Chitosan hydrophobic domains are favoured at low degree of acetylation and molecular weight

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ABSTRACT

The aggregation of chitosan (CS) has been studied as a function of concentration, degree of acetylation (DA), and degree of polymerization (DP) by means of pyrene fluorescence and rheology. Fluorescence experiments show that aggregation of CS involves hydrophobic domains (HD) which are more favoured as lower the DA and DP. Consistent with these results, the viscosity of CS solutions decreases continuously on increasing DA, in the whole range of DP. These results, which rule out the participation of the acetyl groups in the HD, have been interpreted by the theory of hydrophobic polyelectrolytes in terms of the electrostatic energy of the aggregates.

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1. Introduction

Chitosan (CS, Fig. 1) is a linear cationic polysaccharide obtained by N-deacetylation of chitin, a structural component of crustacean shells and fungal cell walls that is commercially obtained at low cost from seafood processing. CS is composed of variable proportions of β(1–4) linked glucosamine and N-acetylglucosamine [1]. Thanks to its low toxicity, and high biodegradability, biocompatibility, and mucoadhesion, CS has emerged as an interesting biopolymer in drug delivery [2,3], tissue engineering [4,5], biofabrication [6], and the food industry [7,8]. The applications of CS mostly depend on its tendency to aggregate in solution, a process controlled by external (pH, ionic strength, temperature) and structural [degree of acetylation (DA), degree of polymerization (DP)] parameters. As a result, much effort has been devoted to study the aggregation of CS by different techniques. Rheological studies have shown that the semidilute regime in CS starts at *ca.* 1 g/L (C^*, depending on DA and DP). At higher concentrations, CS chains start entangling (5–13 g/L, C^{**}) to finally form gels at *ca.* 45 g/L [9,10]. Liquid crystal properties for CS have been also observed by polarizing microscopy in more concentrated solutions [11,12].

The presence of aggregates in solutions of CS has been reported by static light scattering (SLS) [13,14] and dynamic light scattering (DLS) [15–19]. In a recent contribution, Boucard and co-workers have analysed a series of CS samples by small-angle synchrotron X-ray scattering (SAXS) in the semidilute regime [20]. In this study, the upturn at low angle range demonstrates that the solutions are heterogeneous and consist of well dissolved polymer chains that coexist with aggregated domains. In addition, the position of the maximum of the polyelectrolyte scattering peak scaled with the polymer concentration as predicted by the theory of hydrophobic polyelectrolytes developed by Dobrynin and Rubinstein [21–23] pointing to a pearl-necklace conformation for CS in solution. Nevertheless, neither the driving force for these ordered structures in solution nor the role of DA and DP on the aggregation of CS are fully understood yet. Thus, while some SLS experiments have shown an increased aggregation of CS with DA (interpreted as resulting from the formation of hydrophobic interactions between N-acetyl groups) [13], more recent DLS studies have resulted in no differences in aggregation with DA [17]. Several research groups have also observed the presence of hydrophobic domains (HD) in solutions of CS by means of fluorescence experiments using pyrene as a polarity probe. Unfortunately, samples with very close DA values were analysed, whereas the influence of DP on aggregation has been a subject of discrepancy [24–27]. In a more recent contribution it has been described the use of pyrene fluorescence with a series of CS samples having similar DP and varying DA (0–56). These experiments did not

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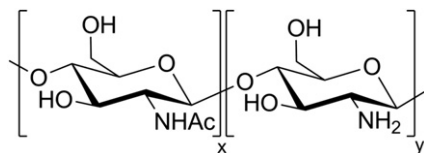
Fig. 1. Chitosan (CS).

show a linear dependence of aggregation with DA, but revealed aggregation for samples with DA 0, indicating that interactions between *N*-acetyl groups might not be the main reason for aggregation [28]. Hydrogen bonds between CS chains which are known in the crystal structure [29] have been also claimed to participate in the aggregation of the polymer in solution [24,25].

In this context, to further develop the applications of CS as a biocompatible polymer, there is an urgent necessity of additional studies to unravel the interactions operating in solutions of CS at a molecular level as a function of DP and DA. In a recent contribution from our laboratory, we have reported a ^{1}H NMR relaxation study on the dynamics of CS (DA 1–70, DP 10–1200) in solution as a function of the temperature, concentration, and ionic strength [30]. This analysis pointed to CS as a semi-rigid polymer with increased flexibility at higher DA, in agreement with a reduced electrostatic repulsion between protonated amino groups. As a step further in our efforts to ascertain the behaviour of CS in solution and its applications in drug delivery [31–33], herein we report a detailed study of the aggregation of CS with different DP (360–1200) and DA (0–70) by means of pyrene fluorescence, rheology, and size exclusion chromatography-multiangle laser light scattering (SEC-MALLS). Notably, our results indicate the aggregation of CS involves HD which are more favoured at lower DA and DP [34].

2. Experimental

Three commercial CS samples with different DP and DA were purchased and denoted as C_{x-y}, where *x* represents the DP, and *y* the DA. Sample CS_{360-7} was obtained from FMC BioPolymer as hydrochloride salt: Protasan Cl 110 (batch number 310-490-01). Samples CS_{730-17} and $CS_{1200-20}$ were obtained from Fluka: CS low MW (catalogue number 22741, lot 407568/1) and CS high MW (catalogue number 22743, lot 371936/1), respectively. These commercial samples were dissolved in 0.5% (*w/v*) AcOH and purified by sequential dialysis against 10^{-3} M HCl, 5.5×10^{-3} M NH$_4$OH, and deionized water. After lyophilization, CS samples were obtained in their deprotonated form.

