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Studies on acetylation patterns of different chitosan preparations

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ABSTRACT

Chitosan is a biopolymer which physicochemical characteristics like fraction of acetylation (F_A) and molecular weight (M_W) depend strongly on preparation conditions. Furthermore, the orientation of acetyl groups along the chain may adopt different patterns like an alternating, random or a block-wise pattern depending on preparations conditions. However, a reliable determination of the pattern of acetylation of unknown chitosan preparations – as a single parameter P_A – is crucial for structure–activity-analysis dealing with biological systems. In this study, an improved method for pattern determination using ^{13}C-NMR data of commercial de-*N*-acetylated and lab-made re-*N*-acetylated chitosan samples is shown. Results indicated a random-dominated pattern (P_A 0.5–1.5) for all 32 samples although different process conditions were used during production of the samples. No evidence for the existence of a clear block-wise or clear alternating chitosan preparation was found. For the first time it could be shown that pattern of acetylation correlates with F_A exponentially $\left(P_A = 1.11 - 0.58 \cdot e^{\left(\frac{-F_A}{0.13}\right)}\right)$.

1. Introduction

Chitin, together with cellulose, belongs to the most abundant biopolymers on earth. It is widely distributed in the exoskeleton of insects, crustaceans and in the cell walls of microorganisms such as fungi and yeast. Chitin is mainly used as a raw material for the production of chitosan, a copolymer comprising variable proportions of *N*-acetylglucosamine (GlcNAc, A-unit) and deacetylated glucosamine units (GlcN, D-unit). To produce chitosan, chitin is typically deacetylated in hot alkali solutions for different time (Lamarque, Lucas, Viton, & Domard, 2005). Chitosan, in contrast to chitin, dissolves in diluted acids, preferably in organic acids and its chloridic form even in aqueous solutions (Weinhold et al., 2009). Due to better solubility chitosan has a higher chemical and biochemical reactivity.

Depending on the source (α- or β-chitin) and process conditions (time, temperature) during alkaline deacetylation, the product shows large varying properties characterized by parameters such as mole fraction of acetylation (F_A) and weight-averaged molecular weight (M_W) (Lamarque, Chaussard, & Domard, 2007). Furthermore, depending on deacetylation conditions, chitosan may show characteristic pattern of acetylation (P_A) that might vary from block over random to an alternating pattern. Theoretically, the P_A should have significant impact on biological activity (enzyme recognition) and even on physicochemical activity (charge density) for a chitosan preparation with exactly the same M_W and F_A but different P_A. However, there is some confusion about the existence of different patterns in the literature and the different scientific approaches using different methods and different samples complicate the comparison of pattern determination.

Already in 1977, Kurita and co-workers Kurita, Sannan, and Iwakura (1977) investigated heterogeneously and homogeneously acetylated chitosan preparations by X-ray diffraction (XRD). They suggested that the homogeneous process should give a random type copolymer while the heterogenous process should result in a block copolymer. The results from Aiba (1991) confirmed these results by studying the swelling properties again with XRD. Later in 1991, Vårum and co-workers described a method for pattern determination using ^{1}H-NMR (Vårum, Anthonsen, Grasdalen, & Smidsrød, 1991a) and ^{13}C-NMR (Vårum, Anthonsen, Grasdalen, & Smidsrød, 1991b). They concluded that tested samples showed similar characteristics of a random Bernoullian distribution. In a subsequent study the composition of soluble parts and insoluble parts of chitosan produced by a heterogeneous process were investigated (Ottøy, Vårum, Christensen, Anthonsen, & Smidsrød, 1996). While a random pattern was clearly observed for the soluble part, a block-wise pattern in the insoluble part was assumed but not proven. Recently, a new production route of chitosan was shown by means of Freeze-pump out-thaw (FPT) cycles yielding in high molecular weight products (Lamarque et al., 2005). Although α- as well as β-chitin were used as starting material, no block-wise pattern could be found in the products with varying F_A.

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Considering the history of the pattern determination two main points should be noted. First, the existence of a block-wise pattern could not be confirmed within an experiment by later working groups. Second, the pattern was determined from laboratory-made chitosan only, which can be sometimes highly different in comparison to commercial samples. Thus, reports about pattern determinations were difficult to prove and results could not be used for further studies using commercially available samples. To avoid these difficulties, we want to show how a more simplified approach for P_A determination can be applied to a pool of commercial samples. Therefore, we want to demonstrate that a general concept of polymer sequence evaluation (Mirau, 2005) can be successfully applied to chitosan pattern analysis avoiding some difficulties of the established pattern analysis as proposed by Vårum et al. (1991b). This previously described method yields in many pattern values for just one sample. Thus, comparison of chitosan preparations between each other becomes tedious. Furthermore, a precise quantitative method is required to obtain accurate pattern values (diad areas) from ^{13}C-NMR spectra, which was not clearly shown by the authors.

