Overview on structural characterization of chitosan molecules in relation with their behavior in solution.

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Summary.

This paper concerns the new results obtained on the characterization of chitins and chitosans. Large series of samples was analyzed covering a wide range of water soluble and insoluble materials. The water soluble polymers were obtained by heterogeneous deacetylation and by homogeneous reacetylation. The calibration of IR spectrum was proposed and shown to be valid in all the range of DA. Application of ^{15}N and ^{13}C solid state NMR was developed to be able to determine DA even *in situ* on insoluble natural materials. All the methods proposed give a very coherent set of results. The molecular weight distribution was established by GPC using cationic porous supports and the good solvent earlier proposed 0.3M AcOH/ 0.2M AcONa. The role of the distribution of acetyl groups along the chain is also discussed and analyzed by NMR ; it is demonstrated clearly the difference between homogeneously acetylated samples and heterogeneous samples coming from different routes of preparation. The dependence of Mark-Houwink parameters allowing to relate the intrinsic viscosity with the molecular weight is also briefly reported. In good concordance with experimental data, molecular modeling helps in understanding the role of the N-acetyl group content and distribution on the stiffness of the chains.

Introduction.

Chitin is one of the most important natural polymer constituting the shells of crustaceans and also the cell walls of many fungi. Commercial chitin can be found with various average degrees of acetylation (DA) ranging from the fully acetylated to the totally deacetylated products. With the higher degree of acetylation, this polymer is soluble in very few solvents which limits its applications. But, after partial deacetylation, when DA is lower than 50%, it becomes water-soluble in aqueous acidic conditions; then it is called chitosan. Usually the reaction is performed in heterogeneous conditions; then, the residual acetyl substituent distribution depends on the source of chitin, on the conditions of deacetylation and on the degree of residual acetylation. It is clear that the solubility of these polymers must directly

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depend on the average degree of acetylation but also on the distribution of the acetyl groups along the chains.

These last years, we have developed experimental work to characterize the chitin and chitosan molecular structure in connection with the physical properties in solution [1)]. The determination of the degree of acetylation as well as its molecular weight remains up to now under discussion but much work is performed due to the large importance of these characteristics on the physical properties of the polymer.

In our work, we have developed and compared different techniques to determine the degree of acetylation (DA). In that respect, IR and ^{1}H liquid state NMR were used. Solid state ^{13}C and ^{15}N NMR were also used to compare their significance and to analyze chitin and chitosan whatever the DA is.

Then, we examine the distribution of acetyl groups along the chain by analysis of the high resolution liquid state ^{13}C NMR spectrum; the diads and triads were directly established on the polymeric material in relation with the origin of the polymers.

The molecular weight distribution was established by gel permeation chromatography equipped with two detectors in line. Using specific columns and solvent, we got the molecular weight distributions on various samples of chitosan with different DA; using the model developed in our laboratory, the interpretation of the experimental results gives not only the average molecular weights but also the persistence length (Lp). Independently, molecular modeling was performed to predict the stiffness of chitin and chitosan for different degrees of acetylation but also for different distributions of the substituents.

Our contribution allows to better understand the behavior in solution on the basis of a proper characterization of the polymers.

Keywords: Chitin; Chitosan; Degree of Acetylation; NMR ; Infrared Spectroscopy ; Molecular Weight Distribution ; Steric Exclusion Chromatography ; Molecular Modeling.

Experimental part.

1. Sample characteristics.

Chitosan samples from different commercial origins were carefully purified as previously described[2)]. They were characterized by their average value of DA determined by liquid state ^{1}H NMR in D_2O acidic conditions (with HCl addition to pH~4)[3)]. The NMR equipment was an AC 300 from Brüker. The spectral measurements were usually performed at 353K after freeze drying and redissolution of the chitosan sample three times to exchange

labile H atoms. The DA is determined, with a precision of 5%, from the integral of the CH_3 signal at 1.97 ppm compared with the integral of H-1 protons considered as an internal standard. The influence of the temperature on NMR response was also investigated by varying temperature in presence of an internal standard (DMSO) added in controlled amount to calibrate the polymer specific signals.

Some samples were prepared by homogeneous reacetylation of highly deacetylated chitosan in a large range of DA (0 to 60%) and different average molecular weights. The conditions used are described below.

2. Preparation of reacetylated chitosans.

The starting chitosan was a sample supplied by Aber Technologies, France (DA=2%). Portions (2 g) of initial or partially hydrolyzed product were redissolved in 0.1 M acetic acid (200 mL) and diluted with methanol (250 mL). Then to each solution was added, with vigorous stirring, a further 50 mL of methanol containing the amount of acetic anhydride required to give the target level of *N*-acetylation (calculated assuming a reaction efficiency of 100%)[4]. After standing for 24 hours at 25°C the *N*-acetylchitosans were precipitated out by addition of concentrated NH_4OH and isolated by centrifugation. The samples were then washed to neutral with 75% aqueous methanol and dried at 60°C under vacuum. It was demonstrated that no O-acetylchitosan is formed from ^{13}C NMR measurements.

3. Chromatography conditions.

SEC was performed using multi-detector equipment; the chromatograph was a 150C Waters with a differential refractometer to determine polymer concentration; a multiangle laser light scattering detector from Wyatt (DAWN DSP-F) was added on line to get the radius of gyration and the molecular weight of the samples during elution.

The solvent adopted was that proposed previously [5] : acetic acid 0.3M/ sodium acetate 0.2M; two columns in series type SynChropak CATSEC 100 and 1000 (USA) were used and the temperature adopted was 25°C. The polymer concentration injected was around 0.5 g/mL. The dn/dc was determined for the different DA values in the same solvent using a special home made loop to work strictly in the same experimental conditions (temperature, solvent, wavelength). All the samples are filtrated on 0.1μm membrane before injection on the columns.

