Simultaneous Multiple Sample Light Scattering for Analysis of Polymer Solutions

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ABSTRACT: An instrument for performing Simultaneous Multiple Sample Light Scattering (SMSLS) is introduced, which allows multiple, independent polymer samples to be analyzed simultaneously. Three demonstrations of SMSLS capabilities are made; proof of the SMSLS ability to make absolute Rayleigh scattering ratio measurements on well-defined standards, the monitoring of a biopolymeric degradation process (hyaluronate degradation using hyaluronidase), with subsequent determination of the Michaelis-Menten constant, and an aggregation process (low concentration gelatin solutions). It is hoped that SMSLS will become a valuable tool for rapid screening and characterization of both equilibrium properties and nonequilibrium processes in polymer solutions. © 2004 Wiley Periodicals, Inc. J Appl Polym Sci 92: 2724–2732, 2004

Key words: light scattering; ageing; degradation; enzymes; particle nucleation

INTRODUCTION

Static light scattering (SLS) has been used for well over a century to determine properties of molecular gases, aerosols, interstellar dust, polymers, and colloids.[1–4] Aided in large part by the progress in high sensitivity photodetection, inexpensive lasers, microcomputers, and chemical filtration technology in the last several decades, the use of SLS has advanced significantly for the characterization of both equilibrium and nonequilibrium properties of polymer solutions.[5] SLS is now routinely coupled to gel permeation chromatography (GPC) columns to provide an absolute characterization of fractionated polymers, without reference to empirical GPC column calibration.[6,7] Time-dependent static light scattering (TDSLS)[8] has been used to follow processes such as aggregation and phase separation,[9–12] degradation,[13–17] microcrystallization,[18,19] and polymerization.[20,21] Use of Automatic Continuous Mixing (ACM) allows properties of complex, multicomponent systems to be characterized by SLS along arbitrary paths in composition space.[22,23] By using very small scattering volumes and using electronic spike recognition algorithms it was recently demonstrated that coexisting solutions of polymers and colloidal particles, traditionally unmeasurable by SLS because of the dominant scattering effect of the large particles, can be usefully measured. This technique has been termed Heterogeneous Time Dependent Static Light Scattering (HTDSLS).[24]

The purpose of the current article is to present a new type of device for Simultaneous Multiple Sample Light Scattering (SMSLS).[25] Its development is spurred by increasing needs for high sample throughput across the polymer industry, and by the continued advance of light sensing, fiber optic, and other technologies that make it technically feasible and economical.

Until now, SLS and TDSLS devices have focused on analyzing single samples at a time. This represents a severe rate-limiting step for characterization if many samples are to be analyzed. The single sample device is particularly ill-suited for characterizing time-dependent properties of multiple polymer solutions, because it requires monopolizing an expensive instrument for indefinite amounts of time for each sample. The ability to simultaneously monitor the time dependence of scattering from many samples should prove useful in areas such as high throughput screening in polymer synthesis, stability studies of pharmaceutical, food, and other complex mixtures, rapid assessment of degradation behavior, bioimmunoassays, etc. Instabilities in polymer solutions, for example, may take minutes, days, months, or longer to achieve a measurable magnitude. An SMSLS device will continuously monitor the state of each sample for any period desired. In fact, samples that have reached a certain criterion can even be removed and replaced with new samples, while older samples continue to be monitored, undisturbed. Because light scattering is exquisitely sensitive to even tiny changes in the molecular mass of scatter-

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ers in solution, changes in aggregation state, polymer mass, and related properties can be detected very early in a process.

The device used to introduce SMSLS in this article has a single, 90 degree detection angle for each sample chamber. Later embodiments can include multiple angles. When used as a high throughput screening device it is important to point out that the purpose of SMSLS will not normally be to provide a complete, absolute characterization of each sample, as is often done in single sample SLS. Rather, the time scale, signature, and magnitude of the changes in intensity will correspond to important features of a process that are being screened for.

For example, if the scattering of light from a concentrated solution of monomers undergoing polymerization is monitored, strong virial coefficient effects may predominate in the scattering over the molecular weight and conversion effects, so that the values of average molecular mass and monomeric conversion cannot be known. In a high throughput screening situation, however, the interest is to know which samples reach certain criteria. Those samples that reach the criteria may then merit further, detailed studies, whereas those that do not can be discarded from further consideration. Hence, the TDSLS signatures obtained by SMSLS contain a bounty of relative information because a change in intensity indicates whether the reaction occurs or not, the time scale of the change is related to the kinetics, the magnitude of the change is related to polymer mass and conversion, and the shape of the signature can reveal mechanistic information.

