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How Do We Set Up or Choose An Optimal Procedure to Determine the Molecular Weight of Polymers with A Good Accuracy Using Size Exclusion Chromatography or Viscometry? A Mini-Review

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Abstract Several procedures have been already developed to determine the average-molecular weight (M) for polymers. However, determination of M with an optimal procedure, given a good accuracy is a major issue for research groups. This mini-review described three following procedures for determination of M with a good accuracy: (a) viscometry using intrinsic viscosity ([η]) data and Mark–Houwink–Sakurada (MHS) equation; (b) size exclusion chromatography (SEC) with a single concentration detector; and (c) SEC equipped with two detectors, concentration and viscometry detectors. The following conclusions were made: (1) in viscometry, the accuracy of M depends on the accuracy for the MHS equation constants, *K* and *a*. The values *K* and *a*, depend on the nature of polymer, quality of the solvent, polydispersity of the polymer samples, molecular weight range, and temperature. of polymer solution; (2) in SEC with a single detector, the value of M was determined, based on the calibration curve (log M against retention time). The polymer standard should have the same conformation in solution with the polymer under investigation, otherwise an unsatisfactory value for the M will be obtained; and (3) SEC equipped with the two detectors, the value of M for the investigated polymer was determined with a good accuracy. The polymer could have any conformation in solution either similar or different from the polymer standard.

Keywords: *Molecular weight, Intrinsic viscosity, Size exclusion chromatography*

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1. Introduction

Polymers or macromolecules are large molecules and composed of long chains and having high molecular weights and high molecular sizes [1]. The unit for molecular weight is usually Dalton (Da: equal to one atomic mass unit). Unlike simple low molecular substances, polymers do not have unique molecular weight. Practically there are no polymers whose molecules are all strictly of the same size or have the same degree of polymerization (DP), this is due to the random nature of polymerization reactions. Most of macromolecules consist of a mixture of chains of differing number of units. Hence, one uses the term average-molecular weight of macromolecules, and that is why in polymer science and technology, the concept of average-molecular weight (M) is used. A series of polymeric compounds of the same chemical structure, and differing only in molecular weights is known as a polymer homologous series [2,3]. Symbols and parameters appearing in this manuscript are given in Table 1. Macromolecules are divided into natural and man-made (synthetic) polymers. When polymers are manufactured in nature by organisms such as plants, animals, bacteria, algae, and fungi, they are called natural polymers, whereas polymers are manufactured in laboratories or industries, they are called synthetic polymers [4,5].

Various procedures and methods have been used for determination of M, resulting in different numerical values, depending on the methods by which they are measured [1,3]. Thus, different numerical values for molecular weights of polymers have been defined as number-average (M_n); weight-average (M_w); and viscosity-average (M_v), molecular weights [6,7]. The value of M_n for a polydisperse polymer does not equal to the same as M_w value. The value of M_w is always greater than M_n, except for the polymer is fully monodisperse.

There is no difference in determination methods for natural and synthetic polymers. Most of methods and procedures used to determine M, for biopolymers are

similar to synthetic polymers [3].

Various properties of synthetic polymers and biopolymers such as melt viscosity, rheological and mechanical properties (tensile strength, modulus, toughness), thermal behavior, chemical resistance, and weatherability are closely related to their Ms and sizes [6,8]. Generally, higher Ms are associated with greater (stronger) physical properties, whereas smaller Ms are associated with lower (weaker) properties. If the M is known along with a good understanding of the polymer conformation, many properties, such as mechanical and rheological properties, can be predicted [9,10].

