Simultaneous Calibration of Size Exclusion Chromatography and Dynamic Light Scattering for the Characterization of Gelatin

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ABSTRACT: A novel method of calibrating size exclusion chromatography (SEC or known as GPC) and dynamic light scattering (DLS or known as QELS or PCS) is proposed for the characterization of gelatin. A conventional calibration of SEC or DLS normally requires a set of narrowly distributed gelatin standards with different molecular weights. By contrast, the new method uses only **one** broadly distributed gelatin sample to calibrate SEC and DLS simultaneously in a single process in which the intrinsic connection between the measured elution volume in SEC and the translational diffusion coefficient in DLS (i.e., both of them are related to the same hydrodynamic volume) is utilized. This new calibration method can also be used for the characterization of other polymers, where the conventional calibration is not applicable.

Introduction

Gelatin has been widely used in the biochemical, pharmaceutical, food, and photographic industries as a binder, stabilizer, and gelling agent. For example, gelatin is used as an important stabilizer ingredient for pulverulent formulations of carotenoids and vitamin A. Quality control of gelatin is very important for its various applications. Characterization of the molecular weight distribution of gelatin is an essential part of quality control. Different methods, such as ultracentrifugation,[1] viscosimetry,[2] size exclusion chromatography (SEC),[3] osmometry,[4] and static light scattering,[5] have been used to characterize gelatin.

For characterizing molecular weight distribution, SEC is a convenient and established method. However, there are a number of difficulties associated with the use of SEC to characterize the molecular weight distribution of gelatin. One of them is to calibrate an SEC column. In a conventional calibration method, the elution volumes of a set of narrowly distributed standards with known molecular weights are measured. However, it is very difficult to obtain such a set of gelatin standards in practice. Therefore, one has to use other methods to calibrate the SEC columns for the characterization of gelatin. Most of the reported calibration methods[6-10] require at least two polymers with different molecular weights or one sample with either two different molecular weight averages or one molecular weight plus intrinsic viscosity data which implies the use of one additional instrument, such as an osmometer or a viscometer.

In this article, we report a newly developed method that simultaneously calibrates both SEC and DLS by using only **one** broadly distributed gelatin, wherein the intrinsic connection between the elution volume in SEC and the translational diffusion coefficient in DLS (i.e., they are related to the same polymer hydrodynamic volume) is utilized.

Basic Principles

An elution volume distribution $C(V)$ and a translational diffusion coefficient distribution $G(D)$ can be respectively measured in SEC and DLS. We can convert $C(V)$ or $G(D)$ into the molecular weight distribution with the calibration

$$V = A + B\log(M) \tag{1}$$

or

$$D = k_D M^{-\alpha_D} \qquad \text{i.e., } \log(D) = \log(k_D) - \alpha_D \log(M) \tag{2}$$

where A, B, k_D, and α_D are the calibration constants. It should be noted that in eqs 1 and 2 we have assumed that both V and $\log(D)$ are linear functions of $\log(M)$, i.e., the first-order approximation, because this will simplify, but not affect, the following discussions. However, if the sample has a special molecular weight distribution or V and $\log(D)$ cannot be linearly scaled by $\log(M)$, eqs 1 and 2 have to be properly modified. In that case, additional information about the molecular weight distribution and the dependence of V and $\log(D)$ on $\log(M)$ is required.

The main task of the calibration is to find A and B or k_D and α_D. Traditionally, the two-sample methods have been used to calibrate SEC or DLS. In this paper, we present a new method to obtain A, B, k_D, and α_D simultaneously by using only one broadly distributed polymer sample. The principle of this one-sample method is in the following: by combining eqs 1 and 2, we have

$$V = \mathbf{A} + \mathbf{B}\log(D) \tag{3}$$

where $\mathbf{A} = A + B\log(k_D)/\alpha_D$ and $\mathbf{B} = -B/\alpha_D$. Further, by taking the square of both sides of eq 3, we obtain