In this work the ability of the SMSLS device to make absolute measurements is first demonstrated by simultaneously measuring the scattering from different concentration solutions of polymers of known mass and showing that the correct masses are obtained. From this, error bars and regimes of accuracy are detailed. Second, an example of degradation is given, in which the effect of hyaluronidase on hyaluronate degradation is assessed at various enzyme and substrate concentrations. A Michaelis-Menten type plot is constructed from these data. Finally, the effect of gelatin concentration on aggregation rate is monitored.

MATERIALS AND METHODS

The SMSLS device

The particular SMSLS prototype used in this work can accommodate eight independent samples. In principle, there is no limit to the number of samples that can be incorporated into an SMSLS device, although a practical limitation per linear CCD array is around 50. The device uses 1-cm square borosilicate sample cuvettes that can be inserted into the device, and later either be cleaned or discarded. A 25-mW, vertically polarized diode laser (LaserMax, Rochester, NY) was used as the light source, and split with a 50% splitter into two beams. Each beam was incident on identical sample holders machined from black nylon, containing milled recesses for each of four cells, and fluid communication channels among the cells, so that an index matching fluid bathes the exterior of the cells. This SMSLS device is, hence, a hybrid of serial and parallel operation, there being two parallel sample banks, each with four sample cells in series. Open faces on opposing sides of the sample holder were milled, and borosilicate microscope glass slides were epoxied onto each face to allow for the laser beam entrance and exit. On a third face, four 0.54-mm holes were bored, aligned with the center of each insertable cuvette. Optical fibers (Polymicro Technology) of diameter 0.48 mm were fixed into the holes using Microtight (Upchurch) HPLC fittings. Each fiber resided about 9 mm behind the interior hole surface, such that its field of view was defined by the hole itself, rather than the fiber's own acceptance angle. The detection was thus termed to be in "pinhole mode," rather than "acceptance angle" mode. The optical fibers were led to a cooled, back thinned Hammamatsu (BC-CCD, HC230-0907). Data from the CCD was continuously monitored via a Labview® data interface provided with the Hammamatsu device. Data reduction software was written separately by the authors.

Computation of the Rayleigh scattering ratio, I_R

The Rayleigh scattering ratio of SMSLS sub-chamber i, $I_{R,i}$, is obtained by subtracting the pure solvent scattering, and relating the scattering detector voltages to the known I_R of toluene:

$$I_{R,i} = \frac{V_i - V_{i,\mathrm{solvent}}}{V_{i,\mathrm{toluene}} - V_{i,\mathrm{dark}}} F I_{R,\mathrm{toluene}}(\lambda, T) \tag{1}$$

where V_i is the scattering voltage from the sample in subchamber i, $V_{i,\mathrm{solvent}}$ is the pure solvent scattering voltage, $V_{i,\mathrm{toluene}}$ is that for toluene scattering, and $V_{i,\mathrm{dark}}$ the dark voltage, each taken from chamber i. For incident light of $\lambda = 677$ nm and at $T = 300$ K, $I_{R,\mathrm{toluene}} = 1.069 \times 10^{-5}$ cm^{-1}, as obtained by $1/\lambda^4$ extrapolation of the value given by Bender et al.[26] As described below, $F = 0.944$ for aqueous samples, and accounts for refractive index differences between toluene and aqueous sample liquid refractive indices.

Reflection losses in series mode

The SMSLS device uses an index matching bath chiefly to minimize stray, or "flare" light as the laser beam passes through each interface, but also benefits

TABLE I
Reflection and Scattering Losses for Series SMSLS Subchambers Where an Aqueous Sample Is in a Sample Cuvette of n_g = 1.533, Which Is Immersed in a Toluene Bath of n_t = 1.496

N	I_N/I_0, due to reflection loss	I_N/I_0, scattering loss[a]
1	0.99498	0.9991
2	0.98999	0.9998
4	0.98007	0.9997
16	0.96054	0.9987
256	0.92264	0.9788

[a] Taking each sample to have a Rayleigh scattering ratio of 10^{-5} cm^{-1}.

from the matching by minimizing the reflection losses at each interface. Light at each interface is normally incident, so that the fraction of light reflected R, in passing from medium of index n_1 to index n_2 is given by

$$R = \left(\frac{n_1 - n_2}{n_1 + n_2}\right)^2 \tag{2}$$

To enter a sample cell the light meets both the bath/glass interface then the glass/sample liquid interface, so that two reflection losses occur per entry into a sample cell. Hence, because the fraction of transmitted light is $T = 1 - R$, the fraction of the incident beam intensity left as it enters the liquid sample in cell number N is

$$\frac{I_N}{I_0} = [(1 - R_1)(1 - R_2)]^N \tag{3}$$

where R_1 is obtained from eq. (1) by substituting n_1 = bath index of refraction and n_2 = sample cell material index of refraction, and R_2 is obtained by substituting n_1 = sample liquid index of refraction, and n_2 = sample cell material index of refraction.