DA values were determined in 2% DCl at 298 K, or in 20% DCl at 343 K for samples with DA over 40 [35,36]. Determination of the GlNAc distribution in commercial samples was carried out by analysis of the relative intensities of the dyad frequencies in ^{13}C NMR [37], using modified acquisition parameters (62.9 MHz, 200.000 scans, 0.1 s acquisition time, no relaxation delay). With this aim, commercial CS samples were dissolved in 0.16 M TFA-*d* (deuterated trifluoroacetic acid) (30 g/L) and treated with 1 M NaNO$_2$ to reduce the DP. The analysis showed a homogeneous distribution of *N*-acetylglucosamine units along the polymer chains.

N-Deacetylation of CS. Finely grounded CS samples were suspended with stirring in an aqueous 40% NaOH solution at 333–353 K, under a N$_2$ stream. After 15–45 min, the reaction mixture was filtered, washed thoroughly with warm deionized water (333 K), and dried overnight under vacuum (P$_2$O$_5$). The process was repeated until the desired DA was obtained [38].

N-Acetylation of CS. CS samples were homogenously *N*-acetylated following known procedures [39]. Briefly, CS samples were dissolved (10 g/L) in a 1:1 mixture 0.5% (*w/v*) AcOH/1,2-propanediol at rt. Then, in order to reach a certain DA value, a freshly prepared solution of Ac$_2$O in 1,2-propanediol was added. The resulting solution was stirred at rt for 2 h. After sequential dialysis against 10^{-3} M HCl, 5.5×10^{-3} M NH$_4$OH, and deionized water, the CS solution was lyophilized.

Fluorescence experiments. 10^{-6} M pyrene solutions in aqueous 0.056 M TFA or 0.2 M AcOH/0.15 M NH$_4$OAc buffer (pH 4.5) were prepared as follows: 200 μL of a 2×10^{-2} M pyrene solution in CH$_2$Cl$_2$ were added to a 5 L round bottom flask followed by 200 mL of CH$_2$Cl$_2$. After mixing, the solvent was evaporated and the flask submitted to high vacuum for 1 h. Then, 4 L of 0.056 M TFA or 0.2 M AcOH/0.15 M NH$_4$OAc were added. After 72 h of stirring, solutions were filtered through a 0.45 μm pore size membrane. These aqueous solutions of pyrene were then used to dissolve CS at a concentration 8–11 g/L. These solutions were subsequently diluted stepwise down to 10^{-4} g/L (before each dilution step, samples were stirred for at least 8 h). Fluorescence experiments were performed on a Spex Fluoromax spectrometer. Emission spectra of pyrene were obtained by exciting samples at 333 nm and measuring the emission between 340 and 550 nm. The slit width was adjusted to 5 nm for the excitation and 1.5 nm for the emission with an integration time of 1 s/nm.

Rheology. The rheological behaviour of the CS samples was determined in a rheometer (Rheolyst AR1000N, TA Instruments, New Castle, DE, USA) equipped with a data analyser AR2500 and a Peltier plate, a cone-plate geometry with a diameter of 60 mm, 2.1° and a 59 μm gap to the Peltier plate. The shear viscosity was measured between 0.1 and 100 s^{-1}. The response to an oscillatory shear stress (*G'* and *G''*, storage and loss moduli) was determined by applying an oscillatory shear force of 0.1 Pa and an angular frequency sweep between 0.05 and 50 rad s^{-1}. CS samples were dissolved in 0.056 M TFA, 24 h before measurements. In addition, samples CS_{1200-3} and $CS_{1200-70}$ were also analysed 12 days after being dissolved.

SEC-MALLS. An Iso Pump G1310A (Hewlett Packard) was connected to two PSS Novema GPC columns (10 μm, 30 Å, 8 × 300 mm; and 10 μm, 3000 Å, 8 × 300 mm). A PSS SLD7000 MALLS detector (Brookhaven Instruments Corporation) operating at 660 nm and a G1362A refractive index detector (Agilent) were connected on line. A 0.15 M NH$_4$OAc/0.2 M AcOH buffer (pH = 4.5) was used as eluent. Polymer solutions were filtered through 0.2 μm pore size membranes before injection. Polymer concentrations were in the range 0.1–5 g/L depending on DP and DA. Refractive index increments d*n*/d*C* were taken from the literature [40–42].

3. Results and discussion

A set of 19 CS samples covering 3 different DP (360, 730, 1200) and DA values 0–70 were prepared from 3 commercial samples, namely CS_{360-7}, CS_{730-17}, and $CS_{1200-20}$, and characterized by size exclusion chromatography-multiangle laser light scattering (SEC-MALLS) (Table 1). The analysis of the relative intensities of the dyads in the ^{13}C NMR spectra of these commercial samples assures a homogeneous distribution of *N*-acetylglucosamine units along the polymer chain [37]. From these samples, CS with varying DA was obtained by *N*-acetylation [39] and *N*-deacetylation [38], following procedures ensuring a homogeneous distribution of *N*-acetyl groups.

Pyrene fluorescence. As mentioned in the Introduction, the presence of HD in solutions of CS has been studied in the past by fluorescence using pyrene as a hydrophobic probe [24–28]. From these studies, however, no clear dependence of aggregation on DA and DP has resulted. With the purpose of shedding light on this relevant property of CS, we have recorded steady state emission spectra of a whole set of CS samples with different DA and DP (CS_{360-1}, CS_{360-19}, CS_{360-39}, CS_{360-70}, CS_{730-0}, CS_{730-6}, CS_{730-17}, CS_{730-70}, CS_{1200-1}, $CS_{1200-20}$, $CS_{1200-70}$), dissolved at different