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Aspects of determining the molecular weight of cyclodextrin polymers and oligomers by static light scattering

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ABSTRACT

Methodic issues affecting the use of static light scattering to determine the average molecular weight of cyclodextrin poly- and oligomers are discussed. The critical features which enable accurate measurement such as aggregation behavior and segment–segment interaction are elucidated. Static light scattering data supported with globule size and aggregate state analysis (as determined by dynamic light scattering) allowed the aggregate-free state of the samples to be justified and the ideal globular conformation of the macromolecules to be corroborated. These molecular characteristics were demonstrated for uncharged, charged and fluorescent randomly crosslinked water soluble cyclodextrin poly- and oligomers.

1. Introduction

Cyclodextrin (CD) poly- and oligomers[1] are compounds of peculiar structure as their building blocks are functional oligosaccharide units in themselves. In this sense, single cyclodextrin rings from which the macromolecule is built may be regarded as 'monomers'. The functionality of these monomers comes from their torus-shaped cyclic structure (6–8 glucose units are linked by 1,4-α-glycosidic bonds, denoted as αCD; βCD; γCD, respectively) enabling the encapsulation of hydrophobic guest molecules within their cavities. It is important to distinguish between cyclodextrin polymers and cyclodextrin pendant polymers. Random structure cyclodextrin polymers are prepared by chemical crosslinking of native or derivatized CD monomers whilst linear polymers by polymerizing CD-containing monomers. Cyclodextrin pendant polymers, on the other hand, are synthesized by grafting cyclodextrin molecules to a preformed polymer chain. The scope of present paper is restricted to elucidating the molecular characteristics of randomly crosslinked cyclodextrin polymers soluble in water, especially those crosslinked with epichlorohydrin.

The potential pharmaceutical applications of water soluble CD polymers as drug solubilizing excipients have been discussed for more than two decades (Szeman et al., 1987a,b; Fenyvesi, 1988) and is the subject of continuing research (Jug, Kosalec, Maestrelli, & Mura, 2011; Jug, Maestrelli, Bragagni, & Mura, 2010; Martin, Sanchez, Cao, & Rieumont, 2006). It is noteworthy that some cyclodextrin derivatives such as (2-hydroxypropyl)-β-cyclodextrin (Liu et al., 2009) possess biological activity of their own. Pending adequate characterization, we anticipate that exploration of the bioactivity of cyclodextrin polymers will also be subject of future scientific interest. Still, it is noteworthy that a number of publications disclosing the properties or behavior of CD polymers lack any mention of the molecular weight of the substance(s) studied. The average molecular weight of different water soluble CD polymer samples can range widely between 2×10^3 to a few million Daltons (Renard, Deratani, Volet, & Sebille, 1997). The consequences of the differences in molecular weight are well illustrated by an in vivo study where a ~100 kDa CD-based macromolecule was found to have greatly superior antitumor efficacy when compared to a ~40 kDa molecular weight analogue (Cheng, Khin, & Davis, 2004).

Mocanu, Vizitiu, and Carpov (2001) reviewed various features of cyclodextrin polymers and relevant characterization methods were detailed. In this review, only references dealing with gel permeation chromatography (GPC) and viscometry were cited with regard to the determination of the molecular weight. Another relevant reference (Yudiarto, Kashiwabara, Tashiro, & Kokugan, 2001)

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[1] Herein, in case the term 'polymer' stands alone it is understood as also referring to 'oligomers' which has molecular weight less than 50 kDa.

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optimized the polymerization reaction of βCD using epichlorohydrin in the presence of sodium hydroxide under a range of reaction conditions. The water soluble fractions separated by ultrafiltration also had their average molecular weight characterized by GPC. In more recent works use of GPC technique is routinely applied for studying the molecular weight of uncharged, crosslinked CD-polymers (Wintgens & Amiel, 2010; Zhang, Chen, & Diao, 2011; Moya-Ortega et al., 2011).

Li, Xiao, Li, and Zhong (2004) reported their results on the synthesis and characterization of cationic CD-polymers where the determination of molecular weight was also performed by GPC. Calibration was made using βCD against standard pullulan samples. High performance size-exclusion chromatography equipped with a multiangle laser-light scattering and refractive index detectors was found to be adequate to determine the weight-average molecular weights (M_w) of amylopectins (You, Fiedorowicz, & Lim, 1999), amylopectin and amylose (Lee, Han, & Lim, 2009) and CD polymer samples synthesized via crosslinking β-CD with maleic anhydride (Girek, Shin, & Lim, 2000).