A typical set of indices of refraction is: batch index = 1.496 (toluene), sample cell material index = 1.533 (borosilicate glass), and sample liquid index = 1.333 (water). Table I shows the decrease in intensity of incident light for this example for different numbers of cells in series. Even after passing through 256 cells in series over 90% of the incident intensity remains.

Scattering losses in series mode

Series mode SMSLS has the advantage of using a single incident light source for all the subchambers in series. Its disadvantage is that significant scattering in any cell(s) prior to a given cell can influence the scattering from the given cell. It is clear that SMSLS cannot be used in series mode in situations where significant turbidity will develop in any given cell. The fraction of the initial intensity I_0 that is incident on the Nth cell is given by the sum of the product of turbidities and path lengths of each previous cell, $\tau_i L_i$ by

$$\frac{I_N}{I_0} = \exp\left[-\sum_{1}^{N-1} \tau_i L_i\right] \tag{4}$$

Hence, for sample cells with pathlengths of 1 cm, turbidities of 0.1 cm^{-1} will cause a 10% drop in intensity in the next cell. Series mode SMSLS will not normally be advisable in the case where solution stabilities are being tested against strong multiple scattering and clouding due to massive aggregation, phase separation, etc. In such cases, fortunately, the incident intensity required for detectable scattering is quite low, so that pure parallel mode SMSLS can be used, wherein the beam from a single laser can be split many times in succession.

To show that series mode SMSLS does not present any serious restrictions for light-scattering measurements in the case of typical single scattering measurements, recall that the Rayleigh ratio I_R is the fraction of incident intensity scattered per cm of path length per steradian of solid angle. For an isotropic scatterer on which vertically polarized light is incident, the scattering in the horizontal plane (often termed the "scattering plane") is isotropic, but falls of as

$$I_R(\phi) = I_{R,0}\sin^2\phi \tag{5}$$

where ϕ is the angle measured with respect to the electric field direction of the incident light. $\phi = 90°$ in the horizontal plane, and $I_{R,0}$ is $I_R(\phi = 90°)$; most scattering measurements are made in the $\phi = 90°$ plane. Integration of eq. (5) over the complete solid angle yields the relation between I_R and turbidity

$$\tau = I_{R,0} \int\int \sin^3\phi d\phi d\theta = \frac{8\pi}{3} I_{R,0} \tag{6}$$

A typical value of $I_{R,0}$ for dilute solution macromolecular characterization is, say, around that of toluene, 10^{-5} cm^{-1}, yielding $\tau = 8.37 \times 10^{-5}$ cm^{-1}. This means that the incident beam can pass through 120 1-cm subchambers and lose only 1% of its intensity. Clearly, scattering losses in typical dilute macromolecular solutions are not a concern for series mode SMSLS.

To get an idea of the type of solution one with $I_{R,0} = 10^{-5}$ cm^{-1} corresponds to, consider that, neglecting virial coefficient effects in dilute solutions of small polymers

$$cM = I_{R,0}/K \tag{7}$$

For a typical water soluble polymer, such as a protein, dn/dc ~0.15 cm^3/g, so that, at $\lambda = 677$ nm, $K = 1.25 \times 10^{-7}$ in water, so that $cM = 80$ $g^2/(cm^3 mol)$. This would correspond, for example, to a 0.0016 g/cm^3 solution of protein of $M = 50{,}000$ g/mol.

Optical correction for calibration using a reference solvent

The determination of molecular masses by light scattering is often termed an "absolute method." This statement is justified insofar as the Rayleigh scattering ratio from the scatterers is related in a model independent way to the scatterer's molar mass and polarizability. Hence, if the polarizability, or related quantity (such as dn/dc) is known, or independently determined, the molar mass is determined from a measurement of I_R. The determination is hence "absolute," and does not depend, for example, on arbitrary assumptions about how, say, a Gel Permeation Chromatography column is "calibrated" to measure "mass" from elution volumes, or correctional procedures, such as "universal calibration."[27] The problem, then, is to determine I_R. The most common way of computing I_R from the scattering signal obtained from a detector is to relate the signal to one obtained when scattering from a substance whose value of I_R is well known. Toluene is one of the most frequently used solvents for this purpose. Hence, the absolute values obtained for masses rely on how well the relationship between a scattering signal and I_R from the reference substance is measured.