$$V^2 = \mathbf{A}^2 + 2\mathbf{AB}\log(D) + \mathbf{B}^2\log^2(D) \tag{4}$$

After integrating both sides of eqs 3 and 4, we have

$$\langle V\rangle = \mathbf{A} + \mathbf{B}\langle\log(D)\rangle \tag{5}$$

and

$$\langle V^2\rangle = \mathbf{A}^2 + 2\mathbf{AB}\langle\log(D)\rangle + \mathbf{B}^2\langle\log^2(D)\rangle \tag{6}$$

where

$$\langle V\rangle = \frac{\int_0^\infty VC(V)\,dV}{\int_0^\infty C(V)\,dV} \tag{7}$$

$$\langle V^2\rangle = \frac{\int_0^\infty V^2C(V)\,dV}{\int_0^\infty C(V)\,dV} \tag{8}$$

$$\langle\log(D)\rangle = \frac{\int_0^\infty \log(D)C(V)\,dV}{\int_0^\infty C(V)\,dV} \tag{9}$$

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and

$$\langle \log^2(D) \rangle = \frac{\int_0^\infty \log^2(D) C(V)\, dV}{\int_0^\infty C(V)\, dV} \quad (10)$$

On the one hand, since $C(V)$ is a weight (or concentration) distribution of the elution volume, we have[6]

$$\int_0^\infty C(V)\, dV \propto \int_0^\infty f_w(M)\, dM \propto \int_0^\infty f_w(M) M\, d(\log(M)) \quad (11)$$

where $f_w(M)$ is a weight distribution. According to eq 1, dV is proportional to $d(\log(M))$. Then, it follows from eq 11 that

$$C(V) \propto f_w(M) M \quad (12)$$

On the other hand, since $G(D)$ is an intensity distribution of the translational diffusion coefficient, we have[11]

$$\int_0^\infty G(D)\, dD \propto \int_0^\infty f_w(M) M\, dM \quad (13)$$

or

$$\int_0^\infty G(D) D\, d(\log(D)) \propto \int_0^\infty f_w(M) M^2\, d(\log(M)) \quad (14)$$

According to eq 2, $d(\log(D))$ is proportional to $d(\log(M))$. Thus,

$$G(D) D \propto f_w(M) M^2 \quad (15)$$

By using eqs 2, 12, and 15, we can rewrite eqs 9 and 10 as

$$\langle \log(D) \rangle = \frac{\int_0^\infty \log(D) G(D) D^{1/\alpha_D}\, dD}{\int_0^\infty G(D) D^{1/\alpha_D}\, dD} \quad (16)$$

and

$$\langle \log^2(D) \rangle = \frac{\int_0^\infty \log^2(D) G(D) D^{1/\alpha_D}\, dD}{\int_0^\infty G(D) D^{1/\alpha_D}\, dD} \quad (17)$$

We are able to calculate **A** and **B** in eqs 5 and 6 with $\langle V \rangle$, $\langle V^2 \rangle$, $\langle \log(D) \rangle$, and $\langle \log^2(D) \rangle$. Furthermore, by using eqs 2, 3, 11, and 13 and the definition of the weight average molecular weight,

$$M_w = \frac{\int_0^\infty M f_w(M)\, dM}{\int_0^\infty f_w(M)\, dM} \quad (18)$$

We have

$$M_{w,SEC} = \frac{\int_0^\infty M C(V)\, dV}{\int_0^\infty C(V)\, dV} = \frac{k_D^{1/\alpha_D} \int_0^\infty 10^{(A-V)/(\alpha_D B)} C(V)\, dV}{\int_0^\infty C(V)\, dV} \quad (19)$$

and

$$M_{w,DLS} = \frac{\int_0^\infty G(D)\, dD}{\int_0^\infty G(D)/M\, dD} = \frac{k_D^{1/\alpha_D} \int_0^\infty G(D)\, dD}{\int_0^\infty G(D) D^{1/\alpha_D}\, dD} \quad (20)$$