4. Viscosity Measurements.

The viscosity measurements were performed with an Ubbelohde capillary viscometer (inner diameter 0.58 mm) at 25 ± 0.01°C after having controlled that no shear rate effect exists in

the range of molecular weights and polymer concentrations covered; the solvent is the same as for SEC experiment.

5. Solid state NMR

The NMR experiments were performed on a Bruker MSL spectrometer operating at a ^{1}H frequency of 200 MHz using the combined technique of proton dipolar decoupling (DD), magic angle spinning (MAS) and cross-polarization (CP). Field strengths corresponding to 90° pulses of 4 μs and 7.5 μs were used for the matched spin-lock cross-polarization transfer for the ^{13}C and ^{15}N respectively. The contact time was 1 ms, the acquisition time 70 ms, the sweep width 29,400 Hz and the recycle delay 4 s for the ^{13}C. A typical number of 10,000 scans were acquired for each spectrum. The chemical shifts were externally referred by setting the carbonyl resonance of glycine to 176.03 ppm. According to Yu *et al.*[6], the contact time was 2ms, the acquisition time 20ms, the sweep width 10,000 Hz and the recycle delay 1 s for the ^{15}N spectra. A typical number of 100,000 scans were acquired. The chemical shifts were externally referred to NH_4^+ of enriched ammonium nitrate. The spinning speed was set at 3,000 Hz for all samples.

6. Molecular modeling.

The computational strategy and the various assumptions used in the modeling protocol have been described in detail elsewhere[7] and will be only summarized here.

As a preliminary study, the conformational characteristics of the glycosidic bond of each dimeric segment that occur in chitosan chains (four disaccharides) has to be established as follows. From standard starting geometries of the disaccharides, the conformational space was explored by rotating the residues around Φ (O-5'—C-1'—O-4—C-4) and Ψ (C-1'—O-4—C-4—C-5) on a 10° grid. At each point of the grid, several orientations of the exocyclic groups were taken into account and geometry optimizations were carried out while constraining the Φ and Ψ torsion angles. At most 81 starting geometries are needed to account for the three staggered orientations of each hydroxymethyl group, the two hydrogen-bonding networks of both the secondary hydroxyl groups and the amino group or acetamido groups. The results are then expressed to as adiabatic potential (Φ,Ψ) maps. The potential energy function and the force field parameters used in this study are those of MM3(92)[8] with a dielectric constant of 78.

From those potential energy surfaces together with the geometries of the energy minima, it is possible to use the program Metropol[7] to generate non-perturbed conformations of long chains. Ensemble-average chain properties are then determined by the conformational

sampling based on a Metropolis Monte Carlo[9] algorithm. The stiffness of the polysaccharide chain is characterized by the persistence length Lp. It is defined as the average projection of the end-to-end vector of an infinite chain in the direction of the first segment of the chain.

The typical calculations involve averaging for 3000 molecules containing 2000 residues each. Such calculations have been repeated ten times. Calculations have been performed at different temperatures. The generated chitosan chains vary in their acetyl content and in their fine structure. The sequence of monomers was either random, in blocks or alternate.

Results and discussion.

The schematic representation of chitin is given in Figure 1. Depending on the molar fraction of acetylated D-glucosamine units, the polymer is named chitin or chitosan as mentioned previously. In addition, intramolecular H-bonds stabilize the conformation. This secondary structure will play a role on solution properties as discussed later.

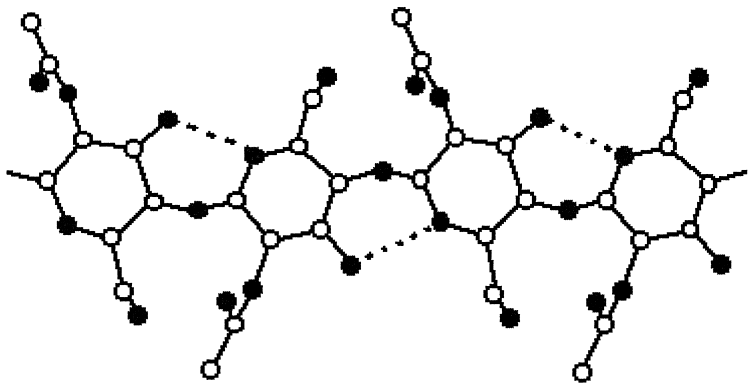

Figure 1. Representation of the chitin molecule in which the intrachain H bonds are represented.

A- Molecular modeling and chain stiffness

A.1.Theoretical approach.

The model adopted has been described previously[10]; the chains are characterized by an intrinsic persistence length Lp, related with the local stiffness of the chain. Lp is determined for the neutral equivalent chain (screening of the electrostatic repulsions by salt addition); but, for a charged molecule, such as chitosan in aqueous acidic solution at a finite ionic

concentration, the effective persistence length is increased due to electrostatic repulsion between neighboring ionic sites by Le, the electrostatic persistence length which can be calculated. Then, the persistence length in our experimental conditions is

$$Lt=Lp+Le \qquad (1)$$

For wormlike chains in θ-conditions the relation between the radius of gyration Rg, the contour length L and Lp is that given by Benoit-Doty:[11]

$$Rg^2= L\,Lp/3 - Lp^2 + 2\,Lp^3/L - 2(Lp^4/L^2)[1- \exp(-L/Lp)] \qquad (2)$$

In the model, for given thermodynamic conditions, two electrostatic contributions are added following the Odijk treatment:[12] 1) an electrostatic excluded volume coefficient which can be calculated for each charge parameter of the polyelectrolyte, each M and each salt concentration; 2) the electrostatic persistence length contribution mentioned previously.

From this approach, we are able to calculate Lp from each set of experimental data.