It was pointed out long ago that, for a given scattering geometry, the scattering signals measured from one solvent must be corrected for geometrical optical effects when comparing them to the scattering from solvents of different index of refraction.[28–30] For example, in upright cylindrical scattering cells whose axis is parallel to the vertically polarized incident electric field, a detector that subtends a fixed solid angle will measure a Rayleigh ratio $I_{R,2}$ from a sample solvent of index n_2 that is related to that of a reference solvent with n_1 by

$$I_{R,2} = \left(\frac{n_1}{n_2}\right)^2 I_{R,1} \tag{8}$$

Hence, measurements of $I_{R,2}$ in a liquid of n_2 must be corrected by the square of the ratio of refractive indices when computing $I_{R,2}$ in terms of the reference value $I_{R,1}$. This correction ignores reflection losses, which are usually negligible compared to the refraction effects represented by eq. (8).

For a scattering geometry where an optical fiber is integrally coupled to the liquid whose scattering is being measured in the "numerical aperture mode" of the fiber, a very different correction factor was found.[20]

The current SMSLS device uses optical fibers in the "pinhole mode," that is, the acceptance angle of detection is determined by the geometry of the fiber anchored in a narrow, deep cylinder, with respect to the laser beam, scattering cell, index matching bath, and associated dimensions, and the indices of refraction of the liquids measured. Let r be the radius of the optical fiber core, d the depth of the pinhole tube holding the fiber, D the distance from the laser beam (assumed to be a line source of intensity) to the center of the pinhole tube, t the half-thickness of the square cell holding the sample liquid, n_s is the refractive index of the liquid sample being measured, and n_r is the index of the reference solvent, which is assumed to be used also in the index matching bath ($n_t = 1.496$, which is both the reference solvent and bath matching fluid for this SMSLS device). A geometrical optical analysis of the system (derivations not included here) yields the following correction factor F, to be used in eq. (1)

$$F = \frac{2}{\left[\frac{r + L'}{r + L} + \frac{\phi'}{\phi}\right]} \tag{9}$$

where

$$\begin{aligned}
\tan\gamma &= \frac{2r}{d} \\
L &= \frac{2rD}{d} \\
L' &= L + t(\tan\gamma' - \tan\gamma) \\
\sin\gamma' &= \left(\frac{n_r}{n_s}\right)\sin\gamma \\
\phi &= \frac{r}{D + d/2} \\
\phi' &= \frac{n_r r}{n_s(D - t + d/2) + t n_r}
\end{aligned} \tag{10a–f}$$

For the current system, $r = 0.027$ cm, $t = 0.5$ cm, $d = 0.90$ cm, $D = 1.25$ cm, and $n_r = 1.496$, so that $F = 0.944$ in eq. (1) for aqueous solutions ($n_s = 1.333$).

Polymer size limits for absolute measurements

The question frequently arises as to what the range of polymer sizes permits accurate M_w measurements at a given, single angle. This can be answered by giving error contours in terms of a polymers mean square radius of gyration $\langle S^2 \rangle$.

To determine M_w, the usual Zimm approximation is made,[31] which, at low concentrations and for $q^2 \langle S^2 \rangle$

$<< 1$, can be written, for a polydisperse polymer population as

$$\frac{Kc}{I_R(q,\,c)} = \frac{1}{M_w}\left(1 + \frac{q^2\langle S^2\rangle_z}{3}\right) + 2A_2c \qquad (11)$$

which directly permits determination of M_w, A_2, and the z-averaged mean square radius of gyration $\langle S^2\rangle_z$. $I_R(q,c)$ is the excess Rayleigh scattering ratio due to the polymers in solution; i.e., the total scattering minus the background scattering from the solvent, as per eq. (1). K is an optical constant, given for vertically polarized incident light by

$$K = \frac{4\pi^2 n^2 (dn/dc)^2}{N_A \lambda^4} \qquad (12)$$

where n is the solvent index of refraction, λ is the vacuum wavelength of the incident light, dn/dc is the differential refractive index for the polymer in the chosen solvent, and q is the usual scattering wave vector $q = (4\pi n/\lambda)\sin(\theta/2)$, where θ is the scattering angle.

From eq. (11)

$$\lim_{c\to 0} \frac{Kc}{I_R(q,\,c)} = \frac{1}{M_w}\left[1 + \frac{q^2\langle S\rangle_z^2}{3}\right] \qquad (13)$$

so that the term $q^2\langle S^2\rangle_z/3$ is the fractional error in the mass determination. M_w will always be underestimated to one degree or another if a single angle is given, and no extrapolation to $q = 0$ is made. For $q^2\langle S^2\rangle_z/3 << 1$, this error assessment is valid for any type of polymer conformation, including globules, rods, microgels, random coils, etc.

