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Chemical Preparation and Structural Characterization of a Homogeneous Series of Chitin/Chitosan Oligomers

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The preparation of a homogeneous series of chitin/chitosan oligomers (chito-oligomers) with the same distribution of degrees of polymerization (DP) ranging from 2 to 12, but with various average degrees of *N*-acetylation (DA) from 0 to 90% is described. This DA-series was obtained according to a two-step chemical process involving (i) the production of a well-defined mixture of glucosamine (GlcN) oligomers obtained by acid hydrolysis of a fully *N*-deacetylated chitosan and after selective precipitations of the hydrolysis products, and (ii) the partial *N*-acetylation of the GlcN units of these oligomers from a hydro-alcoholic solution of acetic anhydride in a controlled manner. The characterization of this series of samples with different DAs by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) allowed us to determine their average DA and identify the main oligomer structures constituting each mixture. Furthermore, MALDI-TOF MS was particularly helpful to study the distribution evolution of the diverse oligomers as a function of DA for the main DPs from 3 to 7. The modeling of these distributions by means of a binomial law displayed that the chemical *N*-acetylation of low DP GlcN oligomers, produced in a homogeneous medium, occurs randomly along the oligosaccharide chains in accordance with a statistical (Bernoullian) arrangement. In this case, the relative proportion of each chito-oligomer present in the mixture can be estimated precisely as a function of DA considering oligomers of same DP.

1. Introduction

Chitosan is a linear copolymer of (1→4)-linked 2-acetamido-2-deoxy-β-D-glucan (GlcNAc) and 2-amino-2-deoxy-β-D-glucan (GlcN) units in varying proportions. Although present to a low extent in biomass, this polysaccharide is chemically obtained by *N*-deacetylation of chitin, the most naturally occurring polymer, mainly present in the cuticles of arthropods, but also in the cell walls of fungi and yeasts. Recently, chitosan has received considerable attention as a functional biopolymer with a wide range of applications in food, agriculture, medicine, pharmaceutics, and cosmetics, taking advantage of its various interesting physicochemical and biological properties.[1] However, its high viscosity in aqueous solution and its insolubility at neutral pH may restrict its use in solution for physiological functional applications, particularly in medicine and food industry. An increasing interest has recently been shown to chitin/chitosan oligomers (chito-oligomers), because these molecules not only are water-soluble, nontoxic, and biocompatible, but also exhibit numerous biological properties, including antibacterial, antifungal, and antitumor activities, as well as immuno-enhancing effects on animals.[2] They also have been shown to elicit increasing protective responses in various plants[3,4] and possess by themselves antimicrobial activities against a wide spectrum of phytopathogens.[5] Since the biological activity of chito-oligomers has often been determined using heterogeneous and/or relatively poorly characterized oligomer mixtures, the size and structure requirements for these compounds to have a biological activity are difficult to determine. However, it seems that chito-oligomers generally must have a DP of at least 4 to induce biological responses,[6] but beyond that requirement, it is not possible to generalize about structural features essential for their biological activity. Therefore, it is of increasing interest to generate well-defined chito-oligomers to determine the relationship between their bioactivity and their structural parameters (DA, DP, and GlcNAc/GlcN distribution).

Conventional methods for preparing chito-oligomers are either chemical or enzymatic.[2] The first consists of a depolymerization of chitin or chitosan including mainly hydrochloric acid hydrolysis,[7] nitrous acid deamination,[8] fluorolysis in anhydrous hydrogen fluoride,[9] and oxidative−reductive reaction by hydrogen peroxide.[10] Additionally, a few total chemical syntheses of chito-oligomers involving multiple protection and deprotection steps have also been reported.[11–13] Enzymatic methods include the hydrolysis of chitin and chitosan with hydrolytic enzymes[2] or the synthesis from smaller oligosaccharides with enzymes having transglycosylation activities.[14–17] However, most of these methods are limited to the elaboration of GlcNAc or GlcN homo-oligomers, and only a very few of them deal with the preparation of partially *N*-acetylated chito-oligomers of low molecular weight.[15,18–20]

In this study, we describe a new efficient method for the production of a homogeneous series of well-defined hetero-oligomers of chitin/chitosan, varying in DA from 0 to 90%, with a narrow distribution of low DPs. This easy-access method involves a simple chemical process and only selective precipitations. The characterization of this series by ^{1}H NMR and MALDI-TOF MS allows the average DAs of the different samples to be determined. In addition to classical analyses reported in the literature, a systematic comparison of these data

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obtained by both techniques is presented here. Furthermore, MALDI-TOF MS is also used to reach the residue distribution in chito-oligomers of same DP as a function of DA. This leads to very interesting information about the mechanisms involved during the *N*-acetylation of low DP GlcN oligomers in homogeneous conditions.

2. Materials and Methods

2.1. Preparation of the Chito-Oligomer Series. *2.1.1. Preparation of the GlcN Oligomer Mixture.* A fully *N*-deacetylated chitosan (Mw = 95000 g/mol) was prepared according to a process involving a multistep heterogeneous *N*-deacetylation, by means of several freeze−pump out−thaw cycles.[21] Then, an acid hydrolysis of the fully *N*-deacetylated chitosan was performed by mixing 2 g of chitosan and 100 mL of concentrated HCl (∼12 M) in a glass reactor. After closing the reactor with a screw cap, the suspension was stirred for 1 h 30 min in a thermostatted bath (72 ± 2 °C). The reaction was then stopped by immersing the reactor in liquid nitrogen for a few minutes. The brown solution obtained was evaporated to dryness, then dissolved in 100 mL of distilled water, and evaporated under vacuum. These operations were repeated twice so as to remove a large amount of HCl. The product was then dissolved in water, and the solution was adjusted to pH 3 with concentrated NaOH in a flask maintained below 20 °C in an ice bath. After an activated charcoal treatment to remove the brown coloration, the solution was filtered through a nitrocellulose membrane (Millipore, 0.22 μm). The resulting solution was then concentrated under vacuum to 50 mL and neutralized to pH 8−9 with concentrated NaOH to remove high DP oligomers by precipitation. The supernatant was then introduced into an Amicon ultrafiltration cell (6.2 cm diam., 200 mL) equipped with a YC05 Amicon membrane, eluted up to 1 L with distilled water to eliminate most of the sodium salt, and then concentrated to 25 mL. Pure ethanol was added to the hydrolysate to precipitate a mixture of low DP GlcN oligomers (sample **1**; 150 mg), which were isolated after centrifugation (11500 rpm; 20 °C; 20 min), evaporation of the ethanol residue, dissolution in distilled water, and freeze-drying.

