Topical Review

Advanced reference materials for the characterization of molecular size and weight

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Abstract
Nano-sized advanced reference materials (RMs) based on synthetic polymers were developed using supercritical fluid chromatography (SFC) and certain size measurement methods. These RMs have reference values of accurate molecular size and molecular weight. One of the RMs investigated was poly(ethylene glycol) (PEG) with no distribution in its degree of polymerization, i.e., its absolute degree of polymerization was determined as 23. SFC was used to separate molecularly uniform polymers from a commercial sample with wide polydispersity in its degree of polymerization. Because of the polydispersity, the average molecular size of the commercial polymer sample showed a distribution. This PEG 23mer RM can provide a uniform molecular size as the degree of polymerization is determined to be precisely 23.

1. Introduction

Practical standard weights are used for calibration or validation of balances in the macro size range. In the micro size range, particularly in the molecular size range, appropriate calibration standards such as a macro weight scale are not available. Standard scales and block gauges are used as accurate length standards in the macro size range; however, in the micro size range such as in the molecular size range, the standards for length measurement are still not well established. This paper summarizes investigations into producing polymer reference materials (RMs) with accurate molecular weight and size by using high-performance characterization technique. The polymers are quite stable and have a molecular weight from thousands to millions with a size range from several to a hundred nanometers. Synthetic polymers typically consist of mixtures of molecules with various degrees of polymerization, because they are generally synthesized by adding repeating units in a stepwise manner. The Federal Institute for Material Research and Testing (BAM) [1–3] in Germany and the National Institute of Standards and Technology (NIST) [4–6] in USA has produced a lot of reference materials; however, they did not develop polymer RMs having all fractions characterized and/or having no polydispersity of molecular weight. By using supercritical fluid chromatography (SFC), we can separate molecularly uniform polymers with no distribution in their molecular weight from polydispersed commercial polymers. Furthermore, by employing certain measurement techniques, the RMs can be determined to have accurate molecular size as well as molecular weight; they can then be used as new standards in the micro size range.

Here, three types of RMs are introduced: 1) monodispersed polymer with reference values of molar mass and size, 2) polymers with all fractions certified by SFC, and 3) molecularly uniform polymers purified by SFC. As the degree of polymerization is not homogeneous, the polydispersity of the molecular weight frequently has significant effects on the physical properties of polymers, such as radius of gyration, diffusion constant, and hydrodynamic radius.

In the next section 2, the monodispersed polymer RM is described. The polydispersity index of weight-averaged molecular weight ($M_w$) over number-averaged molecular weight ($M_n$), $M_w/M_n$, is around 1.05. Static light scattering (SLS) and dynamic light scattering (DLS) were used to obtain the reference values.
of molar mass, radius of gyration, and hydrodynamic radius. Since polydisperse commercial polymers have the values of $M_w/M_n$ around 2, this RM is a monodispersed polymer with $M_w/M_n < 1.1$; however, its molecular weight distribution is observed to affect the reference value of the hydrodynamic radius. A polymer with all fractions certified by SFC is reported in section 3; this RM exhibits reference values for the fractions of all components instead of the polydispersity parameter $M_w/M_n$, because SFC can completely separate all polymers in this RM. Finally, in section 4, a molecularly uniform polymer as an RM developed using an SFC fractionation system is described. This RM has only one degree of polymerization index (23) and provides an accurate molecular size without being affected by the polydispersity of molecular weight. The purity of the uniform polymer is 99.8%. When the parameter of polydispersity index is applied, the value of $M_w/M_n$ is 1.000003.

2. Monodispersed polymer

Polymers have sizes of nanometer or sub-micron range dissolved in water or organic solvents. Polystyrene is a stable and easy-to-synthesize polymer that has been used for a long time as a reference material for molecular weight. We aimed to synthesize a RM made from polystyrene having an accurate reference value of molecular weight as well as molecular size (including radius of gyration and hydrodynamic radius). Those microscopic values can be obtained by using laser light through the application of the Rayleigh-scattering theory. SLS is the essential technique that can be applied to measure the absolute both of molecular weight and size of polymers based on the Rayleigh-scattering theory [7–9]. Using this method, the scattering intensities measured at different angles based on the Rayleigh ratio can be able to obtain the radius of gyration, $R_g$, (molecular size) and the molar mass, $M$, (molecular weight).

In the SLS measurement, the most important parameter for accurate measurement is the Rayleigh ratio. We need the value of Rayleigh ratio for calculating the absolute scattered light intensity from the relative scattered light intensity. Since it is difficult to determine the Rayleigh ratio for each case, reference values for organic solvents (usually toluene or benzene) are used as reported in the literature [10]; however, these values vary by approximately 20%. Synthetic polymers are suitable materials for the determination of the Rayleigh ratio, because their molecular weights can be calculated from other methods, such as mass spectrometry, osmometry, sedimentation, or certain types of chromatography [11–14].

To determine the Rayleigh ratio, we chose appropriate RMs with reliable certified values of molecular weights. These RMs should have suitable sizes for the Rayleigh theory, as well as accurate molecular weights. The scattering angle and the size of the sample are important factors when selecting suitable samples or conditions. The selected RMs had a size of less than approximately 40 nm; however, they must be large enough to exhibit excess scattered light intensity values above those of pure toluene. Additionally, an uncertainty evaluation of the molecular weights measured by SLS is conducted; these uncertainties have never been accurately determined earlier because the uncertainty in the Rayleigh ratio was not clear. The uncertainty in the Rayleigh ratio was evaluated first by using a RM with a narrow mono-distribution, which was synthesized by living anionic polymerization.

2.1. Materials and instrumentation

The samples were two RMs supplied by the National Metrology Institute of Japan (NMIJ) (NMIJ CRM 5008-a [15], NMIJ RM 5009-a [16]) and one standard RM (SRM) supplied by the National Institute of Standards and Technology (NIST) (SRM 705a) [17]; all these RMs have certified molecular-weight values. NMIJ RM 5012-a [18] as the candidate RM was used for uncertainty analysis, which was a polystyrene with a narrow molecular-weight distribution. The polystyrene RMs were dissolved in spectroscopy-grade toluene filtered through a 0.2 µm filter by gently shaking the solution for one day.

The relationship between the excess scattered light intensity $R(q)$ from the pure solvent and the molecular weight is given by equation (2–1) called a Zimm plot [19, 20], when $R_gq ≪ 1$ in the Rayleigh region.

$$\lim_{C \to 0, q \to 0} \frac{KC}{R(q)} = \frac{1}{M} \left[ 1 + \frac{1}{3} (R_gq)^2 \right]. \quad (2-1)$$

Here $K$ is the optical constant, $C$ is the mass concentration, $M$ is the molecular weight and $R_g$ is the radius of gyration of the sample. The value of $K$ is given by equation (2–2) as follows:

$$K = \frac{4\pi^2 n^2 (dn/dc)^2}{\lambda^2 N_A}, \quad (2-2)$$
where $N_A$ is Avogadro’s number, $dn/dC$ is the refractive index increment of the solution, $n$ is the refractive index of the solvent, and $\lambda$ is the wavelength of the laser. In the case of $R_0q \approx 1$, the Berry plot [21] shown in equation (2–3) can be applied:

$$\lim_{{C \to 0}} \left[ \frac{KC}{R(q)} \right]^{1/2} = \frac{1}{M^{1/2}} \left[ 1 + \frac{1}{6} (R_0q)^2 \right],$$ (2-3)

where $q$ is the amplitude of the wave vector as follows:

$$q = (4\pi n/\lambda) \sin (\theta/2).$$ (2-4)