Our main focus was to simplify the analysis and to generate a value, which can be compared easily between different chitosan preparations. Received results can then be used and understood by biologists, who often discuss an impact of change of pattern on enzyme recognition but are not well trained in polymer analysis. Since chitosan research has a very interdisciplinary focus, our aim was to describe the analysis in a simpler way than it is usually discussed in the polymer scientist community. Within this study the quantitative extraction was made using standard NMR spectrometers (^{13}C 90 MHz) instead of expensive high field devices (^{13}C 125 MHz) at much lower temperatures than proposed earlier. Collected data have been revised and increased in quality by curve fitting of the NMR data and by control of the molecular weight. Based on the Vårum method, we propose a new single parameter P_A describing the pattern of acetylation of chitosan. Although this simplification can only be made at the expense of accuracy, we think this simplification has some advantages. Using the pattern parameter P_A enables a faster and straightforward comparison of unknown chitosan preparations for a more thorough structure–activity analysis dealing with e.g. biological systems.

Throughout this work we use the nomenclature proposed by the European Chitin Society (EUCHIS) (Roberts, 2007). Chitin and chitosan will be classified on the basis of their solubility and insolubility in 0.1 M acetic acid. Insoluble material is named chitin, soluble material is defined chitosan. The different types of acetylation will be expressed as the mole fraction of acetylation F_A. It is given in brackets after every distinct chitosan preparation, e.g. a 8% acetylated chitosan A will be written as Chi A [0.08].

2. Experimental

2.1. Material

Chitosan produced by partial de-*N*-acetylation (heterogeneous conditions): Chitosan A–D were purchased from Chipro (Bremen, Germany), chitosan E from Vink (Berlin, Germany), chitosan F from Heppe Biomaterial GmbH (Landsberg, Germany), chitosan G from Polymar (Braschaat, Belgium), chitosan H, J, K were received from EUTEC (Emden, Germany), chitosan I from Kraeber GmbH (Ellerbek, Germany), chitosan L–N from Fluka (Buchs, Switzerland), chitosan O from ABCR (Karlsruhe, Germany), chitosan P from Sigma Aldrich (Seelze, Germany), chitosan Q from Roth (Karlsruhe, Germany) and chitosan R–Y from Bioneer (Hørsholm, Denmark). Samples AX, AY, AZ, BA were produced by Mahtani chitosan (Veraval, India) from chitin particles of different sizes to vary conditions from heterogeneous to more homogeneous de-*N*-acetylation (0.25 up to 2 mm).

Chitosan produced by partial re-*N*-acetylation (homogeneous conditions): chitosan BR, BS, and BT were first fully de-*N*-acetylated, then partially re-*N*-acetylated homogeneously in solution according to the procedure described previously (Lamarque et al., 2005). Re-*N*-acetylated samples and samples generated from different particle sizes were kindly provided by Prof. Moerschbacher (Münster, Germany).

Sodium acetate, ethanol, ethylene glycol and acetic acid were obtained from Fluka (Buchs, Switzerland). Ammonia (25%) was purchased from Merck (Darmstadt, Germany). All reagents used were of analytical grade.

2.2. Purification of chitosan

To ensure a pure raw product the chitosan was cleaned before use and insoluble matter was removed. The chitosan powder was dissolved in 0.5 M acetic acid (10 g/L) for 24 h. The clear solution was centrifuged for 10 min (3577 g) with an ultracentrifuge (Heraeus Instruments, Germany) and the supernatant was filtered through a 0.45 μm cellulose nitrate filter (Sartorius, Germany). The final products were then lyophilized.

2.3. Hydrolysis of chitosan for NMR analysis

First, all chitosan preparations were dissolved at room temperature for 24 h by adding 100 mg chitosan under shaking in 10 mL of 0.07 M HCl. Second, 5 mg $NaNO_2$ were added and the solutions were stored for 4 h and subsequently freeze-dried. The residue was dissolved in D_2O and then lyophilized. This step was repeated twice to exchange labile protons with deuterium.

2.4. NMR spectroscopy

All samples (ca. 5 mg ^{1}H-NMR, and ca. 50 mg ^{13}C-NMR experiments) were dissolved in 0.7 mL 99.95% D_2O (Deutero GmbH, Kastellaun, Germany); one drop of DCl (37%) was added to receive an ambient pH for dissolution and the solutions were transferred to 5 mm NMR tubes. The ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Varian Mercury 400 and on a Brucker Avance 360 (WB 360) spectrometer at 400 MHz and 360 MHz for protons and 100 MHz and 90 MHz for carbon, respectively. ^{1}H-NMR as well as ^{13}C-NMR spectra were recorded at 80 °C, 60 °C and at room temperature (20 °C). 32 and 30,000 scans were acquired for proton and carbon spectra, respectively. Chemical shifts were reported relative to internal acetone (δ_H 2.225, δ_C 31.45). MestRec-4.9 software for PC was used for processing of the spectra. Prior to signal integration in ^{1}H-NMR spectra, a linear drift correlation between 1 and 6 ppm for ^{1}C-NMR and between 0 and 110 ppm for ^{13}C-NMR was applied, respectively. Integration boundaries were set manually by inspection of the spectrum.