This parameter Lp is a characteristic of the local stiffness of the polysaccharides which behave as a wormlike chain. The higher the Lp, the higher the viscosity of a solution for a given molecular weight chain dissolved at a given concentration.

The value of Lp increases slightly with DA and changes with the distribution of the N-acetyl groups along the chain. This point will be discussed in the following.

A.2. Molecular modeling

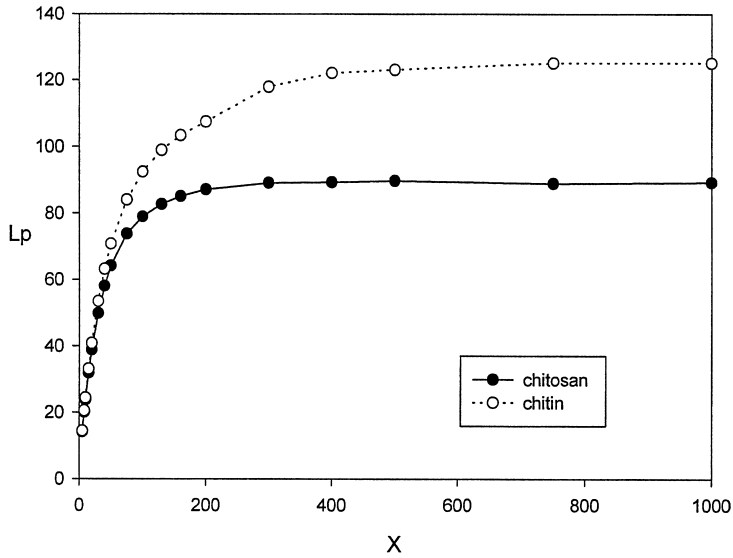

Figure 2. Influence of the polymerization degree (X) on the the persistence length for DA=0 and 100%.

Idealized 3,000 homopolymers chains of chitosan and chitin having a degree of polymerization of 2,000 each (corresponding to the range of molecular weights of our samples) were generated according a Metropolis Monte Carlo procedure at 50° C. This conformational sampling, repeated ten times, allows the computation of the average persistence length (Lp). Figure 2 shows the chain length dependence of the Lp. The estimated asymptotic limit of the Lp is 90 Å for chitosan and 125 Å for chitin, indicating that chitin favors a more extended conformation when compared to chitosan.

To address the question of the effect of the degree of *N*-acetylation on chain dimensions, chains differing in the amount of *N*-acetyl groups (10, 20, ...90%) were generated assuming a random distribution of the substituents [13]. The dependence of Lp on DA for the chitosan and chitin chains is shown in Figure 3. Several points of interest emerge from this dependence.

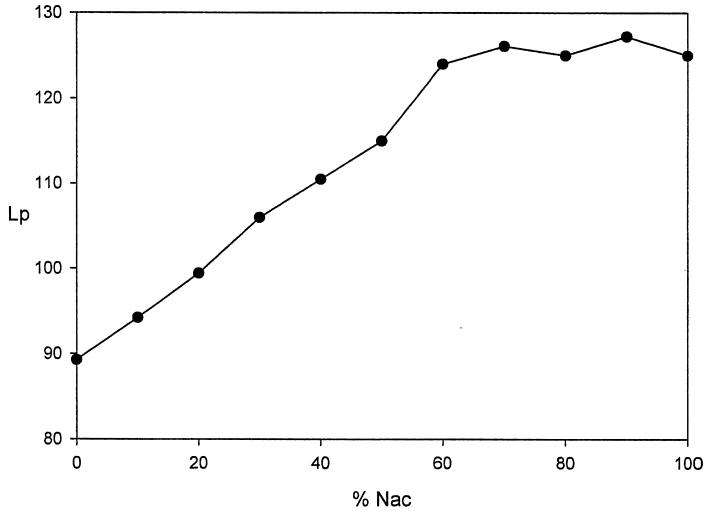

Figure 3. Role of the degree of acetylation on the persistence length obtained at 50°C.

First, at low *N*-acetyl content, between 0 and 60%, the calculated Lp is sensitive to the *N*-acetyl group content and the values increase as the amount of *N*-acetylated residue increases. Second, values are not significantly different for the range of *N*-acetylation between 60 and 100% for which the curve shows a plateau indicating that, in this range of acetylation, the chain extension is globally the same.

Finally, to investigate how the monomeric distribution influences the chain properties, calculations have been performed for chains having 50% glucosamine and 50% *N*-

acetylglucosamine residues with different patterns of monomeric distribution either random, alternate and in blocks at 50°C. Persistence length of a random (Bernouillian) chain is predicted to be 115 Å as calculated previously. Alternate AB copolymers increase the Lp to 135 Å, whereas it decreases to 97 Å for the A_2B_2 ones. It should be noted that there is no significant variation of the Lp for copolymers having from 2 to 20 consecutive glucosamine and *N*-acetylglucosamine monomers. This suggests that the chain dimensions would be the same for samples having either a random or a block pattern of substitution of the *N*-acetyl groups. On the contrary, the alternate AB copolymer is stiffer when compared to the others.

A-3. Role of temperature.

The stiffness of the chitosan depends on the temperature due to change in the local mobility due to destabilization of intramolecular H bonds. The first evidence was obtained from analysis of NMR spectrum obtained for different temperatures; for this approach, we added an internal standard for signal calibration. DMSO was chosen as a small and mobile molecule in an amount in the same range as the $-CH_3$ content of the polymer. The signal of the polymer (or more precisely the ratio X between the polymer signal and that of the standard) increases as the temperature increases as shown in Figure 4.