2.1.2. N-Acetylation of the GlcN Oligomer Mixture. The partial *N*-acetylation was performed in dissolving 20 mg of the GlcN oligomer mixture in 5 mL of a methanol/water (50:50, v/v) solution. Various amounts (2.9, 4.7, 7.1, 9.4, and 10.6 μL) of pure and fresh acetic anhydride (from Aldrich) were added stoichiometrically to reach the expected DA (25, 40, 60, 80, and 90%, respectively). After 15 min of magnetic stirring at room temperature, solutions were evaporated to dryness, then dissolved in 10 mL of distilled water, and evaporated under vacuum. This treatment was repeated twice. Then each sample of chito-oligomer series was dissolved in 10 mL of 0.01 M HCl and, after freeze-drying, was isolated as a white powder in a quantitative yield (samples **2**−**6**, corresponding to the expected DAs: 25, 40, 60, 80, and 90%, respectively).

2.2. Characterization of the Chito-Oligomer Series. *2.2.1. ^{1}H NMR Spectroscopy.* Average DAs of the fully *N*-deacetylated chitosan and chito-oligomers were determined by ^{1}H NMR spectroscopy. Spectra were recorded with a Bruker ALS 300 spectrometer (300 MHz for ^{1}H) at 298 K. Fully *N*-deacetylated chitosan was dissolved in a dilute acid solution of D_2O (10 mg of chitosan for 1 mL of D_2O and 5 μL of concentrated HCl (∼12 M)), while chito-oligomers were dissolved in D_2O (10 mg/mL) only. The signal of HOD (δ 4.80 ppm) was used as reference.

2.2.2. MALDI-TOF Mass Spectrometry. All mass spectra were acquired with a Voyager-DE STR (Applied Biosystems, Framingham, MA) equipped with a nitrogen laser emitting at 337 nm with a 3 ns pulse. The instrument was operated in the reflector mode. Ions were accelerated to a final potential of 20 kV. The positive ions were detected in all cases. Spectra were the sum of 200 shots and an external mass calibration of mass analyzer was used (mixture of peptides from Sequazyme standards kit, Applied Biosystems, Framingham, MA). The matrix used for all experiments was 2,5-dihydroxybenzoic acid (DHB) purchased from Sigma-Aldrich (St Louis, MO) and used directly without further purification. The solid matrix and chito-oligomer samples were dissolved at 10 g·L^{-1} and 1 g·L^{-1} in water, respectively. A volume of 20 μL matrix solution was then mixed with 20 μL of chito-oligomer solutions. An aliquot of 1 μL of each resulting solution was spotted onto the MALDI sample plate and air-dried at room temperature.

2.2.3. ESI Mass Spectrometry. The collision-induced dissociation (CID) mass spectra were acquired with a triple quadrupole mass spectrometer (QqQ, Quattro Micro, Waters, Milford) in positive mode. Samples of chito-oligomers were prepared in water/acetonitrile (50:50, v/v) and directly infused in the source. The cone voltage was 100 V and the collision cell was maintained at 40 eV.

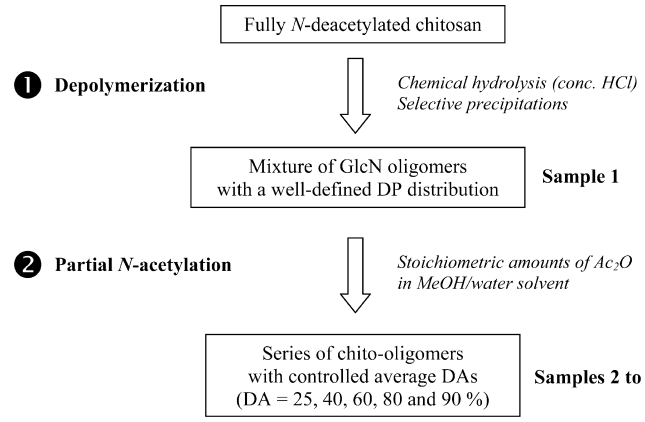

Figure 1. General pathway for the preparation of the series of chito-oligomers with a well-defined DP distribution and varying DAs.

3. Results and Discussion

3.1. Preparation of the Chito-Oligomer Series. The preparation of a homogeneous series of chito-oligomer mixtures ranging from DP 2 to 12 with DAs varying from 0 to 90% was carried out according to a two-step chemical process presented in Figure 1. Thus, a fully *N*-deacetylated chitosan was used as starting material. It was prepared from a heterogeneous *N*-deacetylation, by means of a freeze−pump out−thaw cycles process previously described in the literature.[21] In a first step of the process, a fully *N*-deacetylated chitosan was partially depolymerized by acid hydrolysis with concentrated HCl (∼12 M) using appropriate hydrolysis conditions (1 h 30 at 72 °C) so as to produce low DP GlcN oligomers.[7] After successive treatments, including the removal of the excess HCl by coevaporation with water, then the bleaching of the solution with activated charcoal, efforts were made to reduce the proportion of both the lowest and the highest DPs in the hydrolysis solution, less interesting for the targeted applications, but also to get a narrower distribution in DPs. The highest DP oligomers (DP > ∼15) were removed by precipitation in increasing the pH of the hydrolysate to 8−9 by addition of concentrated NaOH. Then the proportion of the lowest DPs (DP < ∼3) was reduced first by ultrafiltration of the hydrolysate over a 500 g/mol cutoff cellulose acetate membrane and then by precipitation in pure ethanol. Finally, subsequent treatments, including centrifugation, ethanol evaporation, and freeze-drying, after dissolution in distilled water led to the expected mixture of low DP GlcN oligomers (sample **1** in Figure 1), in the free amine form, accounting for about 7.5% in weight of the starting material. The ^{1}H NMR analysis carried out on sample **1** confirmed that all repeating units of chito-oligomers were fully *N*-deacetylated, because no signal at 2 ppm corresponding to

