PROCEDURE FOR DETERMINATION OF THE MOLECULAR WEIGHT OF CHITOSAN BY VISCOMETRY

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Abstract

The aim of this publication is to facilitate the estimation of chitosan molecular weight (MW) in laboratories with no access to sophisticated analytical instruments, by applying the easily accessible and economical capillary viscometry. The procedure of viscosity-average molecular weight (M_v) determination is described in details. The examples provided encompass testing of the experimental procedure for determination of the M_v of chitosan with a low-molecular weight of 7.7 kDa and 88 kDa, after verification with a high-molecular weight polymer (477 kDa). The experimental work demonstrated the importance of the initial concentration of low-MW chitosan for the accurate determination of intrinsic viscosity and, as a consequence, the viscosity-average molecular weight.

Keywords: *chitosan, viscosity-average molecular weight, Mark-Houwink equation, intrinsic viscosity, capillary viscometry*

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1. Introduction

The applications of chitosan are usually contingent upon the degree of deacetylation (DD) and molecular weight (MW) of the polysaccharide. Various studies on chitosan indicate that low-MW chitosans have a greatly enhanced biological activity when compared to the high-MW polymers. Low-MW chitosans, in the order of several tens of kDa, exhibit more pronounced antimicrobial and antifungal effects [1,2]. Moreover, they stimulate plant growth and simultaneously enable the plants to acquire resistance to pathogens [3].

Many research centres in countries that are major producers of chitosan have carried out extensive research into various types of chitosan, and also oligochitosan. To characterise and compare the manufactured polysaccharides, it is important to accurately determine two essential parameters distinctive of chitosan, i.e., the average molecular weight and degree of deacetylation. Methods of DD determination, including FTIR and UV-Vis spectroscopy, NMR spectroscopy, and a few titration approaches, were extensively compared and critically discussed in our previous report [4]. The precise determination of molecular weight requires sophisticated and expensive equipment, such as HPLC-GPC or laser-light scattering, which is not available in most quality-control laboratories. Nevertheless, one can obtain a good approximation using popular and low-cost methods of viscometry to determine the viscosity-average MW. Unfortunately, the commonly used molecular weight determination procedures are best suited for high- to medium-MW chitosans [5].

The purpose of this work was to test the procedures of molecular weight determination by viscometric methods for their suitability for the evaluation of low-MW chitosans. In the first stage, standard measurements were applied to high-MW chitosan (c.a. 500 kDa), and afterwards, the verified procedure was employed for chitosans of relatively low MW (< 100 kDa).

1. Materials and Methods

2.1 Chemicals/Materials

Medical grade chitosans (CS) were obtained from Heppe Medical Chitosan GmbH (Germany): type 90/5, 90/10 and 90/1000. The nominal weight-average molecular weight of CSs (90/5), (90/10) and (90/1000) was in the range 10–80 kDa, 20–100 kDa and 200–500 kDa, respectively, as stated by the manufacturer, and the deacetylation degree was 90%, 84% and 86%, respectively, as determined by potentiometric methods [4,6]. Acetic acid and ammonium acetate (analytical grade) (Avantor Performance Materials Poland) were used as received. Ultrapure water from the TKA Micropure Water system (1.15 μS/cm) was used for the preparation of solutions.

2.2. Preparation of solutions

Chitosan is a hygroscopic material which may contain over 10% water when stored in ambient conditions of laboratory air humidity. For molecular weight measurements, the concentration of chitosan solutions should be precisely known [6]. For the preparation of solutions, this requires chitosan to be thoroughly dried for accurate analytical sample weighting, or water content in a given sample to be known in order to calculate the appropriate correction. Chitosan should be dried in a vacuum drying oven at 40ºC until the difference in weight in subsequent measurements is lower than 0.5%.

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In the first stage of the procedure, the solvent was prepared. For this purpose, 2.891 g of ammonium acetate was weighed and placed in a 250 cm^3 flask and then 50 cm^3 of distilled water was added. After this, the calculated volume of acetic acid was added to a final concentration of 0.05 mol dm^{-3}, and then the flask was made up to the mark with water, resulting in a pH of 4.5. Ca. 0.100 g of dry chitosan was weighed precisely using an analytical balance and transferred into a 100-ml volumetric flask. Then, ca. 80 cm^3 of the solvent of 0.2 M acetic acid/0.15 M ammonium acetate (pH 4.5) was poured into the flask. The flask was closed and the contents were stirred at room temperature to ensure the complete dissolution of the polysaccharide. After 24 h, the flask was made up to the mark with solvent.