Sainz-Rozas, Isasi, & Gonzalez-Gaitano, (2005) studied the hydrodynamic radii of different epichlorohydrin crosslinked βCD polymers by dynamic light scattering. The equivalent molecular mass was obtained based upon the Mark–Houwink–Sakurada equation (Harding, Satelle, & Bloomfield, 1992).

To the best of our knowledge there is no previous work available which characterizes crosslinked cyclodextrin polymers which addresses methodic features of static light scattering when used uncoupled from other techniques. In this work we demonstrate the utility of this method to characterize uncharged, charged and fluorescent cyclodextrin polymer samples as an alternative to GPC technique. The accuracy of GPC results depend on the selection of adequate certified standards which is often a matter of compromise, especially in case of novel materials. To ensure accuracy of the light scattering technique, complementary aggregation state studies were performed using dynamic light scattering.

2. Experimental

2.1. Materials

2.1.1. Neutral cyclodextrin polymer samples

Water soluble neutral CD-polymers were synthesized from α-, β- and γCD by chemical crosslinking in basic media using epichlorohydrin. The obtained products are denoted as PACD, PBCD and PGCD, respectively. The in-detail preparation method is published elsewhere (Fenyvesi, 1988).

2.1.2. Anionic cyclodextrin poly- and oligomers

Carboxymethyl βCD poly- or oligomers (PCMβCD-Na or OCMβCD-Na) may be prepared from carboxymethyl βCD sodium salt (CMβCD-Na) in analogous manner as PBCD. These substances are commercialized products of CycloLab Ltd.

2.1.3. Fluorescent (rhodaminyl) cyclodextrin oligomer sample

OCMβCD-Na was prepared in the following way: CMβCD-Na (DS = 3.7) (9 g, 6.3 mmol) was dissolved in water (8 mL) and the temperature was increased to 60 °C. A solution of NaOH (3 g dissolved in 12 mL) and epichlorohydrin (6 mL) was gradually added. After 2 h stirring at 60 °C the reaction mixture was cooled to room temperature.

OCMβCD-Na was labeled with fluorophore in the same vessel. Rhodamine B isothiocyanate (RBITC, 130 mg, 0.24 mmol) was added and the pink solution was stirred for a further 2 h. The reaction was followed by TLC using 1,4-dioxane:ammonium hydroxide (25%)–10:7 (v/v) as eluent. The reaction mixture was neutralized with HCl 5 N and the unreacted (or decomposed) RBITC was removed by extracting the aqueous phase with dichloromethane till the color of the extract became from dark red to pale pink. The aqueous phase was dialyzed using cellulose membrane (MWCO approx. 1000, from Sigma Aldrich) and freeze drying yielded rhodamine-labeled CMβCD oligomer sodium salt (Rh-OCMβCD-Na) as a pink powder (8.4 g).

The rhodaminyl content in Rh-OCMβCD-Na was estimated by UV–vis spectrophotometry using a calibration curve of rhodaminyl isothiocyanate solutions of 0.03–0.3 mg/L concentrations in MilliQ® water at 260 nm. 0.4 w% rhodaminyl content was obtained.

Fig. 1 represents the structural illustration of the characterized substances.