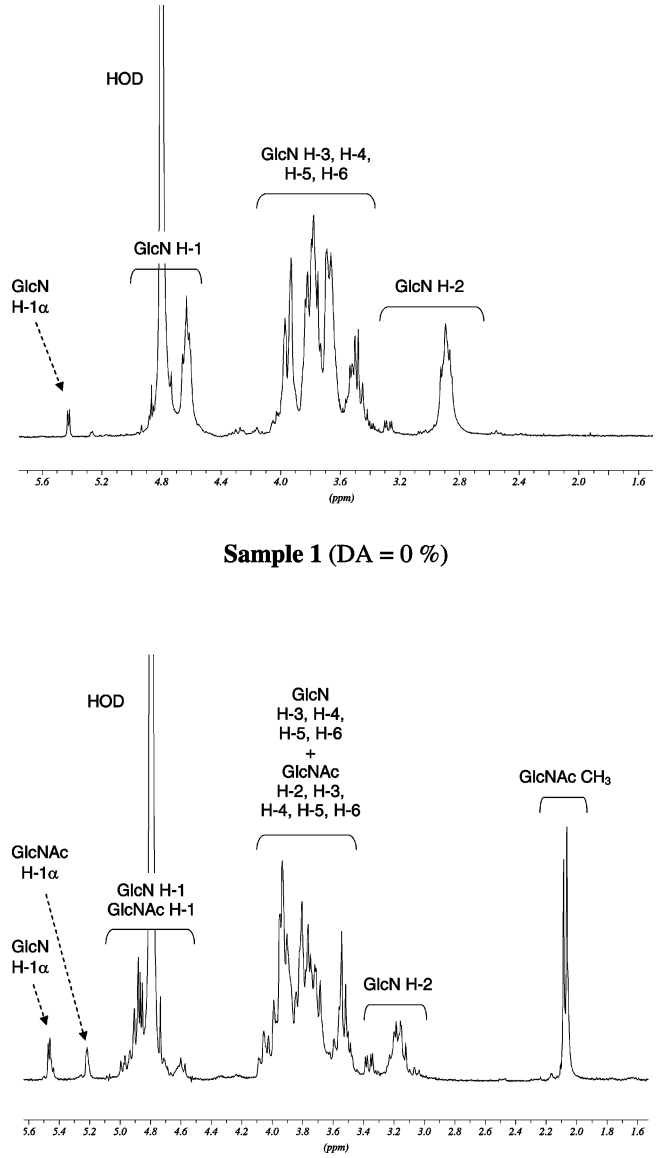

Figure 2. ^{1}H NMR spectra (300 MHz, in D_2O) of fully *N*-deacetylated (DA = 0%, sample 1) and partially *N*-acetylated (DA = 25%, sample 2) chito-oligomers. The assignment of peaks is deduced from literature.[31]

methyl protons of the *N*-acetyl groups was present in the spectrum (Figure 2). The DP distribution of the GlcN oligomer mixture was analyzed by MALDI-TOF MS, as detailed in paragraph 3.2.

The second step of the process was devoted to the partial *N*-acetylation of the GlcN oligomer mixture in a homogeneous medium. *N*-acetylation reactions were performed at room temperature in a hydro-alcoholic solution using various stoichiometric amounts of acetic anhydride as acetylating reactant to get different DAs from 25 to 90%. These conditions were sufficiently soft to preserve the DP distribution and avoid the acetylation of hydroxyl functions of the chito-oligomers. After about 15 min of reaction, solutions were evaporated to dryness, dissolved in water, and coevaporated several times with water to remove acetic acid. Then the acetylated compounds were dissolved in a dilute HCl solution to exchange the acetate form of the glucosamine residues by the hydrochloride. This was followed by the freeze-drying of the resulting solutions leading to the series of chito-oligomers with varying DAs in quantitative yields (samples **2**−**6** in Figure 1). Thus, in these conditions, all acetylated chito-oligomers were isolated in the hydrochloride form, more stable than the free amine.[7] The average DA of each sample of this series and the identification of the main oligomer structures constituting each mixture will be discussed in paragraphs 3.3 and 3.4, respectively.

3.2. Study of the DP Distribution of the GlcN Oligomer Mixture. MALDI-TOF mass spectrometry was chosen to characterize the mixture of GlcN oligomers. This powerful technique, introduced by Karas and Hillenkamp in 1988,[22] was extensively used to analyze many types of macromolecules (such as proteins,[23] DNA,[24] and monodisperse polymers[25]) and was already applied to the characterization of low molecular weight chitosans prepared by acid or enzymatic depolymerization.[8,15,18,26–29] Bahrke et al.[20] reported an interesting sequence analysis by MALDI-TOF post source decay mass spectrometry of chito-oligomers prepared by chemical and enzymatic degradation of chitin and chitosan. On the basis of the analysis conditions detailed in these studies, the MALDI-TOF mass spectrum of the mixture of our GlcN oligomers was performed and is illustrated in Figure 3. Oligomers with different DPs are clearly resolved, and the peak-to-peak mass difference is 161 mass units, matching with the GlcN repeating unit ($C_6H_{11}O_4N$). The end-groups, deduced from the monoisotopic mass, correspond well to the expected one (H and OH groups). The DP distribution of the mixture of GlcN oligomers ranged from 2 to 12 and was centered on DP 5 (from calculation, 5 × 161.07 ($C_6H_{11}O_4N$) + 1.00 (H) + 17.00 (OH) + 22.99 (Na, the cationization ion) = 846.3 mass units; from experiment, 846.4 mass units). Another population (marked with an asterisk in Figure 3) at −18 mass units with regard to expected species is detected, and its proportion increases with the mass. The origin of this population will be identified and discussed hereafter. Finally, the species observed below 500 *m*/*z*, particularly the peak at 413.3 mass units, do not come from the sample, but mainly from impurities of the matrix used. The enlarged region of the mass spectrum (Figure 3) evidence other minor populations. To assign these peaks, a collision-induced dissociation (CID) investigation was achieved with an electrospray ionization (ESI) source. This kind of mass spectrometry analysis consists in the dissociation of an ion (named the "parent ion", Figure 4) after a collision excitation with neutral gas molecules (argon in this case). The obtained fragments were consecutively examined and their mass could provide a structural information about the weak bonds of analyzed products. This investigation was carried out on "parent" peaks at *m*/*z* 524 (DP 3), 685 (DP 4), and 846 (DP 5) (Figure 4). In each case, the fragments with losses of 18, 59, and 119 mass units with regard to each "parent ion" were obtained. This phenomenon pointed out the bonds likely to break in the oligosaccharide during the classical mass spectrometry analysis. On the basis of this CID study and works already performed on the fragmentation of carbohydrate structures (occurring during the mass spectrometry analysis),[30] the identification of various peaks of the Figure 3 enlargement could be determined (Table 1). The losses of 119 and 59 mass units (peaks 1 and 2 in Figure 3) correspond to a fragmentation (as illustrated in the scheme of Figure 4) of $C_4H_9O_3N$ and C_2H_5ON groups, respectively. Moreover, losses of H_2O molecules (i.e., −18 mass units, peak 3 in Figure 3) are clearly detected in Figure 4. As a result, peaks 1, 2, and 3 do not correspond to species present in the mixture of GlcN oligomers, but are related to fragmentation phenomena caused by the analysis technique. Conversely, peaks 4 and 5 represent the expected GlcN chains