2.5. Determination of the F_A

The F_A was determined by ^{1}H-NMR spectroscopy according to the method of Hirai et al. (Hirai, Odani, & Nakajima, 1991). Chitosan samples were dissolved in D_2O/DCl and F_A values were obtained as described previously (Weinhold et al., 2009).

2.6. Determination of the P_A

Chitosan, as linear copolymer of GlcNAc (A) and GlcN (D), can be characterized by different sequences of the monomers along the chains. The possible copolymer architecture includes alternating, random or block-wise distribution of acetyl groups (Fig. 1) and can be determined by ^{13}C-NMR spectroscopy. In the ^{13}C-NMR spectrum the carbon signals have different chemical shifts, depending

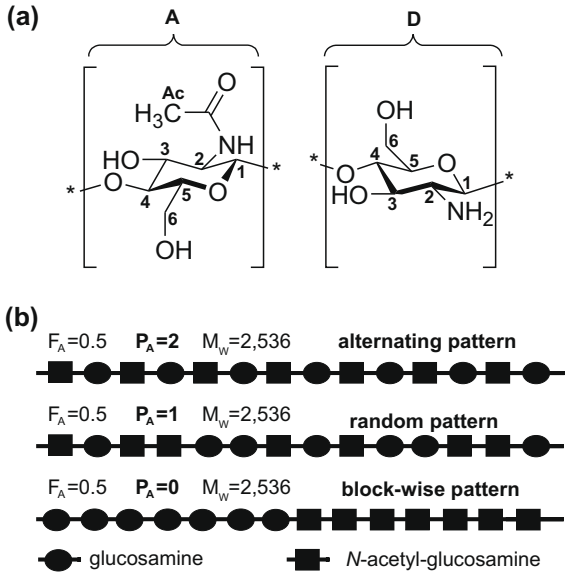

Fig. 1. (a) Structural formulas of the A-unit *N*-acetyl-glucosamine and the D-unit glucosamine. (b) Theoretical patterns of acetylation illustrated as pictographs. A square represents a *N*-acetyl-glucosamine and a circle a glucosamine unit. A pattern can adopt the following extreme values: an alternating, a random and a block-wise distribution.

on the chemical structure of the neighboring units. The intensities of the ^{13}C resonances for the different sequences can be used for a more detailed sequential characterization of chitosan.

In this paper, ^{13}C-NMR data was compared to sequences obtained by theoretical random statistics (Bernoullian model for trial propagation (Mirau & Bovey, 1996; Mirau, 2005)). In this approach, stereochemistry of the chain end is not taken into account and it is either unimportant whether it is a A or a D unit. For a transfer of the Bernoullian model to chitosan characterization we have to consider the possibility that A or D can be added to an unknown initial group and followed again by A or D (described as fraction of acetylation F_A and deacetylation F_D). Adding now an A unit or D unit to another A or D unit, four different sequences (F_{AD}, F_{DD}, F_{AA} and F_{DA}) can be identified and were specified as diad intensities. From the F_A and F_D value alone, chitosan can not be tested for consistency or inconsistency with Bernoullian model, only the diad (or triad) information allows this comparison. If F_A is determined experimentally, it is thus possible to calculate the F_D and all theoretical diad values for a perfect random distribution as shown below:

$$F_{AA} = F_A^2 \quad (1)$$

$$F_D = 1 - F_A \quad (2)$$

$$F_{DD} = F_D^2 \quad (3)$$

$$F_{AD} = F_{DA} = 2 \cdot F_A \cdot F_D \quad (4)$$

In Eqs. (1)–(4) F_{AA} (F_{DD}) is the probability that two A (D) groups are adjacent to each other and F_{AD} (F_{DA}) the probability that one group A has a D neighbor, vice versa. The relative experimental intensities of ^{13}C resonances in the analyzed chitosan were determined and normalized according to Bernoullian statistics and presented as:

$$F_{AD} = \frac{I_{AD} + I_{DA}}{I_{AD} + I_{DA} + I_{AA} + I_{DD}} \quad (5)$$

$$F_{AA} = \frac{I_{AA}}{I_{AD} + I_{DA} + I_{AA} + I_{DD}} \quad (6)$$

$$F_{DD} = \frac{I_{DD}}{I_{AD} + I_{DA} + I_{AA} + I_{DD}} \quad (7)$$

where I_{AD}, I_{DD}, I_{AA} and I_{DA} indicate the experimental area of the frequency F_{AD}, F_{DD}, F_{AA} and F_{DA} in the analyzed samples, respectively. The conformity to Bernoullian statistics can be tested by application of Eq. (8) (Mirau, 2005):

$$P_A = P_\Sigma = \frac{F_{AD}}{(2 \cdot F_{AA}) + F_{AD}} + \frac{F_{AD}}{(2 \cdot F_{DD}) + F_{AD}} \quad (8)$$

If the statistic is consistent with the bernoullian model, a random pattern is found and the P_Σ value becomes 1. For a complete block-wise pattern the value drops to 0 and for a complete alternating pattern the value increases to 2 as shown in Fig. 1. As long as diad areas are extracted from sometimes poorly resolved spectra with help of a peak fitting procedure, P_Σ values may vary depending on spectra quality. Influence of different fitting routines and their impact on the standard deviation on the P_A parameter will be shown in a separate paper (Kumirska et al., 2009). In this paper, the general pattern parameter for polymer analysis P_Σ is denoted as P_A to express the functionality of the acetyl groups in chitosan pattern analysis.

