

Tuning the properties of high molecular weight chitosans to develop full water solubility within a wide pH range.

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The preparation of chitosans soluble in physiological conditions has been sought for years, but so far solubility in non-acidic aqueous media has only been achieved at the expense of lowering chitosan molecular weight. In this work, we applied the multistep ultrasound-assisted deacetylation process (USAD process) to β -chitin and obtained extensively deacetylated chitosans with high molecular weights ($\overline{M}_w \geq 1,000,000 \text{ g mol}^{-1}$). The homogeneous *N*-acetylation of a chitosan sample resulting from three consecutive USAD procedures allowed us to produce chitosans with a high weight average degree of polymerization ($\overline{DP}_w \approx 6,000$) and tunable degrees of acetylation (\overline{DA} from 5 to 80%). *N*-acetylation was carried out under mild conditions to minimize depolymerization, while preserving a predominantly random distribution of 2-amino-2-deoxy-D-glucopyranose (*GlcN*) and 2-acetamido-2-deoxy-D-glucopyranose (*GlcNAc*) units. This close to random distribution, inferred with deconvolution of nuclear magnetic resonance (¹H NMR) spectra, is considered as responsible for the solubility within a wide pH range. Two of the highly *N*-acetylated chitosans ($\overline{DA} \approx 60 \%$ and $\approx 70 \%$) exhibited full water solubility even at neutral pH, which can expand the biomedical applications of chitosans.

Abstract

1. Introduction

Many uses have been reported for the natural polymer chitosan, a linear $\beta(1\rightarrow4)$ -linked copolymer of 2-amino-2-deoxy-D-glucopyranose (*GlcN*) and 2-acetamido-2-deoxy-D-glucopyranose (*GlcNAc*) units, mostly owing to its polycationic character (Chang, Lin, Wu, & Tsai, 2015; Muñoz-Bonilla & Fernández-García, 2012; Roberts, 1992). Chitosan is found on the cell walls of some fungi, but is usually produced from *N*-deacetylation of the abundant polysaccharide chitin under alkaline conditions (Fiamingo, Delezuk, Trombotto, David, & Campana-Filho, 2016; Lamarque, Lucas, Viton, & Domard, 2005; Roberts, 1992). Chitin exists in crustacean shells, mollusks and in the exoskeletons of insects and fungi (Ravi Kumar, 2000; Roberts, 1992), being insoluble in aqueous media and in most organic solvents. It is converted into chitosan via hydrolysis of the acetamido groups of *GlcNAc* units, producing *GlcN* units. The resulting polymer is referred to as chitosan when there is predominance of *GlcN* units (> 50 %), which enhances

polymer solubility as the amino groups are protonated in dilute acid aqueous solutions, i.e. chitosan is a polycationic polymer in such media (Rinaudo, Pavlov, & Desbrieres, 1999; Rinaudo, 2006). The physicochemical properties and biological activity of chitosans depend on the polymer characteristics. The most relevant factors are the average degree of acetylation (\overline{DA}), which expresses the amount of *GlcNAc* units, the pattern of acetylation (*PA*), related to the distribution of *GlcN* and *GlcNAc* units, the average molecular weight, and dispersity (\overline{D}). The structural parameter that most affects chitosan solubility at a fixed value of molecular weight is its degree of acetylation (\overline{DA}), but physicochemical parameters, mainly the pH and ionic strength of the medium are also important (Sorlier, Denuzière, Viton, & Domard, 2001).

Chitosan is one of the few natural aminopolysaccharides, whose biological properties include biocompatibility, antimicrobial, antioxidant and wound-healing acceleration activities (Ishihara et al., 2001; Kong, Chen, Xing, & Park, 2010; Ono et al., 2001; Shariatinia et al., 2019). It is also versatile for it can be processed as films, fibers,

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nanoparticles and hydrogels (Fiamingo & Campana-Filho, 2016; Pillai, Paul, & Sharma, 2009; Valente, Ferreira, Henriques, Borges, & Silva, 2019). However, there are important limitations to the potential chitosan applications in the biomedical field, especially its limited solubility in neutral and alkaline media. Attempts to circumvent this difficulty include chemical modification with functional groups *via* carboxymethylation, quaternization, PEGylation, among other possibilities (Facchinatto, Fiamingo, dos Santos, & Campana-Filho, 2019; Jia, Shen, & Xu, 2001; Rinaudo, 2006; Roberts, 1992, Fiamingo et al. 2017). Unfortunately, many of these procedures decrease the polycationic character of chitosan and increase its cytotoxicity (Jintapattanakit, Mao, Kissel, & Junyaprasert, 2008; Tan, Ma, Lin, Liu, & Tang, 2013). Another route to improve solubility of chitosans or chitin is the *N*-deacetylation of chitin followed by depolymerization *via* ultrasound treatment. Indeed, water-soluble chitin and chitosan were obtained with $\overline{DA} \sim 50\%$ but the average weight molecular weight (\overline{M}_w) was very low due to depolymerization (Cho, Cho, Chung, Yoo, & Ko, 1999, Du, Zhao, Dai, & Yang, 2009; Kubota, Tatsumoto, Sano, & Toya, 2000; Kurita, Koyama, Nishimura, & Kamiya, 1989; Lu, Song, Cao, Chen, & Yao, 2004; Qin et al., 2006).