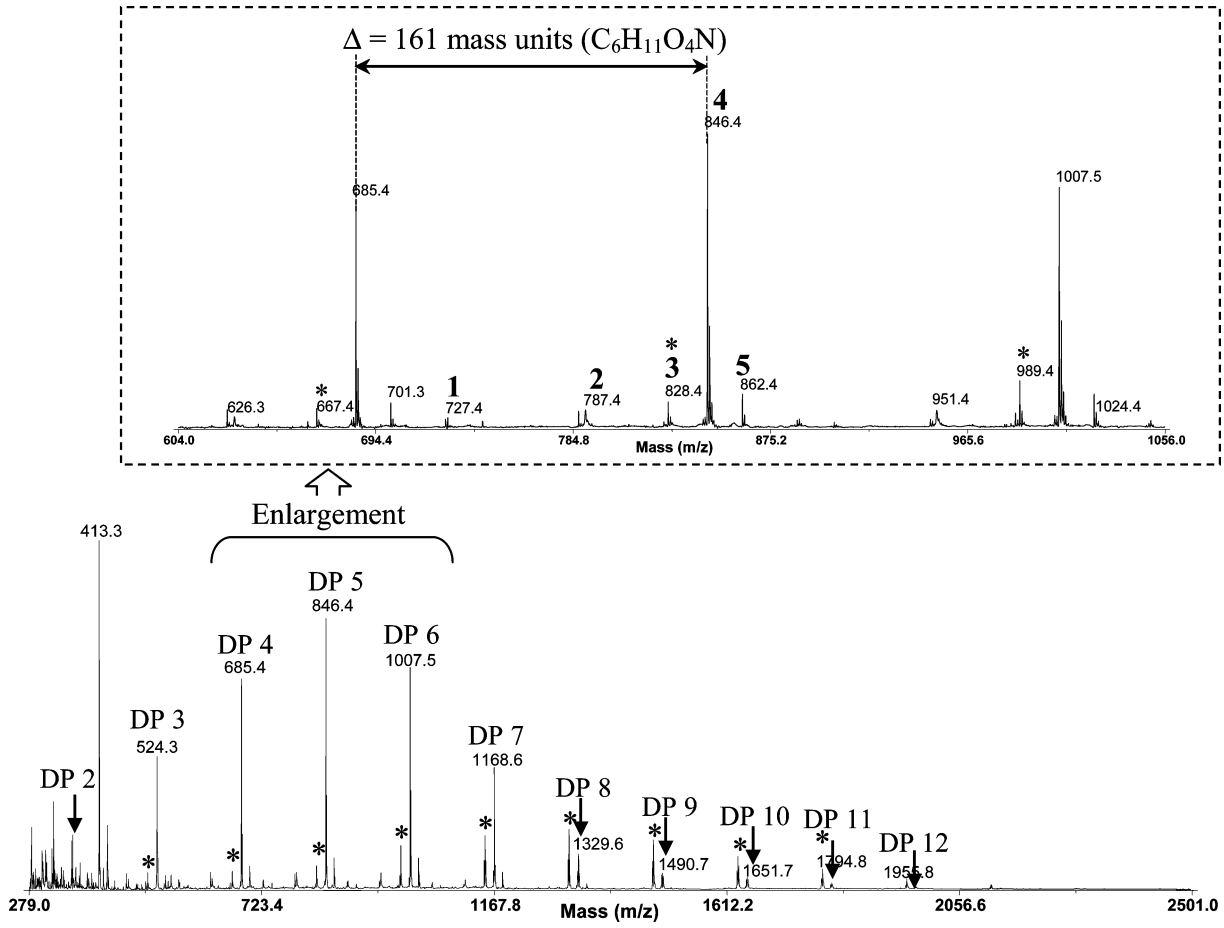

Figure 3. Positive ion MALDI-TOF mass spectrum of the mixture of glucosamine oligomers in reflector mode (with DHB matrix). (*) Peaks corresponding to 18 mass unit losses due to the analysis technique (see explanation in text). The peak at *m*/*z* 413.3 represents an impurity resulting from the MALDI experimental conditions. The inset shows an enlarged zone (between *m*/*z* 604 and 1056) of the global spectrum. The structural assignments of the peaks are presented in Table 1.