In order to remove any undissolved residues, filtration was applied. For this purpose, a filter paper (Munktell type, 389) was vacuum dried in a weighing bottle until constant mass. The vessel was always closed with a lid upon removal from the drier, which prevented moisture absorption. The chitosan solution was filtered through the paper filter placed in a glass funnel. The filter was rinsed once with solvent and twice with distilled water. Then, the wet filter was transferred to the weighing bottle and dried in an air dryer; after two days, it was dried further under vacuum to a constant mass. The mass of undissolved chitosan was determined and applied for calculation of the concentration correction. The filtrate was used for viscosity measurements.

2.3 Procedure of determination of intrinsic viscosity of chitosan by Ubbelohde viscometer

Viscosity measurements were carried out with the AVS-310 automatic viscometer (Schott Geräte) equipped with the Ubbelohde type 531 10/I capillary. The temperature of the water bath was stabilised at 25.0 ± 0.1ºC and monitored for at least 10 minutes before the first measurement. Directly before measurements, the chitosan solutions and the solvent were filtered, respectively, twice through a 0.45 μm pore size filter (Minisart, 16555K) and three times through a 0.2 μm pore size filter (Minisart, 16534K). Then, 10 ml of filtered solvent (acetic acid/ammonium acetate) was placed into the Ubbelohde viscometer using a glass pipette. The measurements of flow time of the solvent were carried out 5 times. The time flow of pure solvent was 98.35 ± 0.15 s. After rinsing and drying the capillary, 10 ml of the filtered chitosan solution was placed in the viscometer. Flow time measurements of chitosan solutions, at a series of concentrations, were conducted. Each solution was measured 5 times. Then, 10 ml of the filtered solvent was added to the viscometer and the flow time of the resulting diluted solution was measured five times. The dilution procedure was repeated three more times, adding 10 ml of solvent each time. Due to the pressure difference at the top and bottom of the column of liquid, the capillary corrections of flow times according to capillary type were introduced in the calculations, using the formula provided by the capillary manufacturer.

In the first step, the arithmetic averages of the five individual flow times were calculated for solvent and chitosan concentrations and denoted as t_0 and t_i, respectively. The measured relative flow times t_i/t_0 approximate the real ratio of dynamic viscosities of η_i/η_0 (dynamic viscosity is expressed in $[Pa{\cdot}s]$). Next, for each chitosan concentration, the following viscosities were calculated using the respective formulas: (formula 1) relative viscosity η_r, (2) specific viscosity η_{sp} , (3) reduced viscosity (viscosity number) η_{red}, and (4) inherent viscosity η_{inh}.

$$\eta_r = \frac{t_i}{t_0} \tag{1}$$

$$\eta_{sp} = \frac{t_i - t_0}{t_0} = \frac{t_i}{t_0} - 1 = \eta_r - 1 \tag{2}$$

$$\eta_{red} = \frac{\eta_{sp}}{c} \quad (3)$$

$$\eta_{inh} = \frac{ln\eta_r}{c} \quad (4)$$

In order to evaluate the intrinsic viscosity [η], two sets of data of reduced viscosity (η_{red}) and inherent viscosity (η_{inh}) were plotted in one graph as a function of chitosan concentration *c*. Then, a rectilinear fitting regression, with a formula y = a*c* + b, was made for each set of data. The value of intercept of each of the fitted straight lines with ordinate axis, i.e. b, resulted from the extrapolation of the concentration to zero, [η], in equations (5) and (6) [5,7]. The intrinsic viscosity used in further calculations was averaged from the two obtained intercept values.

$$[\eta] = \lim_{c \to 0} \eta_{red} \quad (5)$$

$$[\eta] = \lim_{c \to 0} \eta_{inh} \quad (6)$$

The intrinsic viscosity [η] depends on the specific volume of the polymer, which is related to its molecular weight, and the polymer-solvent interactions. The relationship between the intrinsic viscosity [η] and viscosity-average MW, that is M_v, is expressed by the Mark-Houwink equation