For a given sample, $M_{w,SEC}$ should be equal to $M_{w,DLS}$. Therefore, on the basis of eqs 19 and 20, we have

$$\frac{\int_0^\infty 10^{(A-V)/(\alpha_D B)} C(V)\, dV}{\int_0^\infty C(V)\, dV} = \frac{\int_0^\infty G(D)\, dD}{\int_0^\infty G(D) D^{1/\alpha_D}\, dD} \quad (21)$$

There is only one unknown parameter, α_D, in the above equations. For a chosen α_D, we can *first* calculate $\langle \log(D) \rangle$ and $\langle \log^2(D) \rangle$ by using eqs 9 and 10, *then* solve **A** and **B** on the basis of eqs 5 and 6, and *finally* calculate both sides of eq 21. By iterating α_D, we are able to find one α_D which can minimize the difference between the left and right sides of eq 21. With this α_D, we can calculate k_D from either eq 19 or 20 by using the M_w determined by static light scattering and $C(V)$ by SEC or $G(D)$ by DLS. After obtaining **A**, **B**, k_D, and α_D, we are ready to calculate A and B in eq 1. In this way, we are able to calibrate not only SEC but also DLS in one single process with only one broadly distributed sample.

Experimental Section

Gelatins. Two pharmaceutical grade gelatin standards were kindly supplied by Dr. Klaus Bräumer and Dr. Wilfried Babel (Deutsche Gelatine-Fabriken Stoess AG, Eberbach, Germany). One is an A-type gelatin (Bloom value 310, Batch No. 50100, M_w(LLS) = 3.71×10^5); the other, a B-type gelatin (Bloom value 270, Batch No. 21020, M_w(LLS) = 2.92×10^5), which are denoted hereafter as gelatin-A and gelatin-B, respectively.

Laser Light Scattering. Formamide (BASF, analytical grade) without further purifications was used as the solvent in LLS. The gelatin solutions of five different concentrations ranging from 1 to 5 mg/mL were prepared by dissolving a certain amount of gelatin in formamide first at 50 °C for 1 h and then at room temperature for at least 1 day. An estimate of 12% water content in gelatin was taken into account for the calculation of the final gelatin concentration. All solutions were filtered by using a 0.22-μm Millipore filter in order to remove dust. The intensities of the light scattered from the gelatin solutions at different scattering angles (30–90°) were measured with a commercial LLS spectrometer (ALV/SP-86, Germany). An argon ion laser (Coherent INNOVA 300, operated at wavelength 488 nm and 300 mW) was used as the light source. The primary beam was vertically polarized. By placing a polarizer in front of the detector, we measured only the vertically polarized scattered light. An ALV 3000 correlator with 240 linear channels was used to measure the intensity–intensity time correlation functions. All LLS measurements were performed at 25 °C. The details of LLS can be found elsewhere.[12,13]

Size Exclusion Chromatography. A combination of Toyo Soda TSK Gel PW columns was used for SEC (precolumn, 2 × PW 30, 1 × PW50). A salt solution (0.01 M NaH_2PO_4, 0.1 M Na_2SO_4, and 1% SDS) was used as the eluting solvent. The flow rate was 1 mL/min, pH = 5.3, and T = 50 °C. A UV-absorption detector operated at 220 nm was used.

Results and Discussion

Figure 1 shows typical measured SEC elution curves of both gelatin-A and gelatin-B, where $C(V)$ values, the weight distribution of elution volume, have not been normalized. All experimental conditions have already been stated in the previous section. It can be seen that both gelatin-A and gelatin-B are broadly distributed. The curvatures on both $C(V)$ imply multimodal distributions, which are known to occur on gelatin.