Table 1. Constants and symbols used in this manuscript

Symbol	Full name	Symbol	Full name
a	Exponent for MHS equation,	M	Average-molecular weight
Da	Dalton	Ms	Average-molecular weights
DP	degree of polymerization	M_w	Weight-average molecular weight
$f_w(X)$	Cumulative weight fraction	M_n	number-average molecular weight
K	Intercept for MHS equation	M_v	viscosity-average molecular weight
LCB	long chain branching	[η]	Intrinsic viscosity
MHS	Mark-Houwink – Sakurada	Ve	Elution volume
MWD	Molecular weight distribution	SEC	Size Exclusion Chromatography

The M is one of the most basic data for studying the properties of polymers such as physicochemical, mechanical, and rheological properties of polymers. In addition, M is also an important characteristic of biological macromolecules such as proteins and polysaccharides. Both natural and synthetic polymers have been widely used in many industries such as petrochemistry, aerospace, textile, cosmetics, pharmaceutics, medicine, and food. Biopolymers with natural renewable resources and biodegradable and biocompatible properties widely used in agriculture, food, nutrition, medicine, pharmaceutical, and cosmetic sectors [8,11,12].

In this mini-review, an effort has been made to show a direction for choosing or setting up a procedure for determination of M for polymers with a good accuracy by two relative methods: viscometry, and size exclusion chromatography (SEC). Different procedures using these two methods are available in the literature. However, research groups may not choose an optimal procedure to achieve the value for M within the experimental uncertainty, indicating that the procedure used should give a reliable value for M. Determination of M for polymers by absolute methods and determination of molecular weight distribution (MWD) of polymers are out of the objectives of this study. A review of MWD for biopolymers has been already published in this journal [7].

2. Relative Methods for Average-Molecular Weight Determination of Polymers

The M obtained from viscometry and SEC are relative values. The measurements were performed in very dilute solutions. This is due to, in very dilute solutions, every macromolecule is sufficiently away from another one, i.e., they are isolated and they do not interact to each other. When the solutions are semi- concentrated or concentrated, macromolecules are aggregated together and macromolecules are closely intertwined [13,14]. In this study, M was determined for synthetic and natural polymers using three procedures: (a) viscometry using intrinsic viscosity- molecular weight relationship ([η]- M relationship), and Mark–Houwink–Sakurada (MHS) equation; (b) SEC with a single concentration detector such as refractive index; and (c) SEC equipped with two detectors (concentration, and viscometry). In these two relative methods, the measurement of M for an investigated polymer in a solution is based on its chemical structure and conformation in solution. Advantages, disadvantages and information obtained for three procedures are presented in Table 2.

2.1. Intrinsic Viscosity Average Molecular Weight

The viscosity of a dilute solution is a function of molecular size. Molecular size is not the same as molecular weight. Two macromolecules having the same M, one may be linear and another one can be a branched polymer. These two polymers have different sizes and shapes, which affects the value of [η] differently and result in different M values. The [η] of a polymer solution is related to its M according to the MHS equation [13,15]:

$$[\eta]=KM_v^a \quad (1)$$

where [η] is intrinsic viscosity, M_v viscosity-average molecular weight, and *K* and *a*, are the constants for a given solute–solvent system. Constructing log [η] versus log M, gives a straight line with intercept of log *K* and a slope of α. The [η] should be determined by using the same solvent as the constants *K* and *a* were found. If the constants, *K* and *a*, are known for a linear polymer in a certain solvent- temperature system, the constants cannot be used for a branched polymer with the same molecular weight. Determination of constants, *K* and *a*, from [η] data, requires a series of mono-disperse polymer samples with known M_w or a series of polydisperse polymer samples with known M_v. In general, M_v is not experimentally accessible, whereas other average molecular weights (M_n, M_W) are experimentally achievable from different experimental methods [13,14,15,16,17].

