Suplementary information to "Strategy to improve the characterization of chitosan for sustainable biomedical applications: SAR guided multi-dimensional analysis"

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I. RESULTS AND DISCUSSION

Dissolution behavior Dissolution of chitosan superstructures is limited to a few solvents because they are stabilized through hydrogen-bonds. The number of hydrogen-bonds increases with molecular mass and becomes tremendous at masses of several 100,000 Da. A complete dissolution of large polymers can only be achieved if the desired dissolution time is rather long. We observed this for a chitosan with a molecular weight of 500,000 g/mol diluted in acetic acid. Here a dissolution time of 24h under slight shaking is mandatory. If the samples had only a few hours time to dissolve we could detect an asymmetric peak by size-exclusion chromatography (SEC) with a cut-off for the high molecular weight molecules (Fig.1a).
After dissolution some chitosan aggregates remain in solution and can be removed by filtration if their size is large enough. Without filtration aggregates have a significant impact on the determination of the M_w and $[\eta]$ (Fig.1b).

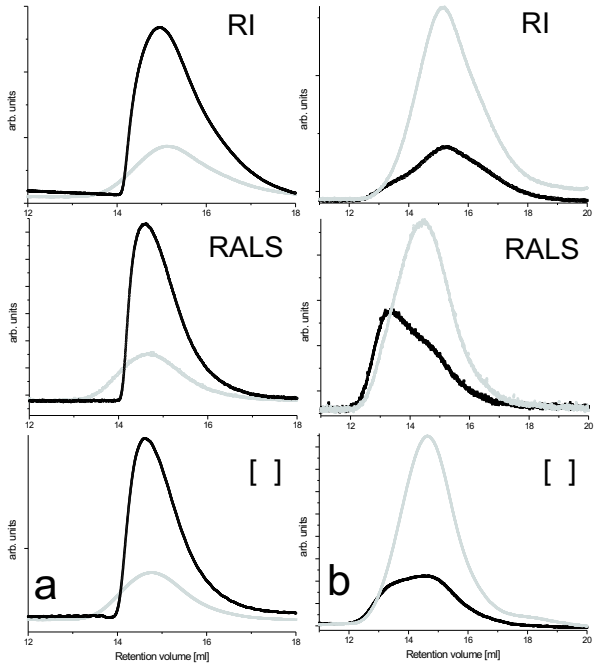

FIG. 1: (a): Comparison between chitosan dissolved for 4h (black) with the same sample after dissolution time of 24h (grey). The picture shows the detector response of the refractive index (RI), light scattering (RALS) and viscosity ($[\eta]$) detector. If the dissolution time is too short the high molecular weight part of the sample will be cut-off by subsequent filtration. This cut-off can be seen in the chromatogram (13-14 ml) and leads to underestimation of the M_w. (b): Detection of aggregates for an unfiltered sample (black) in comparison to a 0.45 μm filtered one (grey). Aggregates occur after dissolution of the chitosan and can be detected as shoulders at low retention volume by all three detectors from a size-exclusion chromatogram. Aggregates can be removed by filtration which yields in symmetric peaks and reproducible results.

Selection of methods for F_A and M_W determination During the last decades of chitosan research many methods were proposed to determine the fraction of the acetyl groups (F_A) and molecular weight (M_W). Although these are two of the most important parameters, no standard technique could be established to generate reliable values. This is mainly deduced by some chitosan typical drawbacks: poor solubility, polyelectrolyte behavior and its polymeric character. Due to the absence of standard analysis techniques many methods were proposed to overcome the deficiency of the unknown parameters. To inform the reader which methods can be applied and which methods

were used throughout this work, we will briefly discuss and summarize the used techniques described in the literature. For the determination of F_A up to 14 different methods have been applied namely IR spectroscopy [1, 2], colloid titration [3], pyrolysis GLC [4], UV-VIS [5], ^{1}H-NMR [6–8], ^{13}C-NMR [9], thermal analysis [10], circular dischroism [11], enzymatic hydrolysis [12], picric acid assay [13], acid hydrolysis GC [14], acid hydrolysis HPLC [15], ninhydrin test [16] and X-ray diffraction [17]. Many methods are limited in their application for different (insoluble/soluble) chitin/chitosan samples. No method is able to study the whole F_A range (from 0 to 1) combined with a very good accuracy. For example, IR spectroscopy has shown to be a fast and useful method for solid samples, but needs to be calibrated with NMR and its applicability is therefore limited. Furthermore there are uncertainties about the best baseline setting and selection of peaks for analysis (amide, amino, hydroxyl). Fast results combined with the best accuracy were found for liquid state ^{1}H-NMR. On the contrary, NMR can not be used for chitin because this technique is limited to soluble material only. Nevertheless NMR became the most used and the most reliable technique for F_A determination in science over recent years. Thus, ^{1}H-NMR was chosen in this study for the F_A determination. Additionally ^{13}C-NMR was used to determine also the pattern of acetylation P_A. Due to good solubility of all our samples this method was applicable without any exceptions.