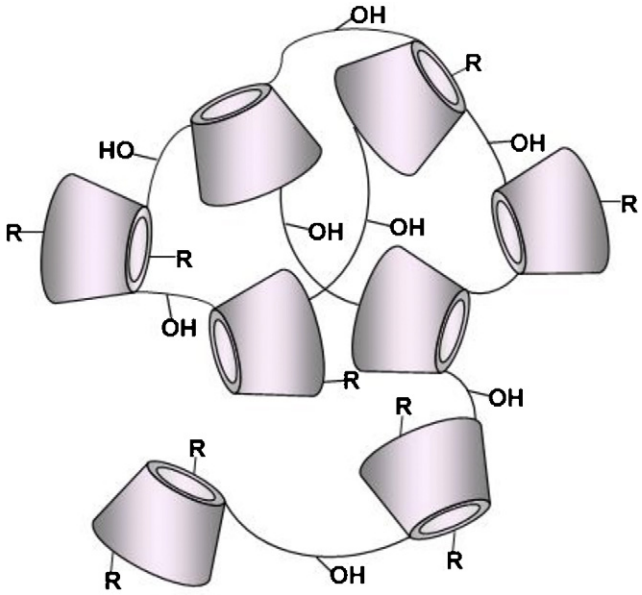

Fig. 1. Schematic structure of crosslinked beta-cyclodextrin oligomers. *R* = —OH: Neutral beta-cyclodextrin oligomer. *R* = —OH or —OCH_2COONa: OCMβCD-Na. *R* = —OH or —OCH_2COONa or rhodaminyl: Rh-OCMβCD-Na.

3. Methods

3.1. Static light scattering

Molecular weight determination was performed using a Malvern Zetasizer Nano ZS instrument running a Static Light Scattering (SLS) method. The samples were studied at different concentrations at 25 °C applying the Rayleigh equation, which describes the intensity of light scattered from the polymer globules in solution:

$$\frac{Kc}{R_\Theta} = \left(\frac{1}{M_w} + 2A_2c\right) P(\Theta),$$

wherein R_Θ, the Rayleigh ratio – the ratio of scattered light to incident light of the sample; M_w, weight-averaged molecular weight; A_2, 2nd virial coefficient; c, concentration; $P(\Theta)$, angular dependence of the sample scattering intensity; K, optical constant as defined as:

$$K = \frac{4\pi^2}{\lambda_0^4 N_A}\left(n_0 \frac{dn}{dc}\right)^2,$$

wherein N_A, Avogadro's constant; λ_0, laser wavelength; n_0, solvent refractive index; dn/dc, the differential refractive index increment.

n_0 and dn/dc were measured using an Abbe refractometer (Karl Zeiss, Jena) at 25 °C. In a case where the polymer globules are

considerably smaller than the wavelength of the incident light, P_Θ will reduce to 1 (Rayleigh approximation). This condition may be verified by determining the diameter of the globules by dynamic light scattering.

M_w and A_2 (indicative of the interaction strength between the polymer segments and the solvent used) can be obtained by measuring the intensity of the scattered light (KC/R_Θ) of various sample concentrations at a single angle which is compared with the scattering produced from a standard sample (pure toluene was used as a reference). This graphical representation is called a Debye-plot, wherein M_w is determined from the intercept at zero concentration ($Kc/R_\Theta = 1/M_w$ for $c \to 0$). A_2 may be determined from the gradient of the Debye-plot. Since cyclodextrin polymers may contain considerable amount of adsorbed water (up to 10 w%), the weighings were corrected with loss on drying data.

3.2. Dynamic light scattering (DLS)

Determination of the diameter of the globules as well as the size distribution of polymer aggregates where present was performed by dynamic light scattering method (also known as photon correlation spectroscopy) using a Malvern Zetasizer Nano ZS instrument. More details on the use of DLS for characterizing the aggregation behavior of CDs have been disclosed recently (Puskás, Schrott, Malanga, & Szente, 2012). Triplicate measurements were carried out for all samples, each averaged of at least 10 runs at 25 °C. The intensity size distribution was utilized for aggregate state analysis, as it is highly indicative of the presence of over-micron size scattering objects. For obtaining accurate M_w data it is essential to assure the absence of particles sized in this range. During globule size analysis it was found that the size distribution by weight was the most indicative of the conformation-dependent size distribution of the macromolecules (sample refractive index: 1.37, absorbance for colorless sample: 0.01, absorbance for fluorescent sample: 0.1).

Instrument specifications: Maximum particle size range (diameter): 0.3 nm–5 μm – Laser: 4 mW He–Ne, 633 nm – Detector: avalanche photodiode.

4. Results and discussion

4.1. General aspects of determining M_w of cyclodextrin polymers

A number of scientific and technical documentations refer to M_w obtainable with static light scattering as 'absolute molecular weight', i.e. there is no need/possibility for calibration (unlike GPC). This can be misinterpreted as the molecular weight obtained by this method being by all means highly accurate. Upon characterizing a number of different cyclodextrin polymer samples, some critical methodic aspects were found.

