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Quantitative Mass Spectrometry and Polydisperse Materials: Creation of an Absolute Molecular Mass Distribution Polymer Standard

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ABSTRACT: The certification of an absolute molecular mass distribution polymer Standard Reference Material is described. SRM 2881 is an *n*-octyl-initiated, proton-terminated, narrow polydispersity, low mass, atactic polystyrene. The absolute molecular mass distribution (MMD) was obtained by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). A new Taylor's expansion method for signal-axis intensity calibration of polydisperse materials is described in detail. The certification includes estimates of uncertainties, both type A (random) and type B (systematic), for each oligomer in the sample having a concentration of at least 0.20% of the total of all oligomers on the low mass tail of the MMD and 0.34% on the high mass tail. Type A uncertainty arising from the MALDI sample preparation was found to be the greatest contributor to the overall uncertainty of the measurement.

1. Introduction

Synthetic polymers are rarely created as a single molecular mass but almost always as a distribution of molecular masses by a process that depends in a complex fashion on the reaction conditions. For this reason no two polymerization reactions (or the product of a continuous reactor as a function of time) yield precisely the same molecular mass distribution (MMD) no matter how carefully reaction conditions are controlled. The shape of resultant MMD impacts all areas of polymer science from fundamental physics to consumer product design because almost all physical properties are dependent on it in one way or another. Subtle aspects of the shape of the MMD can have a profound impact on a polymer's properties. In particular, the low and high mass tails of the MMD, which may contain very little material in and of themselves, may have a disproportionate effect on properties. It is for this reason that measuring molecular mass accurately is a central theme in polymer science and has been since its earliest years.¹

In current practice the molecular mass distribution of a polymer is usually measured by some form of chromatography, often size exclusion chromatography (SEC). However, chromatography requires mass calibration to determine the MMD.² Calibration is performed using narrow polydispersity standards. In turn, these standards are created using absolute methods of measurement, that is, methods such as light scattering or osmometry where a polymer is not required for mass calibration. However, all absolute methods return only a single moment of the distribution and not the entire distribution. Standards where the entire distribution is certified represent the next frontier in the history of MMD measurement.

Since its inception matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS) has been touted as a way to measure the MMD of synthetic polymers;³ however, a rigorous procedure to determine the type A (random) and type B (systematic) uncertainties is lacking. Without measures of these uncertainties, determination of the MMD by MALDI–TOF MS cannot be considered quantitative.^{4,5} The

work described here, and in a related reference,⁶ provides a means to create absolute molecular mass distribution standards of any low mass, narrow polydispersity polymer (including proprietary materials). The Standard Reference Material (SRM) created in developing this method, SRM 2881, in addition to calibrating chromatographs can be used as an operational calibrant for signal-intensity of mass spectrometers. Furthermore, it may be used to show proficiency in the application of international documentary standards. Standards in polymer chromatography include ASTM D5296-05 "Standard Test Method for Molecular Weight Averages and Molecular Weight Distribution of Polystyrene by High Performance Size-Exclusion Chromatography," as well as International Organization for Standardization (ISO) 16014-1:2003. Standards in polymer mass spectrometry include ASTM D7034-05 "Standard Test Method for Molecular Mass Averages and Molecular Mass Distribution of Atactic Polystyrene by Matrix Assisted Laser Desorption/Ionization (MALDI) Time of Flight (TOF) Mass Spectrometry (MS)", as well as Deutsches Institut für Normung DIN 55674 and ISO CD 10927.

SRM 2881, and the method developed to certify it, is the culmination of a multiyear effort that required creation of new methods in reproducible MALDI sample preparation,⁷ extensive testing of the robustness and repeatability of the MALDI method through an international interlaboratory comparison,⁸ numerical optimization of mass spectrometer operational parameters,⁹ and unbiased analysis of mass spectra.^{10–12} This paper covers the final uncertainty determination that employs a Taylor's expansion methodology developed specifically for the purpose of calibrating the signal axis of the mass spectrum, a necessary step in the certification of the absolute MMD of any polydisperse sample, synthetic or natural. As with any standards development effort the following question is paramount: How well does the method (MALDI-TOF MS) measure the quantity it is purported to measure (the MMD)? This can be rephrased as: What are the magnitudes of the uncertainties in the measurement, both random and systematic?

2. Gravimetric Blending for Signal Axis Quantitation of Polydisperse Materials

To certify a polymer molecular mass distribution the gravimetric ratios of polymers with different molecular masses are

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compared to the measured mass ratios from MALDI-TOF MS. The difference between the two gives the bias of the MALDI-TOF MS measurement. By using a variety of polymers whose mixtures span a broad molecular mass range, the bias of the MALDI-TOF MS measurement can be determined as a function of molecular mass. Ultimately with this approach, the molecular mass distribution is derived from the kilogram, the SI unit of mass.

There are several works in the literature that use the blending of polymers of different molecular mass to obtain some estimate of how polydispersity affects the predicted versus found properties of the MMD or its moments. Shimada et al.^{13,14} fractionated low molecular mass polystyrenes and polyethylene glycols into single-oligomer samples using preparative supercritical fluid chromatography (SFC). Oligomers with degree of polymerization up to 25 were separated. Then by mixing five or six single-oligomer fractions from a given polymer they were able to obtain a calibration curve to convert the mass spectrum to the MMD. This approach depends on being able to fractionate the polymers into individual oligomers, which is only currently possible for low mass oligomers. Furthermore, as discussed later, it is important to be working under experimental conditions where there is a linear relationship between signal intensity and analyte concentration. This is necessary to make quantitative measurements of the MMD. However, linearity was not demonstrated in the work of Shimada, et al.

Yan et al.¹⁵ looked at mass ratios of two polydimethylsiloxane polymers (average masses 2200 u and 6140 u)¹⁶ by MALDI–TOF MS. After initially optimizing the laser power to give the strongest signal intensity for both the low and high molecular mass polymers, they found that the mass ratios estimated from MALDI–TOF MS were very close to those predicted from gravimetric ratios. Chen, et al.^{17,18} performed a similar experiment using polyethylene glycols with a variety of end groups. They found for molecular masses from 800 u to 3300 u the gravimetric amount of polymer mapped well onto the MALDI–TOF MS measured values.

In a more comprehensive series of studies Li, et al.^{19–21} began by looking at equimolar blends of polystyrenes with molecular masses below 20 000 u in order to optimize the instrument parameters and sample preparation conditions. They did this by varying instrument parameters as well as the matrix:analyte: salt ratios to find where the measured M_n best matched the M_n expected of the equimolar mixtures. Next, using blends of narrow polydispersity polystyrenes with average molecular masses of 5050 u, 7000 u and 11 600 u, and assuming Gaussian distributions for each individual component, they were unable to detect systematic uncertainties in the mixtures' MMDs or their moments within 0.5%.²¹ This was deemed to be a positive indication that the mass spectrum represented the correct MMD for these low mass, low polydispersity materials.