In this paper, we report experimental procedures to prepare *N*-acetylated chitosans with very high molecular weights ($\overline{M}_w \approx 1,000,000 \text{ g mol}^{-1}$) and controlled average degrees of acetylation ($5\% < \overline{DA} < 70\%$), which display water solubility in a wide pH range, including in neutral and alkaline aqueous media. This was made possible by optimizing a method to prepare high molecular weight, extensively deacetylated chitosans ($\overline{DA} \approx 5\%$). Different average degrees of acetylation are then obtained with homogeneous *N*-acetylation. Hydrogen nuclear magnetic resonance spectroscopy and size-exclusion chromatography are used to characterize the β -chitin and chitosans. Zeta potential measurements are also carried out to evaluate the polymer charge. Aggregation and solubility of chitosans as a function of the pattern of acetylation and degree of acetylation are also discussed.

2. Experimental

2.1. Ultrasound-assisted deacetylation of β -chitin

The polymorph β -chitin, extracted from squid pens (*Doryteuthis spp.*) (Chaussard & Domard, 2004; Fiamingo et al., 2016; Lavall, Assis, & Campana-Filho, 2007), was ground in a knives mill (MA-048, Marconi/Brazil) and the fraction containing particles with average diameter (*D*) in the range $0.125 \text{ mm} < D < 0.250 \text{ mm}$ was submitted to the ultrasound-assisted deacetylation (USAD process) (Fiamingo & Campana-Filho, 2016; Fiamingo et al.,

2016). The β -chitin particles were suspended in 40 % aqueous NaOH at a ratio β -chitin/NaOH aqueous solution of 1/10 (g mL^{-1}). This suspension was transferred to a double-walled cylindrical glass reactor ($\theta_{\text{int}} = 3.5 \text{ cm}$) coupled to a circulating thermostat and then submitted to ultrasound irradiation in a Hielscher Sonifier UP400S ultrasonic device ($\nu = 24 \text{ kHz}$) with the ultrasound probe H22D (diameter = 22 mm). The geometry and dimensions of the glass reactor, and the position of the ultrasound probe inside the glass reactor were fixed as in (Delezuk, Cardoso, Domard, & Campana, 2011). The glass reactor containing the β -chitin suspension (50 mL) was thermostated at $60 \pm 1 \text{ }^\circ\text{C}$, the ultrasonic device was adjusted to emit pulsed irradiation (0.5 s) and surface intensity of 52.6 W cm^{-2} during 50 min. The suspension was submitted to constant stirring during the USAD processing and the reaction was interrupted by quickly cooling the suspension to $0 \text{ }^\circ\text{C}$ and adding hydrochloric acid until $\text{pH} \approx 8.5$. The resulting chitosan, referred to as USAD chitosan Chs1 ($\overline{DA} \approx 36 \%$), was isolated by filtration, extensive washing with deionized water and freeze-drying. To obtain extensively deacetylated chitosans with high molecular weight, Chs1 was submitted to additional one, two, three and four consecutive USAD steps to yield USAD chitosans CHs2 ($\overline{DA} \approx 15 \%$), CHs3 ($\overline{DA} \approx 5 \%$), CHs4 ($\overline{DA} \approx 2 \%$) and CHs5 ($\overline{DA} \approx 1 \%$), respectively.

2.2. Homogeneous *N*-acetylation of chitosan

N-acetylation of USAD chitosan CHs3 ($\overline{DA} \approx 5 \%$) was attained by reacting it with acetic anhydride as reported (Sorlier et al., 2001; Sorlier, Viton, & Domard, 2002; Vachoud, Zydowicz, & Domard, 1997). An aliquot of 0.5 g of the USAD chitosan CHs3 was dispersed in 50 mL of an aqueous acetic acid solution (0.5%) and the suspension was kept under constant mechanical stirring (500 rpm) in a double-walled cylindrical reactor coupled to a circulation thermostat at $25 \pm 1 \text{ }^\circ\text{C}$ for 24 h. To the resulting chitosan solution, 40 mL of 1,2-propanediol were added to prevent *O*-acetylation. Then, a given volume of acetic anhydride was slowly added and the reaction proceeded under constant mechanical stirring for 24 h at $25 \pm 1 \text{ }^\circ\text{C}$. Such a mild reaction condition was used to minimize depolymerization. Distinct acetic anhydride/GlcN molar ratios were employed to yield *N*-acetylated chitosans (A-CHs) with various average degrees of acetylation \overline{DA} (Table 2). At the end of the *N*-acetylation procedure, the reaction medium was neutralized by addition of aqueous ammonium chloride and ethanol was added to promote the precipitation of the

N-acetylated chitosan, which was then extensively washed with deionized water, frozen and freeze-dried.