2.7. Curve fitting

^{13}C-NMR spectra were fitted using the curve-fitting tool in the MestRec software (www.mestrec.com). In order to get a spectrum with good signal to noise ratio the fourier transformation settings were optimized. All diad peaks were fitted with the same curve widths, however, the peak widths were decreased with an increase of temperature (5–6 at 60–80 °C and 7–9 at 20 °C). An unknown peak appeared between F_{DD} and F_{AA} signal and may be attributed to one of the mannose signals. To fit this peak properly a much smaller width was used than for the diad signals. A detailed description of the curve fitting and its analyst dependent impact on the standard deviation will be shown in Kumirska et al. (2009).

2.8. Triple detection size exclusion chromatography (SEC^3)

The biopolymer analysis was performed with a triple detection size exclusion chromatography system (SEC^3, Viscotek, USA) consisting of an online two channel degasser, a high pressure pump, an autosampler (all parts integrated in the GPCmax, Viscotek, USA), a 0.5 μm stainless steel in-line filter with a nylon membrane, two serially connected ViscoGEL columns (PWXL mixed bed 6–13 μm methacrylate particles, 7.8 × 300 mm), a temperature controlled triple detector array (TDAmax 305, Viscotek, USA) with a differential refractometer at $\lambda = 660$ nm (RID 3580), a right angle (90°) light scattering detector (RALS) with a semiconductor laser diode at $\lambda = 670$ nm and a four capillary, differential Wheatstone bridge viscometer. The SEC conditions were as follows: a degassed 0.3 M CH_3COOH/0.3 M CH_3COONa buffer (pH 4.5) with 1% ethylene glycol was used as eluent, the sample concentration was 0.3–1 mg/mL and samples were dissolved for 24 h under shaking, injection volumina varied from 10 to 100 μL, flow rate was maintained at 0.7 mL/min, and the column and detector temperature were kept at 30 °C. Before injection, the sample solutions were filtered through a 0.45 μm cellulose nitrate disposable membrane (Sartorius, Germany). To ensure a low light scattering noise level the eluent was filtrated through a 16–40 μm glass filter. A polyethyleneoxid standard ($M_W = 22,411$ g/mol, $[\eta] = 0.384$ dL/g, $M_W/M_N = 1.03$) was used to normalize the viscometer and the light scattering detector. Data acquisition and processing were carried out by use of OmniSEC 4.1 software (www.viscotek.com). A dn/dc of 0.163 was used for the M_W calculation (Rinaudo, Milas, & Dung, 1993). For large molecules ($R_g > 10$ nm), which are affected by an angular dependance of the scattering light, missing RALS intensity was viscosity corrected by the OmniSEC software. Received molecular weight and intrinsic

viscosity values were used to improve the molecular weight by an iterative method through calculation of the radius of gyration R_g:

$$\left(\frac{1}{6}\right)^{0.5} \cdot \left(\frac{M \cdot [\eta]}{F}\right)^{0.3} = R_g \quad (9)$$

the calculated radius is used to recalculate the scattering function $P(\theta)$ which is then used to determine the viscosity corrected molecular weight.

$$\frac{8}{3}\left[R_g \frac{\Pi n}{\lambda} sin(\theta/2)\right]^2 = x \quad (10)$$

$$2\left[\frac{e^{-x} - (1-x)}{x^2}\right] = P(\theta) \quad (11)$$

3. Results and discussion

This paper is organized as follows: In the first section, the general procedure of P_A analysis using ^{13}C-NMR data is briefly discussed. In the second section, the molecular weight analysis of degraded samples is reported. After that we focus on the resolution dependance of NMR spectra at different temperatures with special emphasis on a modified peak fitting procedure, which still enabled a quantitative P_A determination at room temperature. Finally, we show that our findings for a pool of up to 32 different chitosan preparations improved the comparison by use of the P_A parameter.

3.1. P_A analysis of chitosan

The determination of the monomer sequences in chitosan using ^{13}C-NMR data requires spectra with sufficient resolution of the diad frequencies. In order to get good resolved spectra we hydrolyzed the samples with HCl/$NaNO_2$. Nitrous acid cleaves the β-glycosidic linkage and generates a new reducing end group (2,5-anhydro-D-mannose) (Tømmeraas, Vårum, Christensen, & Smidsrød, 2001). This end group gives rise to additional peaks in the ^{13}C-NMR spectrum (Fig. 2) which can complicate NMR interpretation and may lead to an overlap with important diad frequencies. Even though this hydrolysis modifies the molecules producing artificial chemical entities (fractions), we assume it to be a prerequisite for NMR analysis due to two facts. Firstly, the hydrolysis reduces drastically the viscosity of the solution allowing an appropriate handling of the sample. Secondly, it reduces the signal width, which would otherwise be too large for the applied polymer analysis using NMR techniques. Hydrolysis gives oligomers of various sizes, thus allowing an estimation of the distribution of *N*-acetyl groups.