2.1. Mathematical Model for Gravimetric Blending. To estimate the type B uncertainty for an instrumental method a mathematical model based on either physical principles or comprehensive phenomenological observations is required. A quantitative, predictive physical model of the MALDI process and the TOF mass spectrometer seems out of reach at this time. However, a heuristic mathematical model is available and will serve as our starting point. The experimental works described in the previous section each implicitly assumed that there is a region in the parameter space of the instrument and the sample preparation conditions where the signal intensity, S_i , for an oligomer of mass m_i is linearly proportional to n_i , the number of polymer molecules at that oligomer mass. Mathematically this is given by:

$$S_i = kn_i \tag{2.1}$$

where it is assumed that k is a constant independent of m_i and that the measurement's linear response range for the number of oligomers, $0 < n_i < n_{max}$, is the same for all oligomers independent of mass. From the work of Goldschmidt and Guttman,²² studying an 8000 u polystyrene in a MALDI matrix of either retinoic acid or dithranol, there was found a linear relationship between total signal intensity and total analyte concentration over a wide concentration range provided there is a large molar excess of silver salt to cationize the polymer. However, at a high polymer-to-matrix ratio, the curve becomes nonlinear and the signal intensity approaches saturation. This cutoff was shown to be a weak function of molecular mass.

If a measurement is performed in the linear concentration region for all the oligomers of a polymer, the total measured mass multiplied by the total signal is given by:

$$\sum_{i} S_{i} m_{i} = k \sum_{i} n_{i} m_{i}$$
(2.2)

with sums taken over all oligomers *i*. This is valid only as long as the polymer is evenly distributed throughout the matrix on the MALDI target. The right-hand side of the equation [2.2] is just the total mass of the polymer multiplied by *k*. Dividing the sum over all oligomers of equation [2.1] by equation [2.2] yields:

$$\sum_{i} S_{i} m_{i} \Big/ \sum_{i} S_{i} = k \sum_{i} n_{i} m_{i} \Big/ k \sum_{i} n_{i}$$
(2.3)

The right-hand side of the equation is the M_n of the polymer independent of k since the k in numerator and denominator cancel out. The same holds for equations for M_w and all higher moments and is broadly true when in the linear range.

From the work of Montaudo, et al.,²³ McEwen, et al.,²⁴ and Li, et al.^{19–21} it has been clearly demonstrated that if the m_i span too great a mass range the values for k and/or n_{max} must change dramatically otherwise the MALDI–TOF MS would be able to obtain the MMD correctly for broad MMD polymers.

In general, a measurement is performed in the linear range for each oligomer *i*, but each oligomer behaves differently. Then it is expected that:

$$S_i = k_i n_i \tag{2.4}$$

where k_i is now a function of *i* or, more simply, a function of oligomer mass m_i for a fixed set of instrument parameters and sample preparation conditions (e.g., matrix material, matrix: polymer:salt concentration ratio, method of sample deposition onto the MALDI target, etc.). An equation similar to [2.4] has been assumed by Sato, et al.²⁵ for the relationship between fractions obtained from an analytical SEC and signals measured from each fraction by MALDI–TOF MS. Sato's model is simply a coarse grained (in mass) version of the present mathematical model.

Now if we assume that k_i is a *slowly varying* function of *i* (and hence of m_i) then we may make a Taylor's expansion around a mass peak near the center of the MMD, termed M_0 . The center of the mass spectrum is used to ensure that the function is changing as little as possible over the entire width of the MMD although from a purely mathematical viewpoint the selection of M_0 is arbitrary. Then:

$$S_i = k_0 n_i + Q(m_i - M_0)n_i + \text{higher order terms in } n_i \text{ and } m_i$$
(2.5)

Here Q and k_0 are functions of M_0 as well as of all the experimental conditions: the instrument parameters, the sample concentrations, and the sample preparation method. In the experimental procedure described later, once the instrument parameters and experimental preparation methods are optimized,

every attempt was made to keep them constant to ensure experimental reproducibility. Later it will be shown how variation in the machine parameters can affect the variation of Q/k_0 and thus the type B uncertainty.

We shall now explore the implications of the model embodied in eq 2.5 (absent the higher order terms) and see how small linear shifts of the calibration constant Q over limited mass ranges effects quantities derivable from MALDI–TOF MS data. First we shall explore the total signal, the total detected mass, and the mass ratios of mixtures and see how these quantities relate to the true MMD of the polymer.

The total signal (S_T) from the polymer is given by:

$$S_T = \sum_i S_i = k_0 \sum_i n_i + Q(M_n^0 - M_0) \sum_i n_i$$
(2.6)

while the total mass of polymer experimentally detected G_T^{exp} is given by:

$$G_T^{\exp} = \sum_i m_i S_i = k_0 M_n^0 \sum_i n_i + Q M_n^0 (M_w^0 - M_0) \sum_i n_i \quad (2.7)$$

where M_n^0 and M_w^0 are the true number average and mass average molecular masses.²⁶

After extensive algebra,²⁷ the ratio of the total gravimetric G_T^0 and the total mass spectrometric G_T^{exp} amounts of polymers A and B in a carefully prepared gravimetric mixture may be derived:

$$\frac{G_{TA}^{\exp}}{G_{TB}^{\exp}} \left| \frac{G_{TA}^{0}}{G_{TB}^{0}} = \left\{ 1 + \frac{(Q/k_0)(M_{wA}^0 - M_{wB}^0)}{1 + (Q/k_0)(M_{wB}^0 - M_0)} \right\}$$
(2.8)

Equation 2.8 is the key result in calculating the correction factor (Q/k_0) to convert the mass spectrum into the molecular mass distribution. The reader should notice that if M_{wB}^0 is close to M_0 the term $(Q/k_0)(M_{wB}^0 - M_0)$ is small compared to 1 which means the slope largely depends on the difference $(M_{wA}^0 - M_{wB}^0)$ and on the ratio (Q/k_0) . This concept will be used later on in the data analysis to obtain estimates of (Q/k_0) in a self-consistent, iterative manner.

We close this section saying that the gravimetric calibration of the signal axis using chemically identical polymers can avoid the issues pertaining to the uncertainties arising from ablation, ionization, and detection. However, uncertainties in repeatability and consistency in sample preparation as well as in data analysis still affect the gravimetric calibration technique. Our earlier work in sample preparation methods⁷ and data analysis¹⁰ that were employed here were aimed at reducing these effects.

3. Experimental Section

3.1. Samples and Reagents. Three *n*-octyl-initiated $(M_n \text{ of }$ 6200 u, 9200 u, and 12 600 u)²⁸ and one *n*-butyl-initiated (M_n of 9100 u) polystyrene samples were synthesized by Scientific Polymer Products Inc. (Ontario, NY)²⁹ by anionic polymerization of styrene initiated with *n*-octyl lithium or *n*-butyl lithium and terminated with a proton. These four polymers will be referred to as OctylB, OctylA, OctylC, and ButylA, respectively. OctylA is the material that was certified to become SRM 2881. In addition, SRM 2888, a butylinitiated polystyrene of about 6800 u made by Polymer Source Inc. (Dorval, Québec, Canada) was used as were two other polystyrenes with butyl end groups: one with an M_n of 10 000 u from Polymer Standards Service GmbH (Mainz, Germany), and one with an M_n of 8000 u from Scientific Polymer Products Inc. (Ontario, NY). The matrix used in these experiments was all-trans retinoic acid (RA) purchased from Sigma-Aldrich Inc. (Milwaukee, WI) and used as received. The retinoic acid was stored in a freezer to preserve it. Silver trifluoroacetate (AgTFA) was purchased from Sigma-Aldrich and used as received. The solvent used for all the experiments was unstabilized tetrahydrofuran (THF) also from Sigma-Aldrich. The THF was checked before each experiment for presence of peroxides using Quantofix Peroxide 100 (Macherey-Nagel GmbH, Düren, Germany) test strips. No THF with more than 1 mg/L peroxides was used.