Figure 2 shows typical translational diffusion coefficient distributions of gelatin-A and gelatin-B, where the gelatin concentration is 1.00×10^{-3} g/mL and the scattering angle is 90°. The CONTIN program (courtesy of Prof. Provencher) has been used to perform the Laplace inversion of the measured time correlation function.[14] The distribution, especially the average diffusion coefficient,

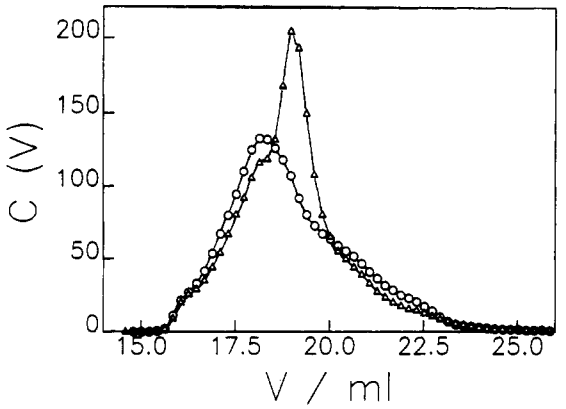

Figure 1. Typical measured SEC elution curves of gelatin-A (triangles) and gelatin-B (circles).

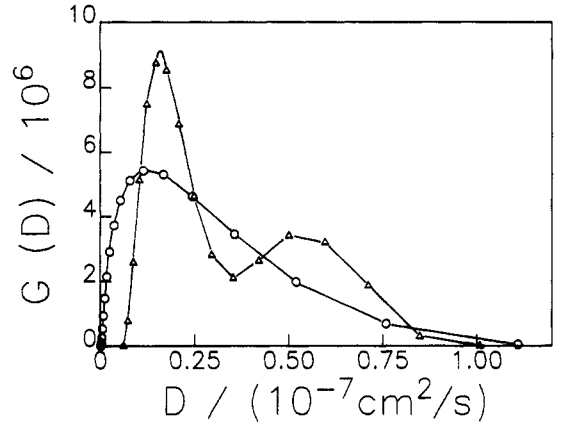

Figure 2. Typical translational diffusion coefficient distributions of gelatin-A (triangles) and gelatin-B (circles), where the gelatin concentration is 1.00×10^{-3} g/mL and the scattering angle is 90°.

Table I. A, B, A, B, k_D, and α_D Calculated from Three Different Methods: (1) LLS + SEC, (2) LLS, and (3) SEC[a]

method		A	B	A	B	$k_D/10^{-5}$	α_D
SEC + DLS	gelatin-A	56.4	5.20	34.7	−2.99	5.54	0.575
	gelatin-B	54.6	4.90	34.0	−2.85	6.22	0.582
DLS		55.7	5.05			5.98	0.580
SEC				34.4	−2.93		

[a] Relative errors of the parameters are the following: A, ±5%; B, ±5%; A, ±5%; B, ±6%; k_D, ±10%; α_D, ±1%.

is quite stable even if there are some uncertainties related to the two ends of the distribution. It should be stated that it is the advantage of this proposed method that it uses the average values instead of the individual fractions in the distribution. The translational diffusion coefficient distributions obtained at finite concentrations and scattering angles were extrapolated to infinite dilution and zero scattering angle.[11] Since the hydrodynamic radius of gelatin is very small (~20 nm), the extrapolation introduced only a few percent of correction. Therefore, the typical extrapolation error is less than 3%.

Table I summarizes all calibration constants obtained in the proposed one-sample method which is denoted as SEC + LLS. For comparison, we also calculated the calibration constants by using the following conventional two-sample methods. On the one hand, in SEC, based on eqs 1 and 19, for two samples, we have

$$\frac{M_{w,SEC,1}}{M_{w,SEC,2}} = \frac{[\int_0^\infty 10^{V/B} C_1(V)\,dV][\int_0^\infty C_2(V)\,dV]}{[\int_0^\infty C_1(V)\,dV][\int_0^\infty 10^{V/B} C_2(V)\,dV]} \quad (22)$$

If we replace $M_{w,SEC}$ with M_w from static light scattering, i.e., knowing the left side of eq 22, we can find one B which