2.3. Average degree of acetylation (\overline{DA})

The average degree of acetylation (\overline{DA}) of β -chitin and chitosans was determined using ^1H NMR spectroscopy (Hirai, Odani, & Nakajima, 1991) with a Bruker AVANCE III spectrometer ($\nu = 400$ MHz), with the composite pulse sequence “ZGCPPR” to suppress the signals of hydrogens from water (Fiamingo et al. 2016). To measure the ^1H NMR spectra, 10 mg of chitosan were dissolved in 1 mL 1 % HCl/D₂O (pD \approx 3-4) upon constant magnetic stirring at room temperature for 24 h, while β -chitin (10 mg) was dispersed in 1 mL 20 % DCI/D₂O (w/w) and kept under constant magnetic stirring at 70 °C overnight to depolymerize it. The spectra of β -chitin and chitosans were acquired at 80 °C and \overline{DA} was calculated from the relative intensities of the signals assigned to the hydrogen atoms bonded to the anomeric carbon of *GlcNAc* (H1' \approx 4.6 ppm) and *GlcN* (H1'' \approx 4.9 ppm) units. From the ^1H NMR spectra it was also possible to estimate the relative abundance of the sequences *GlcN/GlcN*, *GlcNAc/GlcNAc* and *GlcNAc/GlcN* (*GlcN/GlcNAc*), which were expressed by the corresponding diad fractions F_{DD} , F_{AA} and F_{AD} (F_{DA}) (Vårum, Antohonsen, Grasdalen, & Smidsrød, 1991). Deconvolution tools were applied to integrate the signals of ^1H in the interval 5.1 ppm – 4.4 ppm (Fiamingo et al., 2016), in order to determine the signal intensity assigned to the diads and the corresponding diad frequencies normalized according to Bernoullian statistics (Kumirska et al., 2009; Weinhold, Sauvageau, Kumirska, & Thöming, 2009).

2.4. Size-exclusion chromatography (SEC)

Size-exclusion chromatography (SEC) was used to determine the weight average molecular weight (\overline{M}_w), weight average degree of polymerization (\overline{DP}_w) and dispersity (\mathfrak{D}) of the samples. Chitosans were dissolved in 0.2 mol L⁻¹ sodium acetate/0.30 mol L⁻¹ acetic acid buffer (pH = 4.5), which was also used as the eluent at constant flow rate (0.6 mL min⁻¹). The chitosan solutions ($C_p = 0.5$ g L⁻¹) were filtered through 0.22 μm cellulose acetate membranes (Millipore) and then injected ($v = 50$ μL) into SB-G (6 mm DI x 50 mm; pre column), SB-803-HQ (8 mm DI x 300 mm) and SB-805-HQ (8 mm DI x 300 mm) columns (Shodex OH pack®) coupled to an online differential refractometer (Shimadzu CTO-10A). The calibration of the columns was carried out by injecting

eight monodisperse standards of polymaltotriosis (pullulan) with distinct average molecular weights.

The weight average degree of polymerization (\overline{DP}_w) was calculated from the average number of *GlcN* and *GlcNAc* units, and the weight average molecular weight (\overline{M}_w) was obtained with Equation 1:

$$\overline{DP}_w = \frac{\overline{M}_w \times 10^2}{(161 \times \overline{DD}) + (203 \times \overline{DA})} \quad (1)$$

where \overline{M}_w (g mol⁻¹), \overline{DD} (%) and \overline{DA} (%) stand for the weight average molecular weight, average degree of deacetylation and of acetylation, respectively, while 161 and 203 correspond to the molecular weights (g mol⁻¹) of *GlcN* and *GlcNAc* units, respectively.

2.5. Solubility in Aqueous Solutions

The solubility of the chitosans in aqueous media was estimated with the turbidimetry test (Chen & Park, 2003). The transmittance of the solution at 600 nm was recorded on a U-2900 Spectrophotometer (Hitachi High-Technologies Co.) using a quartz cell with an optical path of 1 cm. The chitosan samples were dissolved in aqueous HCl (1%) at 1.5 g L⁻¹ and the pH was adjusted by adding aqueous NaOH (or HCl).

2.6. Zeta potential

The average size and surface zeta potential of the *N*-acetylated chitosans were measured in a Zetasizer Nano ZS (Malvern, UK) using diluted aqueous solutions ($C_p = 1.0$ g L⁻¹). The samples were previously solubilized in hydrochloric acid at stoichiometric protonation condition for 24 h. The resulting solutions were buffered, which resulted in samples with $C_p = 1.0$ g L⁻¹ in 0.2 mol L⁻¹ sodium acetate/0.30 mol L⁻¹ acetic acid buffer (pH = 4.5) or phosphate-buffered saline solution (PBS) (pH = 7.4). The measurements to determine the average size were performed with dynamic light scattering at 25 °C and 173° as scattering angle. The zeta potential measurements were carried out with a bias voltage of 150 V on the driving electrodes of the capillary electrophoresis cell.

3. Results and Discussion

The main aim in this study was to produce and characterize high molecular weight chitosans with controlled degree of acetylation. To this end, the multistep USAD process (Fiamingo et al., 2016) was adapted to

prepare an extensively deacetylated chitosan, namely sample CHs3, which was then submitted to homogeneous *N*-acetylation. The consecutive execution of the USAD processes converted β -chitin ($\overline{DA} \approx 80.5$ %) into five USAD chitosans displaying high weight average molecular weight ($\overline{M}_w > 0.98 \times 10^6$ g mol⁻¹) and average degree of acetylation in the range 1 % < \overline{DA} < 37 % (Table 1). The conversion efficiency of β -chitin into chitosans CHs1, CHs2 and CHs3 is close to that reported in our previous study (Fiamingo et al., 2016), thus confirming the reproducibility of the USAD process. However, the USAD chitosans produced in the present

study display higher weight average molecular weights (\overline{M}_w) and weight average degrees of polymerization (\overline{DP}_w) than those of the previous report (Fiamingo et al., 2016). These differences may result from the use of a less severe drying procedure than in the previous work. Indeed, in the earlier study the chitosan samples were dried overnight in a circulation oven at 30°C, which lead to depolymerization (≈ 12 % / step). In contrast, the USAD chitosans reported here were first frozen and then freeze-dried, minimizing the thermally-induced depolymerization (≈ 6 % / step).

