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Formation and characterisation of a physical chitin gel

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Abstract

The formation of *N*-acetyl chitosan gels in an acetic acid–water–propanediol solution was studied. Side reactions arising from the esterification of 1,2-propanediol by acetic anhydride, and hydrolysis of acetic anhydride were studied, as well as their possible role on the gel formation. The gels were studied by FTIR and ^{1}H NMR spectroscopy. Independently of the initial acetylation degree of chitosan, a minimal acetylation degree is required for gelation (80%). The final acetylation degree of the gels increases with the molar ratio acetic anhydride/amine, but does not reach 100%. An increase in temperature favours the molecular mobility, inter- and intra-molecular hydrogen bondings and hydrophobic interactions, responsible for gelation which appears to depend on two parameters: a critical acetylation degree and a critical molecular weight allowing the aggregation of the polymer chains. The nature of the solvent used to avoid chitosan side reactions during gelation (ethanol or propanediol) does not influence the chemical structure of the gel but only the kinetics of gelation. © 1997 Elsevier Science Ltd.

Keywords: Chitin; Gelation; Degree of acetylation

1. Introduction

Formation of polysaccharide gels based on different frameworks (covalent, ionic, hydrogen bondings, etc.) [1–3] has been widely reported. Chitosan, a copolymer of (1 → 4)-2-amino-2-deoxy-β-D-glucan and (1 → 4)-2-acetamido-2-deoxy-β-D-glucan with an acetyl content generally below 30%, is found in some micro-organisms but is more usually prepared from naturally occurring chitin by *N*-deacetylation with alkali [4]. The first chitosan gels, based on covalent links, were obtained by Broussignac [5] in dilute acetic acid. Then, Hirano et al. prepared *N*-acylchitosan gels by acylation of chitosan with various anhydrides in aqueous alcoholic acetic acid solutions [6].

N-Acylchitosan gels are of interest for various potential applications, particularly in the field of pharmacy or biomaterials, because they exhibit good biocompatibility and bioresorption properties. They can also be used in chromatography as a stationary phase [7].

This kind of gel has been studied extensively [8–11], especially the influence of different parame-

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ters on their formation, such as the nature of the acylating agent [10–13], the solvent [8], and cosolvent [10], the concentration of the acylating agent and chitosan content [13], and the temperature [13]. Some gelation mechanisms were proposed [9,12,13], but this problem can be considered as incompletely solved.

In the present report, the gels studied were prepared by *N*-acetylation of chitosan by means of acetic anhydride in a water–alcohol solution [14], and 1,2-propanediol was used as cosolvent. This alcohol has never been used before and its relatively high viscosity offers relatively slow kinetics of gelation, allowing an improved study of it. The aim was a better understanding of the gelation process. First of all, the possibility of side reactions when the acetylating solution was stored before use was considered, and the kinetics of disappearance of acetic anhydride in relation with the formation of the gel was investigated. The relation between the acetylation degree of the reactive solution and gelation was further studied. The influence of various parameters on gelation, such as the temperature, the molecular weight, the initial degree of acetylation of chitosan, the stoichiometry by means of the molar ratio *R* (anhydride over free amine of chitosan), and the nature of the solvent was then examined. The acetylation degrees of the gels were determined by different methods such as ^{1}H NMR spectroscopy and FTIR spectroscopy.

2. Results and discussion

Side reactions in the acetylation process.—Acetic anhydride, which is the acetylating agent used for the formation of chitin gels, is well known to be hydrolysed in the presence of water. On the other hand, the esterification reaction between an alcohol and an acid anhydride is relatively easy even at room temperature, the yield of the reaction being higher with primary than with secondary alcohols. As a consequence, we noticed that ageing of the acetylating solution had an influence on the gel formation and on its physical properties.

In order to control this problem, the evolution of the composition of an alcoholic (1,2-propanediol) solution of acetic anhydride (10:1 v/v) by FTIR spectroscopy was considered.

We noticed simultaneously the disappearance of acetic anhydride [ν(CO) 1826 cm^{-1}], the formation of acetic acid [ν(CO) 1718 cm^{-1}], and the esterification of the alcohol [ν(CO) 1740 cm^{-1}]. Two monoesters were identified by GC–MS and quantified by ^{1}H NMR spectroscopy (δ 2.05, s, 3 H and δ 2.1, s, 3 H in $CDCl_3$) which correspond to the 2-hydroxypropyl-1-acetate and the 1-hydroxypropyl-2-acetate, respectively. After 150 h, the amount of the former reached about 30 mol% and the latter about 10 mol%. In fact, hour after the preparation of the acetylating solution, less than 5 mol% of acetic anhydride had reacted. Thus, if the acetylating solution is used within 0.5 h after its preparation, the reproducibility of the gel formation is preserved.