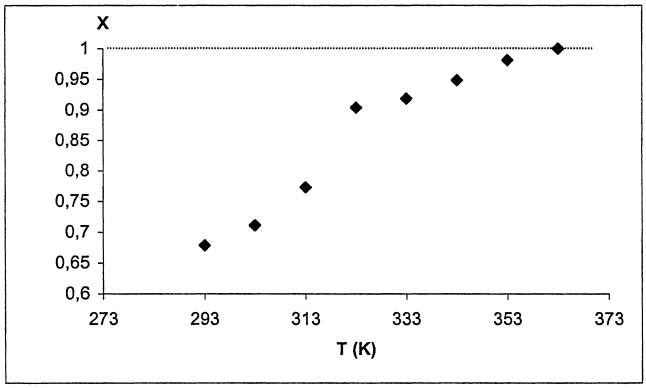

Figure 4. Evolution of relative amplitude of the ^1H NMR signal as a function of the temperature.

This evolution indicates an increase of the chain mobility. This result is perfectly reflected by the evolution of the intrinsic persistence length Lp predicted from molecular modeling (data not shown).

The dimensions of the chains are related with Lp and can be determined by the evolution of the intrinsic viscosity as a function of the temperature.

B-Average degree of acetylation

Different techniques have been proposed to evaluate the average degree of acetylation of chitosans, including infrared, ^{13}C solid state NMR, ultraviolet spectrometry, potentiometric titration, ^{1}H liquid state NMR or elemental analysis. But only infrared, ^{13}C solid state NMR and elemental analysis may be also used to determine the average degree of acetylation of chitins due to the fact that these techniques do not need the solubilization of the polymer. However, some experimental difficulties arise in each technique. For instance, the deconvolution of the amide band (1655 cm^{-1}) in the infrared spectrum is rather difficult. Among these techniques, ^{13}C solid-state NMR appears to be the most reliable for the evaluation of the acetyl content. However, it needs a high level of purification of the studied material.

In a recent communication, Yu *et al.*[6] showed that the evaluation of the acetyl content is possible through the ^{15}N CP-MAS NMR technique. As among polysaccharides the ^{15}N nucleus is only present in chitin and chitosan, this technique appears very promising to evaluate the acetyl content *in situ*, without any strong purification processes.

B.1.Evaluation of DA by ^{1} H NMR

It is easy to characterize chitosan in acidic conditions using liquid state NMR. To avoid the overlap between acetic acid and acetyl groups signals, we add few HCl to D_2O to solubilize the polymer.

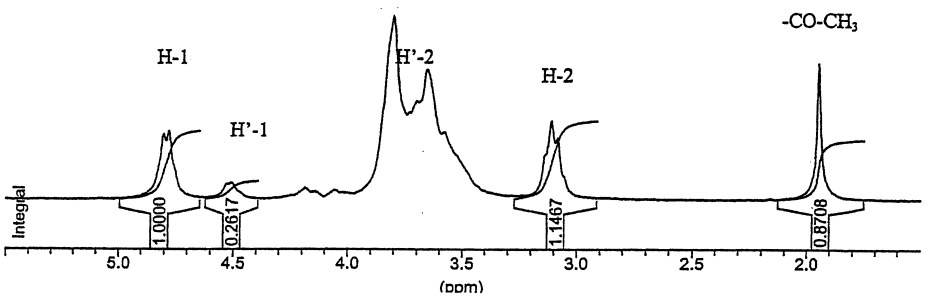

Figure 5. ^{1}H NMR spectrum obtained for a chitosan DA=23% at 353K in D_2O with HCl. Polymer concentration C=10mg/mL.

Figure 5 represents the ^{1}H NMR spectrum obtained for a heterogeneous chitosan with DA=23%. The acetylated units give specific signals H'-1; the DA is determined from the ratio of the integrals of H'-1 and (H-1+ H'-1) or from the ratio between the integrals of CH_3 and 3(H-1+H'-1). The assignments for the protons given in the literature were adopted and are recalled in Table 1.

Table 1. Proton assignment in liquid state NMR.

Proton	δ ppm (TMS)
H-1	4.79
H'-1	4.50
H-2	3.10
H'-2	3.70
H-3 to H-6	3.58-3.80
$-CH_3$	1.95

Nevertheless, this technique is just useful for soluble samples of chitosan.

B.2.Evaluation of DA by ^{13}C CP-MAS NMR

Special care was taken for the quantitative analysis of the NMR measurements. As aforementioned, the DA is evaluated from the relative integrals of methyl or carbonyl groups compared to the carbon integrals of the polysaccharidic backbone. The assignments of the different carbons are listed in the Table 2.

Table 2. Chemical shifts of carbon atoms for α-chitin in ^{13}C CP-MAS NMR[14].

Carbon	**δ (ppm)***
C-1	104.5
C-2	55.6
C-3	73.6
C-4	83.6
C-5	76.0
C-6	61.1
C=O	173.7
CH_3	23.1

* reference TMS.

However, the kinetics of the cross-polarization process should not be the same for each group. The contact time usually used in ^{13}C CP-MAS is 1ms. The rise of the magnetization with contact time has been followed for the highly acetylated chitin sample.

The attribution of the peaks were done according to Tanner *et al.*[14]. It has been found that after 1ms, the carbons of the methyl and polysaccharidic backbone magnetization reach a value of 88% of the theoretical maximum magnetization M_0, whereas the carbonyl only reach 84%. By considering the carbonyl peak, a systematic error of a least 4% could be done. In the following, we will only consider the DA measured by integration of the methyl peak.

Figure 6a displays the ^{13}C CP-MAS spectra of a sample obtained by homogeneous reacetylation.