Table 2. Advantages, disadvantages and information obtained from three procedures: (a) SEC with a single detector; (b) viscometry; and (c) SEC equipped with two detectors (concertation and viscometry)

Methods	Advantages	Disadvantages and restrictions	Remarks
SEC with a single detector	The value of M is obtained from the SEC chromatogram with a single concentration detector (refractive index), and a calibration curve; the calibration care is obtained from a series of	The correct molecular weight is obtained from SEC, if the polymer standard and the investigated polymer has the same chemical structure and conformation in solution; a limited information is	In SEC with a single concentration detector (refractive index): the average-M was determined, based on the calibration curve (log M against retention time) as a reference; The polymer standard should have the

	monodisperse polymer standards; the procedure is suitable with a good accuracy, if the investigated polymer has the same conformation in solution and chemical structure with the polymer standard	obtained from SEC using a single detector.	same conformation in solution with the polymer under investigation, otherwise wrong M values will be obtained for the investigated polymer samples.
Viscometry	Viscometry is a simple technique to perform; requires easy-to-use apparatus; no need special attention to operate the capillary viscometer; no need special attention for preparation of polymer solutions; the MHS equation is a suitable way to study on conformation of polymers in solutions.	The correct value for M_v is obtained, if accurate values for the constants are used in the MHS equation; the constants can be determined correctly if all parameters (nature of the polymer, quality of the solvent, polydispersity of the polymer, molecular weight range, and temperature) affecting them are taken into consideration.	The values of *K* and α for a given solvent and polymer combination at a certain temperature and molecular weight range are constant; the value of α gives information about the conformation of the polymer molecules in solution; applying the correct values of the constants in MHS equation enable one to achieve the M_v value with a good accuracy.
SEC with two detectors (concentration and Viscometry)	The value of M with a good accuracy is obtained using two detectors (concentration and viscometry); it is a suitable technique for the quantitative study of branched polymers such as long chain branching;	SEC equipped with two detectors are relatively a complicated procedure, it is needed enough knowledge on mathematics to process the data for calculation of various average- values of Ms (M_n, M_v, M_w)	The following information is obtained from SEC equipped with two detectors: (1) the [η] is determined from; (a) viscosity of polymer solution relative to viscosity of the solvent; and (b) the solution viscosity of the polymer as a function of polymer concentration eluting from the column; and (2) the combined information [η] and V_e obtained from the SEC enable one to calculate molecular weight of investigated polymer having conformation in solution and chemical structure different from the polymer standard.

2.2. Determination of Viscometric Constants for A Polymer in A Solvent

The SEC can be used to determine viscometric constants for a polymer in a solvent at a certain temperature. The hydrodynamic volume [η] M, correlates with retention volume (elution volume), Ve. According to the universal calibration theory, at a given Ve, two polymers 1 and 2 have the same hydrodynamic volume, [η] M.

$$[\eta]_1.M_1 = [\eta]_2.M_2 \quad (2)$$

Substation of the value for [η] using equation (1)

$$K_1 M_1^{1+a1} = K_2 M_2^{1+a2} \quad (3)$$

Rearranging the equation (3) into a log form, the following equation is obtained

$$\log M_2 = \frac{1}{1+a_2}.\log\frac{K_1}{K_2} + \frac{1+a_1}{1+a_2}.\log M_1 \quad (4)$$

Generally, K_1 and a_1 are known in the literature, and K_2 and a_2 are either known in the literature or can be obtained via measurements by intrinsic-viscosity, osmotic-pressure or light-scattering technique [13,15,18,19].

2.3. Size Exclusion Chromatography

In the SEC, macromolecules (synthetic and natural polymers) in solutions were separated in the column of SEC on the basis of their sizes (but not with molecular weight), and the amounts of materials eluting from the column was determined by the detector as a function of retention time.

The macromolecules are separated on the basis of hydrodynamic molecular volume or size.

The retention time was converted into molecular weight by the use of a calibration curve [19,20]. The elution fractions were analyzed by the SEC detector. Monodisperse polymer standards are required to transform from elution volume (Ve) to M. A series of monodisperse polymer standard samples with Ms are required to determine the M of unknown samples. From the SEC distribution curve, various molecular weight averages (see Figure 1) as follow can be also calculated [16,17,18,19]