Before certification, the within-lot homogeneity and approximate MMD of the candidate polymer, OctylA, were verified by size exclusion chromatography (SEC). Its end-group composition and number-average molecular mass were obtained by nuclear magnetic resonance (NMR). NMR found the expected *n*-octyl and proton end groups, an M_n in close agreement with the value found by SEC, and indications of a trace (<1% by mass) of an aromatic solvent, likely a residual of the synthesis. Since the certification of SRM 2881 depends on comparisons between gravimetric and mass spectrometric compositions of polymer mixtures, all polystyrenes used were vaccuum-dried at 60 °C for 72 h to remove excess solvent.

3.2. MALDI-TOF Mass Spectrometry. All experiments were performed on a Bruker Daltonics (Billerica, MA) Reflex II MALDI-TOF mass spectrometer. The total flight distance is nominally 1.5 m from source to microchannel plate detector. A Laser Science Inc. (Franklin, MA) model LSI-337 nitrogen gas laser operating at 337 nm with an approximately 3 ns pulse width was used. The laser pulse energy was tested both before and after the experiments on each gravimetric composition using a Laser Probe Inc. (Utica, NY) universal radiometer (model RM-3700) with a model RJP-465 energy probe to check for laser intensity drift. A total of 20 events were averaged to compensate for the inherent shot-to-shot variation found in nitrogen gas lasers. All mass spectra in this experiment were obtained in positive-ion reflectron mode. See section 3.4 for further details on instrument parameter selection.

3.3. Mass Axis Calibration Procedure. Mass axis calibration is more easily performed than signal axis calibration. Calibration is usually done with monodisperse biopolymers of known molecular masses. These biopolymers are selected because they typically provide a single major peak whose mass is known accurately; thus, mass axis quantification is quite straightforward. Collecting data with 2 ns time intervals, better than single mass unit accuracy on an instrument with a 1.5 m flight tube in reflectron mode at a mass of about 7000 u is easily achieved.

Calibration of the mass axis was done by combining a single biopolymer with a polymer calibrant. The oligomeric mass of the polymer calibrant, m_j , with *j* repeat units of mass *r*, and the two end groups of total mass m_{end} , is given by:

$$m_i = jr + m_{end} + m_{cation} \tag{3.1}$$

where m_{cation} refers to the mass of the metal cation adducted to the polymer.

Calibration of the mass axis was accomplished through use of the biopolymer mass as follows. The main peak from the biopolymer bovine insulin is assigned to its mass of 5730.61 u. The biopolymer peak will either lie between the masses of two oligomers of the polymer calibrant, or exactly correspond to the mass of an oligomer. If it is at exactly the same mass as one of the oligomers of the polymer calibrant, use eq 3.1 to find the degree of polymerization, *i*, for the oligomer. If the peak of the biopolymer lies between the masses of two oligomers of the polymer calibrant, use eq 3.1 to find m_1 , the mass of the oligomer closest to the mass of biopolymer. Next we find additional calibration points by selecting polymer peaks at intervals between five and 10 repeat units both less than and greater than m_1 and compute their masses from eq 3.1. Generally, a total of four or five calibration masses were selected.

For this work, mass accuracy of only a few mass units was necessary because the mixtures of polydisperse homopolymers used had different end groups with mass differences on the order of tens of mass units. All that was required was calibration close enough to positively identify each oligomer. Furthermore, the main uncertainty in converting from the mass spectrum to the MMD lies in the signal axis quantitation. Achieving greater mass axis accuracy nets no increase in the accuracy of the MMD measurement.

3.4. Instrument Optimization for Signal Axis. The instrument was tuned for quantitation by numerical optimization using a

stochastic gradient approximation method to find a region in instrument parameter space with the least mass bias in the mass range spanned by OctylA, the polymer that would become SRM 2881. This is described in full detail in a previous publication.⁹ The method is described here briefly so it may be referred to it in the discussion of type B uncertainties.

An implicit filtering algorithm was shown to be a viable method to find the best instrument settings while simultaneously minimizing the total number of experiments that need to be performed. This includes considerations of when to halt the iterative optimization process at a point when statistically significant gains can no longer be expected. An algorithm to determine the confidence intervals for each parameter was also given. To do this a mixture of OctylB, ButylA, and OctylC in a mass ratio of 10:70:20 respectively was used. Laser intensity, extraction voltage, extraction delay time, lens voltage, and detector voltage were each varied in order to find their optimal instrument values for measuring the correct molecular mass distribution of the polymer mixture. The ion accelerating voltage and reflectron voltages were held constant. The function was minimized to look for the optimum instrument parameters was J(x)where x is a vector of all the machine parameters:

$$J(x) = \left(\frac{G_{TOB}^{\exp}}{G_{TBA}^{\exp}} - \frac{G_{TOB}^{0}}{G_{TBA}^{0}}\right)^{2} + \left(\frac{G_{TOC}^{\exp}}{G_{TBA}^{\exp}} - \frac{G_{TOC}^{0}}{G_{TBA}^{0}}\right)^{2}$$
(3.2)

where, similar to eq 2.7, G_{TOB}^{exp} is the MALDI computed total mass of OctylB (OB) and G_{TOB}^{0} is the gravimetric mass of OctylB in the mixture and equivalently OC in the subscripts refer to OctylC and BA in the subscripts refer to ButylA. When J(x) = 0 the integrated mass spectral peak ratios equal the gravimetric ratios and the instrument settings are at their optimum values. J(x) was minimized to a small, but nonzero value.⁹ Later, in the discussion of uncertainty in section 5.2, it will be noted that the derivatives of J(x) at the function minimum play a critical role in determining type B uncertainties for the instrument parameters.

3.5. MALDI Sample Preparation. All samples were deposited onto the MALDI sample target by electrospray to increase signal repeatability and reduce "hot spots" following the work of Owens, et al.^{30,31} Electrospray sample preparation was found to be essential for experimental stability and reproducibility. The samples were electrosprayed using a voltage of 5 kV and a flow rate of 5 μ L/ min. The target was placed (4.0 ± 0.2) cm from the tip of a 100 μ L glass syringe with a blunt stainless steel needle (o.d. 0.5 mm, i.d. 0.2 mm). Electrosprayed films on the order of several micrometers thick were made to avoid depletion of the target during laser ablation. The target was divided in half-by placing Teflon film across one side. The right half of the target was electrosprayed with RA and AgTFA only (no polymer) to be used in the creation of background spectra. Reversing the film, the left half of the target was electrosprayed with polystyrene mixture, RA, and AgTFA in a 6:50:6 mass ratio. Each mass spectrum was the sum of 500 laser shots. Five repeats each on both the "blank" (no polymer) and the sample side of the target and was taken from randomized positions on the target.

Concentration of total polymer to retinoic was held about at the three-quarter point in the range of linearity found by Goldschmidt and Guttman.²² This range was rechecked in preliminary experiments. In addition, the silver trifluoroacetate concentration from the above recipe was found to work well for the linear range as long as there was a molar excess of silver. This was checked by making measurements at concentrations of silver trifluoroacetate ranging from 25% to 200% of that value using the 10:70:20 of OctylB:ButylA:OctylC gravimetric mixture employed in the instrument optimization experiments⁹ and described in section 3.4. Within the noise limits of the repeatability of the experiment there was no change of ratio of OctylB/ButylA or OctylC/ButylA either as a function of silver trifluoroacetate concentration or as a function of the time the experiment was done.