The Zimm or the Berry plot can be used to determine the molecular weight, $M$, from the double extrapolation about $C \to 0$ and $q \to 0$:

$$M = \frac{R(0)}{KC}.$$ (2-5)

In general, $R(q)$ is obtained by using the reported value of the absolute Rayleigh ratio of toluene, $R_{VV}$. The observed scattered light intensity, $I(q)$, in the user’s apparatus is compared to the reported value of $R_{VV}$:

$$R(q) = \frac{[I_{\text{solution}}(q) - I_{\text{medium}}(q)] \cdot \sin \theta}{I_{\text{toluene}}(q)}.\]$$ (2-6)

where $I_{\text{solution}}(q)$, $I_{\text{medium}}(q)$, and $I_{\text{toluene}}(q)$ are respectively the scattered light intensities of the solution, the medium (water or solvents), and toluene. An accurate value of $R_{VV}$ is required for reliable SLS measurements, but the reported values for the $R_{VV}$ are markedly depending on the literature source.

The electric-field time-correlation function $g^{(1)}(\tau)$ in DLS measurements, is calculated from the correlation function of the scattered light, $g^{(2)}(\tau)$, by the Siegert relationship [22]:

$$g^{(2)}(\tau) = A \left( 1 + B \left| g^{(1)}(\tau) \right|^2 \right).$$ (2-7)

The $g^{(1)}(\tau)$ can be calculated using the cumulant method, as described in International Organization for Standardization (ISO) 22412 [23]. The average decay rate $\Gamma$ is determined:

$$\left| g^{(1)}(\tau) \right| = \exp(-\Gamma \tau + \frac{1}{2} \mu_2 \tau^2 - 1).$$ (2-8)

The diffusion coefficient $D$ can be obtained by the first ordered cumulant $\Gamma$ as follows:

$$\Gamma = D q^2.$$ (2-9)

Using the value of $D$, the hydrodynamic radius of the sample $R_h$ can be analyzed according to the Stokes–Einstein relationship:

$$R_h = \frac{k_B T}{6 \pi \eta D}$$ (2-10)

where $k_B$, $\eta$, and $T$ are the Boltzmann constant, solvent viscosity, and absolute temperature, respectively. The measured apparent diffusion coefficients $D_{app}$ were extrapolated to infinite dilution $C \to 0$ and to zero scattering angle $\theta \to 0$ to obtain $D(0)$. The hydrodynamic radius of samples ($R_h$) are calculated using by $D(0)$ and equation (2–10).

The light-scattering apparatus has a goniometer system equipped with a yttrium-aluminum-garnet laser with a wavelength of 532 nm as shown in figure 2-1. SLS measurements were carried out by varying the scattering angle, $\theta$, from 30° to 150° at 5° intervals for each concentration. DLS measurements were taken place by scanning the angles $\theta = 30°$, 50°, 70°, 90°, 110°, 130°, and 150°. The sample cell (20 Ø) was set in a toluene bath (80 Ø) kept the temperature at 25.00 °C ± 0.01 °C. Details of the experiment are described in a previous paper [24].
2.2. Accurate Rayleigh ratio

Figure 2.2 shows typical SLS results for polystyrene (PS) in toluene for determining the $R_{vv}$ in this instrument. The two extrapolations to infinite dilution $C \to 0$ and to lower angles $\theta \to 0$ can determine the point of intersection related to an inverse value of the molecular weight.

The three RMs for molecular weight were used for getting accurate Rayleigh ration $R_{vv}$, which are listed in table 2.1. The two RMs are supplied by NMIJ and the one is by NIST. The Rayleigh ratio $R_{vv}$ can be calculated using these molecular weights from the equations (2–5) and (2–6). The values, $2.44 \times 10^{-5}$ cm$^{-1}$, $2.29 \times 10^{-5}$ cm$^{-1}$, and $2.30 \times 10^{-5}$ cm$^{-1}$ listed in table 2.1 are good agreement with each other compared to previous values reported in the literature [25–27]. The Rayleigh ratio $R_{vv}$ is notably dependent on the wavelength of the using light and its polarization. The subscription, $VV$, means that both incident and scattered lights are vertically polarized. The average value at 532 nm using the three synthetic RMs is $R_{VV}(532 \text{ nm}) = 2.34 \times 10^{-5}$ cm$^{-1}$ with a small uncertainty. The methods for evaluating each and the total uncertainty are described in the next section.

2.3. Uncertainty analysis

The uncertainty of the SLS measurement of NMIJ RM 5012-a was evaluated according to the International Organization for Standardization’s ‘Guide to the Expression of Uncertainty in Measurement’ [28], which is
Table 2.1. List of reference values of three RMs and redetermination values of the \( R_x \), for toluene at 25 °C and 532 nm \[29\].

| Measurement methods of \( M \) | Measurement values \( M \) / g mol\(^{-1}\) | Uncertainty of \( M \) / g mol\(^{-1}\) | \( R_x \) / cm\(^{-1}\) | Uncertainty of \( R_x \) / cm\(^{-1}\) |
|-------------------------------|---------------------------------------------|---------------------------------------------|---------------------------|---------------------------------------------|
| NMIJ CRM 5008-a               | SEC-MALS\( ^a \)                            | 276 000                                     | 15 000                    | \( 2.44 \times 10^{-5} \)                      | \( 0.07 \times 10^{-3} \)                      |
| NMIJ CRM 5009-a               | MALDI TOFMS                                  | 8670                                        | 190                       | \( 2.29 \times 10^{-5} \)                      | \( 0.03 \times 10^{-3} \)                      |
| NIST SRM 705a                 | Osmometry, Light scattering\(^b\), Sedimentation | 170 900                                     | 580 740 2100              | \( 2.30 \times 10^{-5} \)                      | \( 0.01 \times 10^{-3} \)                      |

\(^a\)This value is measured by light scattering with another wavelength laser-source.

Figure 2.3. Contributions of each parameter for molecular weight, \( M \), measured by SLS.

hereafter referred to as GUM. Using equations (2–2) and (2–5), the uncertainty of the molecular weight, \( u(M) \), can be calculated by the following equation:

\[
M = \frac{R(\theta)}{KC} = f(I, \theta, R_{VV}, \lambda, N_A, n, dn/dC, C) = \frac{I \cdot \sin \theta \cdot R_{VV} \cdot \lambda^4 \cdot N_A}{4\pi^2 \cdot n^2 \cdot (dn/dC)^2 \cdot C}. \tag{2-11}
\]