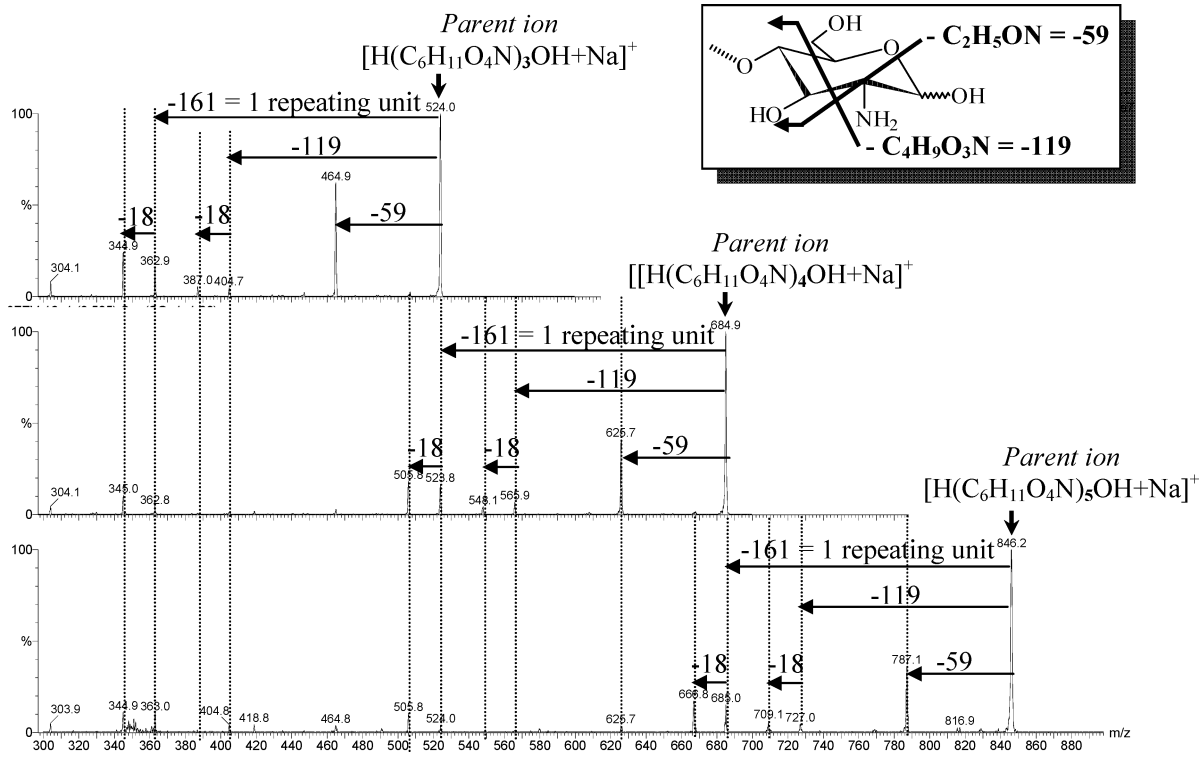

Figure 4. Fragmentation (or CID) analysis of glucosamine oligomer ions (= "parent ions", peaks at *m*/*z* 524, 685, 846), and scheme of glucosamine oligomer fragmentation deduced from this analysis and literature.[30]

Table 1. Structural Assignments of the Peaks Appearing in the Enlarged Region of the MALDI-TOF Mass Spectrum of the Mixture of Glucosamine Oligomers (Figure 3)

peak	monoisotopic mass (*m*/*z*) calculated	monoisotopic mass (*m*/*z*) experimental	formula	comment
1	727.3	727.4	$[H(C_6H_{11}O_4N)_5OH - (C_4H_9O_3N) + Na]^+$	fragmentation of $C_4H_9O_3N$ group
2	787.3	787.4	$[H(C_6H_{11}O_4N)_5OH - (C_2H_5ON) + Na]^+$	fragmentation of C_2H_5ON group
3	828.3	828.4	$[H(C_6H_{11}O_4N)_5OH - (H_2O) + Na]^+$	fragmentation of H and OH groups
4	846.3	846.4	$[H(C_6H_{11}O_4N)_5OH + Na]^+$	glucosamine chain cationized by Na
5	862.3	862.4	$[H(C_6H_{11}O_4N)_5OH + K]^+$	glucosamine chain cationized by K

Table 2. Average Degrees of *N*-Acetylation (DA) of the Series of Chito-Oligomers

sample	DA (%) expected [a]	DA (%; ^{1}H NMR)[b]	DA (%; MS)[c]
2	25	24 ± 1	29 ± 2
3	40	41 ± 1	43 ± 2
4	60	60 ± 1	61 ± 2
5	80	78 ± 1	77 ± 2
6	90	90 ± 1	88 ± 2

[a] Calculated from the proportion Ac_2O versus GlcN residues used (mol/mol). [b] Determined by ^{1}H NMR spectroscopy. [c] Determined by MALDI-TOF mass spectrometry.

cationized by sodium and potassium, respectively. To summarize, this thorough analysis displayed that the mixture of GlcN oligomers was pure and its distribution ranged from the 2- to 12-mers and centered on DP 5.

3.3. Determination of the Average DA of Chito-Oligomer Samples. The determination of the average DA of the different samples of partially *N*-acetylated chito-oligomers was preferentially carried out by ^{1}H NMR spectroscopy. The proton assignment of the diverse signals was deduced from ^{1}H NMR data of GlcN and GlcNAc homo-oligomers (from DP 2 to 6) published in the literature.[31] Compared with the ^{1}H NMR spectrum of the GlcN oligomer mixture, spectra of *N*-acetylated chito-oligomers differed essentially by the presence of characteristic signals of the GlcNAc units, that is, (i) two singlets at δ 2.06/2.08 ppm assigned to the N-acetyl protons and (ii) a broad signal at 5.20 ppm corresponding to H-1 protons of the reducing end α anomer residues, as illustrated in Figure 2. Furthermore, slight downfield chemical shift variations ($\Delta\delta \sim 0.10-0.25$ ppm) were also observed for H-2 protons of GlcN units related to the reducing end α anomer residues (δ 3.35 ppm), the middle residues, and the nonreducing end residues (δ 3.25−3.05 ppm), due to the presence of the amine function in the ammonium form for *N*-acetylated chito-oligomers only. However, signals of GlcN H-2 protons were sufficiently well separated to not overlap with signals of other repeating unit protons. Consequently, the average DA of the different acetylated samples could be determined considering both signal areas of H-2 protons of GlcN units ($A_{GlcN\ H\text{-}2}$) and acetyl protons of GlcNAc units (A_{CH3}) according to eq 1.

$$DA\,(\%) = \frac{\frac{1}{3}A_{CH_3}}{\frac{1}{3}A_{CH_3} + A_{GlcN\ H\text{-}2}} \times 100 \qquad (1)$$

Results given in Table 2 show clearly that the average DA values determined by NMR are in close agreement with the expected values calculated from the molar ratio Ac_2O versus GlcN units used for the *N*-acetylation reaction. To confirm these results, we attempted to determine the average DA of each *N*-acetylated chito-oligomer sample from the study of its MALDI-TOF mass spectrum displayed in Figure 5. Thus, the determination was carried out taking into account the relative ion intensity of each oligomer species identified in each series, as given in Supporting Information. It is indeed widely accepted in the literature that the relative ion intensity corresponding to one oligomer can reflect its relative proportion in a mixture, despite the fact that no linear relation between the relative ion