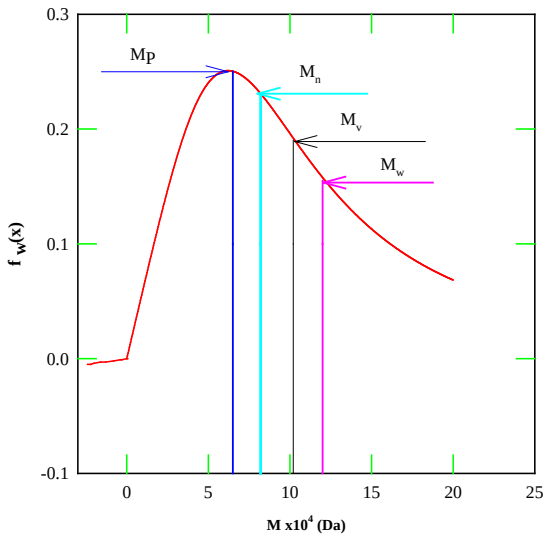

Figure 1. A typical molecular weight distribution profile with a semi-qualitative comparison of different average molecular weights: (a) molecular weight at the highest point of the distribution curve (Mp); (b) number-average molecular weight (Mn); (c) weight-average molecular weight (Mw); and (d) viscosity-average molecular weight (Mv)

The calculation procedure of different average-molecular weights (M_n, M_v, M_w) in SEC may be performed as follows: the height of chromatogram of each fraction ($h_{i,}$), is proportional to its weight fraction, w_i, which in turn is proportional to the product of number of molecules, n_i, and their molecular weight, M_i according to:

$$h_i = k.w_i = k.n_1.M_1 \tag{5}$$

where k is a constant and represents the relationship between the signal height and its weight fraction [21]. Practically, each chromatogram is divided into several equal segments (at least into 500 equal segments of molecular weight) and the corresponding heights are determined. Average-molecular weights (M_n, M_v, M_W) are expressed as summation of individual segments and calculated from the following equations:

$$\overline{Mn} = \frac{\sum_{i=1} wi}{\sum_{i=1} ni} = \frac{\sum_{i=1} ni\,Mi}{\sum_{i=1} ni} = \frac{\sum_{i=1} hi}{\sum_{i=1} (hi/Mi)} = \tag{6}$$

$$\overline{Mw} = \frac{\sum_{i=1} wi\,Mi}{\sum_{i=1} wi} = \frac{\sum_{i=1} ni\,Mi^2}{\sum_{i=1} ni\,Mi} = \frac{\sum_{i=1} hi\,Mi}{\sum_{i=1} hi} \tag{7}$$

$$\overline{Mv} = \left(\frac{\sum_{i=1} wi\,Mi^a}{\sum_{i=1} wi}\right)^{1/a} = \left(\frac{\sum_{i=1} ni\,Mi^{1+a}}{\sum_{i=1} ni\,Mi}\right)^{1/a} = \left(\frac{\sum_{i=1} hi\,Mi^a}{\sum_{i=1} hi}\right)^{1/a} \tag{8}$$

where h_i and M_i are the peak height and molecular weight of compound i. The value of h_i is read directly from the chromatogram.

One can also calculate the average-molecular weight, using the area for compound i,

$$\overline{Mn} = \frac{\sum_{i=1} wi}{\sum_{i=1} ni} = \frac{\sum_{i=1} ni\,Mi}{\sum_{i=1} ni} = \frac{\sum_{i=1} Ai}{\sum_{i=1} (Ai/Mi)} \tag{9}$$

$$\overline{Mw} = \frac{\sum_{i=1} wi\,Mi}{\sum_{i=1} wi} = \frac{\sum_{i=1} ni\,Mi^2}{\sum_{i=1} ni\,Mi} = \frac{\sum_{i=1} Ai\,Mi}{\sum_{i=1} Ai} \tag{10}$$

$$\overline{Mv} = \left(\frac{\sum_{i=1} wi\,Mi^a}{\sum_{i=1} wi}\right)^{1/a} = \left(\frac{\sum_{i=1} ni\,Mi^{1+a}}{\sum_{i=1} ni\,Mi}\right)^{1/a} = \left(\frac{\sum_{i=1} Ai\,Mi^a}{\sum_{i=1} Ai}\right)^{1/a} \tag{11}$$

where A_i is the peak area. The two methods seem to give the same values for average molecular weights. However, the area method is much more accurate, due to the two following reasons: (a) if the chromatogram is symmetrical, one can treat it as a Gaussian distribution (see Figure 2), i.e. the area method is just as good as the height method. Actually, the two methods are identical in this case; (b) if the chromatogram is not strictly Gaussian [13,18,19]. The maximum height does not necessarily represent the median and the area method would minimize the error.