Disappearance of acetic anhydride in the aqueous alcoholic solution.—After having proved the existence of side reactions in the acetylating solution, which do not disturb the gelation under certain conditions, the disappearance of acetic anhydride in the aqueous alcoholic solution by ^{1}H NMR spectroscopy was followed. We used the same conditions of concentration of acetic anhydride as those used to prepare a sample with a molar ratio *R* (acetic anhydride over amine) of 1.4, but without chitosan. One h and half after its preparation, the aqueous alcoholic solution contained only 12 mol% of the initial amount of acetic anhydride, and after 6 h, the acetic anhydride was fully hydrolysed. However, in these conditions of concentration, gelation occurred only between 8 and 18 h. Thus, it can be assumed that the acetylation reaction is achieved before the gelation occurs.

Kinetics of the acetylation reaction.—In order to confirm these results, the evolution of the acetylation degree during gelation was followed. Two samples with molar ratios *R* of 1.8 and 8 were prepared, and their acetylation degrees were determined by ^{1}H NMR spectroscopy on samples at different reaction times until the gelation occurs. The acetylation reaction was stopped in each case by immersion in an ammonia aqueous solution. The samples were then hydrolysed with hydrochloric acid in deuterium oxide (20% w/w) at room temperature.

The kinetics of gelation has already been studied by means of viscosity experiments [12]. In the present report, the kinetics of gelation was followed by Light Scattering (LS), by measuring the intensity of the scattered light vs. time at Θ 45 °C. Upon formation of the gel, the intensity of the scattered light increased steadily (Fig. 1a) and seemed to reach a constant maximum value. Since the difference of molecular weights between a (1 → 4)-2-amino-2-deoxy-β-D-glucan and its *N*-acetylated form is only about 10%, the changes in the scattered intensity due to this chemical modification can be neglected compared to the molecular weight increase resulting from

gelation. Only one transition was observed and the gelling point (G) could be determined by extrapolating the linear portion of the curve in the transition domain back to the time axis (Fig. 1a, b).

For the Sample with R 1.8 (Fig. 1a), 10 min after addition of the acetylating solution to the aqueous alcoholic solution of chitosan, the acetylation degree had already reached about 80% although gelation had not begun and remained stable even after gelation, i.e. beyond 40 min (Fig. 1a). This result confirms that, for this kind of R value, the acetylation degree does not increase significantly as soon as the gel is formed. Thus, acetylation of chitosan occurs very quickly and much sooner than gelation. This relatively high value (80%) can be explained by the involvement of the (1 → 4)-2-amino-2-deoxy-β-D-glucan residues. At low acetylation degrees, due to electrostatic repulsions, the protonated amine groups are sufficiently numerous along the polymer chains to prevent the aggregation and the ordering of the polymer chains required for the gel formation [9,13].