From the law of propagation of uncertainty described in the GUM, equation (2–11) can be expanded [29]:

\[
u^2(M) = \left(\frac{\partial f}{\partial I}\right)^2 u_I^2(I) + \left(\frac{\partial f}{\partial \theta}\right)^2 u_\theta^2(\theta) + \left(\frac{\partial f}{\partial R_{VV}}\right)^2 u_{VV}^2(R_{VV}) + \left(\frac{\partial f}{\partial \lambda}\right)^2 u_\lambda^2(\lambda) + \left(\frac{\partial f}{\partial N_A}\right)^2 u_{N_A}^2(N_A)
+ \left(\frac{\partial f}{\partial n}\right)^2 u_n^2(n) + \left(\frac{\partial f}{\partial (dn/dC)}\right)^2 u_{dn/dC}^2(dn/dC) + \left(\frac{\partial f}{\partial C}\right)^2 u_C^2(C)
= f \left\{ u_I^2(I) + \frac{u_\theta^2(\theta)}{\tan^2 \theta} + \frac{u_{VV}^2(R_{VV})}{R_{VV}^2} + 16 \frac{u_\lambda^2(\lambda)}{\lambda^2} + \frac{u_{N_A}^2(N_A)}{N_A^2} + 4 \frac{u_n^2(n)}{n^2} + 4 \frac{u_{dn/dC}^2(dn/dC)}{(dn/dC)^2} + \frac{u_C^2(C)}{C^2}\right\}. \tag{2-12}
\]

The Zimm plot established using equation (2–1) resulted in a value of \( 6.06 \times 10^5 \) g mol\(^{-1}\) for the molecular weight, whereas the Berry-type plot established using equation (2–3) provided a result of \( 5.94 \times 10^5 \) g mol\(^{-1}\). These values were in reasonable agreement and were within the range of uncertainty, that we discussed this range in the next section.

2.3.1. Uncertainties of various components

The uncertainties of each parameters in equation (2–12) are summarized in table 2–2 and figure 2–3. Details, other than the uncertainties in the Rayleigh ratio, the sample concentration, and the scattered light intensity, are provided in [29].

2.3.1.1. Uncertainty of the Rayleigh ratio

The uncertainty of the \( R_{VV} \) could be evaluated with the uncertainty of the three RMs, as shown in table 2–1. The uncertainty for NIST SRM 705a was specified by the repeatability of the measurements. The uncertainty in the weight-averaged molecular weight of NMIJ RM 5009-a was recalculated from the uncertainty in the
number-averaged molecular weight. The standard deviation of the three measurements of $R_{CV}$ for the three RMs was $8.4 \times 10^{-7}$ cm$^{-1}$. The standard uncertainty, $u_R(R)$, was estimated using the following equation:

$$u_R(R) = 8.4 \times 10^{-7}/\sqrt{3} = 4.8 \times 10^{-7}(\text{cm}^{-1}).$$  \hfill (2-13)

### 2.3.1.2. Uncertainty of sample concentration

The uncertainty of the sample concentration was calculated mainly using the calibration uncertainty of the microbalance, the sample size, and the density of the solvent. The standard uncertainty in calibration of the balance was 0.025 mg and the sample size was 10 mg. Then, the uncertainty of weighing the samples was determined to be

$$0.025 \text{ mg}/10 \text{ mg} = 0.0025.$$  \hfill (2-14)

The density of toluene at 0.1 MPa and 25 °C is 0.86224 g ml$^{-1}$ [30], and the uncertainty of toluene density can be calculated by a rectangular distribution with a whole width of 0.001 g ml$^{-1}$. The uncertainty of sample concentration can be calculated:

$$u_C(C) = 1 \times \sqrt{ \left( \frac{0.025}{10} \right)^2 + \left( \frac{0.001/2 \sqrt{3}}{0.86224} \right)^2 } = 0.0025 \text{ (g/ml)}.$$  \hfill (2-15)

### 2.3.1.3. Uncertainty of scattered light intensity

The uncertainties of the scattered light intensity, $I$, include repeatability of measurements, differences due to the Zimm plot or Berry plots, and variance in the double extrapolations ($C \to 0$ and $\theta \to 0$), which are called Type A uncertainties arising from statistical variances of actual experiments. Other experimental uncertainties are included such as the drift in the instruments and the non-homogeneity of the solution. The standard deviation of the intersection by the Berry plot is extremely small (~1%). As can be seen from equations (2-1) and (2-3), there is no large difference in the values of the molecular weights obtained by a Zimm plot ($6.06 \times 10^{5}$ g mol$^{-1}$) and by a Berry plot ($5.94 \times 10^{5}$ g mol$^{-1}$). This is because the $R_{D}$ of the sample is approximately 30 nm, which is in the Rayleigh region. Uncertainty, $u_I(I)$, is most significantly affected by variances within-bottle and between-bottle. The uncertainty of intensity, $u_I(I)$, was determined using an analysis of the variance (ANOVA) [31]. A more detailed explanation of homogeneity testing methods for the NMIJ RM 5012-a [29] and other typical examples according to ISO Guide 35 [32] are given in the references [32, 33].

### Table 2.2. List of uncertainties for molecular weight measurement by SLS [29].

| Variable $x$                                      | Standard uncertainty, $u_x(x) \partial f/\partial x$ | Contribution $(\partial f/\partial x)^{-1} \cdot u_x(x)$ | Uncertainty type |
|---------------------------------------------------|-----------------------------------------------------|--------------------------------------------------------|-----------------|
| The Avogadro constant $N_A$ $u_N(N_A) = 2.7 \times 10^{16}$ [mol$^{-1}$] | $M/N_A$                                              | $2.8 \times 10^{-2}$                                    | B               |
| Sample concentration $C$ $u_C(C) = 2.5 \times 10^{-3}$ [g ml$^{-1}$] | $M/C$                                                | $1.6 \times 10^{3}$                                    | B               |
| Refractive index increment $dn/dc$ $u_{dn/dc}(dn/dc) = 5.8 \times 10^{-4}$ [mol/dl] | $2M/(dn/dc)$                                         | $6.5 \times 10^{3}$                                    | B               |
| Refractive index of solvent $n$ $u_n(n) = 2.9 \times 10^{-4}$ [g ml$^{-1}$] | $2M/n$                                               | $2.4 \times 10^{2}$                                    | B               |
| Scattering angle $\theta$ [rad] $u_\theta(\theta) = 3.0 \times 10^{-5}$ | $M\tan(\theta)$                                      | $6.0 \times 10^{-6}$                                  | B               |
| Wavelength of laser $\lambda$ [cm] $u_\lambda(\lambda) = 1.7 \times 10^{-5}$ | $4M/\lambda$                                         | $7.8 \times 10^{2}$                                    | B               |
| Rayleigh ratio $RVV$ [cm$^{-1}$] $u_{RVV}(RVV) = 4.8 \times 10^{-7}$ | $M/RVV$                                              | $1.3 \times 10^{4}$                                    | A               |
| Scattered light intensity $I$ $u(I) = 2.0 \times 10^{5}$ | $M/I$                                                | $2.1 \times 10^{4}$                                    | A               |
| Combined uncertainty of molecular weight $M$ [g mol$^{-1}$]$^A$ | $u(M) = 0.25 \times 10^{5}$                         |                                                         |                 |
| Expanded uncertainty of molecular weight $M$ [g mol$^{-1}$] (coverage factor $k = 2$) | $U(M) = 0.51 \times 10^{5}$                         |                                                         |                 |

$^A$Measured molecular weight $M = 6.18 \times 10^{5}$ g mol$^{-1}$
Table 2.3. Particle size corresponding twice of hydrodynamic radius in toluene at 25 °C with concentration and angular dependences at a laser-wavelength of 532 nm.