Table 1. Characteristics of chitosans obtained with different numbers of consecutive USAD procedures (N_{USAD}): \overline{DA} = Average degree of acetylation; \overline{M}_w = Weight average molecular weight; \overline{D} = dispersity; \overline{DP}_w = Weight average degree of polymerization.

Samples	N_{USAD}	\overline{DA} (%)	$\overline{M}_w \times 10^6$ (g mol ⁻¹)	\overline{DP}_w
β -chitin	-	80.5 \pm 1.8 ^a	n.d.	n.d.
CHs1	1	36.3 \pm 1.2 ^b	*1.31	*7,651
CHs2	2	15.6 \pm 1.4 ^c	1.20	7,162
CHs3	3	5.4 \pm 0.7 ^d	1.14	7,038
CHs4	4	2.3 \pm 0.4 ^e	1.08	6,614
CHs5	5	1.2 \pm 0.2 ^f	0.98	6,066

^adue to the presence of insoluble aggregates, Ch-1x was purified before SEC analyses; n.d.: not determined;

3.1. Homogeneous *N*-Acetylation of chitosans

To carry out the *N*-acetylation of the USAD chitosan, the sample CHs3 was chosen as only three consecutive USAD procedures were needed to prepare it. Moreover, this USAD chitosan has low average degree of acetylation ($\overline{DA} \approx 5$ %) and possesses an unusually high weight average molecular weight, being a “quasi-ideal” substrate to carry out random *N*-acetylation and prepare high molecular weight chitosans. Mild conditions were used to carry out the *N*-acetylation of USAD chitosan CHs3, with which high molecular weight chitosans could be produced with variable average degrees of acetylation, depending on the molar ratio of acetic anhydride/*GlcN* used in *N*-acetylation. As indicated in Table 2, these *N*-acetylated chitosans have very high weight average molecular weights ($\overline{M}_w \approx 1,000,000$ g mol⁻¹) and weight average degrees of polymerization ($\overline{DP}_w \approx 6,000$). Some depolymerization nevertheless occurs for chitosan A-CHs3_00, in spite of the mild conditions, and their \overline{M}_w and \overline{DP}_w are ca. 16 % lower than those of CHs3. Moreover,

a fraction of the highly *N*-acetylated chitosans may have been excluded during the isolation procedure, as the *N*-acetylation percentage weight yield decreases with increasing molar ratio of acetic anhydride/*GlcN* employed in *N*-acetylation of CHs3. Indeed, the average degree of acetylation of the *N*-acetylated chitosans produced with low acetic anhydride/*GlcN* molar ratio ($R < 0.65$) increases sharply with increasing *R* (Figure 1) while the corresponding *N*-acetylation percentage weight yield (*Y*) remains relatively high (> 85 %) (Table 2). However, with a higher acetic anhydride/*GlcN* molar ratio ($R > 0.65$), the average degree of acetylation of the *N*-acetylated chitosans increases smoothly with increasing *R* (Figure 1) and the *N*-acetylation percentage weight yield (*Y*) is much lower ($Y < 55$ %).

Figure 1 also shows that the value of \overline{DA} of the *N*-acetylated chitosans increases with the molar ratio of acetic anhydride/*GlcN* without reaching a plateau. Interestingly, these results are markedly different from previous works, including the study by Vachoud et al. (1997) who reported the gelation of chitosan in aqueous

Table 2. *N*-acetylation percentage weight yield (*Y*) and characteristics of the *N*-acetylated chitosans (A-CHs) as a function of the molar ratio of acetic anhydride/GlcN (*R*) employed in the *N*-acetylation of CHs3. \overline{DA} = Average degree of acetylation; M_w = Weight average molecular weight; \overline{D} = dispersity; \overline{DP}_w = Weight average degree of polymerization.

Samples ^(#)	<i>R</i>	<i>Y</i> (%)	\overline{DA} (%)	$\overline{M}_w \times 10^6$ (g mol ⁻¹)	DP _w
*A-CHs3_05	0.00	-	5.6 ± 0.5 ^a	0.96	5,901
A-CHs3_15	0.10	99.6 ± 5.1 ^a	15.5 ± 0.9 ^b	0.99	5,903
A-CHs3_24	0.20	94.2 ± 2.3 ^{b,c}	24.1 ± 0.6 ^c	1.00	5,829
A-CHs3_35	0.32	92.6 ± 1.5 ^c	34.8 ± 0.6 ^d	1.05	6,040
A-CHs3_45	0.46	86.6 ± 1.4 ^d	44.7 ± 0.7 ^e	1.10	6,142
A-CHs3_60	0.65	84.7 ± 0.4 ^d	59.8 ± 0.3 ^f	1.15	6,187
A-CHs3_70	1.21	57.1 ± 2.2 ^e	72.3 ± 1.0 ^g	1.20	6,283
A-CHs3_80	1.72	45.7 ± 2.1 ^f	81.3 ± 0.9 ^h	n.d.	n.d.