For the Sample with R 8, the results are quite

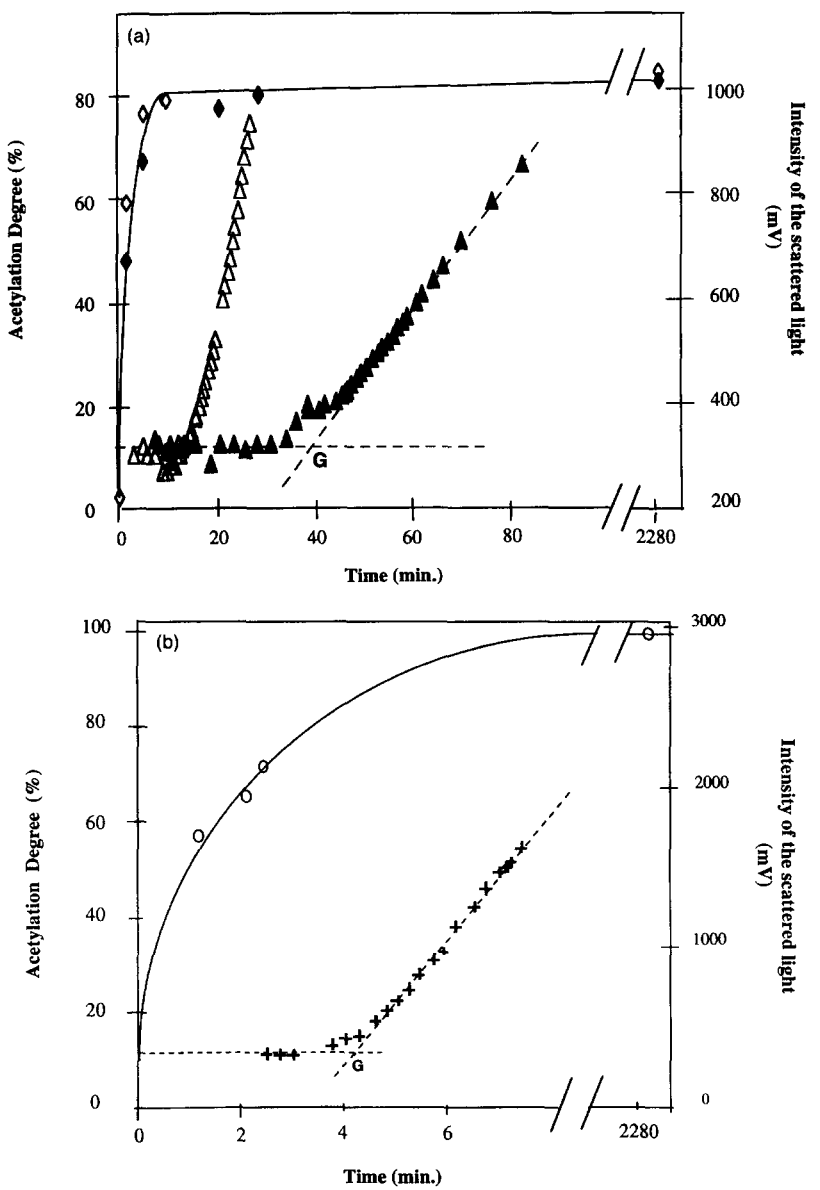

Fig. 1. (a) Kinetics of acetylation reaction (the degree of acetylation is determined by ^{1}H NMR spectroscopy in D_2O after hydrolysis by HCl) at 21 °C (◆) and at 30 °C (◇), and kinetics of gelation by Laser Light Scattering at 21 °C (▲) and 30 °C (△) for a molar ratio R 1.8. (b) Kinetics of acetylation reaction (○) (the degree of acetylation is determined by ^{1}H NMR spectroscopy in D_2O after hydrolysis by HCl) and kinetics of gelation by Laser Light Scattering (+) at 21 °C for a molar ratio R 8.

Table 1
Final degree of acetylation (da) measured by: i) ^{1}H NMR spectroscopy on hydrolysed samples using 20% HCl; ii) FTIR with the methods of Baxter et al. [22] and Shigemasa et al. [21]. *R* is the molar ratio acetic anhydride/free amino groups of chitosan

R	da (%) [a]			Gelation [b]
	NMR ^{1}H	Baxter	Shigemasa	
0.2	25±1	26±2 [p]		−
0.4	39±1	39±3 [p]		−
0.6	55±2	54±3 [p]	51±3 [p]	−
0.8	65±2	69±3 [p]		−
1	69±2	69±3 [p]	73±4 [p]	−
1.25	77±2	68±3 [p]	89±3 [p]	−
1.3	79±2	68±3 [p]	88±3 [p]	+
1.45	79±2			+
2	88.0±2,5	85±3 [f]		+
2.6	92.5±2,5		> 100 [p]	+
4	96.0±2,5	87±4 [f]	> 100 [p]	+
6	99.5±2,5	93±4 [f]		+
10	97.5±2,5	92±4 [f]	> 100 [p]	+
15	96.0±2,5	93±4 [f]		+
20		93±4 [f]	> 100 [p]	+

[a] [p]: Sample examined in the powder form; [f]: sample examined in the film form.
[b] +: Sample gelled; −: sample did not gel.

different (Fig. 1b), because gelation occurs very quickly before the acetylation reaction has reached its maximum. Nevertheless, gelation occurs only at an acetylation degree over 80%.

Thus, two phenomena can be distinguished: firstly, the *N*-acetylation of the amine functions, and secondly, due to the formation of a sufficient number of acetylated residues which are quite hydrophobic and the disappearance of the (1 → 4)-2-amino-2-deoxy-β-D-glucan residues, particularly hydrophilic and charged [9], there is a change in the chain organisation to reach a particular situation. This organisation could be due to inter-molecular hydrogen bondings or hydrophobic interactions [15]. The chemical structure of *N*-acetylglucosamine residues can allow both possibilities. On the other hand, depending on the value of *R*, the acetylation can continue after gelation to reach a value slightly below 100%.