$$[\eta]=KM_v^{\alpha} \quad (7)$$

where K and α are constants that are characteristic for a particular polymer–solvent system at a specific temperature and [η] is the intrinsic viscosity of the polymer in that solution. Constants K and α are dependent on the temperature and deacetylation degree of chitosan and are valid for a certain range of molecular weights [8]. They are tabulated for common polymer-solvent pairs or available in the literature referring to individual polymers. For the utilised chitosans, with respect to DDs and molecular weights, and the applied solvent of acetic acid/ammonium acetate, the respective K and α (Table 1) parameters were determined by interpolation of the data collated in Figure 3 of reference [9], showing the dependence of log[η] = f(log M_w), where M_w stands for weight-average MW for various DD values. The absolute method, i.e. laser light scattering, was used for the determination of molecular weight; thus, M_w values served for [η] determination in a wide range of MW. Calculations of K and α based on the empirical equation provided by Kasaai in [8], elaborated based on literature data for chitosans of various molecular weight and DA in diverse solvents, may also be valid for the currently examined samples. Nevertheless, the certain Mark-Houwink parameters for particular solvent-polymer pairs may differ somewhat from those derived from the general equation, i.e. there may be a relatively high error. In fact, we verified the Mark-Houwink parameters calculated based on ref. [9], with those derived from the approach proposed in [8]. We found them to be very consistent – the example for 90/10 chitosan: K and α calculated form the Kasaai formula was equal to $1.50 \cdot 10^{-5}$ dm^3/g and 0.776, respectively. Thus, this also confirms the appropriateness of a general approach proposed in [8] for the currently used solvent.

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3. Results and Discussion

The straightforward procedure defined above can be used to easily determine the molecular weight of chitosans in laboratories without sophisticated analytical instruments. This method is relatively cheap, and the analysis can be performed using a common Ubbelohde capillary viscometer. The methodology described in point 2.3 is basically well suited for the determination of high- and medium-MW chitosan, particularly for the direct product obtained from chitin deacetylation. However, it is was not known whether the method can be used for chitosans with molecular masses of several tens of kDa. In many large-scale chitosan applications, such as plant protection, for instance, polysaccharides of low MW are preferred due to the enhanced biological activity of short chains; thus, the degraded chitosan is produced and available commercially. Therefore, it is important to establish a verified methodology for the determination of MW, within a wide range, as the universal tool, in order to enable the direct comparison of chitosan specifications from various laboratories. This can be effectively utilised by professionals, both in science and in industry laboratories. The above-mentioned procedure is based on the measurement of intrinsic viscosity of chitosan in a common solvent. The 0.2 M acetic acid/0.15 M ammonium acetate is probably among the best solvents elaborated so far, with respect to the molecular dissolution of chitosan and prevention of aggregation [9,10].

One may also try to estimate the quality of the solvent. Using equations 5 and 6, Huggins constant, K_H and Kraemer constant K_K can be determined [11-13]. It is assumed that for a good solvent and flexible polymer, K_H is of c.a. 0.4, and the theoretical relation of $K_H + K_K$ should equal 0.5. The values of K_H calculated for presently used polymer-solvent pairs are in the range from 0.54–0.78 and values of the $K_H + K_K$ sum equal to 0.47–0.74 (Table 1). This, combined with the knowledge that the α values are close to 0.8, indicates that the chosen solvent is thermodynamically good for the chitosan samples. For inflexible polymers like chitosan, even though the high charge density along the chain is partially neutralised by counter-ions, polysaccharides have a relatively rigid structure; also, with the reduction of molecular weights of chitosan, K_H are normally higher [14,15]. Moreover, the second virial coefficient A_2 for used solvents and for the different chitosan samples of the DD over 80% (mainly with respect to molecular weight) was always greater than 0, typically in the order of c.a. $5 \cdot 10^{-4}$ mol cm^3/g^2, as determined by the static method of laser light scattering [16].

In order to demonstrate the applicability of the viscometric method for the evaluation of low-MW chitosan, in the first stage, measurements according to the original procedure were made for high molecular weight chitosan (CS 90/1000; nominal M_w of 200-500 kDa). Figure 1 presents the data of reduced and inherent viscosities as a function of chitosan concentration in the acetic acid/ammonium acetate solvent. Extrapolation of the experimental values of reduced and inherent viscosities to zero concentration according to equations (5) and (6) enables the determination of intrinsic viscosity. In Figure 2, data based on using the same approach to determine [η] for low molecular weight chitosan (CS 90/10, nominal M_w of 20-100 kDa) are presented in the said coordinates. The initial concentration of both chitosans was 1 g/dm^3.

In the case of 90/1000 chitosan, the data can be fit relatively well with a straight line; characterised by a relatively high result, as for this experimental method, Pearson's correlation coefficient (r) for reduced viscosity was equal to 0.94. In the case of low-MW chitosan (90/10), the obtained data obviously do not follow a straight line, but are rather arranged in a parabola (r = 0.78) [Fig. 2]. This indicates that the outflow times from the capillary for these polymer solutions are too similar to the solvent outflow time. Calculations based on theseresults would be burdened with a large error.