The M_w is particularly sensitive to the presence of larger species, whereas the M_n is sensitive to the presence of lower molecular weights. The value of M_v is different from other average molecular weights. It is not a unique value, and varies from one solvent to another, since it is a function of a, K, and [η] [16,17,18]. The value of M_v can be obtained experimentally. It can be calculated once the values of the constants are known in a polydisperse polymer samples.

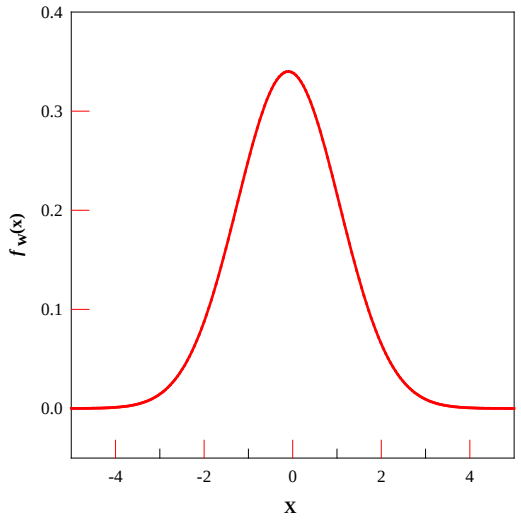

Figure 2. A Gaussian distribution profile of a polymer in size exclusion chromatography. The detector response, $f_w(X)$, as a function of X

2.4. SEC with Two Detectors: Concentration and Viscometry Detectors

Coupling of a SEC with two detectors; one known concentration (refractive index) detector: and another one, an automatic capillary viscometer, result in more information and accurate data for the M in comparison with SEC with one detector. Initially, a calibration curve is plotted for a series of monodisperse standard polymers, i.e., log hydrodynamic volume (intrinsic viscosity x molecular weight, [η]M) versus retention volume, *Ve*.

$$[\eta]M = f(Ve) \tag{12}$$

that is equivalent to the (hydrodynamic volume versus elution volume) for the polymer under investigation. Thus, the same curve can be constructed (hydrodynamic volume versus elution volume) for the polymer under investigation. The basic assumption of universal calibration is that polymer standard and polymer under investigation of identical hydrodynamic volumes are detected at identical elution volumes.

$$[\eta]_i M_i = [\eta]_1 M_1 = f(Ve) \tag{13}$$

where 1 and *i* refer to the polymer standard and the polymer under investigation, respectively. Substitution of the MHS relationship yields

$$K.M_i^{a+1} = f(Ve) \quad (14)$$

$$\log M_i = \frac{\log f(Ve) - \log K}{1+a} \quad (15)$$

The advantage of two detectors in SEC is: enable us to determine the M value for the investigated polymer with a good accuracy, regardless having conformation in solution and chemical structure the same as the polymer standard. Two polymers may have exactly the same M, but they may have very different molecular sizes/ dimensions. Branched polymers have smaller [η] and more compact conformation in solution than that of the linear ones [16,17,18,19,20,21,22].

3. Advantages and Disadvantages of Viscometry and Size Exclusion Chromatography Procedures

This study is valid for all polymers. The only limit for this study is: each polymer should have a solvent to prepare dilution polymer solutions. This study shows an optimal procedure to determine the molecular weight of polymers with a good accuracy using size exclusion chromatography or viscometry. If the research groups choose an unsatisfactorily polymer standard or inappropriate procedure for determination of the M for the investigated polymer, a wrong M may be obtained. Under such unsatisfactorily conditions, the experimental data for M can be even more than double of the correct value for M (overestimated), or can be even less than half of the correct value (underestimated).