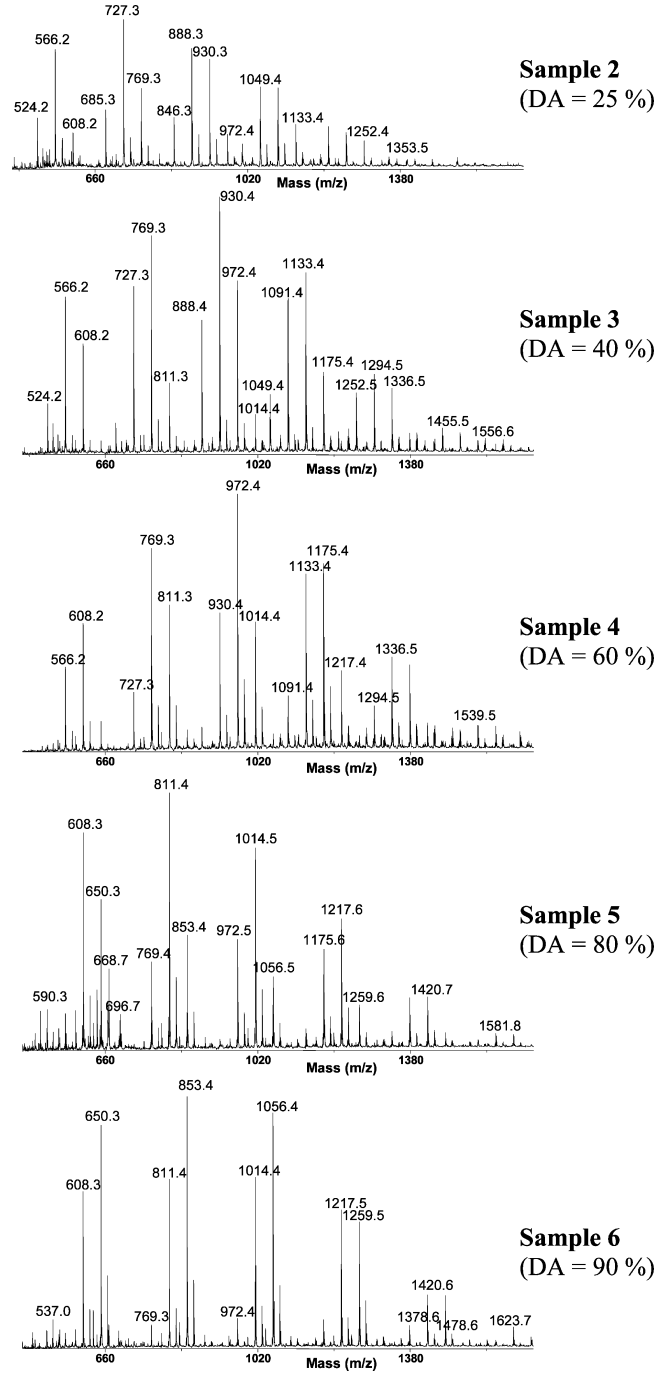

Figure 5. Same regions of positive ion MALDI-TOF mass spectra of the series of chito-oligomers (in reflector mode, with DHB matrix).

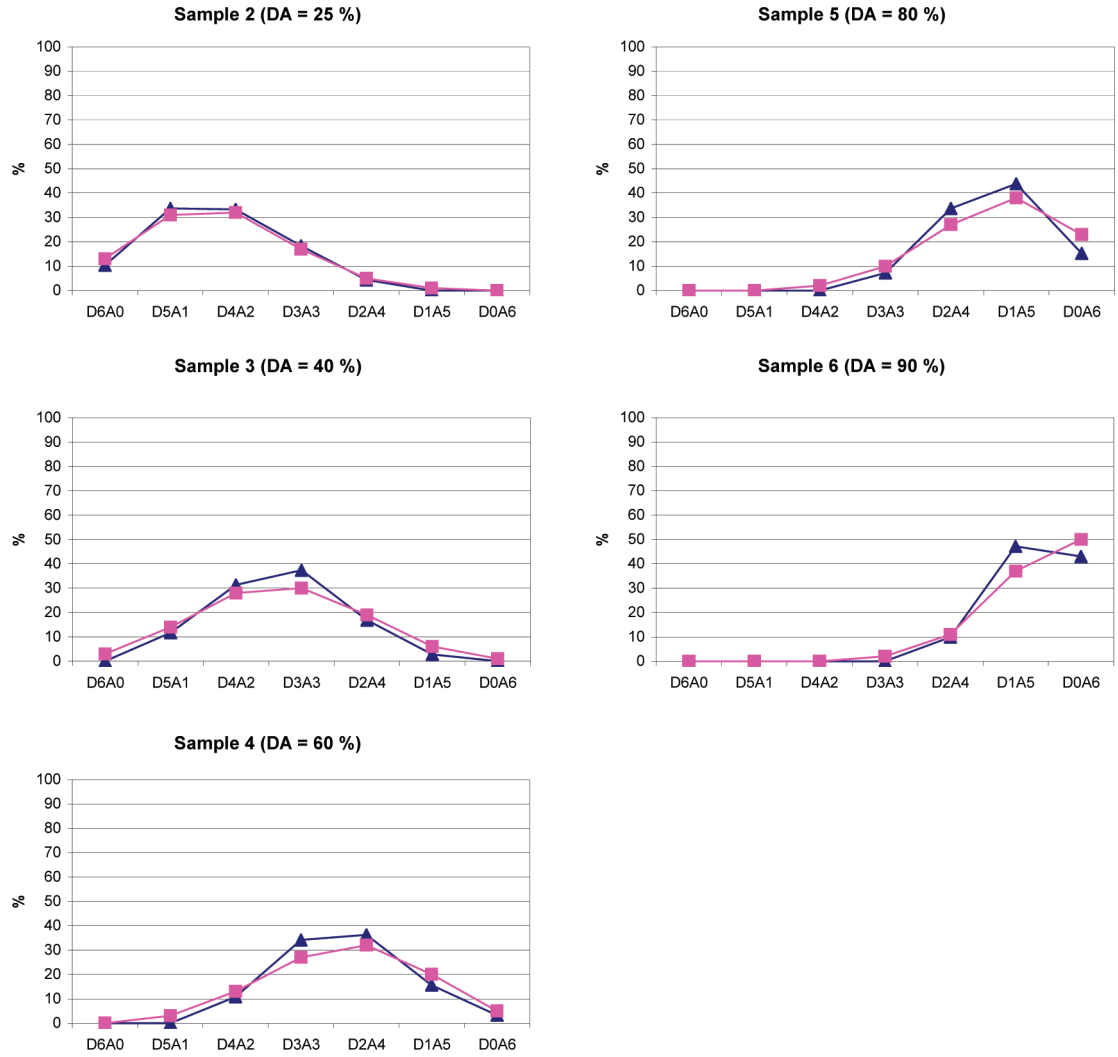

Figure 6. Distributions of D_xA_y species for chito-oligomers of DP 6 as a function of the average DA (-▲-, modeling distribution from MALDI-TOF MS analysis, -■- modeling distribution, D, for GlcN, and A, for GlcNAc).