In general, the experimental data of the sequences in the chitosan samples could be calculated from all carbon signals in the ^{13}C-NMR spectra (Vårum et al., 1991b) (Fig. 2). Inspection of these spectra showed that the highest resolution and only low overlapping signals for the four diad frequencies – F_{AA}, F_{AD}, F_{DA} and F_{DD} – were achieved from the carbon C5 signals (Fig. 2). For other carbon regions diad peaks showed superimposition with neighboring peaks, which inhibited a quantitative extraction of signal areas (for more information about extraction of triad frequencies for C1 and diad frequencies for C6 the reader is referred to the electronic supplement). Furthermore, the shape of the spectra changed upon changing of the F_A, and parts of ^{13}C-NMR spectra with different F_A values are shown in Fig. 3. While a sample with high F_A (Fig. 3 Chi V [0.48]) showed relatively intense signals for all four diad signals, three diad signals are rather weak for a high de-*N*-acetylated sample (Fig. 3 Chi C [0.06]). All signals showed a significant overlapping, thus, a typical integration of these signals yielded in an overestimation of the tiny AA signal intensity, especially for high de-*N*-acetylated samples. In order to extract the real signal intensity we applied a peak fitting procedure for the whole diad region, which allowed a quantitative determination of every signal area.

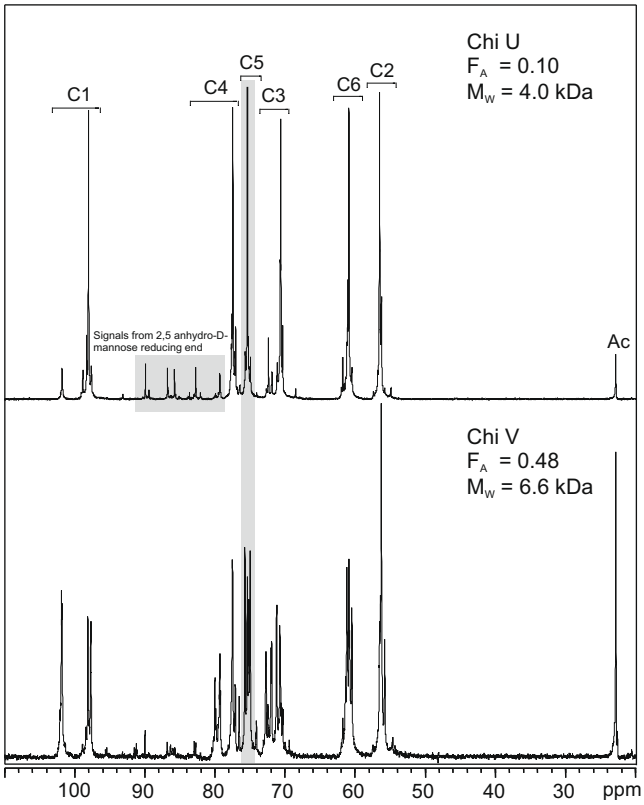

Fig. 2. ^{13}C NMR spectra (100 MHz) of two chitosan samples with different F_A. The C5 area, which was used for pattern analysis, is highlighted in light grey. Signal assignments are based on Vårum et al. (1991b), Tømmeraas et al. (2001).

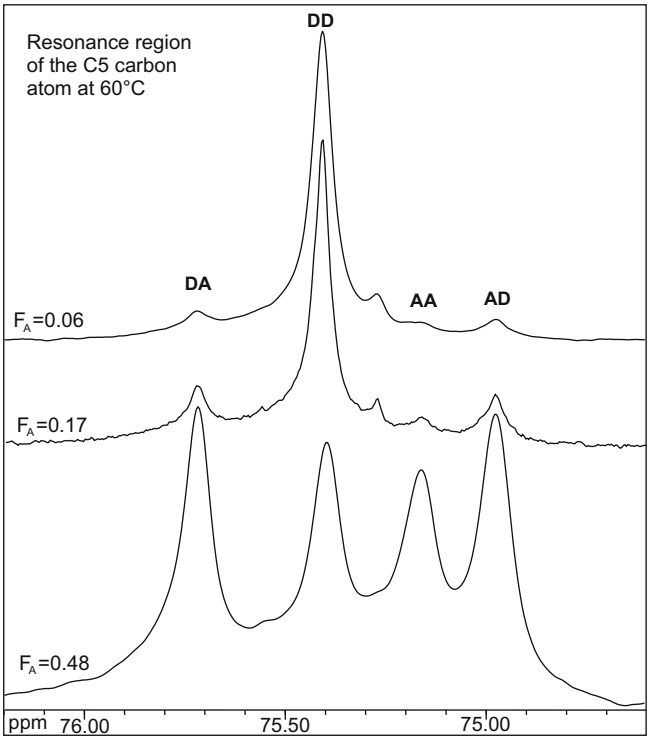

Fig. 3. Extracts of the C5 resonance region of ^{13}C-NMR spectra. Chitosan with different F_A values are shown: Chi C [0.06], Chi O [0.17] and Chi V [0.48].