| Concentration (mg/ml) | Scattering angle (°) |
|-----------------------|----------------------|
|                       | 90       | 100      | 110      | 120      | 130      | 140      | 150      | 160      | 170      | 173     |
| 0.10                  | 44.8     | 44.3     | 43.8     | 43.4     | 43.0     | 42.7     | 42.4     | 42.2     | 42.1     | 42.0     |
| 0.20                  | 44.5     | 44.0     | 43.6     | 43.2     | 42.8     | 42.5     | 42.2     | 42.0     | 41.9     | 41.9     |
| 0.30                  | 44.2     | 43.7     | 43.3     | 42.9     | 42.6     | 42.3     | 42.0     | 41.8     | 41.7     | 41.7     |
| 0.40                  | 43.9     | 43.5     | 43.1     | 42.7     | 42.4     | 42.1     | 41.8     | 41.7     | 41.6     | 41.5     |
| 0.50                  | 43.6     | 43.2     | 42.8     | 42.5     | 42.2     | 41.9     | 41.7     | 41.5     | 41.4     | 41.4     |
| 0.60                  | 43.3     | 42.9     | 42.6     | 42.2     | 41.9     | 41.7     | 41.5     | 41.3     | 41.2     | 41.2     |
| 0.70                  | 43.0     | 42.6     | 42.3     | 42.0     | 41.7     | 41.5     | 41.3     | 41.1     | 41.1     | 41.0     |
| 0.80                  | 42.7     | 42.4     | 42.1     | 41.8     | 41.5     | 41.3     | 41.1     | 41.0     | 40.9     | 40.9     |
| 0.90                  | 42.4     | 42.1     | 41.8     | 41.5     | 41.3     | 41.1     | 40.9     | 40.8     | 40.7     | 40.7     |
| 1.00                  | 42.1     | 41.8     | 41.6     | 41.3     | 41.1     | 40.9     | 40.7     | 40.6     | 40.6     | 40.5     |

2.3.2. Combined uncertainty of SLS

The details of the estimation of each uncertainty component are described above. The combined uncertainty of the molecular weight, \( u(M) \), calculated by equation (2–12) is

\[
u(M) = 0.25 \times 10^5 \text{ (g mol}^{-1}\text{)}.
\]

The expanded uncertainty, \( U(M) \), is calculated from the combined uncertainty by a coverage factor \( k = 2 \),

\[
U(M) = 0.51 \times 10^5 \text{ (g mol}^{-1}\text{)}.
\]

The expanded uncertainty, \( 0.51 \times 10^5 \text{ g mol}^{-1} \), corresponded to approximately 10% of the value of \( M \).

From table 2-2 and figure 2–3, it is noticeable that the main sources of uncertainty is from the Rayleigh ratio, \( R_{VV} \), the scattered light intensity, and the refractive index increment. Those are directly observed values and make large contributions to the uncertainty of molecular weight obtained by SLS measurements. Especially, \( R_{VV} \) has a wavelength dependence and should be determined for each apparatus by using RMs with molecular weights as certified standards.

2.4. Reference values

The value of the mass-averaged molar mass \( M_w \) of polystyrene was determined by SLS as \( M_w = 6.18 \times 10^5 \) (g mol\(^{-1}\)) under the condition of \( R_{VV} \) (of toluene, at 25 °C, at the scattering angle of 90°, with vertical-vertical polarized laser of 532 nm) used to determine the reference value was \( 2.34 \times 10^{-5} \text{ (cm}^{-1}\text{)} \) as calculated.

The radius of gyration \( R_g \) in toluene at 25 °C also determined by SLS was 31.9 nm. The \( R_g \) was obtained using a Berry plot considering the scattering angle and concentration dependencies of scattered light with a laser wavelength of 532 nm. According to the Stokes–Einstein relationship shown in equation (2–10), the particle sizes (twice that of hydrodynamic radii) were determined by DLS measurements at 25 °C in toluene with concentration and angular dependencies. The polydispersity index is enough narrow (\( M_w/M_n \sim 1.05 \)), but particle size is influenced by the polydispersity of the molecular weight. The results are summarized in table 2-3 for a wavelength of 532 nm. In the actual data sheet of NMIJ RM 5012-a, particle sizes measured at other concentrations with two wavelengths of 532 nm and 633 nm are also provided.

2.5. Short summary

We accurately determined the \( R_{VV} \) at a wavelength of 532 nm using SLS measurements for RMs with the uncertainty analysis for the first time. The uncertainty of the \( R_{VV} \) had remained unclear for long time and it was therefore, not previously possible to accurately determine the uncertainty of molecular-weight measurements by SLS measurements. We established the \( R_{VV} \) and determined the value of uncertainty. We also explained a standard method for evaluating the uncertainty of molar mass measurements by SLS. These results allow us to use NMIJ RM 5012-a to obtain accurate molar mass measurements and also provide reference values for the radius of gyration and the hydrodynamic radii.

3. Polymers of all fractions certified

Synthetic polymers always exhibit polydispersity in their molecular weight because they are synthesized by adding repeating units in a stepwise manner. By using evaporative light scattering detector (ELSD) in-lined
high-performance SFC, we can precisely observe all of the mass fractions of the polymers that are needed for certified RMs (CRMs). The uncertainties of the CRMs were estimated according to the GUM [28].

Poly(ethylene glycol) (PEG) is the simplest water-soluble synthetic polymer that is widely used in daily life in the form of sanitary goods and industrial products, especially in the medicine and bioscience fields [34, 35]. PEG is considered important owing to its excellent safety properties and can be used in oral medicine. It has been applied to drug delivery systems and is used as a base material in the modification of proteins. Under these situations, we have planned to produce CRMs based on PEG with certified values not only for the averaged molecular weights but also for all of the mass fractions of components with different degrees of polymerization.

These PEG CRMs were prepared at the NIST and at the Federal Institute for Material Research and Testing (BAM) in Germany. These CRMs are, however, polydispersed samples with only certified mean value of the molecular weight. The molecular weight distribution of the polymers often makes the physical and chemical properties difficult to be precisely measured. Uniform oligomers with no molecular weight distributions are quite useful in determining the exact molecular weight dependence of the physical properties of polymers to avoid the effects of polydispersity, which is an inherent property of synthetic polymers.

SFC is a powerful technique used to separate the uniform oligomers, however, the detection process in SFC is complex compared with that in normal liquid chromatography; this is because, for SFC, the detector must be capable of operating at the high pressures [36–38]. Ultraviolet (UV) detectors are often used for SFC as they can provide good quantitativeness. However, PEGs do not have UV absorbance. Therefore, an ELSD is used for the detection of UV-invisible and non-volatile samples such as PEGs, but it is ability in case of quantitative analysis of molecular weight, has not yet been established [39–41]. We investigated calibration method of SFC-ELSD using equimolar mixture of uniform PEG oligomers with no molecular weight distribution.

3.1. Materials and instrumentation

3.1.1. Materials for RMs
The PEG CRMs with molecular weights of 1500, 1000, and 400 were prepared by Wako Pure Chemical Industries Ltd. (Osaka, Japan). The general chemical formula can be written as HO(CH_{2}CH_{2}O)_{n}H, where n is the index of different degrees of polymerization.