N-acetylated chitosans are named as A-CHs3_XY, where the last two digits stand for their average degrees of acetylation. * In this case, chitosan CHs3 was submitted to the same reaction conditions, however no acetylating reagent was added; n.d.: not determined;

acetic acid when $\overline{DA} \approx 80\%$ and a plateau at $\overline{DA} \approx 96\%$ with homogeneous *N*-acetylation. Indeed, here gel formation was observed already during preparation of *N*-acetylated chitosan A-CHs3_60 ($\overline{DA} \approx 60\%$) while polymer insolubility occurred when preparing chitosan A-CHs3_80 ($\overline{DA} \approx 80\%$). Both phenomena, *i.e.* gel formation and polymer insolubility, are attributed to the very high molecular weight of chitosan CHs3 ($\overline{DA} \approx 5\%$), which is converted during *N*-acetylation into highly acetylated chitosan still exhibiting a high weight average molecular weight.

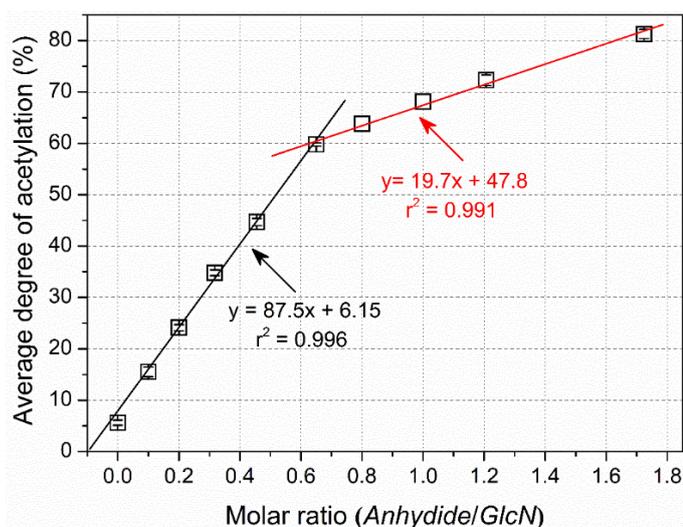


Figure 1. Average degree of acetylation (\overline{DA}) of *N*-acetylated chitosans versus molar ratio of anhydride/GlcN employed in *N*-acetylation.

3.1.1. Chitosan aggregation and solubility in aqueous solutions

As most natural polymers, chitosan exhibits a self-associative behavior leading to aggregation in aqueous solutions. Ottøy et al. (1996) reported increasing chitosan self-aggregation with increasing average degree of acetylation and/or molecular weight, with aggregation raising from 3% to 18% when the molecular weight varied from 61,000 g mol⁻¹ to 270,000 g mol⁻¹. Philippova et al. (2001) observed from fluorescence measurements using pyrene as hydrophobic probe that chitosan aggregation starts at concentrations ≥ 1 mg mL⁻¹ and that the addition of urea or ethanol led to a reduction in the hydrophobic domains. From these results they concluded that neither hydrogen bonds nor hydrophobic interactions were responsible for chitosan aggregation, in contrast to the conclusion of Ottøy et al. (1996). Indeed, according to this latter study (Ottøy et al., 1996), for a fixed molecular weight, the more acetylated chitosans had higher tendency toward self-association, suggesting that aggregation is caused by hydrophobic interactions involving acetyl groups of GlcNAc units. In another study (Anthonsen et al. (1994), the use of acetate buffers with suitable ionic strengths and low polymer concentration were suggested to decrease chitosan self-aggregation. Summarizing the results from the literature mentioned in this paragraph, one infers that chitosan aggregation in solution is favored with high values of molecular weight, average degree of acetylation and polymer concentration.

Aggregation in the aqueous solutions of USAD chitosans and *N*-acetylated chitosans was investigated here by preparing dilute solutions ($C_p = 1.0 \text{ g L}^{-1}$) in 0.2 mol L^{-1} sodium acetate / 0.3 mol L^{-1} acetic acid (AcOH/AcONa buffer; pH = 4.5). The solutions were filtered and the amount of insoluble fractions was determined by gravimetry. Thus, 100 mL aliquots of these solutions were kept under constant magnetic stirring at room temperature for 24h and then filtered through previously weighted $0.22 \mu\text{m}$ membranes (mixed cellulose esters, Millipore), which were dried and weighted again. Table 3 shows that chitosans produced with at least two consecutive USAD procedures, *i.e.* samples CHs2, CHs3, CHs4 and CHs5, contained negligible amounts of insoluble fractions ($> 1\%$). In contrast, 15% of the initial weight of chitosan CHs1 was recovered as an insoluble fraction upon filtration. All the *N*-acetylated chitosans (up to $\overline{DA} \approx 70\%$) were fully soluble in the buffer but the solution of chitosan A-CHs3_80 ($\overline{DA} \approx 80\%$) presented a high amount of insoluble fractions ($\approx 85\%$). The molecular weight of the samples fully soluble in AcOH/AcONa buffer, namely USAD chitosans CHs2, CHs3, CHs4, CHs5, and the *N*-acetylated chitosans with $\overline{DA} \leq 70\%$ was determined by SEC analysis. This analysis was carried out by dissolving these chitosan samples in AcOH/AcONa buffer, resulting in solutions at different concentrations ($C_p = 0.5$ and 1.0 g L^{-1}), which were filtered through a $0.22 \mu\text{m}$ Millipore membrane. Monomodal molecular weight distribution curves and low dispersity ($1.4 < \overline{D} < 1.8$) were observed in all cases, indicating no significant aggregation in solution. In fact, a small value for aggregation ($\approx 6\%$) could be estimated for A-CHs3_70 chitosan by comparing its \overline{DP}_w with the value for A-CHs_05. However, this difference is not significant if one considers the data dispersion ($\approx 10\%$).