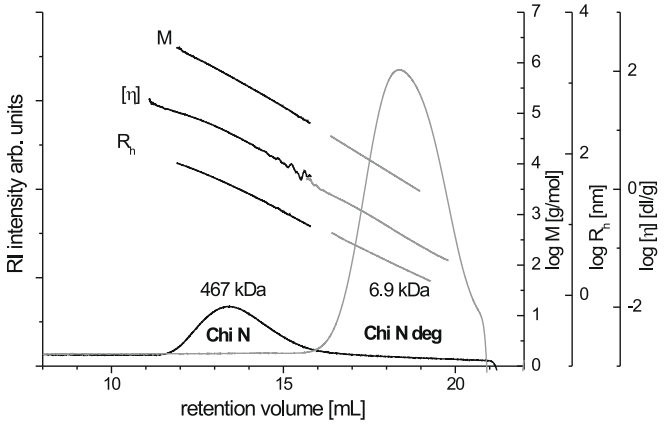

Fig. 4. Typical chromatograms including refractive index response (RI) of two chitosan samples. Initial chitosan N (black) has been hydrolyzed with $NaNO_2$ resulting in a decrease of the molecular weight (grey).

3.2. Molecular weight determination of initial and degraded samples

Determination of the molecular weight was performed by size-exclusion chromatography (SEC[3]). Initial samples as well as $NaNO_2$ degraded samples were analyzed to monitor the change in the molecular weight within the reaction. A typical chromatogram of an initial sample compared to a degraded sample is shown in Fig. 4.

In every case the reaction led to a successful depolymerization of samples, however, the final weight varied between samples. Twenty preparations yielded in M_W values below 10 kDa, seven to M_W values between 10 and 20 kDa and five samples to a final weight of more than 20 kDa (see Table 1). In none of these cases the higher molecular weight had a significant impact on ^{13}C-spectra resolution. Even for sample Chi G with a final weight of 33 kDa the P_A analysis was still possible. This means samples with initial M_W equal to this value do not need to be degraded beforehand and can be measured directly.

3.3. Temperature impact on spectra resolution and P_A analysis

Peak width in NMR spectroscopy is influenced by the viscosity of the sample. Due to the lack of sensitivity for the NMR spectroscopy relative high amounts of sample material are required. However, higher concentration of a polymeric substance led to an increase in viscosity and decrease of spectral resolution, which is strongly affected by the molecular weight of the sample. Starting with high temperature measurements (80 °C) for Chi M [0.12] we obtained spectra with good resolution, as expected (Fig. 5). However, a continuous operation of NMR systems at elevated temperatures for long measurements is far from ideal, a lower temperature would lead to a more simplified measurement procedure. Inspection of the spectra revealed again in overlapping signals, hence, line-fitting had do be applied even at high temperatures. Due to this we decided to check the spectra quality at lower temperatures. Again sample Chi M [0.12] was measured at room temperature and the resolution thus obtained turned out to be still sufficient to obtain separated peaks (Fig. 5). The following line-fitting procedure was readapted slightly (line widths were increased) and calculated P_A values showed only small changes. The P_A value decreased up to 2% after decreasing temperature down to 60 °C during the measurement. Hence, the P_A determination can be simplified by conducting measurements at lower temperatures

Table 1
Physicochemical parameters of different chitosan preparations. Molecular weight before (M_W) and after degradation (M_W deg), fraction of acetylation (F_A) and pattern of acetylation (P_A) as well as all diad frequencies ($F_{AA}, F_{AD}, F_{DA}, F_{DD}$) are shown for all investigated chitosan samples.