3.1.2. Separation by SFC
SFC instrument SUPER-201 are supplied by JASCO Co., Tokyo, Japan with a SF Cpak SIL-5 silica gel column (4.6 mm I.D. × 250 mm, JASCO Co., Tokyo, Japan). The detection of PEG in-lined with SFC was carried out by ELSD (ELSD 2000, Alltech Associates, IL, USA), and the concentrations of separated PEGs were determined by total organic carbon (TOC) measurements by SHIMADZU TOC-500 apparatus (Shimadzu Corporation, Kyoto, Japan). The TOC apparatus was calibrated by using potassium hydrogen phthalate which was a certified reference material with the certified value of purity (NMIJ CRM 3001-a No.31, Tsukuba, Ibaraki, Japan).

A typical chromatogram for separation of PEG was shown in figure 3–1, where all fractions containing in PEG 1500 were completely separated. The details of the separation conditions were listed in table 3-1 and have been reported earlier [42, 43].

3.1.3. Calibration method of SFC-ELSD
As illustrated in figure 3–2, at first, uniform oligomers were fractionated by SFC. And then, concentrations of fractionated uniform oligomers were determined by TOC measurements, and they were prepared into solutions of equiweight mixtures of 8 uniform oligomers with the degree of polymerization n = 6, 8, 12, 18, 21, 25, 30, and 42. The equiweight mixtures of the uniform PEG oligomers were used for calibration of SFC-ELSD. The equiweight mixtures were dissolved in methanol at different concentrations of 2, 4, 6, 8, and 10 mg ml^{-1} for checking the linearity of the response of SFC-ELSD. The other SFC conditions were the same as table 3-1.

The concentration dependence of the response of SFC-ELSD was not large and can be included in the uncertainty of the experiments; however, the dependence of the degree of polymerization were so significant that should be corrected by the calibration curve. From the nonlinear fitting by the exponential curve, the response of SFC-ELSD against the degree of polymerization, i, was calibrated as follows:

\[ a_i (\text{SFC} - \text{ELSD}) = 0.38162 \cdot \exp(-0.060144 \cdot i) \]  

(3-1)
Figure 3.1. Typical SFC-ELSD chromatograms of the PEG 1500 under the condition of modifier gradient. The index ‘\( i \)’ is the degree of polymerization.

Table 3.1. The SFC-ELSD conditions for separation of PEGs.

| Conditions                        | PEG 400       | PEG 1000      | PEG 1500      |
|----------------------------------|---------------|---------------|---------------|
| Column                           | SFCpak SIL-5 (4.6 mm I.D. \( \times \) 250 mm Long) |               |               |
| Pressure                         | 20 M Pa       |               |               |
| Flow rate of CO\(_2\)            | 2.0 ml min\(^{-1}\) (Constant) |               |               |
| Initial flow rate of modifier    | 0.40 ml min\(^{-1}\) | 0.60 ml min\(^{-1}\) | 0.60 ml min\(^{-1}\) |
| Gradient rate of modifier        | 0.013 ml min\(^{-2}\) | 0.0067 ml min\(^{-2}\) | 0.0067 ml min\(^{-2}\) |
| Modifier                         | methanol/water (9/1 in volume) |               |               |
| Temperature                      | 50 °C (Constant) |               |               |
| Inject concentration             | 600 mg ml\(^{-1}\) | 600 mg ml\(^{-1}\) | 200 mg ml\(^{-1}\) |
| Sample size                      | 2 \( \mu \)l in methanol |               |               |
| Detector                         | ELSD with silicon photodiode |               |               |
| Light source of ELSD             | laser diode 5 mW, wavelength of 670 nm |               |               |
| Tube temperature of ELSD         | 40 °C (Constant) | 60 °C (Constant) |               |
| Nebulizer gas                    | Compressed air at 0.80 l min\(^{-1}\) | Compressed air at 0.60 l min\(^{-1}\) |               |
| Impactor                         | off           |               |               |

Figure 3.2. A schematic representation of the calibration method of SFC-ELSD by using an equiweight mixture of uniform oligomers.
The \( a_i \) is the sensitivity coefficient of the SFC-ELSD response. The details are described in the references [44, 45].

### 3.2. Uncertainty analysis

#### 3.2.1. Uncertainties of three components

The homogeneity testing and uncertainty analysis of the PEG CRMs were taken place according to the international documents of Guide 35 [31] and GUM [28], respectively. The uncertainty for determination of molecular weight by SFC-ELSD mainly arises from three factors as follows; 1) relative peak intensity of the SFC-ELSD chromatogram \( u(s_i) \), 2) the sensitivity coefficient of the SFC-ELSD response \( u(a_i) \), and 3) the atomic weight from the periodic table \( u(M_i) \).

#### 3.2.1.1. Uncertainty of relative peak intensity

The uncertainty of relative peak intensity \( u(s_i) \) is including the homogeneity of 300 bottles of each CRM. In general, homogeneity testing should be taken placed by using larger number of bottles, \( m \); \( m > \sqrt{\text{total number of bottles}} \).

In this case, 7 bottles were used for homogeneity testing and 4 repeatability measurements (within bottle) were carried out. The 28 measurement results were randomly taken placed and estimated by analysis of variance (ANOVA) against the \( 7 \times 4 \) matrix [44]. The uncertainty of relative peak intensity \( u(s_i) \) was finally evaluated including the ANOVA result, long time stability, ratio of signal/noise of the detector, and fluctuation of baseline on the chromatogram.

#### 3.2.1.2. Uncertainty of sensitivity coefficient

The uncertainty of the sensitivity coefficient \( u(a_i) \) is estimated from the standard deviation of the concentration dependence of SFC-ELSD response and the 95% confidence level of the nonlinear fitting of calibration curve as shown in equation (3–1).

#### 3.2.1.3. Uncertainty of the atomic weight

The uncertainty of the atomic weight \( u(M_i) \) is necessary for calculating the molecular weight. The \( u(M_i) \) can be calculated by assuming a rectangular \( a \ priori \) probability distribution from the atomic weights of the elements listed in the IUPAC Technical Report (2005) [46]. The \( u(M_i) \) is calculated from the chemical structure of PEG [OH-(CH\(_2\)-CH\(_2\)-O)\(_i\)-H] as follows:

\[
\begin{align*}
\text{u}^2(M_i) &= (2j)^2u^2(C) + (4i + 2)^2u^2(H) + (i + 1)^2u^2(O) \\
u^2(C) &= 0.0008^2/3 = 2.13 \times 10^{-7} \\
u^2(H) &= 0.0007^2/3 = 1.63 \times 10^{-9} \\
u^2(O) &= 0.0003^2/3 = 3.00 \times 10^{-10}.
\end{align*}
\]