3.6. Data Analysis Methods. Peak picking and peak integration were performed using the *MassSpectator* computer $code^{10-12}$



Figure 1. Linearity of mass spectrometric total signal intensity, S_T , vs polymer concentration in MALDI target. Example graph for ButylA ($M_n \sim 9100$ u) with inset showing a typical mass spectrum.

developed at NIST for polymer standards production by mass spectrometry. MassSpectator makes no assumptions about peak shape and requires no smoothing or preprocessing of the data (a process which has been shown to distort peak area)¹⁰ but does require a background spectrum for statistical purposes. The algorithm is based on a time-series segmentation routine that reduces the data set to groups of three strategic points where each group defines the beginning, center, and ending of each peak located. The peak areas are found from the strategic points using a commonplace polygonal area calculation routine. Peaks with statistically insignificant height or area are then discarded. The determination to discard is made by segmenting the corresponding "blank" background spectrum. This analyte-free spectrum by definition has no polymer peaks in it thus giving a good measure of noise in the analyte mass spectrum. Using spreadsheet software the data were then separated into series by the polymer end group and the appropriate numbers such as total mass, total area, M_n , M_w , and M_z of each series were computed.

4. Experimental Results

First linearity with respect to polymer concentration was demonstrated. That is, for a fixed mass of matrix as the amount of polymer increases so should the mass spectral signal from the polymer. This was performed for two polystyrenes: OctylA and ButylA. A representative example of those data, with a typical spectrum, is shown in Figure 1.

Linearity in the concentration versus signal intensity relationship for single analytes is necessary but not sufficient to demonstrate that the proper MMD was necessarily derived from the mass spectrum. The next step is to study mixtures of two polymers-one of which is held at a constant polymer to matrix ratio while the other is varied. In this way the mixture can be used to control the polydispersity. Starting with polymers having two different end groups but with similar molecular mass distributions, specifically OctylA and ButylA each with an average mass of approximate 9000 u, OctylA was held at a fixed concentration of 25 mg polymer/ 1 g matrix while ButylA was varied from 0 mg of polymer/1 g of matrix to 50 mg of polymer/1 g of matrix. This result is shown in Figure 2 where, because the polymers are virtually identical except for end group, there is a direct correspondence between gravimetric ratio and MALDI signal intensity ratio. To check this in more detail at a fixed total polymer-to-matrix mass ratio and the octyl-tobutyl ratio was varied. The slope of the ratios of the two sets of data is again close to one. This demonstrated that the alkyl end group does not affect the mass spectrometry in any measurable way. In cases using polystyrenes with octyl end groups with



Figure 2. OctylA and ButylA mass spectrometric total signal intensity ratio vs gravimetric concentration ratio in MALDI target. There is no measurable bias indicating that the *n*-butyl and *n*-octyl end groups have no effect on the mass spectrometry. Inset: Typical reduced mass spectrum showing relative ion intensity. (OctylA as filled bars, ButylA as open bars.)



Figure 3. OctylB and ButylA mass spectrometric total signal intensity ratio vs gravimetric concentration ratio in MALDI target. OctylB is overcounted with respect to ButylA. The dashed line indicates no measurable bias. Inset: Typical reduced mass spectrum showing relative ion intensity. (OctylB as filled bars, ButylA as open bars.)

average masses of 6000 u and 12 000 u, the polystyrene with the butyl end group was held fixed the polystyrene with the octyl end group was varied. This is shown in Figures 3 and 4. By repeating this method with various combinations of octyl and butyl initiated polymers listed in section 3.1, the value of the slopes of the gravimetric ratio versus the MALDI–TOF MS ratio of masses was determined. In all mixtures at least one of the polymers had a molecular mass near 9000 u, the value chosen for M_0 .

From eq 2.8, the slopes $(G_{TA}^{exp}/G_{TB}^{exp})/(G_{TA}^0/G_{TB}^0)$ are expected to be a function of molecular mass difference of the polymers, $(M_{wA}^0 - M_{wB}^0)$. This dependence of the experimental slopes on $(M_{wA}^0 - M_{wB}^0)$ is shown in Figure 5. We have plotted the slopes versus the difference in the experimental values, M_{wA}^{exp} and M_{wB}^{exp} , instead of the difference in the true values, M_{wA}^{0} and M_{wB}^{0} , demanded by equation [2.8]. This was the first step in a iterative procedure where for the values of M_w^0 in eq 2.8 we took $Q/k_0 =$ 0 initially in the term $1 + (Q/k_0)(M_{wi}^0 - M_0)$ and $1 + (Q/k_0)(M_{vi}^0 - M_0)$ Information). With the computed value of Q/k_0 from Figure 5 we got a new value for the terms $1 + (Q/k_0)(M_{wB}^0 - M_0)$ and



Figure 4. OctylC and ButylA mass spectrometric total signal intensity ratio vs gravimetric concentration ratio in MALDI target. OctylC is undercounted with respect to ButylA. The dashed line indicates no measurable bias. Inset: Typical reduced mass spectrum showing relative ion intensity. (OctylC as filled bars, ButylA as open bars.)



Figure 5. Estimation of Q/k_0 for all data sets.

for M_{wA}^0 and M_{wB}^0 . A new Q/k_0 was then computed and this was carried on until an unchanging value of Q/k_0 was found. This took about five iterations. Over those iterations the value of the slope went from 6.37×10^{-5} to 6.47×10^{-5} , which was the final value for Q/k_0 with an estimated type A uncertainty of 40%.

5. Discussion of Uncertainties

5.1. Type A Uncertainties in the MALDI–TOF Mass Spectrometry Measurement. There are two sources of type A uncertainty that affect the MMD. The uncertainty from Q/k_0 , the mass spectrum to MMD conversion factor, that was estimated in the previous section, and the uncertainty of the intensity of each individual oligomer in the mass spectrum:

$$\beta_i^0 = S_i / \sum_i S_i \tag{5.1}$$

where β_i^0 is the fraction of the signal for the *i*th oligomer with mass, m_i . In the type A uncertainty analysis, averages were calculated for each individual β_i^0 over the approximately 500 spectra measured in this study where OctylA (the polymer that became SRM 2881) was one of the two polymers mixed, or when OctylA was measured alone. It is upon these measurements that the type A uncertainty for β_i^0 was based.

Table 1. Estimated Type A and Type B Uncertainties for Q/k_0

Type A uncertainty	all sources	$\approx 40\%$
Type B Uncertainty		
instrumentation related uncertainties		
	acceleration voltage	< 0.5%
	detector voltage	0.245%
	laser attenuation	0.15%
	delay time	<0.5%
	extraction voltage	0.029%
	lens voltage	0.014%
sample preparation uncertainties		
	weighing uncertainties	$\approx 3\%$
	syringe uncertainties	$\approx 4\%$

An analysis of variance (ANOVA)³² was done on each oligomeric β_i^0 assuming they were independent of each other. It found that the sample-to-sample variance for the fraction of a given oligomeric mass is much greater than the within-sample variance where "sample" refers here to a specific gravimetric mixture and the MALDI target prepared from it. Thus, the *p*-values run much higher than those generally expected from the F distribution for $\alpha = 0.05$. (Recall that the *p*-value is the probability that the variation between conditions may have occurred by chance, so spectra with smaller *p*-values vary more significantly.) This high p-value (often as high as 10 when the p-value predicted for $\alpha = 0.05$ is typically closer to 2) is due to the large number of the degrees of freedom in the within-sample variance owing to the fact that the data are not truly independent run to run.³³

5.2. Type B Uncertainties in the MALDI–TOF Mass Spectrometry Measurement. In section 3.4, an approach for the selection of optimal instrument parameters that yield a mass spectrum which best replicates the molecular mass distribution of a synthetic polymer was described. From this method an algorithm to determine the confidence intervals for each parameter was presented.⁹ From these confidence intervals the type B uncertainties for the instrument parameters can be determined.