It is well known that cyclodextrin monomers are susceptible to self-aggregation (Coleman, Nicolis, Keller, & Dalbiez, 1992; González-Gaitano et al., 2002). We supposed that a similar self-aggregation effect might apply for the cyclodextrin polymer globules which should be controlled for by selecting appropriate conditions such as solvent polarity and concentration. Presence of aggregates (even in small quantity) can manifest as poor reproducibility of the obtained M_w (error > ±10%) and by increases in the 'apparent molecular weight' as the static light scattering method cannot distinguish between aggregates and individual polymer molecules. Aggregates may form via intermolecular hydrogen bond interaction between the cyclodextrin moieties, and in the case of Rh-OCMβCD-Na the rhodaminyl substituents may interact with one another by π–π stacking. The absence of aggregates might be ascertained by polymer globule diameter analysis (as measured by DLS), which should result in a monomodal intensity size distribution curve for statistically crosslinked polymer samples. This means that less than 0.1% of the scattered intensity is attributed to the fraction of the species forming aggregates. These associates have typically more than 1 μm diameter. Based on aggregate-state studies by DLS, filtration of the test samples presented in this paper was necessary since a small amount of species susceptible to form aggregates were always found in detectable quantities in distilled water.

Upon filtration it must be demonstrated that the filtrate is still representative of the sample, (only contamination is filtered out) since excessive removal might result in sample fractionation. Having prepared the samples in the corresponding solvent, Millipore Durapore® 0.45 μm nominal pore size membrane filters were used to remove large aggregates. It was assumed that upon the synthesis of the polymers, a small fraction of macromolecules with irregular chemical composition (statistically occurring segments of high degree of substitution or crosslinking) or due to aggregating by-products might have been formed. The presence of these aggregates could be attributed to a specific fraction of the sample, since the aggregates could be entirely filtered off, i.e. the filtrate contained less than 0.1 volume% of aggregating species as verified by DLS. Possible re-association after filtration was excluded as no aggregates were observed in the filtrates on standing. The ~1% concentration change due to the filtration, whilst less than the experimental error of the molecular weight determination, was still taken into account.

The Rayleigh approximation (Section 3.1) requires that the polymer globule volume average size obtained by DLS should be less than one tenth of the wavelength of the laser source used. The use of this approximation is justified as assumption was confirmed for all samples described in this work.

4.2. Determining M_w of neutral cyclodextrin polymers

Simple aqueous solutions of neutral cyclodextrin polymer solutions were found to be adequate for the SLS measurement according to the criteria stated in Section 4.1. A variety of PACD, PBCD and PGCD samples could be successfully tested in the molecular weight range of 20–260 kDa (measuring parameters: $dn/dc = 0.14$, measuring concentration range: 2.0–10.0 g/L). The correlation coefficients of the Debye plots were higher than 0.9. The adequacy of the used conditions was verified by determining M_w of (2-hydroxypropyl)-β-cyclodextrin (HPβCD) samples of known average degree of substitution as determined by NMR according to European Pharmacopoeia 7.4. HPβCD was regarded a structural analogue of a hypothetical monomer of BCDPS. From the degree of substitution of HPβCD M_N (number average molecular weight) could be calculated. The M_w obtained by DLS were 5–6% higher than the declared M_N values (1380–1480 Da). This result was in good agreement taking the polydispersity of the multicomposite HPβCD samples into account.

4.3. Determining M_w of a carboxymethyl cyclodextrin polymer

Due to the polyelectrolyte nature of PCMβCD-Na, the selection of the solvent suitable for the analysis was found to be highly critical. Debye plot analysis was not possible using distilled water. By changing the polarity, the ionic strength and pH of the solvent used, a range of 'apparent' M_w values (88–258 kDa) were obtained for the same sample of relatively high molecular weight. Therefore apart from the criteria listed in Section 4.1, an additional requirement was set regarding the solvent to be used for these polymers.