Chitosan preparation	M_W [kg/mol]	M_W *deg* [kg/mol]	F_A	F_{AA}	F_{AD}	F_{DA}	F_{DD}	P_A
Chi A	122	6.4	0.08	0.0430	0.1847	0.1847	0.7723	0.79
Chi B	98	6.0	0.02	0.0182	0.0287	0.0287	0.9531	0.46
Chi C	145	7.3	0.06	0.0250	0.1399	0.1399	0.8351	0.81
Chi D	149	6.1	0.13	0.0229	0.2180	0.2180	0.7592	0.96
Chi E	303	26.0	0.04	0.0414	0.1302	0.1302	0.8284	0.68
Chi F	210	8.5	0.15	0.0410	0.2581	0.2581	0.7009	0.91
Chi G	238	33.6	0.18	0.0631	0.3002	0.3002	0.6367	0.89
Chi H	201	15.8	0.02	0.0309	0.0704	0.0704	0.8988	0.57
Chi I	281	16.3	0.13	0.0263	0.2180	0.2180	0.7557	0.93
Chi J	230	12.1	0.22	0.0465	0.3339	0.3339	0.6195	0.99
Chi K	353	6.9	0.07	0.0179	0.1123	0.1123	0.8698	0.82
Chi L	151	10.2	0.14	0.0274	0.2342	0.2342	0.7383	0.96
Chi M	314	12.2	0.12	0.0250	0.2101	0.2101	0.7648	0.93
Chi N	467	6.9	0.16	0.0497	0.2799	0.2799	0.6704	0.91
Chi O	428	28.7	0.16	0.0694	0.2861	0.2861	0.6445	0.85
Chi P	244	8.9	0.03	0.0301	0.1412	0.1412	0.8287	0.78
Chi Q	317	17.1	0.13	0.0595	0.2222	0.2222	0.7183	0.79
Chi R	10.3	6.2	0.48	0.2109	0.5591	0.5591	0.2300	1.12
Chi S	9.7	3.3	0.10	0.0372	0.2344	0.2344	0.7284	0.90
Chi T	19.3	5.5	0.46	0.2057	0.5623	0.5623	0.2320	1.13
Chi U	16.8	4.0	0.10	0.0340	0.2028	0.2028	0.7632	0.87
Chi V	29.7	6.6	0.48	0.2096	0.5536	0.5536	0.2368	1.11
Chi W	26.8	6.2	0.10	0.0294	0.2069	0.2069	0.7637	0.90
Chi X	43.1	5.8	0.44	0.2192	0.5582	0.5582	0.2226	1.12
Chi Y	40.9	5.1	0.10	0.0260	0.2299	0.2299	0.7441	0.95
Chi AX	223	11.0	0.14	0.0754	0.2765	0.2765	0.6481	0.82
Chi AY	194	23.2	0.13	0.0396	0.2353	0.2353	0.7251	0.89
Chi AZ	270	24.1	0.14	0.0300	0.2080	0.2080	0.7620	0.90
Chi BA	245	8.3	0.16	0.0501	0.2601	0.2601	0.6898	0.88
Chi BR	29.9	5.5	0.36	0.1447	0.5328	0.5328	0.3226	1.10
Chi BS	155	3.0	0.38	0.1590	0.5374	0.5374	0.3036	1.10
Chi BT	491	3.8	0.39	0.1356	0.4898	0.4898	0.3745	1.04

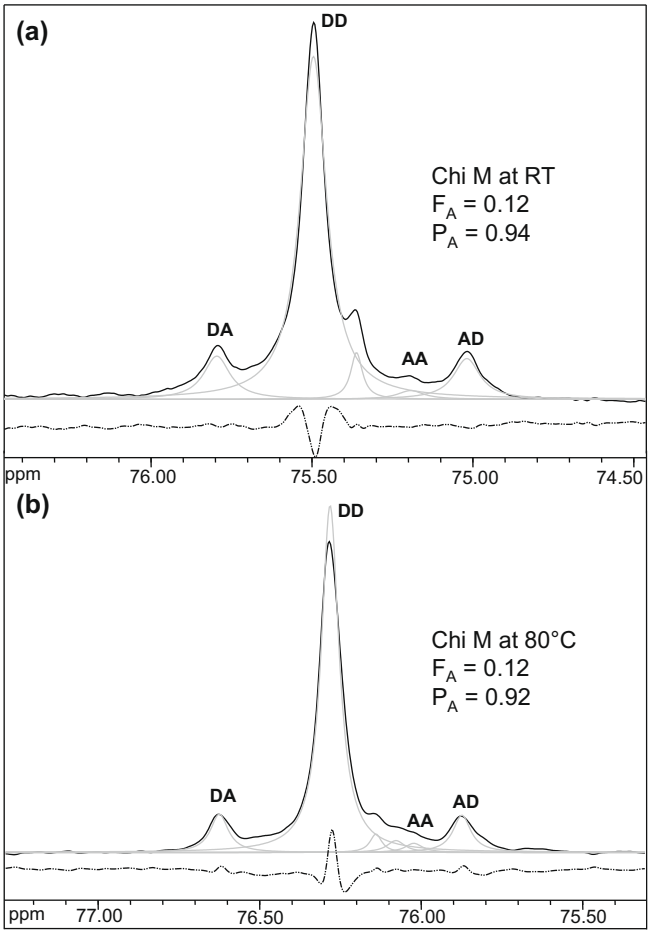

Fig. 5. The picture shows extracts of the C5 resonance region of ^{13}C-NMR spectra (90 MHz) at room temperature (a) and 80 °C (b). For both spectra fitted curves (light grey) and residual of the fit (dash-dotted line) is shown. Line widths of the fitted curves are reduced at elevated temperature but only slight changes in the P_A value could be found when the experiment was conducted at room temperature.

without worsen the accuracy. Lower temperatures have the advantage that possible interferences like temperature induced degradation, evaporation of the solvent and equilibrating delays can be avoided. Furthermore, acceptable spectra resolution were obtained using standard devices (^{13}C 90 MHz) instead of high field devices (^{13}C 125 MHz) as reported by Vårum et al. (1991b). Together with this finding we emphasize that this method is more suitable as a standard technique because high temperature as well as high field devices are not necessary for the P_A determination.