3.1. Discussion on Viscometry Procedure

The exponent *a* for MHS equation has a wide range, less than 0.5 up to greater 1.00. For flexible chain conformation the exponent *a* varies $0.5 \leq a < 1.0$. For polymers having rod-like conformation, the value for exponent *a* may exceed unity. The plot of log [η] versus log M_v for a wide M_v range of a polymer in a solvent at a certain temperature may not be a linear and deviation from linearity could increase with an increase in M_v. Thus, the MHS equation for a polymer-solvent system at a certain temperature can be used for a limited range of M_v. The accuracy of M_v values depends on dispersity of the investigated polymer [23,24].

For instance, the exponent *a* for pullulan samples with M in the range of 5–1000 kDa, having narrow MWD in 0.05M Na_2SO_4 at 30°C were found to be 0.667 [17]. Pullulan behaves like a flexible random coil in this solvent. The plot log [η] versus log M for entire M range for pullulan was a linear. No deviation from linearity was observed. Whereas the plot log [η] versus log M for entire M range of dextran (0.18-5900 kDa) in 0.05M Na_2SO_4 at 30°C was not linear and deviation from linearity increased with an increase in M_w [24]. The dispersity of the experimental points also increased significantly in upper part of the plot. The macromolecule first consists of linear chains and latter the branch density increases slowly. The asymptotic region with a significant increase in chain branching was apparently attained when the M was enough large. The values of 0.512, 0.425, and 0.273 were found for exponent *a* for dextran in 0.05M Na_2SO_4 at 30 ◦ C. A value of 0.273 for ultra-high M indicates that the samples possess highly branched structures [24]. Ioan et al. [23] also have reported that the plot of log [η] versus log M for dextran was non-linear for a wide range of M. They reported that for hyperbranched polymers the ratio, M_w/M_n did not remain constant for different M range.

The structure and conformation of methyl cellulose (MC) (M_w = 37- 79 kDa) and hydroxypropyl methyl cellulose ether (HPMC) (M_w = 50 -71 kDa) samples dissolved in dilute aqueous (D_2O) solutions at a temperature of 25°C were determined. In both macromolecule structures contain several strong hydrogen bonds caused by the presenting hydroxy groups in the MC and HPMC macromolecules. The presence of several hydrogen bonds among hydroxyl groups are the characteristics of cellulose derivatives. The formation of several hydrogen bonds (among intra- and inter macromolecules chains) in these polymers would yield in rigid rod structures and highly extended conformations [25,26]. For these polymers having rigid rod structures and rod-like conformation in solutions, the value for exponent *a* should exceed unity.

3.2. Discussion on Size Exclusion Chromatography Procedures

In the SEC method, the macromolecules are separated on the basis of hydrodynamic molecular volume or size. The value of M from the SEC chromatogram with a single concentration detector (refractive index) is determined using a calibration curve, log M against retention time. The calibration curve is obtained from a series of monodisperse polymer samples. In the SEC instrument equipped with a single detector, a research group should choose a satisfactory polymer standard. Such satisfactory polymer standard should have similar structure and conformation in solution with the investigated polymer, otherwise a wrong value for M value can be determined. This is due to, linear, branched and highly branched polymers with the same M will be eluted differently. The highly branched polymer will be eluted the first and the linear polymer will be the last one. Thus, choosing the correct polymer standard having similar structure and conformation in solution with the polymer under investigation is highly recommended [16,17,24,27].

In the SEC instrument equipped with two detectors (refractive index and viscometry), a research group measures the value of [η] for the two polymers. The hydrodynamic volume and elution volume are taken into consideration for the both standard and investigated polymers. The M can be determined using equation 15 [16].

