreement is comparable to that attained when using the total scattered intensities to determine $N(t)$ (filled-in symbols in Fig. 4). An exponent equal to unity would indicate linear nucleation rates. An exact determination of these exponents is however difficult even in homogeneously crystallizing systems (Schätzel, 1996). The observed lysozyme clusters may serve as precursors for the growth of larger clusters observed in recent calorimetric and small-angle static light-scattering studies (Georgalis *et al.*, 1998; Umbach *et al.*, 1998).

The described experiments were conducted in a routine manner, *i.e.* without taking precautions to avoid dust and using disposable square cells throughout. We believe that routine diagnostic experiments should deliver reliable answers without the manipulations involved in careful DLS experiments. Whereas some residual dust contamination was evident in some spectra, the backscattering geometry rendered spectra remarkably insensitive to dust. The overall NIBS performance was very satisfactory, and only a small percentage of the spectra from either experiment had to be discarded. This is advantageous since a set up of very clean samples in large scale diagnostic experiments is difficult, cumbersome and very expensive. The effects captured with the NIBS detector are subtle, *i.e.* the quoted differences in particle radii are small, and similar observations in the presence of larger clusters are unlikely to be successful with standard DLS instrumentation, at least at the higher concentrations examined.

† For these simple computations we have assumed that the scattered intensity $I(m)$ of a cluster with mass m can be approximated as $I(m) \propto \int_0^\infty N(m)R_h^6(m)P(q)S(q)\,dm$ where $N(m)$ denotes the number of clusters and $P(q)$, $S(q)$ the static, form and structure factors of the cluster, responsible for shape and intraparticle interactions, respectively. We have assumed both $P(q)$ and $S(q)$ to be equal to unity.

4. Conclusions

The increasing demand for quick and non-invasive light-scattering techniques and the rapid progress of modern optics and electronics have led to a considerable expansion in the field of laser light scattering. The use of polarization maintaining optical fibers for beam launching and detection of scattered light provides means towards the use of miniature optics with small size sources and detectors.

The NIBS microprobe incorporates many interesting features besides its miniature size. The instrument performs very well in a wide range of concentrations covering transparent to very turbid samples and permits investigations in both DLS and DWS regimes. Even at high solute contents, the instrument offers excellent suppression of multiply scattered light still using a homodyne detection scheme. The signal detection is very efficient due to special optics and typically only 1 mW of laser power is required or low concentrations of small scatterers like lysozyme to obtain usable spectra within short times. These preliminary experiments indicate that crucial information on protein nucleation can be obtained in relatively short periods of time. Therefore, NIBS is a promising platform for establishing high-throughput screening by combining experimental (Eberstein *et al.*, 1994) and computational (Soumpasis & Georgalis, 1997) schemes.

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References

Aberle, L. B., Wiegand, S., Schröer, W. & Staube, W. (1997). *Prog. Colloid Polym. Sci.* **14**, 121–124.

Auweter, H. & Horn, D. (1985). *J. Colloid Interface Sci.* **105**(2), 399–409.

Brown, R. G., Burnett, J. G., Mansbridge, J. & Moir, C. I. (1990). *Appl. Opt.* **29**, 4159–4169.

Brown, R. G., Burnett, J. G., Mansbridge, J., Moir, C. I. & Lowans, B. S. (1990). *Appl. Opt.* **29**, 3291–3302.

Brown, R. G. W. & Grant, R. (1987). *Rev. Sci. Instrum.* **58**, 928–931.

Brown, R. G. W. & Jackson, A. P. (1987). *J. Phys. E*, **20**, 1503–1507.