We can write J(x) from eq 3.2 in terms of the calibration equation for the signal axis:

$$J(x) = \left(\frac{G_{\text{TOB}}^0}{G_{\text{TBA}}^0}\right)^2 \left(\left[\frac{G_{\text{TOB}}^{\text{exp}}}{G_{\text{TBA}}^{\text{exp}}} / \frac{G_{\text{TOB}}^0}{G_{\text{TBA}}^0}\right] - 1 \right)^2 + \left(\frac{G_{\text{TOC}}^0}{G_{\text{TBA}}^0}\right)^2 \left(\left[\frac{G_{\text{TOC}}^{\text{exp}}}{G_{\text{TBA}}^{\text{exp}}} / \frac{G_{\text{TOC}}^0}{G_{\text{TBA}}^0}\right] - 1 \right)^2 (5.2)$$

Substituting eq 2.8 into eq 5.2 yields:

$$J(x) = (Q/k_0)^2 \left[\left(\frac{G_{\text{TOB}}^0}{G_{\text{TBA}}^0} \right)^2 \left(\frac{(M_{wOB}^0 - M_{wBA}^0)}{1 + (Q/k_0)(M_{wBA}^0 - M_0)} \right)^2 + \left(\frac{G_{\text{TOC}}^0}{G_{\text{TDA}}^0} \right)^2 \left(\frac{(M_{wOC}^0 - M_{wBA}^0)}{1 + (Q/k_0)(M_{wBA}^0 - M_0)} \right)^2 \right]^2 (5.3)$$

From this we obtain partial derivatives of Q/k_0 as:

$$\frac{\delta \ln(J(x))}{\delta p} \approx 2 \frac{\delta \ln(Q/k_0)}{\delta p} \tag{5.4}$$

where *p* is any instrument parameter. Notice as before we have taken the term $1+(Q/k_0)(M_{wBA}^0 - M_0)$ to be very near 1 and second order in all corrections to the derivative. All other terms are independent of Q/k_0 .

In Table 1 the effect on the type B uncertainty of the MALDI–TOF MS calibration parameter Q/k_0 for each of adjustable instrument parameter is given. Table 1 also shows that the type A uncertainty dominates all type B uncertainty. The acceleration voltage uncertainty was taken as 0.5% based on the fluctuations in the power supply voltage. The delay time

for ion extraction is a discrete variable whose uncertainty has been discussed previously.⁹ Its value was taken to be 0.5% as well.

It should be pointed out that the numerical optimization method along with the Taylor expansion calibration model allows us to estimate the type B uncertainty and is much simpler that what had to be done on previous generation NIST polymer molecular mass standard reference materials created using light scattering³⁴ or membrane osmometry.³⁵

5.3. Type A and Type B Uncertainties in Sample Preparation. This section considers the estimated uncertainty involved in sample preparation, particularly the gravimetric aspects. Specifically, this involves the masses of each of the polymers in solution, the volumes of solution used to make up the concentration ratio, and finally the repeatability of the MALDI made from two different solutions of the apparently same concentration ratio.

First consider the mass and volume effects in preparing the polymer solutions. Initial solutions were made from polymer samples weighing from 5 to 6 mg precise to 0.1 mg and added to 4 mL of THF. The weighing, being precise to 0.1 mg, gives an estimate of the uncertainty of $\sqrt{2} \times (0.1/5.0) \approx 3\%$. Once the stock solutions were prepared, volumes were combined to create various polymer mixtures. Although pipettes were initially used to measure out solutions, these were found to poorly reproduce volumes in the μ L range. Instead, glass syringes of 100 μ L total volume were used and found to reproduce the volumes well. The reproducibility of the 100 μ L syringe was determined to be about 3%. Repeatability of a 5 mL glass syringe for a 4 mL volume used to make up the initial solutions of polymers was determined to be about 4%. To avoid contamination and calibration problems separate syringes were used for each polymer throughout the experiments.

The repeatability of the electrospray part of the sample preparation and repeatability of the ablation was difficult to determine. Once a target was sprayed, it as placed in the instrument and 500 randomly placed laser shots for the sample side of the target were taken followed by 500 randomly placed laser shots of the "blank" (no polymer) side of the target. This was repeated five times. Mass peaks were then integrated and separated into their appropriated groups by end group mass. Next the ratios from these integrated peak sums taken to give the integrated ratios between the two polymers in the sample. These integrated ratios are displayed on each graph in Figures 2, 3, and 4. Uncertainty in the results can be traced to the repeatability of the MALDI-TOF MS measurement. Even when going to great lengths in sample preparation and by using a large number of laser shots per spectrum, the MALDI process itself proved to be highly variable.

6. Final Certified Values

The fraction β_i^0 for each oligomer is defined as:

$$\beta_i^0 = n_i / \sum_i n_i \tag{6.1}$$

The final certified values for β_i^0 (with associated uncertainties) for SRM 2881 are given in Table 2 and shown in Figure 6.

The type B uncertainty is small compared to the two forms of type A uncertainty, gravimetric and instrumental. Why is the type B uncertainty small compared to the type A? In earlier SRM work using light scattering to determine the M_w ,³⁴ or osmometry to determine the M_n ,³⁵ experiments could be designed such that the type A uncertainty was comparable or somewhat smaller than the type B uncertainty. Here this is not the case and is likely due to lack of control over some parts of the experiment, in particular, the sample preparation. The sample preparation was done with a spraying technique where it was



Figure 6. Final oligomer concentrations for Standard Reference Material 2881 with total (type A and type B) uncertainty.