intensities and their absolute amounts has yet been established.[27,32] Thus, average DAs were calculated considering more precisely the contribution of the main chito-oligomer species of each series, that is, chito-oligomers from DP 3 to 7 (it was indeed not possible to take into account oligomers in minority such as DP 2 and DPs 8−12 because of the matrix peaks and the low signal/noise, respectively), according to eq 2, where the DA_{th} (%) represents the theoretical DA value directly deduced from the D_xA_y structure (D, for GlcN, and A, for GlcNAc) of the corresponding oligomer (see Supporting Information for details) determined by MALDI-TOF MS as presented in paragraph 3.4.

$$\mathrm{DA}\,(\%) = \frac{\sum_i (\mathrm{DA}_{th}(\%))_i \times (\text{ion intensity})_i}{\sum_i (\text{ion intensity})_i} \qquad (2)$$

Compared to DA values determined by ^{1}H NMR, average DAs calculated by MALDI-TOF MS were found to be very similar for most of the samples and tend to confirm NMR results (Table 2). Even if a slight difference of 5% was observed in sample **2**, the good correlation obtained with these two techniques suggests that the MALDI-TOF MS can also be used as a quantitative analytical method for a precise determination of the average DA of mixtures of low DP chito-oligomers, particularly appropriate for a large range of DAs.

3.4. Determination of the Oligomer D_xA_y Distributions. MALDI-TOF MS was also used to characterize the chemical *N*-acetylation of GlcN oligomers and in identifying the various chemical structures of acetylated oligomers produced during the reaction. Indeed, the comparison of mass spectra of the different series reported in Figure 5 allowed us to study the predominance of specific species, as a function of DA. As expected, the mass spectrum of the high DA series (sample **6**; DA = 90%) revealed mainly the presence of fully acetylated oligomers (D_0A_3, D_0A_4, D_0A_5, D_0A_6, D_0A_7), while monoacetylated oligomers (D_2A_1, D_3A_1, D_4A_1, D_5A_1, D_6A_1) were mostly present in the mass spectrum of the low DA series (sample **2**; DA = 25%), as detailed in Supporting Information.

Owing to the relative complexity of other mass spectra, further interesting information was obtained especially in examining the distribution of all D_xA_y species related to a same DP, according to the average DA. More precisely, these distributions were evaluated in determining for each D_xA_y species, its relative proportion compared to other oligomers of same DP, by means of their peak intensities. In this case, the different distributions preferentially obtained for the main DPs ranging from 3 to 7 (Figure 6 and Supporting Information) showed clearly that, for each DP, the preponderant oligomers correspond to those for which the theoretical DA is the closest to the average DA of the mixture. For example, in sample **5**

(DA = 80%), species predominating for DP 3 to 7 were respectively D_1A_2 (DA_{th} = 67%), D_1A_3 (DA_{th} = 75%), D_1A_4 (DA_{th} = 80%), D_1A_5 (DA_{th} = 83%), D_2A_5 and D_1A_6 (DA_{th} = 71 and 86%, respectively).

Furthermore, it appears that some of these distributions displayed particular shapes, which could be described as pseudo bell-shaped curves. This is especially observed for chito-oligomers ranging from DP 3 to 7, for average DAs between 25 and 80% (samples **2**−**5** in Figure 6 and Supporting Information). Because bell-shaped curves are generally observed in statistical distributions, attempts at the modeling of these distributions were carried out considering that the acetylation of GlcN units occurred randomly along the oligosaccharide chain, as previously shown for the homogeneous acetylation of chitosan polymers.[33] Thus, for a given DP, the theoretical proportion of D_xA_y species was determined as a function of the average DA, calculating the probability (P (%)) to randomly acetylate a number x of GlcN units among a total number DP of GlcN (x) and GlcNAc (y) residues (DP = $x + y$), according to a binomial law illustrated by eq 3, where C_{DP}^{x} represents the number of combinations to pick x unordered outcomes from DP possibilities.

$$P(\%) = C_{DP}^{x}\left(\frac{DA(\%)}{100}\right)^{x}\left(1-\frac{DA(\%)}{100}\right)^{DP-x}\times 100 \quad (3)$$

The modeling of the oligomer D_xA_y distributions was carried out at different DAs for DPs ranging from 3 to 7 as displayed in Figure 6 and Supporting Information (square, modeling distribution; diamond, experimental distribution), respectively. The comparison of these modeling distributions with those determined by MALDI-TOF MS evidenced clearly the good to very good correlation between experimental and theoretical values. These results suggest that the chemical *N*-acetylation of a mixture of low DP GlcN oligomers occurred randomly in our homogeneous conditions, leading to statistical (Bernoullian) distributions of *N*-acetylated chito-oligomers. In this case, the relative proportion of the different D_xA_y species corresponding to a same DP could be simply estimated as a function of DA by means of a binomial law.

Conclusion

A homogeneous series of chitin/chitosan oligomers (chito-oligomers) varying from DA 0 to 90% with a narrow distribution of DPs within 2 and 12, centered on DP 5, was easily prepared by quantitative *N*-acetylations of a mixture of GlcN oligomers (previously obtained by a controlled acid hydrolysis of fully *N*-deacetylated chitosan). The determination of the DP distribution of the GlcN oligomer mixture was carried out by MALDI-TOF MS. For each sample of the series, the average DA was determined by ^{1}H NMR and confirmed by MALDI-TOF MS analyses, taking into account the relative ion intensity of the main chito-oligomers of each series, that is, species from DP 3 to 7. MALDI-TOF MS was also helpful to establish for the same DP range, the evolution of the distribution of all D_xA_y species corresponding to a same DP as a function of DA. The modeling of these distributions by a binomial law was found to be consistent with a random (Bernoullian) arrangement, suggesting that, in our experimental conditions, the chemical *N*-acetylation reaction of fully *N*-deacetylated chito-oligomers occurred statistically along the oligosaccharide chains. In this case, a precise estimation of the relative proportion of all D_xA_y species related to a same DP could be achieved whatever the DA and DP from 3 to 7.