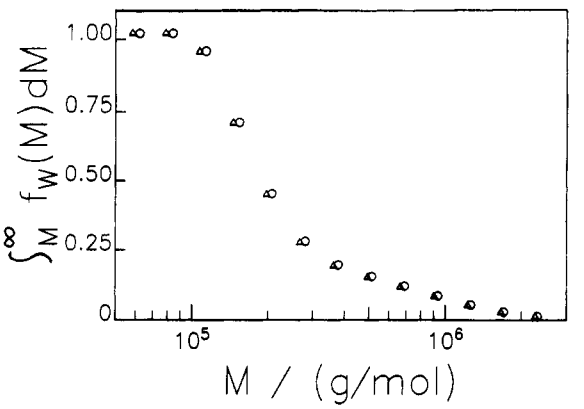

Figure 3. Cumulative weight distributions ($\int_M^\infty f_w(M)\,dM$) of gelatin-A calculated from one $G(D)$ but with two different pairs of k_D and α_D. The circles represent k_D = 5.54E-5 and α_D = 0.575, and the triangles, k_D = 6.22E-5 and α_D = 0.582.

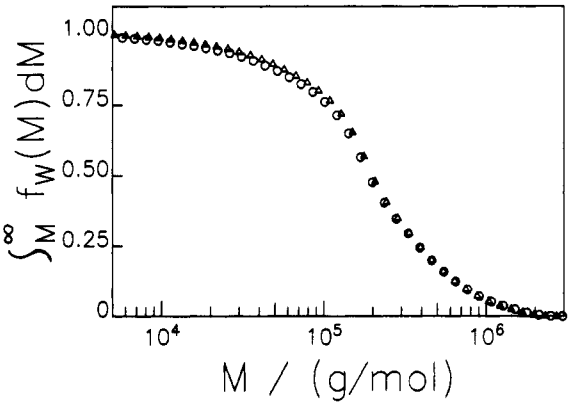

Figure 4. Cumulative weight distributions ($\int_M^\infty f_w(M)\,dM$) of gelatin-A calculated from one $C(V)$ but with two different pairs of A and B. The circles represent A = 34.7 and B = −2.99, and the triangles, A = 34.0 and B = −2.85.

minimizes the difference between the left and right sides by iterating B. With this B, we can calculate A by using eq 22 and the weight average molecular weight M_w from static laser light scattering measurement. On the other hand, in DLS, based on eq 20, we have

$$\frac{M_{w,1}}{M_{w,2}} = \frac{[\int_0^\infty G_1(D)\,dD][\int_0^\infty G_2(D) D^{1/\alpha_D}\,dD]}{[\int_0^\infty G_1(D) D^{1/\alpha_D}\,dD][\int_0^\infty G_2(D)\,dD]} \quad (23)$$

By iterating α_D, we can find a value of α_D which minimizes the difference between the left and right sides of eq 23. With this α_D, we can calculate k_D from $G(D)$ and M_w according to eq 20. These two two-sample methods are denoted by subscripts LLS and SEC, respectively. The calculated calibration constants by the two-sample methods are also listed in Table I. They are close to the ones obtained by our proposed one-sample method.

Figure 3 shows two cumulative weight distributions of gelatin-A obtained from the same measured $G(D)$ but with two different pairs of k_D and α_D, where the circles represent $k_D = 5.54 \times 10^{-5}$ and $\alpha_D = 0.575$ calculated from the experimental data of gelatin-A and the triangles $k_D = 6.22 \times 10^{-5}$ and $\alpha_D = 0.582$ calculated from the experimental data of gelatin-B. In Figure 3, the two distributions are basically the same except for some small difference in the low molecular weight tail.