For the case of linear polymers, the "worm-like chain" formula relates the mean square radius of gyration $\langle S^2\rangle$ to persistence length L_p and total polymer contour length L. The mass of the polymer M is just $L(m/b)$, where m/b is the linear mass density (monomer mass/contour length of the monomer); for example, for hyaluronate, m/b is 40 g/mol-Ang. The worm-like chain formula[32] is

$$\langle S^2\rangle = \frac{LL_p}{3} - L_p^2 + 2L_p^3/L - 2\left(\frac{L_p^4}{L^2}\right)[1 - \exp(-L/L_p)] \qquad (14)$$

Although $\langle S^2\rangle$ in eq. (14) refers to the dimension of the polymer in the absence of excluded volume interactions (often referred to as $\langle S^2\rangle_0$), it is the fully perturbed value of $\langle S^2\rangle$ that is measured by light scattering, and most other techniques, so that it has become practical to speak of an "apparent persistence length" L_p', which includes excluded volume effects, and use it in place of the unperturbed L_p in eq. (14).[33]

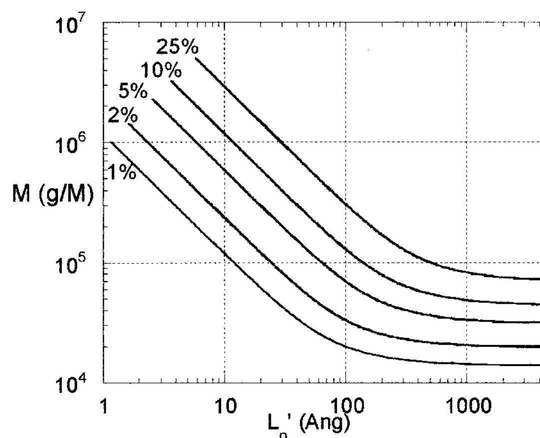

Figure 1 Error contours by which M_w is underestimated by a single-angle light-scattering measurement, for any given pair of values of apparent persistence length L_p' and M, using 40 g/mol-Angstrom. The values for q are $\theta = 90°$, $n = 1.333$, and $\lambda = 6770$ Angstroms.

Figure 1 shows selected error contours of M vs. L_p' for a value of m/b of 40 g/mole-Angstrom, appropriate for Hyaluronic acid, and selected errors; 1, 2, 5, 10, and 25%. The values for the current SMSLS device are used for q; $\lambda = 677$ nm and $\theta = 90°$. The case of aqueous samples is used, $n = 1.33$. In the random coil limit, the relationship between M and L_p for a given fractional error, Err, becomes

$$M = \frac{3\ \mathrm{Err}(m/b)}{q^2 L_p'} \qquad (15)$$

which allows a quick estimate of errors for any values of m/b and q.

Error considerations

The main sources of error in the determination of M_w and A_2 (and any higher virial coefficients) stem from systematic errors in I_R and the constant K in eq. (11), and random errors in I_R, and, to a lesser extent, c. Most notably, the value of dn/dc in K leads to a systematic error in M_w, which varies as the inverse square of dn/dc in any light-scattering experiment where the concentration is not determined independently by a refractometer (in this latter case the error in M_w is inversely proportional to dn/dc). Here, the focus is on the random errors introduced by the SMSLS device itself.

The random SMSLS errors arise from the inherent signal to noise limitations of the detection portion, and the run to run errors involved in removing and replacing nonidentical cells. The signal-to-noise ratio was assessed as (standard deviation)/average value, and

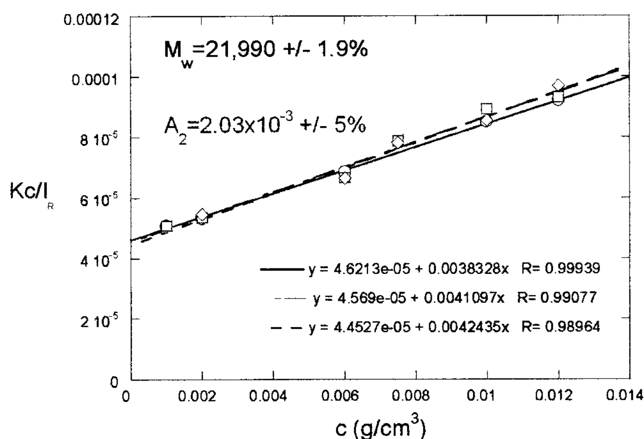

Figure 2 Determination of M_w and A_2 for a narrow polydispersity PEG standard in water.