Influence of the molar acetylation ratio on the kinetics of gelation.—The chitosan A32E03 samples acetylated with *R* values between 0.2 and 1.25 do not gel, even after 15 d at room temperature. As reported in Table 1, only samples obtained with *R* values higher than 1.3 can gel.

We studied the kinetics of gelation by LS in batch at 32 °C, for samples with molar ratios *R* varying between 2 and 8. The higher the molar ratio *R*, the faster the gelation occurred, confirming the results obtained by Roberts [12] (Fig. 2), i.e. the gel is achieved after 13 min for *R* 2 and only after nearly 4 min for *R* 8. These results confirm that two factors play an important role: the stoichiometry necessary to obtain the acetylation allowing gelation and the concentration of acetic anhydride which, when in excess, contributes to increase the kinetics of acetylation and thus of gelation.

Relation between the molar ratio R and the final acetylation degree of the gels.—In a first step, the final acetylation degree of samples obtained by acetylation of chitosan A32E03 with various molar ratios *R* varying between 0.2 and 20 was determined by ^{1}H NMR spectroscopy in DCOOD as solvent. In these conditions, acetylation degrees over 100% were always found. As discussed in the literature [16], this unusual value can be related to the degradation of the gels in formic acid, providing cleavage of the glucosidic bond, then a decrease of the intensity of the glucosidic ring protons, and then an apparent increase of the acetylation degree. It could be also ascribed to a possible *O*-formylation as reported in the literature [17,18]. Nevertheless, these two possibilities were not pointed out by Hirano [6,8–10] who only attributed degrees of substitution over 1 to an *O*-acetylation. The *O*-formylation was confirmed by an FTIR study of our chitin gels in the film form [ν(CO) 1720 cm^{-1}].

The comparison of different methods of analysis of the gels by ^{1}H NMR spectroscopy allowed us to check the accuracy of the method using hydrochloric

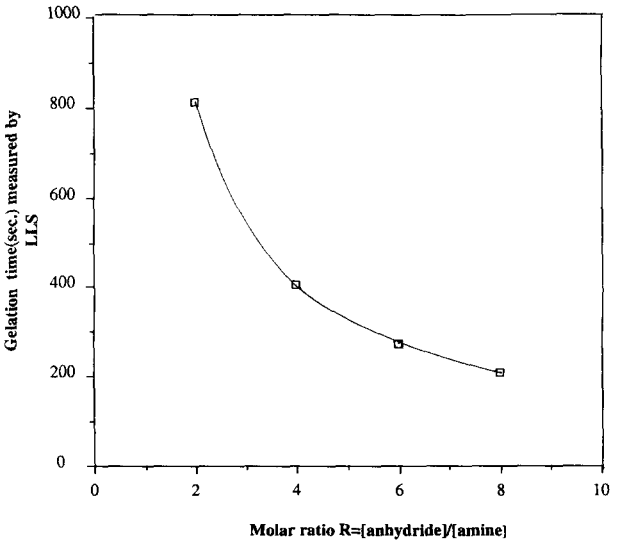

Fig. 2. Kinetics of gelation followed by Laser Light Scattering for different molar ratios *R*. θ = 45°, λ = 632.8 nm.

acid hydrolysis. 20% Hydrochloric acid hydrolyses the glycosidic bonds, allowing the solubilisation of the gels in water, without any further degradation of the polymer. Furthermore, the NMR spectral resolution, due to the low molecular weight, was improved and the experimental errors were minimised (Fig. 3). Some deacetylation is likely [19], leading to the formation of acetic acid (^{1}H NMR in D_2O, δ 2, s, 3 H), but this should not influence the determination of the acetylation degree. The integral intensity of the methyl protons used to calculate this parameter [19] is defined as the sum of the integral intensities of all the methyl signals originating from *N*-acetyl groups and of acetic acid. Hydrolysis by sodium nitrite [20] gave the same result as hydrochloric acid hydrolysis, but the latter is more efficient and allows a better NMR spectral resolution.

FTIR experiments gave approximately the same acetylation degrees as obtained by ^{1}H NMR spectroscopy after hydrolysis by hydrochloric acid (Table 1), but the upper limit of the methods used for the interpretation of the spectra [21,22] is reached, particularly with the method of Shigemasa [21]. The results deduced from the method proposed by Baxter et al. [22], obtained with samples in the film form, are relatively close to those obtained by ^{1}H NMR spectroscopy. Since acetic anhydride could also react with the primary alcohol (C-6) of the glucosidic ring, leading to the formation of esters, we checked, in the IR spectra of the gels, the absence of the vibration band near 1730 cm^{-1}, corresponding to the ν(CO) of the esters. Furthermore, this vibration band was actually observed in the IR spectrum of a sample obtained in the same conditions as those used for preparation of the gels, but without alcohol. The absence of alcohol is well known for inducing an *O*-acylation [9]. This is conclusive evidence confirming the absence of *O*-acetylation in the gels.