An additional insight from the MWD, and [η] of polymers has been obtained using the SEC in combination with refractive index, viscometry, and multiangle light scattering detectors. A more quantitative interpretation of the MHS equation was obtained by assessing the variation of the slope for the plot log [η] versus log M_v [27]. The curvature in the plot can be correlated to the structural and chemical properties (e.g., branching, composition,

randomness) of the polymer. This information can be used to assess the effect of the solvent system and conditions on the solvation behavior of polymers. In addition, the information can be used to investigate whether an industrial polymer batch (under processing) contains unwanted branched species or exhibits particular solvation behavior. The significant advantages of such procedure are: no prior information on the inter- and intramolecular interactions of the polymer is needed. The curvature of the plot can be used to make comparisons between polymer samples and to explain potential differences in polymer branching or intramolecular interactions [27].

4. Compare Viscometry and Size Exclusion Chromatography

The SEC method requires relatively a complicated apparatus equipped with column, detector and a heating system to adjust temperature for the column and detector. It is needed special attention to operate SEC instrument. The SEC equipped with two detectors is needed enough knowledge on mathematics to process the data for calculation of various average- values of molecular weights (M_n, M_v, M_w)., whereas viscometry requires an easy-to-use apparatus and no need special attention to operate the capillary viscometer. No need special attention for the preparation of polymer solutions in viscometry. In the SEC method, A package of monodisperse polymer samples as polymer standards are needed to construct calibration curve. Different average-molecular weights (M_n, M_v, M_w) can be calculated using SEC chromatograms. In addition, the MWD and dispersity (M_w/M_n) of the investigated polymer can be also calculated in the SEC procedures. Furthermore, one can perform fractionation using the SEC method. In the fractionation, a polydisperse macromolecule can separate into its individual fractions (homologous series), based on their molecular sizes. Thus, monodisperse fraction can be obtained in the fractionation procedure by the SEC method. Whereas in viscometry, only M_v can be calculated using MHS equation. In viscometry, molecular weight of polymers can be determined, when polymerization, degradation, or fragmentation are performing. In other words, the molecular weight can be measured when chain scission or progress in chain formation (polymerization) are under performing.

5. Conclusions

This manuscript showed: "How do we set up or choose an optimal procedure to determine the molecular weight of polymers with a good accuracy using size exclusion chromatography or viscometry method"? Two relative methods viscometry and SEC for determination of M of polymers (synthetic and natural), with three procedures: (a) viscometry using intrinsic viscosity- molecular weight relationship, and MHS equation; (b) SEC with a single concentration detector; and (c) SEC equipped with two detectors (concentration and viscometry) were studied. The following conclusions were made: (1) in the SEC equipped with a single concentration detector, the M was determined, based on the calibration curve (log molecular weight against retention time). The polymer standard should have the same structure and conformation in solution with the polymer under investigation, otherwise unsatisfactory molecular weight values will be determined; (2) in viscometry, the value of M_v was determined using MHS equation with known the equation constants, *K a*nd *a*, for the polymer solute- solvent system. The accuracy of M depends on the accuracy for the constants. The values of *K* and *a*, depend on the nature of the polymer, the quality of the solvent, the polydispersity of the polymer samples, molecular weight range, and temperature. In viscometry, molecular weight of polymers can be determined, when polymerization, degradation, or fragmentation are performing. This is due to viscometry requires an easy-to-use apparatus and a simple technique to perform. No need special attention to operate the capillary viscometer; (3) the SEC equipped with two detectors (concentration and viscometry) is a reliable technique to determine molecular weights of polymers having any conformation in solution and chemical structure either similar or different from the polymer standard. In this procedure, the value of M with a good accuracy is obtained; and (4) several polymers having monodisperse distribution commercially are available as polymer standards. They have different structures, linear, branched and highly branched. The polymer standard can have any chemical structure and conformation in solution either similar or different from the polymer under investigation.

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Conflict of Interests

The author of this manuscript declares that there is no conflict of interests regarding the publication of this manuscript

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