B.3.Evaluation of DA by ^{15}N CP-MAS NMR

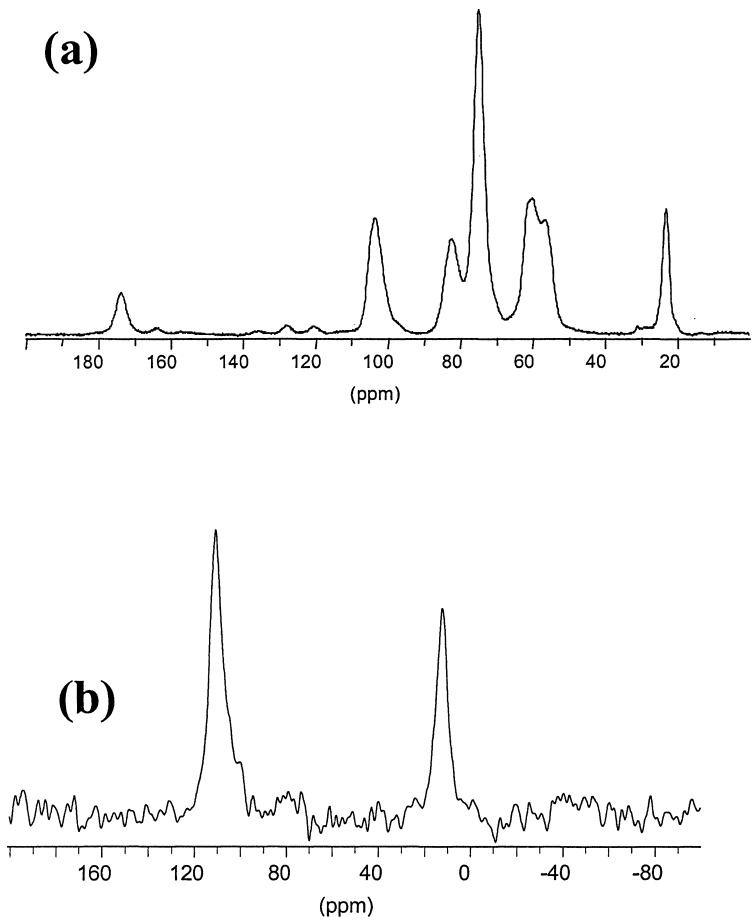

Figure 6. NMR spectra of chitosan DA=58% prepared in homogeneous conditions.
(a) ^{13}C CP-MAS ; (b) ^{15}N CP-MAS.

Figure 6b displays the ^{15}N CP-MAS spectra obtained with the same sample as used in the ^{13}C experiment. It was shown previously that chitin and chitosan display two unique peaks at respectively c.a. 110 ppm for the amide and c.a. 10 ppm for the amine groups. Cross-polarization kinetics has been followed for the two peaks. This corresponds to the observation of Yu *et al.* on partially deacetylated chitin [6]. The two groups share the same $T1\rho(^1H)$ of 5 ms. At a contact time of 2 ms, both groups reach 66 % of the theoretical magnetization M_0, so that the quantisation by direct integration is reasonable. Although the confidence interval should not be greater than 5%, data from liquid state ^1H NMR, solid state ^{13}C and ^{15}N CP-MAS are in good agreement in the whole range of DA[15]. Additionally, due to the line broadening effect in ^{15}N CP-MAS, one could not expect to detect acetylation level lower than 10% with this technique. In that sense, solid state ^{15}N NMR is less sensitive than ^{13}C. However, the line-widths in ^{15}N should give a good indication of the cristallinity of the sample. Additionally, the lines are isolated and easier to treat in ^{15}N NMR in the general case in which there is no other nitrogenated species.

B.4.IR determination.

The use of infrared spectroscopy for the characterization of the composition of chitin and chitosan covering the entire range of degree of acetylation (0<DA<100%) and a wide variety of raw materials is further examined. First, in Figure 7, the IR spectrum of chitosan clearly shows that the degree of hydration of the sample has no influence on the spectrum.

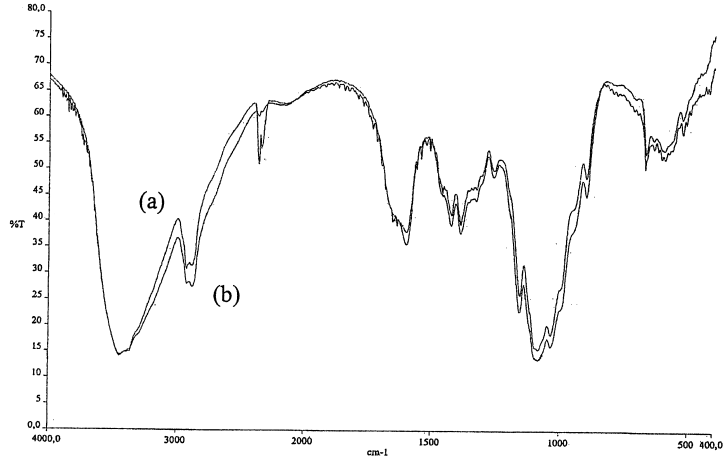

Figure 7. IR spectra for chitosan.(a) dried; (b) stabilized at 98% RH.

The ratio of absorbance bands selected was calibrated using ^1H liquid and ^{13}C CP-MAS solid-state NMR as absolute techniques. IR spectra of the structural units of these polymers

validated the choice of baselines and characteristic bands. The bands at 1650 and 1320 cm^{-1} were chosen to measure the DA. As internal reference the intensities at 3450 and 1420 cm^{-1} were evaluated. The absorption band ratios involving the reference at 3450 cm^{-1} had poorer fit. The absorption ratio A_{1320}/A_{1420} shows superior agreement between absolute and estimated DA-values (DA% = 31.92·A_{1320}/A_{1420} – 12.20; r = 0.990). The results are given in Figure 8. The only deviation comes from two samples of β-chitin; this may be due to the difference in morphology compared to the α-chitin samples[16].