in all cases of varying signal levels led to an error no greater than 0.5%. This error can enter on three separate occasions for determining I_R, as seen in eq. (1). The variations in scattering due to repetitively taking a sample cell out and replacing it was assessed, and found to be 0.9%. Hence, there are six possible sources of random error in a complete measurement of I_R (a signal fluctuation and sample insertion error each for the sample, toluene, and pure solvent), and, because each measurement is made independently of the others, the errors add in quadrature, that is

$$\text{error} = \sqrt{3x(0.5)^2 + 3x(0.9)^2} = .0178.$$

The total of all random errors in a complete SMSLS determination of I_R can, hence, be conservatively rounded up to ±2%.

A further source of random error in computing Kc/I comes from c itself. The solutions in the following experiments were prepared by serial dilution of a stock solution. By repetitive pipetting, the random error was found to vary from 0.1 to 1%, depending on the volume being measured.

RESULTS AND DISCUSSION

Absolute molar mass determinations

The capability of the SMSLS device to make absolute molar mass determinations was assessed by using water soluble and organosoluble polymer standards.

Polyethylene glycol (PEG) was used for aqueous determinations. It was supplied by Polymer Laboratories (2071-1001). The value of dn/dc = 0.132 was taken from the Polymer Handbook.[34]

Figure 2 shows the Kc/I_R vs. c plot for PEG. The results of three separate measurements made on a PEG standard, nominally of M_w = 21,450 (from the Polymer Laboratories Certificate of Analysis), and M_w/M_n = 1.04, are shown. From this, M_w = 21,990 ± 1.9%, where the 1.9% reflects the standard deviation of three different complete determinations, using the same samples, permuted among the various subchambers. This error falls within the limits expected from the above analysis of random errors. The deviation of 3.5% with respect to the nominal M_w is presumed to be comprised of both the random errors assessed above, including concentration determinations, and systematic errors in the value of dn/dc, as well as in the nominal value itself. Independent measurements by SEC (using a Brookhaven Instruments BI-MwA multiangle light-scattering detector, a homebuilt viscometer, and Shimadzu refractometer) yielded M_w = 22,700.

The value of A_2 from these data is 2.03×10^{-3} cm^3-g^2/mol +/5%, which is within the range of 1×10^{-3} to 3×10^{-3} reported in ref. 34.

Polymer Laboratories Inc. polystyrene (PS) standard of nominal M_w = 65,800 g/mol and M_w/M_n = 1.02 (PL 2013-4001) was used for testing performance with organic solvents. The PS was dissolved in toluene, and the index matching solvent was also toluene. dn/dc = 0.105 for PS in toluene was taken from ref. 34. Figure 3 shows the Kc/I_R data for PS. The same level of error discussed previously can be assigned to the result of M_w = 66,500 g/mole +/1.9% and A_2 = 1.156×10^{-3} cm^3-g^2/mol. This value of M_w falls within 1% of the nominal value, and A_2 reported for PS of M_w = 95,400 in ref. 34 is 1×10^{-3}.

These results establish that the SMSLS device is capable of making absolute molecular mass measurements, exactly like single sample devices, based solely on the scattering behavior of a well characterized organic solvent (toluene in this case).

Degradation experiments

When polymers undergo degradation, the intensity of the light they scatter diminishes. A detailed series of

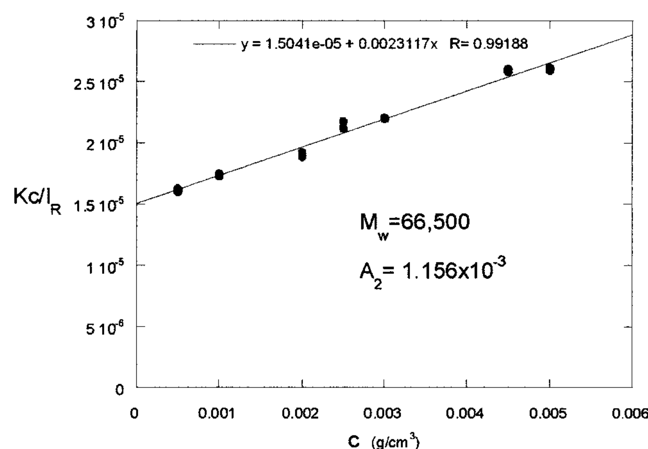

Figure 3 Determination of M_w and A_2 for a narrow polydispersity PS standard in toluene.

theoretical and applied work in this area has been published over the past 15 years.[15–17,35] Among many significant results is the fact that the absolute degradation rate of a polymer that resembles a random coil and undergoes random degradation can be determined by monitoring the light scattered at a single, arbitrary angle.