Unfortunately no method has received general acceptance for the molecular weight determination of chitosan like NMR for the F_A determination. There is still a lot of uncertainty about the proper method giving realistic molecular weight values. Usually values from different methods are incomparable to each other and vary dramatically. Here methods like batch viscosity measurements [18], batch SLS measurements [19–22], RI-SEC (Pullulan calibration) [23–25], SEC-MALS [21, 26–33], batch MALS [34–36], SEC-LALS [37–39] and ultracentrifugation [20, 32, 40] are proposed in the literature. Some of the methods are applicable for synthetic and natural polymers but difficult to use in the context with chitosan due to the fact that chitosan is a naturally occurring polyelectrolyte, with attributes like self-aggregation and column adsorbing behavior. The solvent conditions have to be adjusted carefully to avoid precipitation (acidic solvent), aggregation (addition of salt, filtration), and adsorption on the column material (addition of organic solvent, selection of appropriate column material, addition of salt). If the chosen solvent supports or can not avoid aggregation, all methods can lead to a higher molecular weight when aggregates were not removed. Chromatography is advantageous in comparison to batch methods because here a separation of aggregate signals from sample signals may possible. Additionally, chromatography opens up another very important parameter which can only be achieved by chromatographic methods: polydispersity.

Throughout this work we apply a triple detector (SEC^3) chromatographic setup which combines the M_W determination with a quick conformational analysis (Mark-Houwink-plot). As already emphasized, aggregates can influence the viscosity measurement (Fig. 1) and due to this fact we prefer to control the measurement of the intrinsic viscosity $[\eta]$ together with the size determination. Online viscometers measure the viscosity distribution for each monodisperse separated fraction of the polydisperse sample while offline batch measurements give only the mean viscosity of the whole molecular weight distribution. Thus, the Mark-Houwink-plot obtained by SEC^3 shows a more reliable conformational analysis than a plot obtained from batch measurements.

Selection of the refractive index increment dn/dc For correct molecular weight determination using light scattering techniques a reliable refractive index increment dn/dc is essential. However, the correct determination of the dn/dc for a polydisperse mixture of different chitosan entities is a delicate thing. Impurities (inorganic material, pigments, water content) and especially an broad molecular weight and F_A distribution can influence dn/dc determination. Published results on dn/dc values differ like the chitosan preparations, used for this analysis, differ for nearly all possible parameters. Values from 0.142 up to 0.208 were found and used for the M_w calculation. Small changes in dn/dc can be explained by its dependance on temperature, solvent and laser-wavelength but the rather huge difference between 0.142 and 0.208 is still unexpected. The fact that there is some uncertainty which dn/dc is useful for chitosan we took a more thorough look to related polysaccharides. If a change in the chemical environment of a polysaccharides occurs one can expect also a change in its dn/dc. Values for Alginate (0.158 - 0.165), Amylopectin (0.142 - 0.156), Amylose (0.146), Carboxymethylcellulose (0.147 - 0.163), Carragenan (0.140), Dextran (0.136 - 0.150), Ethylcellulose (0.154), Hyaluronic acid (0.155 - 0.176), Starch (0.146 - 0.152) and Pullulan (0.137 - 0.147) were found for laser wavelength between 436 - 633 nm in aqueous solution with varying salt contents (all values were taken from [41]). The comparison revealed that completely different polysaccharides show no big disparity in their dn/dc. Expecting the same for chitosan, a value comparable to those polysaccharides values was taken (0.163 reported from Rinaudo et al. [42]). Substance recovering rates, based on this value, were determined after every SEC run and proved to be between 75 % and 110 %. Comparing these recovery rates with the standard deviation of the weighing procedure and with the impurity content of the samples, the dn/dc selection was confirmed.

Impurities of chitosan preparations

TABLE I: Heavy metal content of different chitosan preparations. Zero values indicate a content below the detection limit.

Chitosan preparation	**Cd [mg/kg]**	**Pb [mg/kg]**	**U [mg/kg]**	**Cr [mg/kg]**	**Co [mg/kg]**	**Ni [mg/kg]**	**Cu [mg/kg]**	**Zn [mg/kg]**	**As [mg/kg]**
Chitosan C	0.02 ±2.6%	1.46 ±1.0%	1.19 ±1.7%	33.11 ±4.1%	0.51 ±4.6%	11.89 ±2.9%	2.99 ±2.6%	9.38 ±2.2%	0.08 ±6.3%
Chitosan H	0.01 ±4.2%	0.09 ±0.9%	0	4.65 ±4.3%	4.58 ±4.6%	4.17 ±3.5%	134.2 ±2.1%	14.41 ±1.2%	0.09 ±6.7%
Chitosan P	0.01 ±5.2%	0.10 ±0.8%	0	15.99 ±0.2%	0.26 ±0.1%	13.09 ±1.2%	0.59 ±5.8%	1.31 ±1.5%	0.03 ±10.7%
Chitosan AF	0.02 ±1.8%	0.25 ±1.5%	0.04 ±2.6%	11.77 ±3.7%	0.48 ±3.9%	8.60 ±2.0%	17.18 ±2.8%	37.99 ±2.3%	0.02 ±15.9%
Chitosan D	0.06 ±1.8%	1.74 ±0.7%	0.14 ±2.4%	12.51 ±1.9%	0.11 ±3.1%	5.53 ±1.8%	1.59 ±1.8%	6.41 ±2.0%	0.49 ±3.1%
Chitosan AQ	0	0.30 ±0.8%	0	1.31 ±3.7%	0.02 ±5.7%	3.60 ±3.8%	8.65 ±3.2%	3.15 ±1.3%	0.02 ±7.8%

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