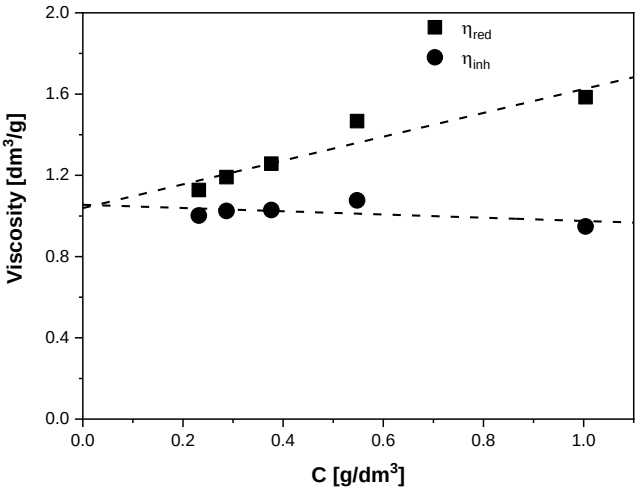

Figure 1. Determination of intrinsic viscosity of high-MW chitosan. Reduced and inherent viscosities as a function of chitosan concentration; initial concentration 1 g/dm^3 in acetic acid/ammonium acetate solvent.

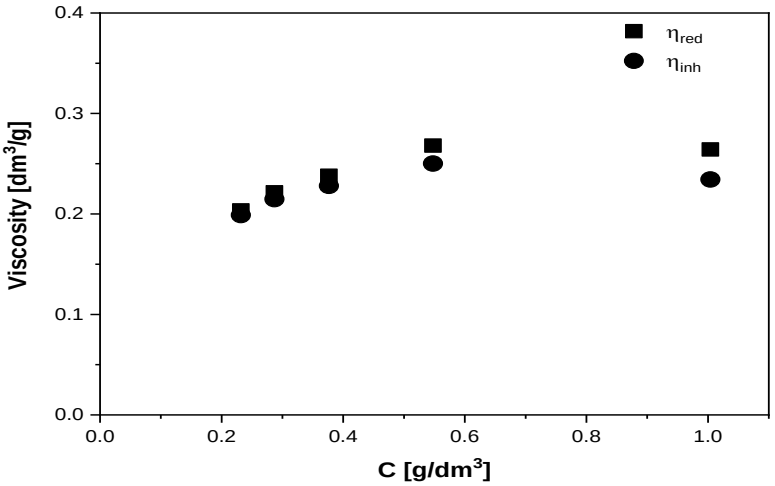

Figure 2. Determination of intrinsic viscosity of chitosan 90/10. Reduced and inherent viscosities as a function of chitosan concentration; initial concentration 1 g/dm^3 in acetic acid/ammonium acetate solvent.

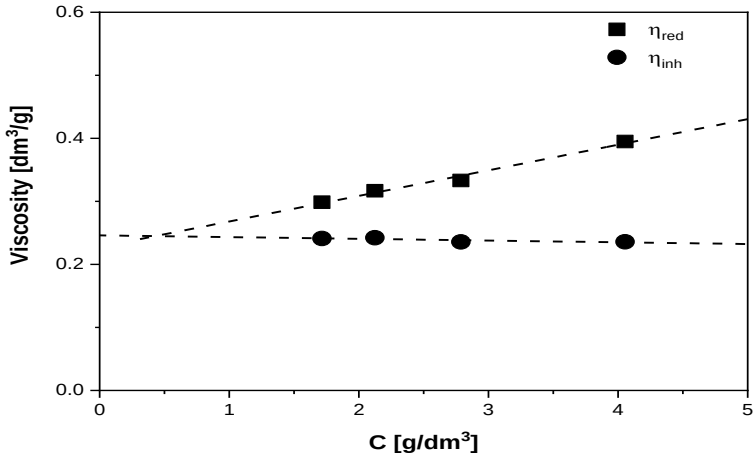

Figure 3. Determination of intrinsic viscosity of chitosan 90/10. Reduced and inherent viscosities as a function of chitosan concentration; initial concentration 4.05 g/dm^3 in acetic acid/ammonium acetate solvent.