We, hence, revisit such a reaction, whose TDSLS behavior was detailed earlier, that of hyaluronate degradation by hyaluronidase.[36] Reed et al.[36] showed that for an ideal random coil undergoing random degradation the number of bonds cleaved per second per g/mol of polymer mass is given by

$$\dot{\beta} = 2Kc\,\frac{d[1/I_R(t)]}{dt} \tag{16}$$

The velocity normally measured in enzymology is in molar bonds cleaved per second, v, which is given by

$$v = 1000\dot{\beta}c \tag{17}$$

where the factor of 1000 ensures that v is in mol/liter-s. The Michaelis-Menten-Henri model is one of the simplest for enzymatic action, and considers the velocity of enzymatic conversion at steady state, where there is a constant concentration of the enzyme–substrate complex, which itself can either dissociate or proceed to final product formation. The measured velocity is related to substrate concentration c, and the Michaelis-Menten rate constant K_M by

$$v = v_{\max}\frac{c}{c + K_M} \tag{18}$$

where $v_{\max}$ is the maximum velocity obtained when the enzyme is completely saturated by substrate, and all the enzyme is in the enzyme–substrate complex.

Figure 4 shows data for several of the simultaneous degradation reactions of sodium hyaluronate (HA) from Streptococcus Zooepidemicus (#H-9390, Sigma-Aldrich, St. Louis, MO), under the action of hyaluronidase from bovine testes (#H-3884, Sigma-Aldrich), in a buffer consisting of 0.15 M NaCl and 0.1 M sodium succinate, at pH = 5.31. There were 40 units of enzyme per mL in each HA solution, and the concentration of HA was different in each solution measured simultaneously by SMSLS.

The data in Figure 4 is the raw data transformed into I_R/Kc form via multiplication by a single constant, comprised in eq. (1), *without subtracting the solvent scattering.* The Rayleigh ratio thus obtained is the total I_R from the solution, $I_{R,\text{total}}$, which, combining eq. (1) and (16) yields

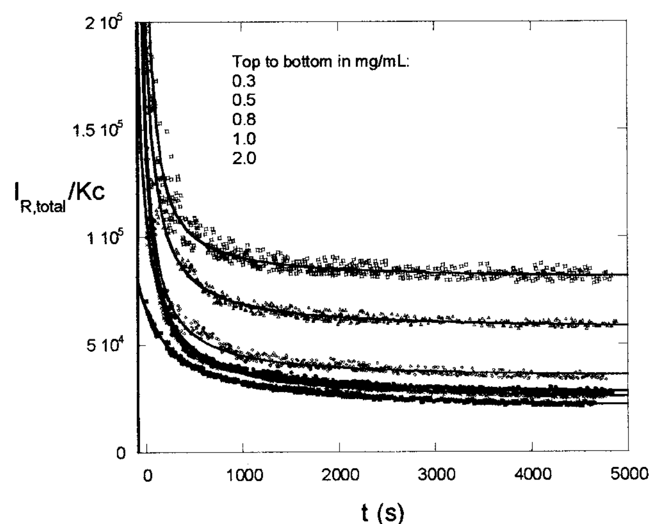

Figure 4 Intensity decay curves for the degradation of sodium hyaluronate by hyaluronidase. $I_{R,\text{total}}/Kc$ shown corresponds to the raw scattering voltage data multiplied by a constant [see eq. (1)].

$$\frac{I_{R,\text{total}}}{Kc} = \frac{1}{\dfrac{Kc}{I_{R,\text{HA}}\,(t=0)} + \dfrac{\dot{\beta}}{2}t} + \frac{I_{\text{solvent}}}{Kc} \tag{19}$$

The fits in figure 4 are to this equation, using $\dot{\beta}$ and I_{solvent}/Kc as the fitting parameters. The advantage of this procedure over a direct linear fit to $Kc/I_R(t)$, such as used by Reed et al. previously, is that the effect of the baseline is handled through fitting, rather than subtracting it in eq. (1). Although the baseline is nominally that of the solvent plus the low concentration of enzyme used, small deviations, due, for example to nonhydrolyzable impurities, can have large effects on the linearity of $Kc/I_R(t)$ and the subsequent fitting, as amply discussed in ref. 36.