Acetylation degree.—The acetylation degree of the samples which do not gel ($0.2 \leq R \leq 1.25$) is particularly low and increases rapidly with R (Table 1). The minimal molar ratio allowing gelation (R 1.3) presents an acetylation degree close to 80%. Over this value, the increase is weaker and it seems that the acetylation degree reaches a plateau at about 96% for molar ratios higher than 4 (Table 1). The acetylation degree vs. the molar ratio obeys a hyperbolic tangent law (Fig. 4). These results confirm that a minimal acetylation degree is required for gelation. Over a sufficient excess of anhydride, this excess does not influence the acetylation degree. The minimal value (80%) agrees with the value published by Roberts [13] (82%), although the experimental conditions are not exactly the same (nature of the alcohol, concentration and initial acetylation degree of chitosan).

Whatever the molar ratio, it seems impossible to reach an acetylation of 100%. This fact can be attributed to the increase of steric hindrance with the

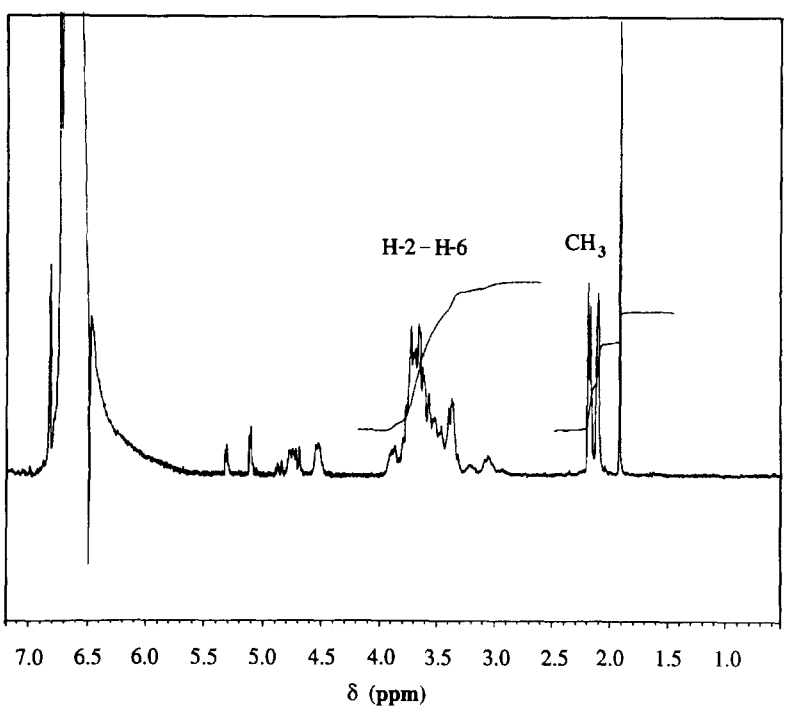

Fig. 3. ^{1}H NMR spectrum (250 MHz) in D_2O of a gel obtained with the chitosan A37C13, hydrolysed by HCl.

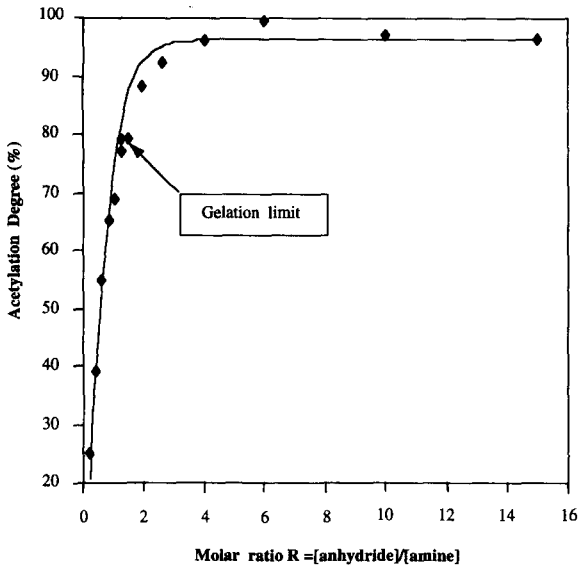

Fig. 4. Evolution of the final degree of acetylation (da) determined by ^{1}H NMR spectroscopy (250 MHz) in D_2O after hydrolysis by HCl, with the molar ratio R.