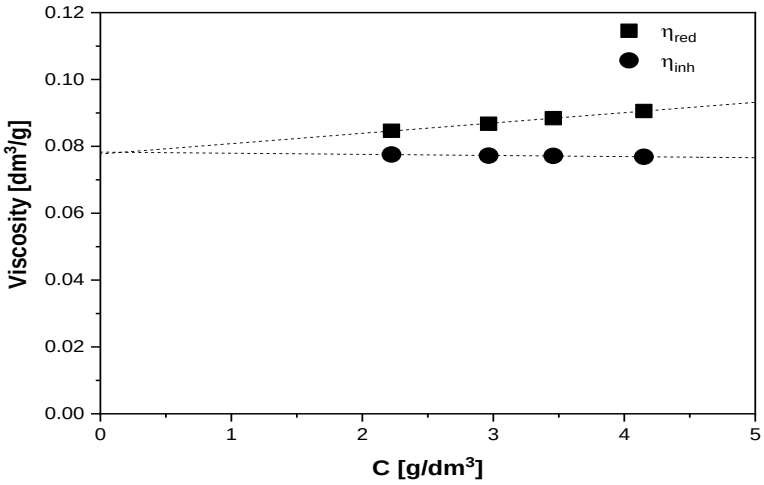

Figure 4. Determination of intrinsic viscosity of chitosan 90/5, Reduced and inherent viscosities as a function of chitosan concentration; initial concentration 4.25 g/dm^3 in acetic acid/ammonium acetate solvent.

In principle, the outflow time of the polymer solution at the initial concentration should be at least 1.5 times higher than that of the neat solvent, and the optimum may be ca. 2.5-fold greater [11]. Therefore, in such cases, it is recommended to considerably increase the initial concentration of low-MW chitosan, while taking care not to exceed the chain overlap concentration c* (critical concentration, $c^* \approx 1/[\eta]$). In the present case, the concentration was increased to 4.05 g/dm^3 for low molecular weight chitosan 90/10; note that the initial concentration in the first experiment was 1 g/dm^3.

Additionally, viscosity measurements have also been performed for chitosan (type 90/5) of still lower molecular weight than chitosan 90/10. The initial concentration of that chitosan was set to ca. 4.25 g/dm^3. The resulting data of reduced and inherent viscosities are shown in Figures 3 and 4 as a function of the low-MW chitosan concentration *c*.

Upon an increase of the initial concentration of low-MW chitosans by 4.05- and 4.25-fold, the values of η_{red} and η_{inh} evidently show a better linear correlation with polymer concentration. The coefficient of determination, r, becomes comparable to that for high-MW chitosan, r = 0.98 (90/10) and 0.99 (90/5). This enables the accurate determination of the intrinsic viscosity. The relatively high initial concentration of low-MW chitosan solution allowed the viscosity of this solution to be increased, and thus the difference between the outflow time of the solution and that of the solvent to also increase; the results therefore become reliable.

The values of intrinsic viscosity obtained from the extrapolation of respective viscosities to zero concentration and the viscosity-average molecular weights calculated using equation (7) are collected in Table 1. It is expected that the polymers with a molecular weight distribution (M_w/M_n, the subscript n stands for the 'number') higher than 1, as for non-fractionated natural polymers, are characterised by lower values of M_v than values of M_w. The obtained results show that the viscometric method may be used for the estimation of viscosity-average MW of chitosans with even a low molecular weight if the initial polymer concentration is adequately adjusted to provide sufficiently high relative viscosity and if the K and α parameters are known or can be computed.

Table 1. Intrinsic viscosities and viscosity-average molecular weights of evaluated chitosans.

Chitosan	**90/1000**	**90/10**	**90/5**
DD [%]	86.1	84.2	90.2
Molecular weight given by producer [kDa]	200–500	20–100	10–80
K_K	-0.071	-0.045	-0.055
K_H	0.538	0.785	0.650
K_K+K_H	0.467	0.740	0.595
K* [dm^3/g]	$4.28\cdot10^{-5}$	$3.64\cdot10^{-5}$	$9.50\cdot10^{-5}$
α*	0.773	0.780	0.750
[η] [dm^3/g]	1.047	0.261	0.078
M_v [kDa]	477	88	7,7

* K and α values were determined from data in Figure 3 of reference [9].

4. Conclusions

Standard protocols for the viscometric determination of average molecular weights are intended for high-MW chitosans, in the order of several hundred kDa; nevertheless, they fail to provide accurate results for low-MW samples. A simple modification of the methodology by a significant increase in the initial polymer concentration enables reliable results to also be obtained for chitosans of molecular weights in the range of a few kDa. This modified method may be well adapted in laboratories not equipped with complex analytical instruments.

5. Acknowledgements

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6. References

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