3.4. P_A comparison of different chitosan preparations

P_A values of all chitosan preparations indicated a random-dominated pattern (0.5–1.5) and are shown in Table 1. Comparison of the P_A value with the F_A of the specific sample revealed an exponential relation with a slight decrease of the P_A value with decreasing F_A (Fig. 6). This general behavior was not only found for heterogeneously prepared chitosan samples but also for the three homogeneously prepared samples, which can be found on the same line (Fig. 6). For highly acetylated samples (Chi R [0.48], T [0.46], V [0.48], X [0.44]) the P_A showed values above 1, indicating a slight tendency to a more alternating structure. Furthermore, highly de-*N*-acetylatied samples (Chi B [0.02], E [0.04], H [0.02]) showed the opposite behavior. Herein the block-wise character increases. However, all preparations show a higher contribution of Bernoullian characteristics so that the pattern can be considered

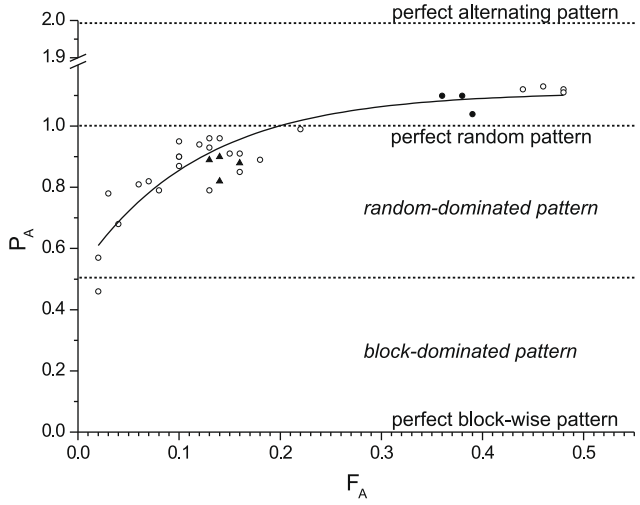

Fig. 6. Dependance of the pattern parameter P_A versus F_A. De-*N*-acetylated chitosan samples (open circles), re-*N*-acetylated samples (full circles) as well as de-*N*-acetylated samples with different particle sizes (full triangles) are plotted. All samples show a random-dominated pattern according to the P_A value. However, high acetylated samples show a slight trend to a more alternating pattern while low acetylated samples show a more block-wise character. The solid lines represents a trend calculated by last square fitting $P_A = 1.11 - 0.58 \cdot e^{\left(\frac{-F_A}{0.15}\right)}$, $R^2 = 0.83$.

as random-dominated in the range 0.5 to 1.5. Only for values lower than 0.5 or higher than 1.5 the block-wise/alternating contribution is more than 50% and dominates the pattern. These patterns can then be described as block-wise-dominated and alternating-dominated, respectively. A completely block-wise or alternating structure is found when the value drops to 0 or reaches 2, respectively.

Although we found a rather small value (Chi B 0.46) for low F_A, the term"block-wise" should be used carefully. In this specific case only 2% acetylated groups are remaining at the polymeric chain (12 A units, 590 D units). If the A units are orientated e.g. in six AA pairs, resulting P_A drops easily to 0.5 due to the higher F_{AA} contribution in the spectrum in comparison to the other diad intensities. These pairs can be distributed randomly on the chain resulting in a general random distribution, but these pairs still show a significant F_{AA} intensity in NMR. For such a low amount of remaining acetyl groups only slight changes can lead to a strong decrease of the P_A value. Generally, we consider a chain with 2% remaining acetyl groups as nearly completely charged with almost identical behavior like a 100% de-*N*-acetylated chain. At F_A values of 0.15 or even at 0.5 we would expect classical block-wise behavior with one charged side and one side covered with uncharged but H-bond active groups. However, a low P_A for F_A values higher than 0.1 could not be found. Hence, the existence of a clear block-wise pattern among commercial de-*N*-acetylated samples, de-*N*-acetylated with varying particle size and re-*N*-acetylated samples, studied in this work, could not be found.

4. Conclusion

Within this study we improved and applied the chitosan pattern determination first shown by Vårum et al. (1991b). Through implementation of a line-fitting procedure for ^{13}C-NMR data a quantitative determination of signal areas could be presented and was used for the polymer sequence evaluation (Mirau, 2005). Furthermore, temperature during NMR measurements was decreased for simplification. The resulting diad frequencies were summarized in one single value – P_A pattern of acetylation – enabling a much faster comparison of unknown chitosan preparations. It was shown that all studied samples had a random-dominated pattern (P_A 0.5–1.5) although high acetylated

chitosan showed ca. 10% higher contribution of an alternating structure and low acetylated samples showed up to 54% stronger contribution of block-wise characteristics than expected from a pure Bernoullian distribution. No evidence for the existence of a completely block-wise or alternating structure was found although samples were produced using different processes including de-*N*-acetylation as well as re-*N*-acetylation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2009.06.001.

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