Figure 4 shows two cumulative weight distributions of gelatin-A obtained from the same measured $C(V)$ but with two different pairs of A and B, where the circles represent A = 34.7 and B = −2.99 obtained from gelatin-A and the

Table II. Weight-Average Molecular Weights of Gelatin-A and -B Measured or Calculated from Different Methods[a]

gelatin	$10^{-5}M_w$	$10^{-5}M_{w,DLS}$	$10^{-5}M_{w,SEC}$	$10^{-5}M_{w,DLS+SEC}$
A	2.92	3.00	3.12	3.02
B	3.71	3.61	3.48	3.65

[a] Relative errors of the above weight-average molecular weights are in following: M_w, ±5%; $M_{w,DLS}$, ±10%; $M_{w,SEC}$, ±10%; $M_{w,DLS+SEC}$, ±10%.

triangles $A = 34.0$ and $B = -2.85$ obtained from gelatin-B. In Figure 4, the two distributions are very similar except for a small difference in the low molecular weight tail.

By using the constants in Table I, we are able to calculate three different weight average molecular weights: $M_{w,DLS}$, $M_{w,SEC}$, and $M_{w,SEC+LLS}$. They are listed in Table II and are comparable to M_w determined in static light scattering. The difference among them is within the experimental uncertainty. Thus it can be concluded that the proposed SEC + LLS one-sample method is comparable to the other two-sample methods and the calculated calibration constants are stable.

In addition, we have calculated the z-average translational diffusion coefficient D_z in two different ways. One is the cumulant fit of the measured time correlation functions, and the other is the integration of the molecular mass distribution by using k_D and α_D in Table I or the integration of the diffusion coefficient distribution according to the definition of D_z. The relative errors of D_z calculated in different ways are within ±5%, which is considered as another check of the proposed method.

After establishing the calibration relation between V and M, i.e., having A and B, we are able to determine the molecular weight distribution of a given practical gelatin sample from a single SEC measurement.

Figure 5 shows the typical weight distributions of five different commercial B-200 gelatin samples (162378, 166401, 176497, 164623, and 172640). Their weight average molecular weights range from 2.41×10^5 to 3.67×10^5, and M_w/M_n values range from 3.7 to 5.3. Both the lower and higher molecular weight tails should not be taken very seriously because of the experimental uncertainties in the baseline.

Conclusions

We have accomplished a simultaneous calibration of size exclusion chromatography (SEC) and dynamic light scattering (DLS) for the characterization of the molecular weight distribution of gelatin, in which the intrinsic connection between the measured elution volume in SEC and the measured translational diffusion coefficient in DLS (i.e., they are related to the same hydrodynamic volume) is utilized. In principle, on the one hand, this new method can be used as an SEC or DLS calibration for other special cases where a conventional calibration cannot be easily used because of the lack of a set of narrowly distributed standards, the existence of only one very broadly distributed sample, or the failure of polystyrene as a standard. On the other hand, it can also be used as a supplementary method to check an SEC or DLS calibration. In comparison with other calibration methods, the experimental time required in the new method is comparable and the only extra instrumentation is a time correlator for dynamic light scattering if we realize that the static laser light scattering spectrometer is required in any case for the determination of the absolute weight average molecular weight.

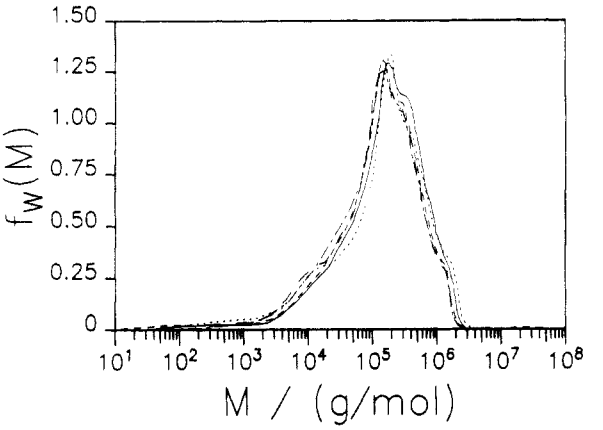

Figure 5. Weight distributions of five different B-200 gelatin samples (162378, 166401, 176497, 164623, and 172640). Their weight average molecular weights range from 2.41×10^5 to 3.67×10^5, and M_w/M_n values range from 3.7 to 5.3.

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