acetylation process which makes access to the amine functionality more difficult. On the other hand, chitosan, in its ammonium form, is a polyelectrolyte whose intrinsic pK_a (pK_0) is relatively low (near 6.5) [23]. Thus, in acidic media, it always exists both in the protonated amine and in the amine form. In addition, the use of a weak acid such as acetic acid as a protonating agent leads to an incomplete formation of the salt [18] and thus reinforces the presence of free amine functions, even in acidic media. These considerations are important with regard to the mechanism of acetylation which can occur only on the uncharged amine functionality. However, when acetylation occurs, the pK_a increases, due to the decrease of the charge density. As a consequence, for a given pH, the protonated amine forms are increased, thus limiting the conversion by a continuous displacement toward NH_3^+ production. In addition, when the acetylation degree becomes very high, the polymer cannot be considered as a true polyelectrolyte but as a chain corresponding to a copolymer of low-molecular-weight oligomers of (1 → 4)-2-amino-2-deoxy-β-D-glucan and long sequences of (1 → 4)-2-acetamido-2-deoxy-β-D-glucan residues. In this case, the pK_0 value increases and tends towards that of the monomer (7.5) [24], thus increasing the protonation in disfavour of the acetylation. These considerations can also be used to explain why the acetylation rate does not exceed 50%, even for R 10, when acetylation of chitosan in hydrochloride salt form was attempted. Indeed, hydrochloric acid is a strong acid which is completely dissociated in water and then protonates nearly all the amino groups of chitosan [18].

Influence of the initial acetylation degree of chitosan on the minimal acetylation degree required for gelation.—The chitosan used for the gelation study (A32E03) had an initial acetylation degree of 2.5, and the gels obtained by acetylation exhibited acetylation degrees higher than 80%. In order to check the possible influence of the initial acetylation degree on the value required for gelation, three other chitosans were tested for gelation, and their acetylation degree was determined by ^{1}H NMR spectroscopy (Table 2). First, we observed that the minimal molar ratio was obviously always over 1, whatever the initial acetylation degree, and seemed to be independent of the initial acetylation degree up to an initial value near 15%. The minimal acetylation degree required for gelation ($\approx$ 80%) seems to be independent of the initial one, although the polymers do not have the same dp_w. In fact, the molecular weight does not seem to influence the degree of acetylation of the gel in the range of molecular weight used. The influence of this parameter on the gelation will be studied later. The distribution of the (1 → 4)-2-acetamido-2-deoxy-β-D-glucan residues in the different samples of chitosan used for gelation was not determined, but this parameter is known to affect the behaviour of the

Table 2
Characteristics of the various commercial samples used: weight average degree of polymerisation (dp_w), degree of acetylation (da), and water content

Origin	Batch no.	dp_w (SEC–LLS)	da (%) (^{1}H NMR)	Water content (%) (TGA)
Aber Technologies	A32E03	810	2.4 [a]	10.8
	A07B15	1070	6 [a]	11.5
	A40D01	1110	16 [a]	11.0
			19 [b]	
	A37C13	340	9 [a]	10.4

[a] In D_2O.
[b] In D_2O after hydrolysis by HCl.

Table 3
Final degree of acetylation (da) measured by: i) ^{1}H NMR spectroscopy (250 MHz) in D_2O after hydrolysis by HCl; ii) FTIR with the method of Baxter et al. [22]

R	da (%) [a]			
	FTIR		^{1}H NMR	
	25 °C	100 °C	25 °C	100 °C
0.6	54 (−)		55 (−)	
0.7		61 (−)		61 (−)
0.8	69 (−)		65 (−)	
0.9		70 (+)		72.5 (+)
1	68 (−)	78 (+)	69 (−)	80 (+)
1.25	68 (−)		77 (−)	
1.3	68 (+)		79 (+)	

[a] (+): Sample gelled; (−): sample did not gel.

polymers in solution [15]. The distribution of (1 → 4)-2-acetamido-2-deoxy-β-D-glucan residues (under investigation) and its relation with the gel formation is certainly an important factor to be considered.