Table 2. Number Fraction β_i^0 for Each Oligomer in the Molecular Mass Distribution with Total Uncertainty for Standard Reference Material 2881

repeat	oligomer	number fraction	uncertainty	cumulative
number, <i>i</i>	mass, u	of MMD, β_i^0	fraction $(k=2)$	of MMD
63	6675.80	0.0020	0.0003	1.0
64	6779.95	0.0025	0.0005	1.2
65	6884.10	0.0033	0.0006	1.6
66	6988.26	0.0041	0.0007	2.0
67	7092.41	0.0053	0.0008	2.5
68	7196.56	0.0067	0.0010	3.2
69	7300.71	0.0084	0.0011	4.0
70	7404.86	0.0105	0.0013	5.1
71	7509.01	0.0129	0.0014	6.4
72	7613.17	0.0157	0.0016	7.9
73	7717.32	0.0186	0.0017	9.8
74	7821.47	0.0217	0.0017	12.0
75	7925.62	0.0254	0.0017	14.5
76	8029.77	0.0287	0.0017	17.4
77	8133.93	0.0321	0.0017	20.6
78	8238.08	0.0353	0.0016	24.1
79	8342.23	0.0383	0.0015	28.0
80	8446.38	0.0410	0.0011	32.1
81	8550.53	0.0431	0.0010	36.4
82	8654.69	0.0449	0.0007	40.8
83	8758.84	0.0456	0.0007	45.4
84	8862.99	0.0459	0.0006	50.0
85	8967.14	0.0457	0.0008	54.6
86	9071.29	0.0450	0.0010	59.1
87	9175.44	0.0437	0.0012	63.4
88	9279.60	0.0416	0.0014	67.6
89	9383.75	0.0397	0.0016	71.6
90	9487.90	0.0368	0.0017	75.2
91	9592.05	0.0339	0.0017	78.6
92	9696.20	0.0310	0.0017	81.7
93	9800.36	0.0279	0.0016	84.5
94	9904.51	0.0249	0.0016	87.0
95	10008.66	0.0219	0.0015	89.2
96	10112.81	0.0191	0.0014	91.1
97	10216.96	0.0164	0.0013	92.7
98	10321.12	0.0140	0.0011	94.1
99	10425.27	0.0118	0.0010	95.3
100	10529.42	0.0098	0.0009	96.3
101	10633.57	0.0081	0.0007	97.1
102	10737.72	0.0066	0.0006	97.8
103	10841.87	0.0053	0.0006	98.3
104	10946.03	0.0042	0.0005	98.7
105	11050.18	0.0034	0.0005	99.1

expected that that the rapid droplet evaporation will yield homogeneity in the sample. However, the electrospray may be affected by a variety of things that were not controlled including humidity in room during sample preparation and observed subtle changes in the electrospray cone character leading to uncontrolled droplet size.³⁶

There is a second source of type A uncertainty. There is the source from the repeatability of the fundamental data, i.e., in β_i^0 , as discussed above. However, there is also a source of uncertainty in the correction term for Q/k_0 . Table C1 (Supporting Information)³⁷ gives the values of the mean value of the experimental β_i^0 , the experimental fraction of the signal in peak of mass m_i , with its statistical standard deviation (the contribution to the type A uncertainty) for the cumulative distribution from approximately 1% to 99%. (Low mass, uncertified oligomers constitute the mass up to 1%, and high mass, uncertified oligomers constitute the mass above 99%). Recall that Table 1 offers a listing of the contributions to the uncertainty from all sources. It is unclear that these are independent uncertainties but having no other knowledge it must be assumed they are independent and thus they were combined in the usual way by using the square root of the sum of squares.³⁸ When doing so, the type B uncertainties have a negligible contribution to the overall uncertainty.

From the data provided in Table 2, the calculated moments of the distribution are as follows: M_n 8923 u, M_w 9006 u, and M_z 9087 u. These numbers give a very small polydispersity. This is due to the material constituting the lowest 1% and the highest 0.9% of the distribution not being certified. The highest fraction would, of course, increase M_w and M_z and increase the calculated polydispersity.

7. Discussion of Method

We conclude with a few final words about the Taylor's expansion mathematical model. The impetus for the model came from earlier published data that suggested that k_i appeared qualitatively to be a slowly varying function of mass. A Taylor's expansion that is linear in mass creates a useful quantitative computational bridge from mass spectrum to molecular mass distribution. The choice of variable for the Taylor's expansion is largely arbitrary and should, in the best circumstances, be chosen as the variable with the longest range of linearity and the smallest slope across the molecular mass distribution to be certified. Thus, if some other variable (e.g., time in time-of-flight mass separation) were shown to be superior then an expansion in that variable would provide a more accurate fitting function. Work in our laboratory on this topic continues.

Our testing of the mathematical model is far from exact but appears to be robust provided a few conditions are met. Most importantly, for a given set of instrument parameters, and for a sample preparation with some modicum of homogeneity and repeatability, a fairly wide region of measurement parameter space where the polymer-to-matrix mass ratio results in a proportional number of polymer ions arriving at the detector must exist. This proportionality constant must be a slowing varying function of mass and oligomer concentration so that it can be linearly approximated. Additionally, this region of concentration space must have a high enough polymer concentration for us to obtain a good signal-to-noise ratio for statistically meaningful data analysis. Improvements to the method could be made in two areas: (1) enhanced reproducibility in the mass spectra through better sample preparation methods, or a quantitative predictive model of the polymer MALDI process on which to base the uncertainty analysis, and (2) an estimate of the functional dependence of Q on the various machine parameters. A quantitative model exists for the MALDI process for small molecule analytes^{39,40} that may well be extendable to macromolecules. The second improvement can only come from a mathematical description of total instrument behavior, from ablation through mass separation and detection to signal digitization. This is currently beyond reach for MALDI-TOF mass spectrometry but is an active area of research worldwide.

8. Conclusion

A new Taylor's expansion approach to the determine the absolute molecular mass distribution of polydisperse samples when individual molecular components are not available was described. In the case presented here an *n*-octyl initiated, proton terminated, narrow polydispersity polystyrene was certified as Standard Reference Material 2881. Certification involved determination of type A (random) and type B (systematic) uncertainties for the concentration of each oligomer in the sample. Type A uncertainty, likely arising from unknown variables in the sample preparation, was greater than the type B uncertainty caused by the instrumentation or the data analysis. Research into better understanding and control of the MALDI sample preparation conditions would yield the biggest payoff in decreasing the overall uncertainty of the measurement.

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Supporting Information Available: Appendix A, text giving a full mathematical development of the Taylor's expansion method, Appendix B, text giving a more detailed description of the type A statistical analysis performed on the data, and Appendix C, consisting of a table of oligomer concentration at each mass of oligomer, mentioned in the main body of the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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- (27) A full mathematical development can be found in Appendix A of the online Supporting Information.
- Values as measured by the vendor using size-exclusion chromatography (28)in tetrahydrofuran with multiple-angle laser light scattering detection.
- (29) Certain commercial materials and equipment are identified in this paper in order to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply necessarily that the item is the best available for the purpose.
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Appendix A Detailed Mathematical Development of the Taylor's Expansion Method

Picking up where the main text leaves off in section 2.1 with equation [2.7]:

$$G_T^{\exp} = \sum_i m_i S_i = k_0 M_n^0 \sum_i n_i + Q M_n^0 (M_w^0 - M_0) \sum_i n_i$$
[2.7]

We define the common molecular mass moments of a polymer distribution:

$$M_{n}^{0} = \sum_{i} m_{i} n_{i} / \sum_{i} n_{i}$$
 [A.1]

$$M_{w}^{0} = \sum_{i} m_{i}^{2} n_{i} / \sum_{i} m_{i} n_{i}$$
[A.2]

$$PD_n^0 = M_w / M_n$$
 [A.3]

$$M_{z}^{0} = \sum_{i} m_{i}^{3} n_{i} / \sum_{i} m_{i}^{2} n_{i}$$
 [A.4]

$$PD_w^0 = M_z / M_w$$
 [A.5]

The experimental moments from MALDI-TOF MS are defined as M_n^{exp} , M_w^{exp} and M_z^{exp} , while the true values are given as M_n^0 , M_w^0 and M_z^0 . PD_n defines the polydispersity index which is a measure of the breadth of the polymer distribution. When PD_n is equal to one (i.e., in statistical terms the variance of MMD is zero), all of the polymer molecules in a sample are of the same molecular mass and the polymer is referred to as monodisperse.