Based on the literature (Anthonsen et al. (1994), Sorlier et al. (2001)), the main probable reason for the solubility of these chitosan samples with unusually high average molecular weights and average degrees of acetylation is the predominating random pattern of acetylation (PA). Anthonsen et al. (Anthonsen et al. (1994)) suggested that strong interchain associations occur for chitosans with long sequences of *GlcNAc* units, with aggregation taking place if there are sequences of at least five consecutive *GlcNAc* units. Therefore, the tendency of a given chitosan to aggregate may be estimated from its pattern of acetylation (PA), which expresses the distribution probability of *GlcN* and *GlcNAc* units along the polymer chains (Weinhold et al., 2009). Chitosans with

predominantly block-like distribution (PA = 0 for an ideal block-like distribution) tend to form large amounts of insoluble aggregates. In contrast, ideally alternated (PA = 2) and random distributions (PA = 1.0) disfavor such interactions, decreasing chain aggregation and yielding fully soluble chitosans.

Table 3 shows that the USAD and the homogeneously *N*-acetylated chitosans had predominantly random distributions (PA ≈ 1) and low aggregation in solution, except for samples CHs1 and A-CHs3_80. Aggregation in CHs1 may be attributed to its very high weight average degree of polymerization ($\overline{DP}_w > 7,500$) originating from a single USAD processing. As for A-CHs3_80, insolubility is due to a combination of two factors: long average length of polymer chains and large number of long sequences of *GlcNAc* units. Significantly, the pattern of acetylation also affects the pK_0 of chitosans, which is ~ 6.4 and 7.3 for $\overline{DA} \sim 0\%$ and 100% , respectively. According to Sorlier et al. (2001), the increase in pK_0 upon increasing \overline{DA} occurs when the neighborhood of protonated *GlcN* units are rich in *GlcNAc* units, decreasing the electrostatic repulsion of positively charged units, and weakening its acid character.

The predominant random patterns of acetylation ($0.7 < \text{PA} < 1.0$) for the chitosans produced here with multistep USAD processes are consistent with earlier reports (Delezuk et al., 2011; Fiamingo et al., 2016). The high intensity of the ultrasound irradiation in the USAD process caused the particles of β -chitin to be highly hydrated with increased accessibility to reactive sites owing to the decrease in particle size (*i.e.* increased surface area). It also led to disruption of crystalline regions. Thus, a quasi-homogeneous *N*-deacetylation reaction occurred and the resulting chitosan (CHs1) had an almost ideal random deacetylation pattern (PA ≈ 1.0 ; Table 3). The values of PA decreased with further consecutive USAD processing because particle hydration decreased, with ensuing decrease in accessibility to reactive sites. Table 3 shows that chitosans resulting from homogeneous *N*-acetylation of CHs3 also had a predominant random pattern of acetylation, with PA values slightly higher than for CHs3. Indeed, PA increases with increasing average degree of acetylation with the homogeneous *N*-acetylation of chitosan. Owing to the steric hindrance due to the *GlcNAc* neighbors, whose number increases with \overline{DA} , amino groups from *GlcN* in a neighborhood rich in *GlcNAc* units are less accessible, and *N*-acetylation occurs mostly at sites far from these regions.

Table 3. Distribution of diad frequencies (F) and pattern of acetylation (PA) of β -chitin, USAD and N -acetylated chitosans determined from ^1H NMR spectra.

Samples	\overline{DA} (%)	CrI (%)	F_{AA}	F_{AD}	F_{DD}	PA
β -chitin	80.5 ± 1.8	44.1	n.d.	n.d.	n.d.	n.d.
CHs1	36.3 ± 1.2	19.7	0.14	0.23	0.41	0.98
CHs2	15.6 ± 1.4	18.9	0.04	0.13	0.70	0.93
CHs3	5.4 ± 0.7	15.0	0.01	0.05	0.89	0.91
CHs4	2.3 ± 0.4	15.1	0.01	0.02	0.96	0.75
CHs5	1.3 ± 0.5	14.5	0.01	0.01	0.98	0.73
A-CHs3_5	5.6 ± 0.5	13.9	0.01	0.05	0.89	0.91
A-CHs3_15	15.5 ± 0.9	15.1	0.02	0.14	0.70	1.03
A-CHs3_25	24.1 ± 0.6	19.1	0.06	0.19	0.55	1.03
A-CHs3_35	34.8 ± 0.6	20.4	0.12	0.23	0.43	1.00
A-CHs3_45	44.7 ± 0.7	21.1	0.19	0.27	0.27	1.09
A-CHs3_60	59.8 ± 0.3	22.1	0.34	0.25	0.15	1.06
A-CHs3_70	72.3 ± 1.0	22.4	0.49	0.23	0.06	1.10
A-CHs3_80	81.3 ± 0.9	23.1	n.d.	n.d.	n.d.	n.d.

a) F_{AD} is the probability that *GlcNAc* and *GlcN* units are neighbors; b) F_{AA} is the probability that two *GlcNAc* units are neighbors; c) F_{DD} is the probability that two *GlcN* units are neighbors; n.d.: not determined.