Influence of the temperature on the gelation and acetylation reaction.—All the experiments concerning gelation were performed at room temperature. At this temperature, the minimal molar acetylation ratio allowing gelation for chitosan A32E03 was *R* 1.3. If the reaction temperature was increased up to 100 °C, gelation was even possible for an *R* value of 0.9, and the same acetylation degree was obtained for the samples *R* 0.9 (100 °C) and *R* 1.3 (21 °C), i.e. near 70% (Table 3). Thus, the increase of temperature enhances the acetylation reaction, and a lower initial quantity of acetic anhydride is then required to reach the same conversion state.

An ungelled sample, obtained with a molar ratio *R* 1.25 (%Ac 77%) at room temperature, gelled when heated to about 90 °C. Thus, the temperature influences not only the acetylation reaction but also the molecular mobility, allowing the acetylated residues to form easier zone junctions. It also favours inter- and intra-molecular hydrogen bondings and hydrophobic interactions.

Influence of the temperature on the kinetics of gelation.—The kinetics of gelation vs. acetylation for two series of samples obtained with a molar ratio *R* of 1.8 at 21 and 30 °C was then compared. The kinetics of gelation was followed by LS in batch, as described above. In order to prevent any problem ascribable to the formation of aggregates in the chitosan solutions, as previously reported in the literature [25], the chitosan solutions used for the study of kinetics of gelation were all prepared at the same time. We observed that gelation occurred more readily at 30 °C (17 min) than at 21 °C (40 min), although the two series presented nearly the same acetylation degrees at the same time (Fig. 1a). This result agrees with that of Roberts [12] who showed that the gelation time decreased twice when the temperature increased from 20 to 25 °C. This means that gelation is more sensitive to temperature changes than to the acetylation degree. As a consequence, as mentioned above, it provides an easier chain mobility, allowing a faster and probably better organisation of the interactions between chain segments, responsible for gelation.

Influence of the molecular weight of chitosan on gelation.—We observed above that the initial acetylation degree of chitosan had no influence on the final acetylation degree of the gels, although the chitosan samples do not have the same dp_w. In order to investigate the influence of the molecular weight on gelation, the gelation with different molecular weights, with always the same concentration of polymer (0.5% w/v), was studied. Various molecular weights were obtained by hydrolysis of chitosan in the presence of sodium nitrite [20], and the weight-average molecular weight of the samples was determined by SEC–MALLS.

We noticed that high molecular weights allow more rapid gelation kinetics. On the other hand, there is a limit for low molecular weights corresponding to a dp_w near 7, for which gelation is no longer possible whatever the molar ratio. Gelation becomes possible for dp_w equal to 30. Nevertheless, for dp_w between 30 and 280, microgels are obtained even with a high molar ratio, whereas macroscopic gels, which extend throughout the whole volume of the reaction volume, are only formed for dp_w over 280. The longer the chains, the easier is the establishment of segment interactions. In addition, the solubility decreases with the molecular weight, which favours segment–segment interactions. Indeed, when the medium becomes a bad solvent, it is well known that the solubility parameter of the polymers decreases when the molecular weight increases. This result shows that there are really two phenomena which are responsible for gelation. On one hand, a critical acetylation degree, close to 80%, allows aggregation of the polymer chains because of hydrophobic interactions or hydrogen bondings, and on the other hand, the aggregation of the polymer chains depends on the chain length. At very low molecular weights, even with a high acetylation degree, physical crosslinking cannot occur.

Influence of the nature of the alcohol.—As proposed by Hirano [9–11], methanol was the first alco-

hol used to prevent the *O*-acetylation, and remains the most commonly used [12,13]. Gelation behaviour in the presence of another alcohol was studied by Roberts [12], but except for some observations on the aspect and the gelation time, no study concerning the cosolvent is known.

We also used ethanol. In the same conditions as used for 1,2-propanediol, the formation of a macrogel was possible, contrary to the observations of Roberts [12] who noticed the formation of 'gelatinous lumps'. The gelation was more rapid in ethanol than in 1,2-propanediol, probably because of the lower viscosity of the alcohol. At room temperature, the minimal molar ratio *R* required for gelation (1.2) was nearly the same as in propanediol (1.3) for an acetylation degree close to 77%. A sample obtained in ethanol with a molar ratio equal to 6 presents an acetylation degree of 100%, which is near the value obtained in 1,2-propanediol. It seems that the two solvents present no significant difference upon gelation, suggesting that the alcohol is necessary to avoid any side reactions but has no other role in *N*-acetylation and gelation.