The solvent must behave as a near-theta solvent, i.e. the second virial coefficient (A_2) should be 0 within 95% confidence interval. This criterion ensures that the polyelectrolyte chain is not highly extended (giving incorrectly high values at low ionic strength

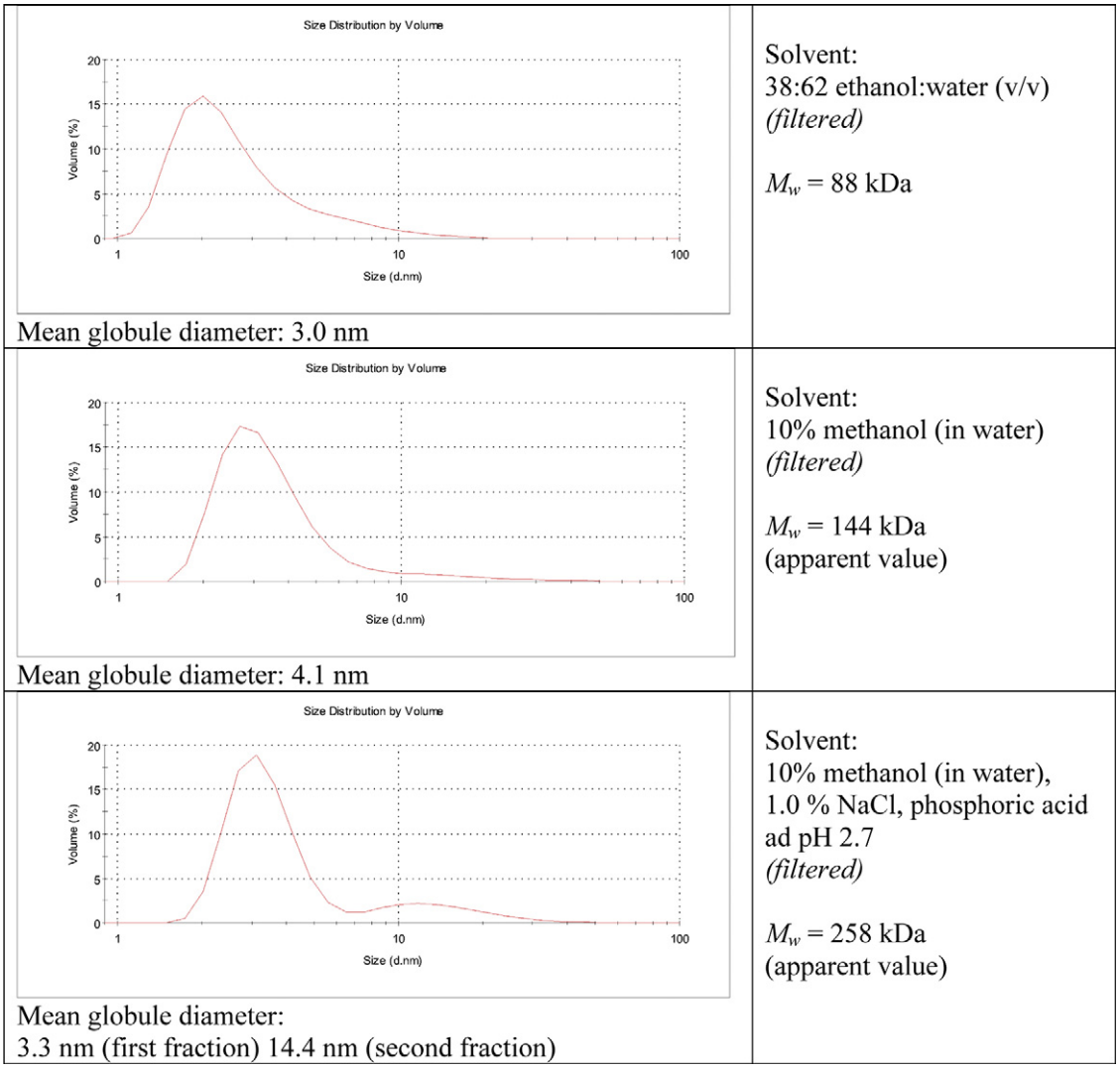

Fig. 2. Globule size distributions of PCMβCD-Na as determined in various dissolution media by dynamic light scattering.

or high relative permittivity) due to solvation and electrostatic repulsion, but rather it was aimed to ensure the ideally globular conformation of the macromolecules. Under these circumstances the segment–segment interactions are very weak allowing the polymer to adopt randomly fluctuating 3D structures or random coil configurations. Various solvent compositions were tested systematically, and 38:62 ethanol:water (v/v) solvent mixture was found to meet this latter criterion. The experimentally determined *dn*/*dc* value was 0.117 mL/g in this solvent.