By multiplying equations [A.1] and [A.2] together we have:

$$M_{w}^{0}M_{n}^{0} = \sum_{i} m_{i}^{2}n_{i} / \sum_{i} n_{i}$$
 [A.6]

Taking the ratio of equations [2.6] and [2.7], we obtain:

$$M_n^{\exp} = \sum m_i S_i / \sum S_i$$
 [A.7]

Combining equations [2.6] and [A.7] gives:

$$M_n^{\exp} = M_n^0 \left\{ \frac{(1 + (Q/k_0)(M_w^0 - M_0))}{(1 + (Q/k_0)(M_n^0 - M_0))} \right\}$$
[A.8]

For use later in this paper and by the same algebra:

$$M_{w}^{\exp} = \sum m_{i}^{2} S_{i} / \sum m_{i} S_{i}$$
 [A.9]

with the result that:

$$M_{w}^{\exp} = M_{w}^{o} \left\{ \frac{(1 + (Q/k_{0})(M_{z}^{0} - M_{0})))}{(1 + (Q/k_{0})(M_{w}^{0} - M_{0}))} \right\}$$
[A.10]

All higher moments may be obtained in a similar way and have a similar form.

Equation [A.10] gives by simple division:

$$M_{w}^{0} = M_{w}^{\exp} \left\{ \frac{(1 + (Q/k_{0})(M_{w}^{0} - M_{0})))}{(1 + (Q/k_{0})(M_{z}^{0} - M_{0}))} \right\}$$
[A.11]

which yields:

$$M_{w}^{0} = M_{w}^{\exp} \left\{ 1 - \frac{(Q/k_{0})M_{w}^{0}(PD_{w} - 1)}{1 + (Q/k_{0})(M_{z}^{0} - M_{0})} \right\}$$
[A.12]

Equation [A.12] states that the deviation of the mass moment measured by MALDI-TOF MS from the true mass moment is a function of the polydispersity (PD) (arising from that moment) divided by a correction term arising from how far that moment is from the mass M_0 around which the Taylor's expansion to obtain Q and k_0 is centered. In equation [A.12] the reader should notice if that M_z^0 is close to M_0 the term in $(Q/k_0)(M_z^0 - M_0)$ is small compared to 1

and the result depends on the polydispersity of the polymer. This result was first demonstrated experimentally by Montaudo, *et al.*¹ and later confirmed experimentally by Li, *et al.*^{2,3}

Since we shall later be gravimetrically mixing polymers to obtain estimates of Q/k_0 , let us look at the equations relating to these mixtures. Equation [2.7], states that the MALDI-TOF MS measured total mass, G_T^{exp} , is proportional to the true mass, G_T^0 :

$$G_T^0 = M_n \sum n_i$$
 [A.13]

$$G_T^{\text{exp}} = k_0 G_T^0 \left\{ 1 + Q / k_0 (M_w^0 - M_0) \right\}$$
[A.14]

Consider now a mixture of the chemically identical polymers with end groups having different masses, or two different molecular mass polymers having distributions that are well separated such that each oligomer in the mass spectrum can be assigned to a specific polymer in the mixture. Call them polymer A and polymer B. Then the measured ratio of the masses of each is:

$$\frac{G_{TA}^{\exp}}{G_{TB}^{\exp}} = \frac{k_{0A}G_{TA}^{0}}{k_{0B}G_{TB}^{0}} \left\{ \frac{1 + (Q_A/k_{0A})(M_{wA}^{0} - M_0)}{1 + (Q_B/k_{0B})(M_{wB}^{0} - M_0)} \right\}$$
[A.15]

Notice the expansions for both polymer distributions A and B are performed around the same M_0 . Note that Q_A, Q_B, k_{0A} and k_{0B} are all functions of M_0 .

Two things are needed for the ratios of the true masses in equation [A.15] to be obtained from the measured ratio of the MALDI-TOF MS. First, the ratio k_{0A}/k_{0B} needs to be determined. This ratio is equal to 1 if polymers A and B are chemically identical only having different average molecular masses because the expansion point for the Taylor's expansion, M₀, is taken as being the same for both polymers. If the repeat groups of polymers A and B are identical but the end group is different then it is possible the ionization probability may be different. In this paper we have chosen end groups with different, but easily distinguishable, masses (58 u and 114 u) and with the same, minimal polarity (*n*-butyl and *n*-octyl) so that sample

preparation, ablation, and ionization should be controlled by the polystyrene repeat unit chemistry because no silver charging on the butyl or octyl end group is expected. Verification of this will be demonstrated is section 5.1. It should also be noted that for polystyrenes with end groups with very different polarities (e.g., -OH and -H) the effect of the end group on ablation and charging has been shown to be minimal.⁴ Furthermore, any corrections would be much less than [number of repeat units]⁻¹. Thus we may assume the ratio of $k_{0A}/k_{0B} = 1$. The same argument applies to Q: that it is independent of the end group, and only depends on M_0 and the instrument parameters and sample preparation conditions.

Thus we have from equation [A.15]:

$$\frac{G_{TA}^{exp}}{G_{TB}^{exp}} = \frac{G_{TA}^{0}}{G_{TB}^{0}} \left\{ \frac{1 + (Q / k_0)(M_{wA}^{0} - M_0)}{1 + (Q / k_0)(M_{wB}^{0} - M_0)} \right\}$$
[A.16]

Simple algebra leads us to:

$$\frac{G_{TA}^{exp}}{G_{TB}^{exp}} = \frac{G_{TA}^{0}}{G_{TB}^{0}} \left\{ 1 + \frac{(Q / k_0)(M_{wA}^{0} - M_{wB}^{0})}{1 + (Q / k_0)(M_{wB}^{0} - M_0)} \right\}$$
[A.17]

The slope, $\frac{G_{TA}^{\circ,\circ}}{G_{TB}^{0}}$, is central to calculating the correction term and is given by:

$$\frac{G_{TA}^{exp}}{G_{TB}^{exp}} = \left\{ 1 + \frac{(Q / k_0)(M_{wA}^0 - M_{wB}^0)}{1 + (Q / k_0)(M_{wB}^0 - M_0)} \right\}$$
[2.8]

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- (4)

Appendix B Details on the Type A Statistical Uncertainty in the Data

The non-independence of spectra from run to run is difficult to take into account statistically. For example, the fractions in the MMD all must sum to one for any one spectrum as well as for the averages of all spectra. Further, there are changes between each concentration data set due to changes in the MALDI target preparation method—although every attempt to control this and make it consistent. This was not due to the concentration change in the ratio of one polymer to another, or to the total polymer, since no drift in the moments of the distribution as a function of concentration was observed. It should be recalled that each concentration set to obtain one slope Q/k_0 is a result of twenty different sample preparations and sprayings of the sample onto a target. Thus, the collection of the data for one slope required two days of intensive work on the mass spectrometer. The approximately twenty-five Q/k_0 slopes were collected over a span of about 12 months. Stable control of sample preparation and of instrument parameters was a challenge for this many experiments and over this time span. Some data were rejected because it was too difficult to determine the slope from the noise in the data. As stated before, the data were checked for concentration dependence of the moments M_n and M_w of the MMD. The concentration was randomized over the two day adapt collection period with 2 or 3 repeats to check for consistency. The data was analyzed as a function of time they were run in the day and/or the order of running. None of these showed any significant trend in the moments. This suggested the MMD was not changing significantly as a function of the overall concentration of polymer in the matrix or what order it was run.