The values of v according to eq. (17), are found from the values of $\dot{\beta}$ from the above fits (including for the data curves not shown). It is customary to determine $v_{\max}$ and K_M from a linear fit of the form

$$\frac{1}{v} = \frac{1}{v_{\max}} + \frac{K_M}{cv_M} \tag{20}$$

Figure 5 shows the data plotted in this form, for which a value of $K_M = 0.00142$ cm^3/g. A wide variety of values for K_M is reported in the literature,37,38 ranging from 0.00001 to about 0.002. These values depend on solution pH and ionic strength conditions, as well as the purity and source of enzyme. The value found here is within the expected range.

It is emphasized that, in the case of degradation of random coils polymers, the absolute rate constant can be obtained *at any angle*,[16,17,34] so the single-angle

SMSLS device is not limited in this particular application by having only 90 degree detection. To do this, however, requires that reliable absolute values of I_R are obtained, which was demonstrated in the first experiments on low polydispersity standards.

Aggregation experiments

Many polymer solutions, especially those containing biopolymers, are often unstable. Their instability frequently manifests itself via self-aggregation of the polymers. The finding that many proteins self-aggregate, and indiscriminately aggregate with many other types of proteins, for example, has caused consternation in the proteomics sector, where widespread aggregation has falsely been interpreted as protein specificity.[39]

The object of this section is merely to demonstrate that SMSLS provides a sensitive and convenient means to monitor aggregation in many samples, not to make an analysis of complex underlying kinetics. A simple system for demonstrating aggregation is gelatin.[40] Figure 6 shows gelatin aggregation experiment results for varying gelatin concentrations. The solvent was water with 0.2% by weight sodium azide. The results are expressed in terms of the fractional change in scattering with respect to the initial gelatin scattering values. In systems where stability in time is to be assessed, for example, of a pharmaceutical formulation or mixture of polymeric species, SMSLS should readily report even small amounts of aggregation.

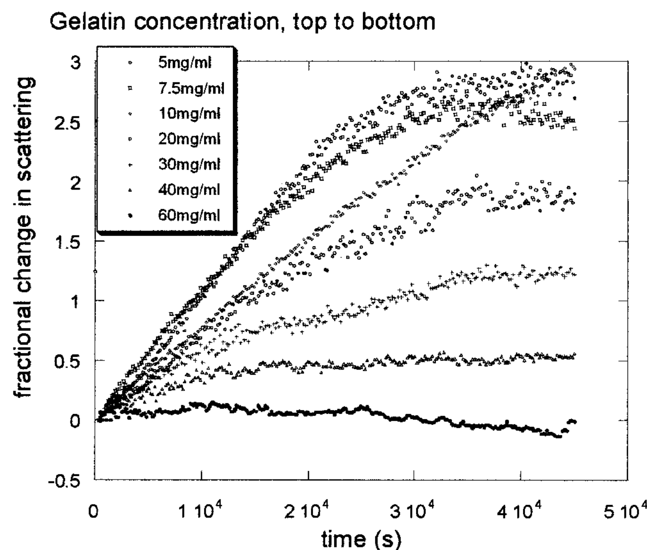

Figure 6 Aggregation of gelatin in water with 0.2% (by weight) sodium azide at different concentrations.

CONCLUSIONS

It has been demonstrated that SMSLS is capable of making absolute molecular mass determinations. The ability of SMSLS to make measurements of the absolute excess Rayleigh scattering ratio I_R allows not only M_w to be determined, to within error limits explored quantitatively above, but also allows absolute degradation rates to be determined independently of q. The main utility of SMSLS is envisioned to lie in high throughput characterization scenarios, where time-dependent changes in polymer solutions allow monitoring phenomena of interest, such as degradation, aggregation, and polymerization. The latter is of keen interest for new materials synthesis, and work is currently underway to discover and interpret TDSLS signatures from concentrated monomer solutions undergoing polymerization.

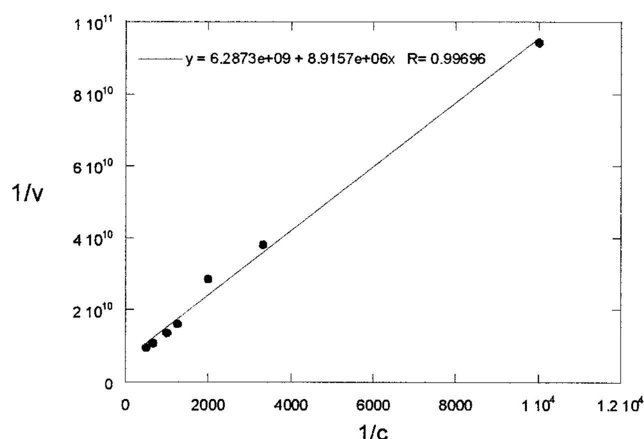

Figure 5 Michaelis-Menten-Henri plot for determination of K_M.

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