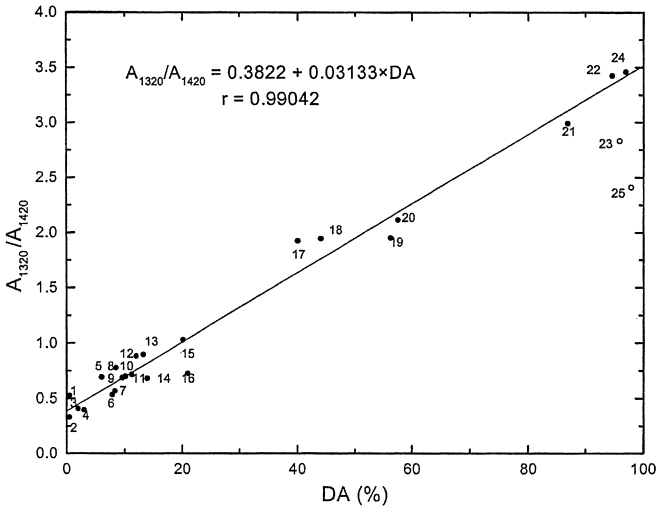

Figure 8. Calibration curve for chitin and chitosan as a function of DA (o are β-chitins).

C. Distribution of acetyl groups

As described previously by Varüm *et al.* [17,18], ^{1}H and ^{13}C NMR on partially hydrolyzed chitosan allow to describe the distribution of N-acetyl groups in chitosan. In their work, samples having the same degree of acetylation but obtained from homogeneous or heterogeneous reaction were compared. The distribution of diads and triads were analyzed and compared with a Bernoullian distribution. Whatever is the origin of the sample for DA<30%, they concluded that the distribution of the acetyl groups was random .

In our work we developed direct NMR investigation on chitosan with the same average degree of acetylation but different distributions of the acetyl substituents. The homogeneous samples were prepared by reacetylation of fully deacetylated chitin in homogeneous conditions. The heterogeneous samples were obtained by solid state deacetylation of chitin.

The ^{13}C NMR analysis was performed on high field NMR equipment; the spectra were analysed to determine the diads and triads characteristic of the distribution; the C-1 and C'-1 were examined. The spectra are given in Figure 9 for DA=22% in homogeneous (2) and heterogeneous (1) structures. The advantage of the conditions adopted is that the distribution is established on the undepolymerized chitosan; the only condition is the solubility in acidic conditions.

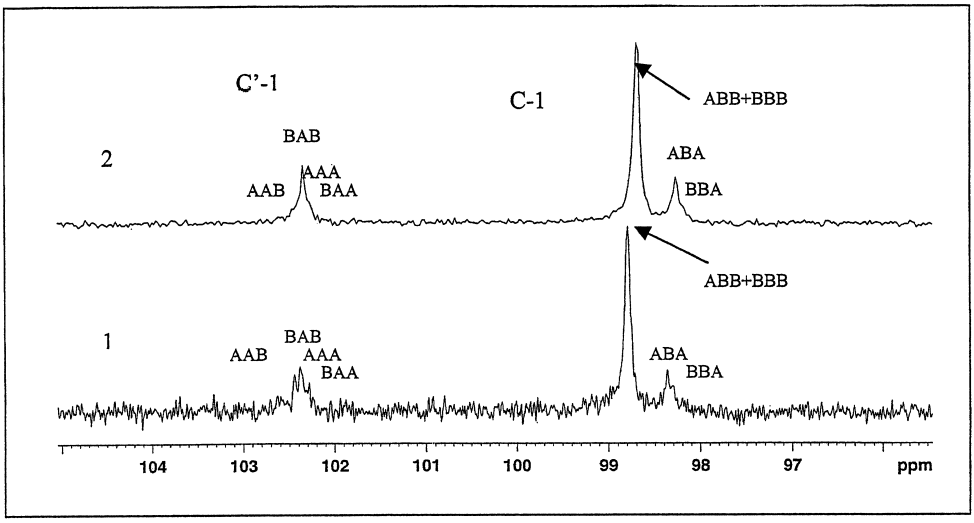

Figure 9. C-1 region of the ^{13}C NMR of two chitosans with DA= 22%.
(1) heterogeneously acetylated; (2) homogeneously acetylated.

The distribution of diads and triads for heterogeneous distribution of acetyl groups reflected a blockwise distribution with the higher frequency of Glu-NAc---Glu-NAc blocks.
On homogeneous samples, the alternated blocks of Glu-NAc----Glu-NH_2---Glu-NAc are most probable. The interesting conclusion is that this result allows to interpret the Lp values obtained by GPC analysis; the Lp is shown to be higher for homogeneously substituted chitosan (Lp~130Å compared to 110 Å for heterogeneous sample) and the same conclusion was established from molecular modeling [13].

D-Molecular weight distribution

The different samples were passed through the equipment to get the molecular weight distribution from which the function Rg(M) is obtained directly. But in a first step, some constants, such as dn/dc, or the second virial coefficient A_2, have been determined to

calculate the exact value of M by SEC coupled with a multi-angle laser light scattering detector with the Wyatt software (Astra 4 for Windows). The value of dn/dc has been studied as a function of DA. This shows that there is no significant influence of DA on dn/dc and so the average value 0.190 ± 0.005 (mL/g) was adopted. This value is in good agreement with those previously given in the literature.