Brown, R. G. W., Jones, R., Rarity, J. G. & Ridley, K. D. (1987). *Appl. Opt.* **26**, 2383–2389.
Brown, R. G. W., Ridley, K. D. & Rarity, J. G. (1986). *Appl. Opt.* **25**(22), 4122–4126.
Brown, W. (1993). *Dynamic Light Scattering, the Method and Some Applications*. Oxford Science Publications.
Casay, G. A. & Wilson, W. W. (1992). *J. Cryst. Growth*, **122**, 95–101.
Chu, B. (1991). *Dynamic Light Scattering*. New York Academic Press.
CRC Handbook of Chemistry and Physics (1984–1985). 65th ed. Cleveland, Ohio: The Chemical Rubber Co.
Dhadwal, H. S. & Chu, B. (1989). *Rev. Sci. Instrum.* **60**(5), 845–853.
Dhadwal, H. S. & Ross, D. A. (1980). *J. Colloid Interface Sci.* **76**(2), 478–489.
Drewel, M., Ahrens, J. & Podschus, U. (1990). *J. Opt. Soc. Am. A*, **7**, 206–210.
Ducruix, A. F. & Giegé, R. (1992). *Crystallization of Nucleic Acids and Proteins*. Oxford: IRL Press.
Dyott, R. B. (1975). *Microwaves Opt. Acoust.* **2**, 13–18.
Eberstein, W., Georgalis, Y. & Saenger, W. (1994). *J. Cryst. Growth*, **143**, 71–78.
Feher, G. & Kam, Z. (1985). *Methods Enzymol.* **114**, 77–112.
Georgalis, Y. & Saenger, W. (1993). *Adv. Colloid Interface Sci.* **46**, 165–183.
Georgalis, Y., Schüler, J., Eberstein, W. & Saenger, W. (1994). *Fractals in the Natural and Applied Sciences, IFIP (A-41)*, edited by M. M. Novak, pp. 139–151. Amsterdam: Elsevier Science.
Georgalis, Y., Schüler, J., Frank, J., Soumpasis, D. M. & Saenger, W. (1995). *Adv. Colloid Interface Sci.* **58**, 57–86.
Georgalis, Y., Umbach, P., Raptis, J. & Saenger, W. (1997*a*). *Acta Cryst.* D**53**, 691–702.
Georgalis, Y., Umbach, P., Raptis, J. & Saenger, W. (1997*b*). *Acta Cryst.* D**53**, 703–712.
Georgalis, Y., Umbach, P., Zielenkiewicz, A., Utzig, E., Zielenkiewicz, W., Zielenkiewicz, P. & Saenger, W. (1998). *J. Am. Chem. Soc.* **119**(49), 11959–11965.
Georgalis, Y., Zouni, A., Eberstein, W. & Saenger, W. (1993). *J. Cryst. Growth*, **126**, 245–260.
Giegé, R., Drenth, J., Ducruix, A. F., McPherson, A. & Saenger, W. (1995). *Prog. Cryst. Growth Charact.* **30**, 237–281.
Ishimaru, A. (1978). *Wave Propagation and Scattering in Random Media*. New York: Academic Press.
Kam, Z., Shore, H. B. & Feher, G. (1978). *J. Mol. Biol.* **123**, 539–555.
Landau, I. M. & Slyojov, V. V. (1965). *J. Phys. Chem. Solids*, **19**, 35–43.
MacFadayen, A. J. & Jennings, B. R. (1990). *Opt. Laser Technol.* **22**(3), 175–187.
Meakin, P. (1988). *Phase Transitions and Critical Phenomena*, Vol. 12, p. 351, edited by C. Domb & L. L. Lebowitz. New York: Academic Press.
Muschol, M. & Rosenberger, F. (1995). *J. Chem. Phys.* **103**(24), 10424–10432.
Muschol, M. & Rosenberger, F. (1996). *J. Cryst. Growth*, **167**, 738–747.
Niimura, M., Minezaki, Y., Ataka, M. & Katsura, T. (1995). *J. Cryst. Growth*, **154**, 136–144.
Overbeek, E., Sinn, C. & Palberg, T. (1997). *Prog. Colloid Polym. Sci.* **14**, 116–120.
Provencher, S. W. (1982*a*). *Comp. Phys. Comm.* **27**, 213–227.
Provencher, S. W. (1982*b*). *Comp. Phys. Comm.* **27**, 229–232.
Pusey, P. N. (1990). *Liquids, Freezing and the Glass Transition*, edited by D. Levesque, J. P. Hansen & J. Zinn-Justin, p. 764. Amsterdam: Elsevier.
Ross, D. A., Dhadwal, H. S. & Dyott, R. B. (1987). *J. Colloid Interface Sci.* **64**(3), 533–542.
Schätzel, K. (1991). *J. Modern Opt.* **38**, 1849–1865.
Schätzel, K. (1996). *Ordering and Phase Transitions*, edited by A. K. Arora & B. V. R. Tata, pp. 17–39. New York: VCH Publishers.
Schätzel, K. & Ackerson, B. J. (1992). *Phys. Rev. Lett.* **68**(3), 337–340.
Schätzel, K. & Ackerson, B. J. (1993). *Phys. Rev. Lett.* **48**(5), 3766–3777.
Schmitz, S. K. (1990). *An Introduction to Dynamic Light Scattering by Macromolecules*. New York: Academic Press.
Segré, P. N., van Megen, W., Pusey, P. N., Schätzel, K. & Peters, W. (1995). *J. Mod. Opt.* **42**, 1929–1939.
Soumpasis, D. M. & Georgalis, Y. (1997). *Biophys. J.* **72**, 2770–2774.
Tanaka, S., Masahiko, Y., Kawashima, K., Kohzo, I., Hayakawa, R. & Ataka, M. (1996). *J. Cryst. Growth*, **168**, 44–49.
Tanaka, T. & Benedek, G. B. (1975). *Appl. Opt.* **14**(1), 189–196.
Umbach, P., Georgalis, Y. & Saenger, W. (1998). *J. Am. Chem. Soc.* In the press.
Van Keuren, E. R., Wiese, H. & Horn, D. (1993). *Langmuir*, **9**, 2883–2887.
Weitz, D. A. & Pine, D. J. (1993). *Dynamic Light Scattering, The Method and Some Applications*, edited by W. Brown, pp. 652–720. Oxford: Clarendon Press.
Wiese, H. & Horn, D. (1991). *J. Chem. Phys.* **94**(10), 6429–6443.
Wiese, H. & Horn, D. (1992). *Ber. Bunsenges. Phys. Chem.* **96**, 1818–1828.
Wilson, W. W. (1990). *Methods Companion Methods Enzymol.* **1**, 110–117.