Due to the increasing interest of chito-oligomers for their specific biological activities, especially in the range of DP within 3−8, our molecules are now tested to establish a structure−function relationship between the DA and DP of chito-oligomers on the one hand and the antimicrobial or eliciting activities inducing disease resistance in plants on the other hand.[6]

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Supporting Information Available. Distributions of D_xA_y species for chito-oligomers from DP 3 to DP 7 as a function of the average DA, and MALDI-TOF mass spectrum assignments of the series of chito-oligomers. This information is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Domard, A.; Domard M. In *Polymeric Biomaterials*; Dumitriu S., Ed.; Marcel Dekker: New York, 1994; p 187.
(2) (a) For a review, see: Jeon, Y. J.; Shahidi, F.; Kim, S. K. *Food Rev. Int.* **2000**, *16*, 159. (b) Kim, S. K.; Rajapakse, N. *Carbohydr. Polym.* **2005**, *62*, 357.
(3) Vander, P.; Vårum, K. M.; Domard, A. E. I.; Gueddari, N. E.; Moerschbacher, B. M. *Plant Physiol.* **1998**, *118*, 1353.
(4) Cabrera, J. C.; Messiaen, J.; Cambier, P.; Van Cutsem, P. *Physiol. Plant.* **2006**, *127*, 44.
(5) Kendra, D. F.; Hadwiger, L. A. *Exp. Mycol.* **1984**, *8*, 276.
(6) Moerschbacher, B. M.; El Gueddari, N. E. In *Advances in Chitin Science*; Domard, A., Guibal, E., Vårum, K. M., Eds.; Montpellier: France, 2007; Vol. IX, p 10.
(7) Domard, A.; Cartier, N. *Int. J. Biol. Macromol.* **1989**, *11*, 297.
(8) Tommeraas, K; Varum, K. M.; Christensen, B. E.; Smidsrod, O. *Carbohydr. Res.* **2001**, *333*, 137.
(9) Defaye, J.; Gadelle, A.; Pedersen, C. *Carbohydr. Res.* **1994**, *261*, 267.
(10) Nordtveit, R. J.; Vårum, K. M.; Smidsrød, O. *Carbohydr. Polym.* **1994**, *23*, 253.
(11) Aly, M. R. E.; Ibrahim, E. S. I.; El Ashry, E. S. H.; Schmidt, R. R. *Carbohydr. Res.* **2001**, *331*, 129.
(12) Kuyama, H.; Nakahara, Y.; Nukada, T.; Ito, Y.; Nakahara, Y.; Ogawa, T. *Carbohydr. Res.* **1993**, *243*, C1.
(13) Barroca, N.; Trombotto, S.; Domard, A. Patent PCT/FR2006/051102, 2006.
(14) Usui, T.; Matsui, H.; Isobe, K. *Carbohydr. Res.* **1990**, *203*, 65.
(15) Akiyama, K.; Kawazu, K.; Kobayashi, A. *Carbohydr. Res.* **1995**, *279*, 151.
(16) Singh, S.; Gallagher, R.; Derrick, P. J.; Crout, D. H. G. *Tetrahedron: Asymmetry* **1995**, *6*, 2803.
(17) Yoon, J. H. *Enzyme Microb. Technol.* **2005**, *37*, 663.
(18) Cabrera, J. C.; Van Cutsem, P. *Biochem. Eng. J.* **2005**, *25*, 165.
(19) Zhang, H.; Du, Y.; Yu, X.; Mitsutomi, M.; Aiba, S. I. *Carbohydr. Res.* **1999**, *320*, 257.
(20) Bahrke, S.; Einarsson, J. M.; Gislason, J.; Haebel, S.; Letzel, M. C.; Peter-Katalinic, J.; Peter, M. G. *Biomacromolecules* **2002**, *3*, 696.
(21) Lamarque, G.; Cretenet, M.; Viton, C.; Domard, A. *Biomacromolecules* **2005**, *6*, 1380.
(22) (a) Karas, M.; Hillenkamp, F. *Anal. Chem.* **1988**, *60*, 2299. (b) Karas, M.; Bahr, U.; Ingendoh, A.; Nordhoff, E.; Stahl, B.; Strupat, K.; Hillenkamp, F. *Anal. Chim. Acta* **1990**, *241*, 175.
(23) Bonk, T.; Humeny, A. *Neuroscientist* **2001**, *7*, 6.
(24) Distler, A. M.; Allison, J. *Anal. Chem.* **2001**, *73*, 5000.
(25) (a) Montaudo, G.; Lattimer, R. P. In *Mass Spectrometry of Polymers*; CRC Press: New York, 2002. (b) Montaudo, G.; Samperi, F.; Montaudo, M. S. *Prog. Polym. Sci.* **2006**, *31*, 277. (c) Pash, H.; Schrepp, W. In *MALDI-TOF Mass Spectrometry of Synthetic Polymers*; Springer-Verlag: Berlin, 2003.
(26) Nah, J.-W.; Jang, M.-K. *J. Polym. Sci., Part A: Polym. Chem.* **2002**, *40*, 3796.

(27) Li, J.; Du, Y.; Yang, J.; Feng, T.; Li, A.; Chen, P. *Polym. Degrad. Stab.* **2005**, *887*, 441.
(28) Kittur, F. S.; Kumar, A. B. V.; Varadaraj, M. C.; Tharanathan, R. N. *Carbohydr. Res.* **2005**, *340*, 1239.
(29) Lee, H.-W.; Park, Y.-S.; Jung, J.-S.; Shin, W.-S. *Anaerobe* **2002**, *8*, 319.
(30) El Rassi Z. In *Carbohydrate Analysis by Modern Chromatography and Electrophoresis, Journal of Chromatography Library*; Elsevier Science: New York, 2002; Vol. 66, p 989.
(31) Sugiyama, H.; Hisamichi, K.; Sakai, K.; Usui, T.; Ishiyama, J. I.; Kudo, H.; Ito, H.; Senda, Y. *Bioorg. Med. Chem.* **2001**, *9*, 211.
(32) Naven, T. J. P.; Harvey, D. J. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 1361.
(33) Schatz, C.; Pichot, C.; Delair, T.; Viton, C.; Domard, A. *Langmuir* **2003**, *19*, 9896 .

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