Light scattering was also used to measure in static conditions the weight average molecular weight (Mw) and the second virial coefficient (A_2) of two samples. This resulted in an average A_2 value of 1.5 $x10^{-3}$ $mol.mL.g^{-2}$ showing that the solvent adopted is a good solvent for chitosan. The influences of the solution concentration, the flow rate and the ionic strength on the molecular characteristics were also examined. It was shown that the initial solution concentration injected, between 0.5 and 1.5 $g.L^{-1}$, does not have any effect on the results obtained. The flow rate was fixed at 0.3 $mL.min^{-1}$ to have good separation from the solvent signal. It is important to note that, generally , more than 95% of the product was eluted from the columns; this implies no adsorption and good solubility. This result permits us to conclude there is no aggregation when the solvent AcOH (0.3M)/AcONa (0.2M) is used.

It must be mentioned that no evidence of aggregation appears on the chromatograms obtained for samples injected at a concentration lower than the overlap concentration. Then, the SEC analysis is performed with no difficulties.

A series of Rg (M) curves for samples in the same average molecular weight range but with different DA values, obtained by homogeneous *N*-reacetylation of chitosan was analyzed. Over the range of average DA values from 0 to 60%, the samples are perfectly soluble and show only a very slight deviation in the curves. The fact that the different curves can be nearly superimposable indicates that the DA has no significant role on the hydrodynamic volume and confirms our previous results. The intrinsic persistence length and the parameters of the relationship:

$$Rg=K'M^{\nu} \qquad (3)$$

were determined for each curve Rg(M) and it was concluded that the exponent ν and the constant K' have average values equal 0.548 ± 0.002 and 0.064 ± 0.002 respectively over the range of DA values studied. From the theoretical treatment, the Lp values are calculated for each Rg(M) curve separately; it is shown that Lp value varies slightly when DA increases with Lp= 110 Å for DA=2% up to 150 Å for DA~60%[19] (Table 3).

Table 3. Experimental values obtained for K' and ν for different DA on homogeneous samples.

DA%	Lp (Å)	K'	ν
2	110	0.063	0.546
12	130	0.062	0.546
24	130	0.065	0.546
41	150	0.063	0.547
61	150	0.063	0.549

These Lp values are larger than those calculated from molecular modeling; at 25°C, the predicted values are Lp= 95Å and Lp= 125 Å for DA= 0 and 50% respectively, assuming a random distribution of the acetyl groups. The values of Lp obtained for homogeneous *N*-reacetylated chitosan are higher than those predicted for the two limits given previously. The reason for these higher values could be related to the acetyl group distribution along the chain; in fact, the molecular modeling also predicted that for an alternating distribution of acetylated and non-acetylated glucosamine units, the chain is stiffer (by about 17%). More investigation on the relation between the chemical modification of chitin and the distribution of substituents along the chain as determined by NMR has to be performed.

From the previous treatment, it is easy to predict the K and a parameters of the Mark – Houwink relation allowing to relate the intrinsic viscosity to the molecular weight. The values are given in Table 4 .

Table 4 . Mark-Houwink parameters predicted for the wormlike chain model from GPC experiments on homogeneous and heterogeneous samples.

DA (%)	K	a
0-3	0.079	0.796
12	0.074	0.800
22-24	0.0695	0.81
40	0.0634	0.823
56-61	0.0574	0.825

In the next table, the values previously given assuming that the DA has a low influence on K and a parameters are recalled [5].

Table 5. Mark-Houwink parameters given previously [5].

DA	K	a
<10%	0.082	0.76
>10%	0.076	0.76

These values are in relatively good agreement, nevertheless their application on intrinsic viscosity values gives larger Mv values than the application of values given in Table 4.

The data Rg(M) given for different samples obtained in heterogeneous conditions have nearly the same position than the first series obtained with homogeneous samples; the data are represented on Figure 10. From all these data it is confirmed that the chitosans with different DA values over the range 0-25% have the same behavior i.e. the stiffness does not change to any significant extent. In this case, the relation between Rg and M (relation 3) gives a value for the constant K' (K'= 0.061± 0.001) slightly lower than the average values given for the previous series of samples; the a parameter equals 0.546 ± 0.001.

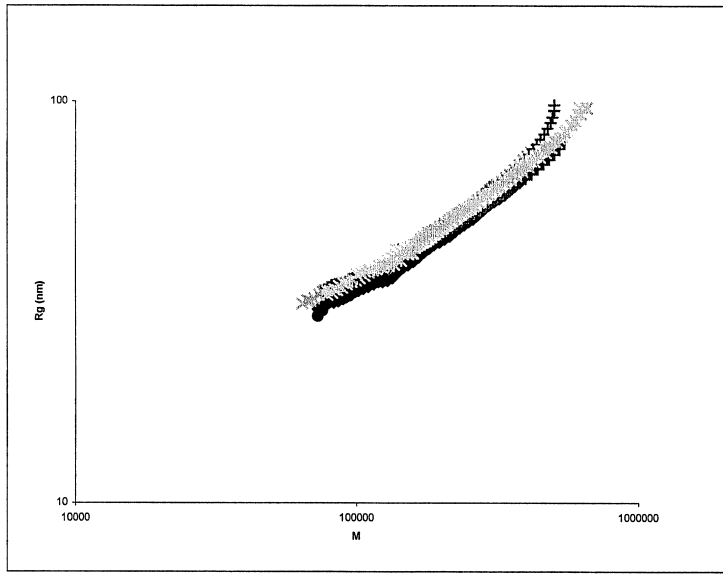

Figure 10. Radius of gyration of heterogeneous chitosan as a function of the molecular weight. Solvent: 0.3M AcOH/ 0.2M AcONa.

The analysis of the data indicates that Lp is nearly independent of DA(in the accuracy range) over the range of 0 <DA<25 % having an average value of Lp =110 Å; this conclusion

confirms our previous results[20]. From these data, it should be predicted that the relation between the intrinsic viscosity and the molecular weight must be nearly independent on DA in a moderate range of molecular weight; nevertheless we were not able to demonstrate in this work from direct measurement of the intrinsic viscosity. The calculated values are given in Table 4.