Two other methods were used to check the reproducibility of the data. First, linearity of concentration versus signal intensity was found for all data sets. Second, all data sets had a few (at least two) sets of data points in which the concentration ratios of the two polymers were repeated. In all these the noise within one repeat of five data sets was larger than the repeatability between the solutions made up the same—indicating the repeatability of our making of the solutions was excellent. This showed that the sample preparation method used within one set of experiments was good.

The data was analyzed in two additional ways to check for self-consistency. In the first, a single peak was given a fixed value (the average value for that peak of all spectra) and the rest of the peaks the spectrum under consideration were scaled accordingly. This did not change the overall distribution of values of the *p*-value computed indicating that spectrum-to-spectrum

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differences were a leading cause of type A uncertainty. In the second, four adjacent data points in a spectrum were summed to one reducing the number of points per peak from 64 points to 16 points. This course graining of the spectrum made the values of the *p*-values computed much higher indicating that within-spectrum noise was not the major source of type A uncertainty.

					total type A	standard		
	. P	number	standard	% correction	and type B	uncertainty	% overall	uncertainty
ropost	oligomer	fraction,	deviation (%)	for β_i^0 from	uncertainty in	on mean of	uncertainty of	of fraction,
iepear	u u	$oldsymbol{eta}_i^0$	for β_i^0	Q/k_0	β_i^0 [note 1]	β_i^0 [note 2]	β_i^0 [note 3]	$oldsymbol{eta}_i^0$
63	6675.80	0.0020	40.25 %	-18.60 %	40.93 %	13.64 %	27.29 %	0.0005
64	6779.95	0.0025	43.42 %	-17.77 %	44.00 %	14.67 %	29.33 %	0.0007
65	6884.10	0.0031	32.80 %	-16.94 %	33.49 %	11.16 %	22.33 %	0.0007
66	6988.26	0.0041	32.21 %	-16.11 %	32.85 %	10.95 %	21.90 %	0.0009
67	7092.41	0.0048	30.62 %	-15.27 %	31.23 %	10.41 %	20.82 %	0.0010
68	7196.56	0.0063	31.14 %	-14.44 %	31.67 %	10.56 %	21.11 %	0.0013
69	7300.71	0.0078	25.63 %	-13.61 %	26.20 %	8.73 %	17.47 %	0.0014
70	7404.86	0.0090	23.52 %	-12.77 %	24.07 %	8.02 %	16.05 %	0.0014
71	7509.01	0.0116	19.95 %	-11.94 %	20.51 %	6.84 %	13.67 %	0.0016
72	7613.17	0.0135	19.99 %	-11.11 %	20.48 %	6.83 %	13.65 %	0.0018
73	7717.32	0.0163	16.52 %	-10.27 %	17.02 %	5.67 %	11.35 %	0.0019
74	7821.47	0.0191	14.39 %	-9.44 %	14.88 %	4.96 %	9.92 %	0.0019
75	7925.62	0.0223	11.30 %	-8.61 %	11.81 %	3.94 %	7.88 %	0.0018
76	8029.77	0.0262	10.26 %	-7.77 %	10.72 %	3.57 %	7.15 %	0.0019
77	8133.93	0.0296	8.34 %	-6.94 %	8.79 %	2.93 %	5.86 %	0.0017
78	8238.08	0.0322	7.52 %	-6.11 %	7.91 %	2.64 %	5.27 %	0.0017
79	8342.23	0.0353	4.95 %	-5.28 %	5.38 %	1.79 %	3.59 %	0.0013
80	8446.38	0.0385	3.92 %	-4.44 %	4.30 %	1.43 %	2.87 %	0.0011
81	8550.53	0.0404	4.85 %	-3.61 %	5.06 %	1.69 %	3.37 %	0.0014
82	8654.69	0.0423	4.14 %	-2.78 %	4.29 %	1.43 %	2.86 %	0.0012
83	8758.84	0.0440	4.09 %	-1.94 %	4.17 %	1.39 %	2.78 %	0.0012
84	8862.99	0.0446	4.18 %	-1.11 %	4.20 %	1.40 %	2.80 %	0.0013
85	8967.14	0.0464	5.49 %	-0.28 %	5.49 %	1.83 %	3.66 %	0.0017
86	9071.29	0.0446	5.89 %	0.56 %	5.89 %	1.96 %	3.93 %	0.0018
87	9175.44	0.0440	6.38 %	1.39 %	6.40 %	2.13 %	4.27 %	0.0019
88	9279.60	0.0424	6.49 %	2.22 %	6.55 %	2.18 %	4.37 %	0.0019
89	9383.75	0.0404	6.92 %	3.06 %	7.02 %	2.34 %	4.68 %	0.0019
90	9487.90	0.0380	7.13 %	3.89 %	7.30 %	2.43 %	4.86 %	0.0018
91	9592.05	0.0359	7.05 %	4.72 %	7.30 %	2.43 %	4.87 %	0.0017
92	9696.20	0.0325	7.91 %	5.55 %	8.22 %	2.74 %	5.48 %	0.0018
93	9800.36	0.0297	8.23 %	6.39 %	8.62 %	2.87 %	5.75 %	0.0017
94	9904.51	0.0268	8.45 %	7.22 %	8.93 %	2.98 %	5.95 %	0.0016
95	10008.66	0.0242	8.47 %	8.05 %	9.07 %	3.02 %	6.04 %	0.0015
96	10112.81	0.0207	9.99 %	8.89 %	10.60 %	3.53 %	7.07 %	0.0015
97	10216.96	0.0182	10.38 %	9.72 %	11.08 %	3.69 %	7.39 %	0.0013
98	10321.12	0.0155	8.24 %	10.55 %	9.26 %	3.09 %	6.17 %	0.0010
99	10425.27	0.0139	14.47 %	11.39 %	15.17 %	5.06 %	10.12 %	0.0014
100	10529.42	0.0113	9.94 %	12.22 %	11.07 %	3.69 %	7.38 %	0.0008
101	10633.57	0.0096	11.89 %	13.05 %	12.99 %	4.33 %	8.66 %	0.0008
102	10737.72	0.0077	7.35 %	13.89 %	9.21 %	3.07 %	6.14 %	0.0005
103	10841.87	0.0063	8.93 %	14.72 %	10.69 %	3.56 %	7.13 %	0.0005
104	10946.03	0.0051	10.61 %	15.55 %	12.30 %	4.10 %	8.20 %	0.0004
105	11050.18	0.0042	17.30 %	16.38 %	18.50 %	6.17 %	12.33 %	0.0005

Appendix C Table C1 Values of oligomer concentration β_i^0 at each mass of oligomer, m_i , with estimations of uncertainty

Table C1 Notes

- 1. Sum of squares of all type A and type B uncertainties to β_i^0 .
- 2. Assuming nine degrees of freedom.
- Following NIST policy, the standard uncertainty of the mean is multiplied by 2 to get an estimate of uncertainty limits. See B. N. Taylor and C. E. Kuyatt, *Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results*, NIST Technical Note 1297, US Government Printing Office, Washington, D.C., 1994. <u>http://physics.nist.gov/Pubs/guidelines/TN1297/tn1297s.pdf</u>