The pH dependence for the chitosan solubility was investigated at a fixed polymer concentration ($C_p = 1.5 \text{ g L}^{-1}$), with a given sample being considered insoluble when its solution transmittance was lower than 90 %. Except for β -chitin and chitosan A-CHs3_80, which were insoluble in the whole pH range, USAD and N -acetylated chitosans displayed good solubility at $\text{pH} \leq 4.5$. Their clear, colorless solutions had high transmittance ($\approx 100 \%$) and high apparent viscosity owing to their high molecular weights. Representative data for transmittance versus pH of aqueous solutions of N -acetylated chitosans are shown in Figure 2 for three samples: A-CHs3_5, A-CHs3_25 and A-CHs3_60, i.e. with low, medium and high average degrees of acetylation, respectively. A-CHs3_5 displays the typical profile for chitosans, with solubility up to $\text{pH} \approx 4.7$. In contrast, A-CHs3_25 remains soluble at $\text{pH} \approx 7.8$ and A-CHs3_60 is fully soluble in the whole pH range. The maps with the pH range of solubility/insolubility of USAD chitosans in Figure 3 reveal that solubility is strongly associated with the polyelectrolyte character, especially for the extensively deacetylated chitosans (CHs3; CHs4 and CHs5). These three chitosans precipitate at low pH (≈ 4.7) due to neutralization of positive charges, while more acetylated USAD chitosans (CHs1 and CHs2) remained soluble at $\text{pH} \approx 7.0 - 7.5$. The N -acetylated chitosans with medium average degree of acetylation

($15 \% < DA < 35 \%$), namely samples A-CHs3_15, A-CHs3_25 and A-CHs3_35, were soluble up to neutral and slightly alkaline media. For the highly N -acetylated chitosans (A-CHs3_60 and A-CHs3_70), Figure 4 shows that they are fully soluble in the whole pH range. These chitosans were made insoluble by adding ethanol, a non-solvent, in which case a hydrogel was formed.

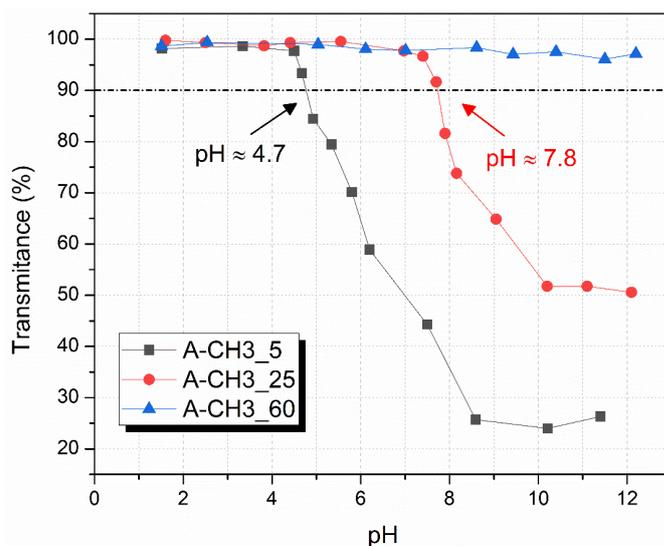


Figure 2. Optical transmittance versus pH for aqueous solutions of N -acetylated chitosans A-CHs3_5, A-CHs3_25 and A-CHs3_60.

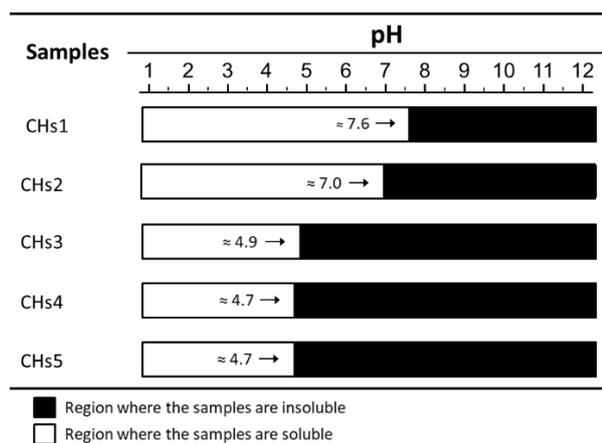


Figure 3. Solubility of USAD chitosans in aqueous solutions as a function of pH.

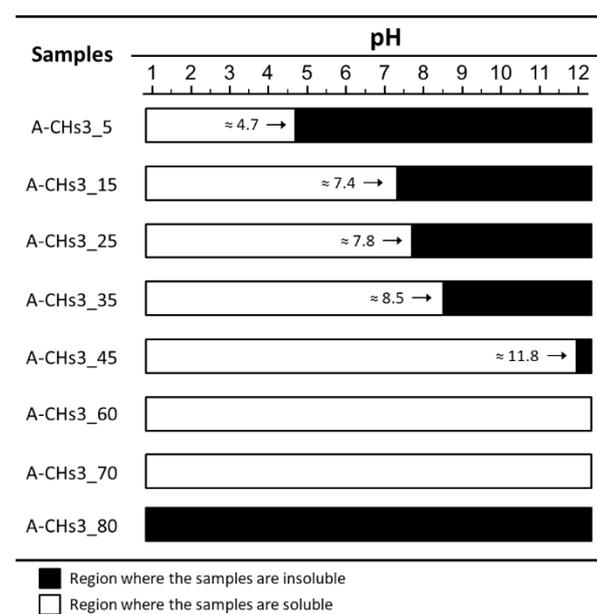


Figure 4. Solubility of *N*-acetylated chitosans in aqueous solutions as a function of pH.