Table 6. Characteristics of chitosans prepared by heterogeneous deacetylation.

Samples	DA (%) by RMN ^1H	$[\eta]$ ($mL.g^{-1}$) by viscometry	Mw ($g.mol^{-1}$) by SEC-MALLS	Mv ($g.mol^{-1}$)
1	17	830	210000	135000
2	12.5	650	193000	171500
3	6	-	105000	-
4	3	300	70000	48800
5	6	700	160000	147500
6	traces	1060	257000	225000
7	22	800	226000	196000
8	23	220	60000	36000
9	12	600	285000	135000
10	3	920	225000	213300
11	25	730	210000	174000
12	13	950	260000	250000
13	8	880	235000	225000

Some results concerning molecular characterization of heterogeneous samples are given in Table 6. The Mv values were obtained using the Mark-Houwink determination proposed previously (Table 5); these values (for samples 2,5-7,10, 12 and 13) are usually slightly lower than Mw obtained by GPC experiment, as predictable. Few cases give Mw much higher than Mv and may be related to some solubility problems: in fact the intrinsic viscosity is too low compared to Mw from light scattering. This may be related to some aggregates formed in the solution due to the block distribution of acetylated units.

Conclusion

This paper described our last experimental data in the field of analysis and characterization of chitosans from solid state NMR (^{13}C and ^{1}H) allows us to determine the average degree of substitution in all the range of DA. The methods proposed are also valid for *in situ* determination. ^{1}H and ^{13}C NMR in liquid state allows to get rapid determination of DA on chitosan, the water soluble form in acidic conditions. In addition, the results were used to calibrate the IR spectra for DA determination on polymer powder whatever is the DA. A new set of reference and analysis bands was proposed.

^{13}C NMR on the polymeric material in solution was analyzed to relate the distribution of diads and triads with the distribution of acetyl groups along the chain, depending on the conditions of preparation of the samples.

The physical properties of a polymer are related with the molecular weight distribution; it was established by GPC using homogeneous or heterogeneous samples in a large range of DA.

In the solvent proposed for good solubility of chitosan (0.2 M AcONa / 0.3 M AcOH), it was found that the hydrodynamic volume is nearly not influenced by the value of DA. Using the wormlike chain model, we determined the intrinsic persistence length of chitosan: for heterogeneous samples (DA<25%), Lp= 110 Å, in good agreement with the value calculated from molecular modeling.

For homogeneous samples, available in a larger range of DA (DA<60%), it was shown that Lp increases slightly with DA (110 Å <Lp<150 Å) just as found by modeling. Especially, the larger probability to alternated distribution of acetyl groups (determined by NMR) justifies an increase of Lp, larger compared to that calculated for a random distribution.

At end, it was clearly shown that the mobility of the chain increases when temperature increases corresponding to a lower stiffness and a lower Lp; this effect will explain the variation of viscosity of a chitosan solution with temperature.

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References.

1. P. Le Dung, M. Milas, M. Rinaudo, J. Desbrières, Carbohydr. Polym. **24**, 209 (1994).
2. M. Rinaudo, M. Milas, J.Desbrières, in: *Applications of chitin and chitosan.* M.F.A.Goosen (Ed.), Technomic Pub Co Inc, Lancaster 1997, p 89.
3. J. Desbrières, M. Milas, M. Rinaudo, in "*Chitin World*". Z.S.Karnicki, M.M. Brzeski, P.J.Bykowski, A. Wojtasz-Pajak,(Eds.) Wirtschaftsverlag NW, Bremerschaft 1994, p. 73.
4. G.G.Maghami, G.A.F. Roberts, *Makromol. Chem.* **189,** 195 (1988).
5. M.Rinaudo, M. .Milas, P. Le Dung,. *Int.J. Biol. Macromol.* 15,281(1993).
6. G.Yu, F.G. Morin, G.A.R.Nobes, R.H.Marchessault, *Macromolecules,* **32,**518 (1999).
7. B. Boutherin, K. Mazeau, I. Tvaroska, *Carbohydr. Polym.*, **32**, 255 (1997).
8. N. L. Allinger, Y. H. Yuh, J. H. Lii, *J. Am. Chem. Soc.*, **111**, 8551 (1989).
9. N. Metropolis, A. W. Rosenbluth, M. M. Teller , E. Teller, *J. Phys. Chem.*, **21**, 1087 (1953).
10. W. Reed, in *Macroion Characterization from Dilute solutions to complex Fluids.* K.Schmitz (Ed.) ACS Symp. Ser.,**548**,297 (1994).
11. H. Benoit, P. Doty, *J.Phys.Chem.* 57,958 (1953).
12. T. Odijk, *Biopolymers* **18,**3111(1979).
13. K.Mazeau, S.Perez, M.Rinaudo, *J.Carbohydr. Chem* (in press).
14. F.S.Tanner, H. Chanzy, M.Vincendon, J.C. Roux, F. Gaill, *Macromolecules*, **23,** 3576 (1990).
15. L.Heux, J.Brugnerotto, J.Desbrières, M-F. Versali, M.Rinaudo, *Biomacromolecules* (in press).
16. J. Brugnerotto, J.Lizardi, F.M.Goycoolea, W.Arguelles-Monal, J.Desbrières, M.Rinaudo, *Polymer* (in press).
17. K.M. Varum, M.W.Anthonsen, H.Grasdalen, O.Smidsrod,*Carbohydr.Res.***211**, 17 (1991).
18. K.M. Varum, M.W.Anthonsen, H.Grasdalen, O.Smidsrod,*Carbohydr.Res.***217**, 19 (1991)
19. J.Brugnerotto, J.Desbrières, G.Roberts, M.Rinaudo (submitted).