#### 3.2.2. Combined uncertainty of molecular weight

The certified values are number-averaged molecular weight, \( M_n \), and weight-averaged molecular weight, \( M_w \). \( M_n \) and \( M_w \) are calculated by the following equations:

\[
M_n = \sum_i x_i M_i
\]

\[
M_w = \sum_i w_i M_i
\]

where \( x_i \) and \( w_i \) are the number fraction and weight fraction for each component with degree of polymerization, \( i \). Because the response of SFC-ELSD is proportional to the weight fraction \( w_i \) and the equiweight mixtures were used for calibrating SFC-ELSD, the following equation can be applied:

\[
x_i = \frac{w_i/M_i}{\sum_j \left( w_j/M_j \right)}
\]

\[
w_i = \frac{s_i/a_i}{\sum_j \left( s_j/a_j \right)}
\]
weights are from 0.5% to 2% of the values of molecular weights. The uncertainties increase at higher $M$. Reference values are shown as expanded uncertainties with a coverage factor (NMIJ CRM 5007-a) with their standard uncertainties. On their actual certificate sheets, the uncertainties are listed for PEG 400 (NMIJ CRM 5005-a), PEG 1000 (NMIJ CRM 5006-a), and PEG 1500 (NMIJ CRM 5007-a) with their standard uncertainties. In the same manner, the uncertainty of the number fraction, $u(x_i)$, is calculated from $u(w_i)$ and equation (3–6) by equation (3–9).

$$u^2(x_i) = \sum_j \left( \frac{\partial x_i}{\partial w_j} \right)^2 \cdot u^2(w_j) + \sum_j \left( \frac{\partial x_i}{\partial M_j} \right)^2 \cdot u^2(M_j)$$

$$= \frac{1}{\left( \sum_j \frac{w_i}{M_j} \right)^4 M_i^2} \left\{ \left( \sum_j \frac{w_j}{M_j} \right)^2 - 2 \left( \sum_j \frac{w_j}{M_j} \frac{w_i}{M_i} \right) \cdot u^2(w_i) + w_i^2 \sum_j \frac{1}{M_j^2} u^2(w_j) \right\}$$

$$+ \frac{1}{\left( \sum_j \frac{w_i}{M_j} \right)^4 M_i^4} \left\{ \left( \sum_j \frac{w_j}{M_j} \right)^2 s_i^2 - 2 \left( \sum_j \frac{w_j}{M_j} \frac{w_i^2}{M_i^2} \right) \cdot u^2(M_i) + M_i^2 w_i^2 \sum_j \frac{w_j^2}{M_j^2} u^2(M_j) \right\}$$

(3–9)

The uncertainty of the $M_w$, $u(M_w)$, is obtained from $u(w_i)$ and equation (3–5) by equation (3–10).

$$u^2(M_w) = \sum_j \left( \frac{\partial M_w}{\partial w_j} \right)^2 \cdot u^2(w_j) + \sum_j \left( \frac{\partial M_w}{\partial M_j} \right)^2 \cdot u^2(M_j)$$

$$= \sum_j M_j^2 u^2(w_j) + \sum_j w_i^2 u^2(M_j)$$

(3–10)

The uncertainty of the $M_n$, $u(M_n)$, is calculated from $u(x_i)$ and equation (3–4) is rewritten as equation (3–11).

$$u^2(M_n) = \sum_j \left( \frac{\partial M_n}{\partial x_i} \right)^2 \cdot u^2(x_i) + \sum_j \left( \frac{\partial M_n}{\partial M_j} \right)^2 \cdot u^2(M_j)$$

$$= \sum_j M_j^2 u^2(x_i) + \sum_j x_i^2 u^2(M_j)$$

(3–11)

A part of a typical example of evaluated uncertainties for PEG 1000 is shown in table 3-2.  

### 3.3. Reference values

In table 3-3, the certified values of weight-averaged molecular weight $M_w$ and number-averaged molecular weight $M_n$ are listed for PEG 400 (NMIJ CRM 5005-a), PEG 1000 (NMIJ CRM 5006-a), and PEG 1500 (NMIJ CRM 5007-a) with their standard uncertainties. On their actual certificate sheets, the uncertainties are shown as expanded uncertainties with a coverage factor $k = 2$. The standard uncertainties of molecular weights are from 0.5% to 2% of the values of molecular weights. The uncertainties increase at higher molecular weights, i.e. at higher degrees of polymerization, $i$. The main reason for the large uncertainty ay higher $i$-values came from the decrease of peak intensities detected by SFC-ELSD as illustrated in figure 3–2.
Table 3.2. A part of uncertainty analysis for certification of PEG 1000 [44].

| i  | $M_i$  | Peak intensity $s_i$ | Sensit. coeff. $a_i$ | Mass fraction $w_i$ | Mole fraction $x_i$ | Type A | Type A | Type B | Contributions |
|----|--------|---------------------|----------------------|---------------------|---------------------|--------|--------|--------|---------------|
| 7  | 326.38 | 0.00110             | 0.2505               | 0.00041             | 0.00130             | 0.00034 | 0.0069 | 0.007  | 0.00013       |
| 8  | 370.44 | 0.00118             | 0.2359               | 0.00046             | 0.00130             | 0.00038 | 0.0057 | 0.008  | 0.00015       |
| 9  | 414.49 | 0.00127             | 0.2221               | 0.00053             | 0.00133             | 0.00036 | 0.0046 | 0.008  | 0.00015       |
| 10 | 458.54 | 0.00161             | 0.2091               | 0.00071             | 0.00162             | 0.00034 | 0.0036 | 0.009  | 0.00015       |
| 17 | 766.91 | 0.04287             | 0.1373               | 0.02896             | 0.03928             | 0.00051 | 0.0010 | 0.016  | 0.00035       |
| 18 | 810.97 | 0.05429             | 0.1293               | 0.03895             | 0.04996             | 0.00060 | 0.0014 | 0.017  | 0.00044       |
| 19 | 855.02 | 0.06539             | 0.1217               | 0.04982             | 0.06061             | 0.00120 | 0.0018 | 0.018  | 0.00088       |
| 20 | 899.07 | 0.07417             | 0.1146               | 0.06001             | 0.06944             | 0.00091 | 0.0021 | 0.019  | 0.00072       |
| 21 | 943.12 | 0.08017             | 0.1079               | 0.06888             | 0.07598             | 0.00093 | 0.0023 | 0.020  | 0.00077       |
| 22 | 987.18 | 0.08372             | 0.1016               | 0.07640             | 0.08051             | 0.00047 | 0.0026 | 0.021  | 0.00047       |
| 37 | 1647.97| 0.00118             | 0.0412               | 0.00265             | 0.00167             | 0.00020 | 0.0032 | 0.035  | 0.00046       |
| 38 | 1692.02| 0.00063             | 0.0388               | 0.00149             | 0.00092             | 0.00007 | 0.0032 | 0.036  | 0.00016       |
| 39 | 1736.07| 0.00042             | 0.0366               | 0.00107             | 0.00064             | 0.00009 | 0.0031 | 0.037  | 0.00023       |
| 40 | 1780.13| 0.00024             | 0.0344               | 0.00065             | 0.00038             | 0.00010 | 0.0030 | 0.038  | 0.00027       |

Uncertainty

$u(s_i) = \frac{\partial w_i}{\partial s_i} \cdot u(s_i)$

$u(a_i) = \frac{\partial w_i}{\partial a_i} \cdot u(a_i)$

$u(M_i) = \frac{\partial w_i}{\partial M_i} \cdot u(M_i)$
### Table 3.3. Weight-averaged and number-averaged molecular weights, $M_w$ and $M_n$, and the uncertainties, $u(M_w)$ and $u(M_n)$, for PEG 400, 1000, and 1500.

|          | Weight-averaged molecular weight $M_w$ | Uncertainty $u(M_w)$ | $u(M_w)/M_w$ | Number-averaged molecular weight $M_n$ | Uncertainty $u(M_n)$ | $u(M_n)/M_n$ |
|----------|---------------------------------------|----------------------|--------------|---------------------------------------|----------------------|--------------|
| PEG 400  | 431.2                                 | 2.8                  | 0.0066       | 409.9                                 | 2.2                  | 0.0054       |
| PEG 1000 | 1084.7                                | 10.4                 | 0.0096       | 1040.6                                | 8.8                  | 0.0084       |
| PEG 1500 | 1601.0                                | 33.4                 | 0.0209       | 1560.6                                | 30.0                 | 0.0192       |

#### Figure 3.3. A schematic representation of the uncertainty contributions for the certification of PEG CRMs by SFC-ELSD.