3. Conclusion

The study of the formation of *N*-acetyl chitosan gels in an acetic acid–water–propanediol solution provides various results. Under mild conditions, side reactions, like hydrolysis of acetic anhydride and esterification of the alcohol cosolvent, can be avoided. Two parameters are responsible for gelation: the hydrophilic character of the polymer and the molecular mobility of the chains. Temperature influences gelation, particularly the interactions between the chain segments which are also influenced by the molecular weight of chitosan. We showed that gelation required a minimal acetylation degree, but the distribution of the (1 → 4)-2-acetamido-2-deoxy-β-D-glucan residues is also an important parameter which could interfere with gelation. The physical properties of the gels (syneresis, swelling) are under investigation.

4. Experimental

The gels were obtained by acetylation of various samples of chitosan from Aber Technologies whose characteristics are given in Table 2.

Formation of the gels.—An aq CH_3COOH (0.5% w/v) chitosan soln (1% w/v) was filtered on Millipore membranes of porosities from 3 to 0.22 μm. 100 mL of this soln were mixed with propanediol (80 mL). This aq alcoholic soln was left to stand overnight without stirring for degazing. The alcohol (20 mL) was then mixed with the desired amount of Ac_2O to prepare the acetylating soln. The latter was slowly added to the aq alcoholic chitosan soln. The mixture was then stirred during ~ 30 s and transferred into a cylindrical mould. The final concn of the polymer in the gel (before syneresis) was 0.5% (w/v).

Before each analysis, the gels were washed in an ammonia soln to remove the protonated amino groups, then repeatedly washed with deionised water in order to eliminate propanediol and CH_3COOH, and finally lyophilised.

^1H NMR spectroscopy.—^1H NMR spectra of chitosan were recorded on a Bruker AC 200 spectrometer (200 MHz for ^1H) in D_2O at 298 K.

The gels, insoluble in water, were hydrolysed by HCl in D_2O (20% w/w) [21], and studied on a Bruker 400 DRX spectrometer (400 MHz for ^1H) at 298 K in D_2O. Some samples were hydrolysed by $NaNO_2$ [20] and studied by ^1H NMR spectroscopy. The corresponding results were compared to those obtained by HCl hydrolysis. Some samples, which do not gel and which are soluble in water, were studied in D_2O (without any hydrolysis step), after lyophilisation and exchange in D_2O, in order to minimise the HOD signal.

The acetylation degree of chitosan was determined from the ratio of the area of methyl protons of (1 → 4)-2-acetamido-2-deoxy-β-D-glucan residues with reference to H-2 to H-6 protons of (1 → 4)-2-acetamido-2-deoxy-β-D-glucan and (1 → 4)-2-amino-2-deoxy-β-D-glucan residues, as proposed by Hirai et al. [19].

FTIR spectroscopy.—The gels were studied by transmission on a Perkin–Elmer 1760-X spectrometer, in the KBr pellet form and/or in the film form [26], after being steeped in MeOH–aq NH_4OH [27] in order to remove the possible protonated amine groups, and dried in an oven at 80 °C under reduced pressure. The acetylation degree of the gels were deduced from the methods of Shigemasa et al. [21] and Baxter et al. [22]. The ratio of the absorbances of the vibration bands at 1560 and 1070 cm^{-1} corresponding to the amide II band and to the (C–O) ether stretching band, respectively, were used as proposed by Shigemasa.

Kinetics of gelation.—Kinetics of gelation were carried out in a LAUDA thermostated bath with an accuracy of ±0,1 °C. The gelation process was moni-

tored by Light Scattering with an angle $\theta = 45°$ by means of the instrument described below.

Thermogravimetric analysis.—The water content of chitosan was obtained on a Du Pont Instrument TGA 2000, using a 10–20 mg sample and a temperature ramp of 2 °C/mn.

Size Exclusion Chromatography (SEC) and Multi-Angle Laser Light Scattering (MALLS).—Size exclusion chromatography was performed on Protein Pack SW-200 and TSK G4000PW columns, connected to a Waters 410 (Waters–Millipore) differential refractometer, using an CH_3COOH–ammonium acetate buffer at pH 4.3 as eluent, and an IsoChrom LC (Spectra-Physics) pump. The flow rate was 0.5 $mL\,mn^{-1}$. The MALLS detection was obtained by means of a Wyatt DAWN F detector on line, operating at 632.8 nm. The polymer solns (0.1%, w/v) were filtered on a 0.22 μm pore size cellulose acetate membrane (Millipore) before injection by means of an injection loop (50 μL).

Gas Chromatography Mass Spectroscopy (GC – MS) analysis.—The side reaction products in the acetylating soln were analysed by GC–MS with a VG 70E mass spectrometer and a DB5 column at 70 °C with helium as flow gas and detection by flame ionisation.

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