Fig. 2 demonstrates how different solvents influence the globule size distribution of filtered samples of PCMβCD-Na. The apparent M_w is over-estimated when analysis is conducted in the methanolic solvent mixture compositions suggested for the analysis of substances similar to anionic CD-polymers by gel permeation chromatography (Tosoh Bioscience, 2011). It was found that in these solvents the globules become highly extended or partially associated (as indicated by e.g. the presence of the second modality over 10 nm, relatively large globule size and wider volume size distribution curve). In less polar solvent: 38:62 ethanol:water (v/v) solvent mixture, the CD polymer molecules take nearly ideal globular conformation as indicated by the slope of the Rayleigh-plot. The monomodal globule size distribution becomes narrower and the mean globule size decreases. It is anticipated that the obtained globule size is representative of the molecular dimensions of the polymer molecules. It is noted, however, that DLS provides size data derived from the hydrodynamic behavior of the light scattering objects and provides the best approximation if these structures are spherical. Due to the randomly crosslinked structure it is considered probable that the globules are largely spherical.

4.4. Determining M_w of a rhodaminyl labeled cyclodextrin oligomer

The method was applied for Rh-OCMβCD-Na which is a fluorescent cyclodextrin oligomer derivative. Laser light scattering methods are only suitable to characterize fluorescent samples if the molecules in the sample are not excited at the wavelength of the laser used since the subsequently emitted radiation might interfere with the scattered light. Fig. 3 shows the UV–vis absorption spectrum of Rh-OCMβCD-Na. It was found that this material does not absorb light significantly at the wavelength of the Zetasizer instrument used (633 nm). Favorably the same solvent composition used for PCMβCD-Na was also found be the most suitable for the analysis of OCMβCD-Na and Rh-OCMβCD-Na over the discussed criteria (absence of aggregates after filtration, $A_2 = 0$ within 95% confidence interval). The experimentally determined *dn*/*dc* value

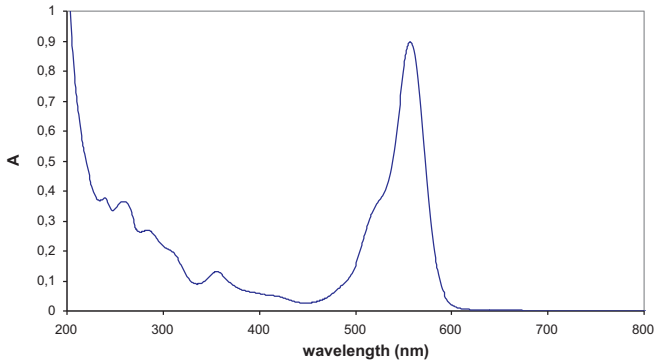

Fig. 3. UV–vis absorption spectrum of Rh-OCMβCD-Na (c = 1.800 mg/ml).

of Rh-OCMβCD-Na was 0.120 mL/g. The average molecular mass of OCMβCD-Na used as the starting material for a rhodaminyl labeled product was found to be 38 ± 5 kDa as determined by the method detailed in Section 4.3. The M_w of the Rh-OCMβCD-Na obtained from this oligomer was found to be 44 ± 4 kDa. Since weight contribution of the rhodaminyl groups (0.4 w%) present in the florescent sample was negligible with regards to the experimental error, the small difference found between the two M_w came within reasonable limits. The result is reassuring considering the optical properties of the two samples were highly different, which builds confidence in the methodic criteria as described.

This fluorophore labeled product is the subject of further investigations including in vitro and in vivo activity as nanocarrier for anticancer drug delivery and investigations by multiphoton fluorescence microscopy. The presented results demonstrate that even the most fundemental feature such as molecular weight of the sample requires a cautious approach. The consideration of these factors will be crucial to the interpretation and the reproducibility of the future investigations.

5. Conclusions

Some factors affecting the applicability of static light scattering in the accurate determination of average molecular weight of cyclodextrin poly- and oligomers were demonstrated. It was shown that the aggregate state, the characteristics of the segment–segment interaction, the spectral properties (in case of a fluorescent sample) all affect the data obtained by this method. Our work indicates that this method which is widely recognized as absolute requires close control in order to obtain valid results. Both appropriate membrane filtration and the selection of solvent proved to be critical in terms of obtaining representative and accurate data on these substances.

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