In figure 3-3, the uncertainty contributions of the atomic weight $u(M_i)$, peak intensity of the chromatogram $u(s_i)$, and calibration of SFC-ELSD $u(a_i)$ are represented schematically. The uncertainty of the atomic weight $u(M_i)$ is almost negligible. At lower i-values, the reproducibility of SFC-ELSD was not so good that the uncertainty $u(s_i)$ is relatively significant as compared to $u(a_i)$ at large peaks around the top of the distribution. The largest contribution of uncertainty is $u(a_i)$ around the top of the distribution and at higher i-values, though that of $u(s_i)$ is getting slightly larger again as the i-values increase. The decrease of SFC-ELSD response at higher i-values is significantly influenced by the certification. Currently, other detectors are investigated which can be connected to SFC, such as a condensation nucleation light scattering detector [47] and a corona charged aerosol detector [48], in order to reduce the uncertainty as compared to that when using the ELSD.

#### 3.4. Short summary

Synthetic polymers always have molecular-weight distributions due to their synthesize process of step-by-step reaction. Certified values of synthetic polymer RMs are usually averaged values of the molecular weight together with the polydispersity index. Here, we reported the NMIJ CRMs of PEG 400, 1000, and 1500, which have the certified values for all fractions of their components as well as weight and number averaged molecular weights. SFC is almost only one technique to separate all components of synthetic polymers and equiweight mixtures made from separated uniform oligomers with various degrees of polymerization were very useful for calibration of the SFC-ELSD. This calibrated SFC-ELSD permitted the certification of all fractions of the entire component in CRMs. Polydispersity index becomes needless because the CRMs have accurate values of the fraction percentages against all the components. This PEG CRMs can be used to calibrate analytical instruments, control quality of measurements, and validate analytical methods for polymers and bio materials.

#### 4. Molecularly uniform polymer

Many artificial products, such as semiconductors and pharmaceuticals, require nanometer-size processing and advanced precise functions. Synthetic polymer manufacturing is reaching the stage of molecular-level controlling. On the field of living macromolecules, such as DNA, the molecular length is uniform to exert precise functions, although their molecular weight is large. On the field of synthetic polymers, narrowing of
molecular weight distribution and precise control of molecular length are expected for modeling the physical properties, however, it is still difficult to synthesize artificial polymers with uniform molecular length and weight.

As described in section 3, SFC is a powerful technique for preparing accurate molecularly uniform polymers from commercial polydispersed materials [49–53]. NMIJ CRMs from 5001-a to 5007-a were certified against all fractions of the components with different degree of polymerization, but the reference materials had polydispersity of the molecular weight. The NMIJ CRM 5011-a PEG 23mer is a unique reference material with no molecular weight distribution, which polymer molecules with a degree of polymerization of 23 were fractionated by preparative SFC. Since the molecular weight of the repeating unit of PEG is 44, which is smaller than 104 for polystyrene or 254 for polycarbonate, the optimization of SFC conditions was very important to separate the molecularly uniform oligomers. The preparative SFC system with microcyclone separator had been developed and enable us to fractionate high-purity molecularly uniform oligomers.

4.1. Material and instrumentation
4.1.1. Material of the RM
The material for the NMIJ CRM 5011-a was a PEG 1000 supplied by Wako Pure Chemical Industries Ltd. (Osaka, Japan), as same as that of NMIJ CRM 5006-a. The fractionated PEG 23mer prepared by SFC was subdivided into 107 bottles for distribution to customers. The bottles were sealed under argon gas and packed into polypropylene bags filled with argon gas, as shown in figure 4-1. The concentration of uniform PEG was accurately measured by TOC analysis in extra-pure water. The amount of PEG in each bottle was approximately 50 µg.

4.1.2. Preparation by SFC
The SFC apparatus was a preparative SFC (JASCO Co., Tokyo, Japan) with two pumps, one column, a Gilson 223 sample changer (Gilson, Inc. WI, USA), and a backpressure regulator BP-2080 (JASCO Co., Tokyo, Japan). The instrumental setup is illustrated in figure 4-2 and the details were described in the reference [54]. The first pump for the supercritical carbon dioxide was PU-2080-CO$_2$ pump (JASCO Co., Tokyo, Japan) with the cooling jacket, and the second one was PU-2080 pump (JASCO Co., Tokyo, Japan) for the modifier solvent. An SFCpak SIL-5 silica gel column (JASCO Co., Tokyo, Japan) with a pore size of 6 nm and a particle size of 5 µm was used; the column had an internal diameter of 4.6 mm and a length of 250 mm. The. ELSD 2000 (Alltech Associates, Deerfield, IL, USA) was used as the in-line detector coupled with SFC. Injected amount was 100 mg ml$^{-1}$ × 5 µl. The microcyclone separator attached to the end point of the eluate reduced contamination and enabled this SFC system to develop efficient fractionation of molecularly uniform oligomers with high purity. Other experimental conditions were same as listed in table 3-1 for separation of PEG 1000. By this SFC fractionation system as shown in figure 4-3, pure PEG 23mer was prepared and able to become a candidate material for the NMIJ CRM.
4.2. Uncertainty analysis

4.2.1. Uncertainty of each component

Uncertainties of the PEG 23mer can be estimated as the same way as described in section 3.2. They consist of three factors: relative peak intensity $u(s_i)$, the sensitivity coefficient of the SFC-ELSD response $u(a_i)$, and the atomic weight from the periodic table $u(M_i)$. The purity and homogeneity of the PEG 23mer were tested by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOFMS) and re-analyzed by SFC-ELSD. The purity of the PEG 23mer were so high that no other oligomers were not detected above the quantitation limit; however, extremely small amount of PEG 22mer was observed under the quantitation limit as fluctuations of base line. The fraction of PEG 22mer was counted into uncertainty analysis. Other fractions were not observed owing to the fact that it was below the threshold for detection limit, that is, it was close to noise level as the relative peak intensity less than 0.01. The uncertainty of PEG 24mer was assumed as a representative value for other PEG components with a rectangular a priori probability distribution [54].

The $u(a_i)$ is the uncertainty of sensitivity coefficient $a_i$ of SFC-ELSD, which can be determined by equiweight mixture of uniform oligomers. The sensitivity of SFC-ELSD has almost the same level for polymers with adjacent degrees of polymerization [45]. In this case, no peaks were quantitatively detectable, and $a_i$ can be set as approximately equal to 1.