Further evidence of the stability of *N*-acetylated chitosan solutions is inferred from the zeta potential results in Table 4. As expected, positive zeta potentials were measured for all chitosan samples and pH of the medium due to protonation of the amino groups in *GlcN* units. The zeta potential was higher in the acetate buffer (pH = 4.5) than in PBS (pH = 7.4), as the chitosan chains are fully charged in the former cases due to protonation of the amino groups. The zeta potential (ZP) for chitosans in the acetate buffer decreased linearly with increasing \overline{DA} (ZP (mV) = $24.7 - 0.34 \overline{DA}$; $r^2 = 0.98$). In PBS solution (pH 7.4), even though the same trend could be observed, the differences are not significant for $\overline{DA} \geq 25\%$. In these cases, the low values of zeta potential and the small changes with increasing \overline{DA} should be ascribed to the low fraction of protonated *GlcN* units, especially at high \overline{DA} . It is also worth noting that the zeta potential of chitosan A-CHs3_70 in the PBS buffer is very low, indicating that chain aggregation may have begun. Accordingly, the average particle size of A-CHs3_70 is considerably higher than for the other chitosans (Table 4). Since the acetate buffer is a good solvent for chitosans, the particle size was independent of \overline{DA} , except for the extensively deacetylated chitosan (A-CHs3_5), which behaves as a "true" polyelectrolyte (Schatz et al. (2003)). It has a larger average particle size than the other chitosans owing to the high positive charge density of its chains. Regardless of chitosan characteristics and buffer, all size distributions were monomodal, except in the case of A-Chs3_5 that was insoluble in PBS buffer.

Table 4. Zeta potentials (ZP) and diameter means of *N*-acetylated chitosans in acetate buffer or PBS.

Samples	\overline{DA} (%)	Acetate buffer (pH 4.5)		PBS (pH 7.4)	
		ZP (mV)	Diameter (nm)	ZP (mV)	Diameter (nm)
A-Ch-5%	5.6 ± 0.5^a	$+23.4 \pm 0.3^a$	22.9 ± 1.5^a	.*	.*
A-CHs3_15	15.5 ± 0.9^b	$+18.8 \pm 1.6^b$	16.8 ± 0.7^b	$+10.9 \pm 3.4^a$	23.7 ± 1.2^a
A-Ch-25%	24.1 ± 0.6^c	$+16.6 \pm 0.8^c$	16.7 ± 1.1^b	$+4.6 \pm 1.4^b$	24.9 ± 1.8^a
A-Ch-35%	34.8 ± 0.6^d	$+10.0 \pm 0.5^d$	16.2 ± 1.7^b	$+3.6 \pm 1.4^b$	26.7 ± 1.1^a
A-Ch-45%	44.7 ± 0.7^e	$+8.2 \pm 0.6^d$	15.7 ± 1.6^b	$+2.7 \pm 1.2^b$	24.5 ± 0.9^a
A-Ch-60%	59.8 ± 0.3^f	$+4.6 \pm 0.2^e$	16.6 ± 2.3^b	$+2.3 \pm 1.6^{b,c}$	22.5 ± 0.8^a
A-Ch-70%	72.3 ± 1.0^g	$+2.5 \pm 0.9^f$	15.4 ± 1.8^b	$+1.3 \pm 0.8^c$	51.2 ± 8.4^b

*due to the presence of insoluble precipitates in this PBS solution, A-Ch-5% did not present significant signal for this analysis.

The whole set of results demonstrate that the *N*-acetylated chitosans reported here exhibit water solubility in a wider pH range than in any other study in the literature, in spite of the very high weight average degrees of polymerization ($DP > 6,000$). In fact, water solubility at relatively high pH (≈ 7.0) was reported for low molecular weight chitosans with $\overline{DA} \approx 50\%$ (Cho et al., 1999; Du et al., 2009; Kubota et al., 2000; Lu et al., 2004; Qin et al., 2006). Kubota et al. (2000), for instance, obtained half-acetylated chitosans ($\overline{DA} \approx 50\%$; $\overline{M}_w \geq 150,000 \text{ g mol}^{-1}$) soluble at $pH \leq 8$, which became soluble in a wider pH range when the molecular weight was considerably lowered ($\overline{M}_w \leq 33,000 \text{ g mol}^{-1}$). It should be mentioned that the acetylated chitosans A-Ch-25%; A-Ch-35%; A-Ch-45%; A-Ch-60% and A-Ch-70% cannot be dissolved in pure water, even though they remain soluble at $pH \geq 7.8$. Indeed, when samples of these chitosans were placed under constant magnetic stirring in pure water, even during two days, they did not dissolve and remained in a hydrated powder/gel form. Thus, complete solubilization of *N*-acetylated chitosans is only reached using acid aqueous solutions because protonation of the *GlcN* units is required to break the crystalline structure.

4. Conclusion

We have shown that chitosans can be produced with controlled average degree of acetylation, a random pattern of distribution of *GlcN* and *GlcNAc* units and unusually high molecular weights, also displaying water solubility in a wide pH range. This was made possible by employing a modified USAD process to yield extensively deacetylated chitosans ($\overline{DA} \leq 5\%$) with higher molecular weight than in previous studies. It is significant that these chitosans maintained their polycationic character even in PBS solutions, which represents a breakthrough that will permit unprecedented studies with cell membrane models by mimicking the physiological conditions. Furthermore, extensively deacetylated chitosans should tend to expose their charged groups at a larger extent than other chitosans, which is expected to impart stronger biological activity, e.g. in antimicrobial applications.

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