The uncertainty $u(M_i)$ was calculated by assuming a rectangular a priori probability distribution [29]. The listed elements in an IUPAC technical report are: C [12.0096; 12.0116], H [1.00784; 1.00811], and O
Based on the chemical structure of PEG, the standard uncertainty $u(M)$ is calculated by equation (4–1).

$$u^2 (M_i) = (2i)^2 u^4 (C) + (4i + 2)^2 u^4 (H) + (i + 1)^2 u^4 (O) u^2 (C) = (0.0020/2)^2/3 = 3 = 3.33 \times 10^{-7} u^2 (H)$$

$$= (0.00027/2)^2/3 = 6.08 \times 10^{-9} u^4 (O) = (0.00074/2)^2/3 = 4.56 \times 10^{-8}$$  (4–1)

### 4.2.2 Combined uncertainty of molecular weight

Since the sensitivity coefficient $a_i$ is equal to 1, the weight fraction of $i$ component $w_i$ can be calculated by equation (4–2).

$$w_i = \frac{s_i}{\sum_j s_j}$$  (4–2)

The uncertainty of the weight fraction $u(w_i)$ can be calculated from same equation (3–8). In the case of $a_i = 1$, equation (3–8) can be simplified as equation (4–3).

$$u^2 (w_i) = \sum_j \left( \frac{\partial w_i}{\partial s_j} \right)^2 u^2 (s_j)$$

$$= \frac{1}{\left( \sum_j s_j \right)^2} \left\{ \left( \sum_j s_j \right) - 2 \left( \sum_j s_j \right) s_i \right\} u^2 (s_i) + s_i^2 \sum_j u^2 (s_j)$$  (4–3)

The contributions of $u(s_i)$ and $u(w_i)$ are summarized in table 4–1. Weight-averaged molecular weight $M_w$ and number-averaged molecular weight $M_n$, and those uncertainties are also calculated from equations (3–5), (3–4), (3–10), and (3–11), respectively.

### 4.3 Reference values

#### 4.3.1 Molecular weight

The purity of the PEG 23mer is as high as 0.998 (kg/kg) and the expanded uncertainty is 0.002 (kg/kg) with the coverage factor $k = 2$. The weight-averaged molecular weight $M_w$ of NMIJ CRM 5011-a is 1031.152, and its standard uncertainty $u(M_w)$ is 1.226. The number-averaged molecular weight $M_n$ is also calculated as 1031.148. The value of $M_w/M_n$ is usually used as an index of the polydispersity of a synthetic polymer. Commonly used commercial synthetic polymers have $M_w/M_n$ values in the range from 1.1 to 2. Monodispersed polymers specially synthesized with the sharpest distribution generally have $M_w/M_n$ values of approximately 1.01. However, NMIJ CRM 5011-a has an $M_w/M_n$ value of 1.000003, which clearly demonstrates that it has a significantly high purity of its degree of polymerization.

#### 4.3.2 Molecular size

During the analyses of common synthetic polymers, the distribution of degree of polymerization often makes it difficult to accurately measure the physical properties of the sample. Molecularly uniform polymers are highly useful in measurements of the exact molecular weight dependence of the polymer properties. NMIJ CRM of the PEG 23mer is also capable of providing information on molecular size. The relationship between molecular weight $M$ and diffusion coefficient $D_0$ for separated uniform PEG oligomers in dilute solutions of deuterium oxide at 30 °C was measured using pulsed-field gradient nuclear magnetic resonance (PFG NMR), as shown in equation (4–4) [59].
Table 4.2 Stokes-Einstein particle size of PEG 23mer in deuterium oxide at 30 °C.

| Molecular weight $M$ | Diffusion coefficient $D_0$/m$^2$ s$^{-1}$ | Stokes-Einstein particle size $d$/nm |
|---------------------|-----------------------------------------------|-------------------------------------|
| 1031.2              | $3.18 \times 10^{-10}$                        | 1.4                                 |

Table 5.1 List of RMs developed by NMIJ for molecular weights of polymers.

| RM No. | Title | Reference Value |
|--------|-------|-----------------|
| 5001-a | Polystyrene 2400 | All Fraction$^a$ |
| 5002-a | Polystyrene 500  | All Fraction$^a$ |
| 5004-a | Polystyrene 1000 | All Fraction$^a$ |
| 5005-a | Poly(ethylene glycol) 400 | All Fraction$^a$ |
| 5006-a | Poly(ethylene glycol) 1000 | All Fraction$^a$ |
| 5007-a | Poly(ethylene glycol) 1500 | All Fraction$^a$ |
| 5008-a | Polystyrene (Polydisperse) | $M_w$, $M_n$, $M_w/M_n$$^b$ |
| 5009-a | Polystyrene 8500  | All Fraction$^a$ |
| 5010-a | Poly(ethylene glycol) Nonylphenyl Ether | All Fraction$^a$ |
| 5011-a | Poly(ethylene glycol) (23mer) | All Fraction$^a$, $R_h$$^c$ |
| 5012-a | Polystyrene (for Light Scattering) | $M_w$, $R_G$, $R_h$$^e$ |

$^a$ Mass and number fractions of each component with different degree of polymerization by SFC
$^b$ Reference values were measured by SEC
$^c$ Mass and number fractions of each component with different degree of polymerization by MALDI TOF-MS
$^d$ Molecular size were measured by PFG NMR
$^e$ Molecular size and weight were measured by light scattering

By using this equation and the viscosity of deuterium oxide at 30 °C, $\eta = 0.973 \times 10^{-3}$ Pa s [60], the particle size $d$ (twice of hydrodynamic radius) was calculated from the Stokes-Einstein relationship shown in equation (2–10). The results are summarized in table 4-2, and the details of the molecular size measurement are described in the reference [59].

4.4. Short summary

A molecularly uniform PEG CRM without a polydispersity in the degree of polymerization was developed by using a preparative SFC system with microcyclone separators. Biomacromolecules usually have narrow molecular-weight distributions, but synthetic polymers generally exhibit a polydispersity in their degree of polymerization which impedes the precise control of their physical properties and material functionalities. NMIJ CRM PEG 23mer was accurately determined the degree of polymerization equal to 23 and is the first CRM that does not have a molecular-weight distribution. The polydispersity index $M_w/M_n$ of the CRM is 1.000003, which is several digits smaller than that of normal RMs of synthetic polymers. Furthermore, this CRM can be used for calibration of instruments, controlling and validation of measurements for synthetic polymers as well as for biomaterials, which have accurate molecular weight and size.

5. Summary

NMIJ has been developing RMs of synthetic polymers, such as polystyrene and poly(ethylene glycol), as listed in table 5-1 [61]. These RMs were produced based on the reference values of weight and number fractions and/or molecular size as well as the averaged molecular weights, for the first case in the RMs. Uniform polymers are highly useful in determining the detector sensitivity and the physical properties of polymers against their molecular weights. SFC is one of the few methods for separating uniform polymers. First, we separated molecularly uniform polymers from commercial polymer samples by means of preparative SFC. Thereafter, we used them for an accurate calibration of the detectors and/or the measurements of molecular size, such as radius gyration and hydrodynamic radius.

Recently, high-performance CRMs have been developed to have multiple reference values such as non-spherical shape, two polydisperse, zeta potential, and so on [62–64]. RMs are required to catch up with advanced industrial materials and those risk assessments. NMIJ CRMs are suitable as a calibration reagent against weights and sizes, instead of biomacromolecules, whose qualities are individual and easily change over time.
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