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New Aspects of the Formation of Physical Hydrogels of Chitosan in a Hydroalcoholic Medium

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New aspects concerning the mechanism of formation of chitosan physical hydrogels without any cross-linking agent were studied. The gelation took place during the evaporation of a hydroalcoholic solution of chitosan. We first demonstrated that it was possible to form a physical hydrogel from a hydrochloride form of chitosan. Chromatographic methods showed that during the gel formation, when the initial concentration is over C^* , the critical concentration of chain entanglement, the water and acid used for the solubilization of the polymer were both eliminated. This particular situation contributed to decrease the dielectric constant of the medium and the apparent charge density of chitosan chains, thus inducing the formation of a three-dimensional network through hydrophobic interactions and hydrogen bonding. In the gelation process, this step was kinetically determining. The speed of evaporation of water and acid were determined and different initial conditions were compared. Thus, we investigated the influence of: the initial polymer concentration, the nature of the counterion and the alcohol, the temperature and the geometry of the reactor. Our results allowed us to confirm the existence of a second critical initial concentration C^{**} , from which the evaporation of water became more difficult. We suggested that C^{**} corresponded to a reorganization of the solution involving the presence of gel precursors. Then, a mechanism of formation of physical hydrogels of chitosan in a hydroalcoholic medium could be proposed. For the first time, we demonstrated that it was possible to generate physical hydrogels in the presence of various diols, which size of the carbonated chain appeared as a limiting factor for the gelation process. These physical hydrogels of chitosan are currently used in our laboratory for tissue engineering in the treatment of third degree burns with the possibility to adapt their mechanical properties from the choice of both the acid or the alcohol used.

Introduction

The use of natural polymers to produce medical devices, for in vivo and in vitro tissue engineering seems more and more promising. Particular attention has been paid to studies on physical hydrogels based on natural polymers¹ thus constituting a new branch of polymer science.

Hydrogels are polymeric networks, hydrophilic in nature, which can contain a very large amount of water or biological fluids while remaining insoluble. Their hydrophilic character is due to the presence of functional groups such as: alcohols,² carboxylic acids, or amines.³ Most of the studies on hydrogels were centered on chemically cross-linked hydrogels; the composition and the cross-linking density being controlled to induce a precise swelling and specific mechanical properties.⁴ The disadvantages of such systems include the difficulty of their processing, the presence of a significant solvent fraction, and their low biocompatibility.

Hydrogels can also be prepared thanks to physical cross-links formed by incorporating on the polymer chains a limited number of hydrophobic groups. Physical gels are continuous and relatively disordered three-dimensional networks possibly formed in associating one or several complementary polymers. They may arise from electrostatic or hydrophobic

interactions, hydrogen bonding, or the combination of several of these low energy interactions.

This work deals with the formation of physical hydrogels from chitosan, a natural polysaccharide possessing the rare property of bioactivity. Chitin and chitosan are extensively studied in pharmaceutical fields for their biodegradability, biocompatibility, bioactivity, and interesting physicochemical properties.⁵ Chitin is found in the cuticles of all of the arthropods, the endoskeletons of cephalopods, and the cell walls of fungi. Its partial or full deacetylation leads to chitosan. Chitin and chitosan belong to the family of glycosaminoglycans and constitute a series of the linear copolymers of *N*-acetyl-D-glucosamine and D-glucosamine with β , (1 \rightarrow 4) glycosidic linkages. When the distribution of the two constitutive residues is random, chitosan corresponds to degrees of acetylation (DA) below 70%. Chemical modifications and various processing methods have produced a variety of physical forms of chitosan corresponding to solutions,⁶ powders, flakes, gels,⁷ microcapsules,⁸ semipermeable membranes,⁹ fibers,¹⁰ etc.

In the case of chitosan, as for synthetic polymers, the literature reports essentially on chemical hydrogels. Yao et al.^{11–14} showed that it was possible to form cross-linked chitosan thanks to glutaraldehyde, interpenetrated with a polyether and co-cross-linked hybrid polymer networks of

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Table 1. Dielectric Constants of Various Water/Alcohol Mixtures at 25, 50, and 60 °C, for Different Alcohols, as Determined by Akerlöf³⁸

% alcohol (w/w)	methyl alcohol		ethyl alcohol		<i>n</i> -propyl alcohol		isopropyl alcohol		<i>tert</i> -butyl alcohol		glycerol	
	25°C	50°C	25°C	50°C	25°C	50°C	25°C	50°C	25°C	50°C	25°C	60°C
0	78.5	69.85	78.5	69.85	78.5	69.8	78.5	69.85	78.5	69.85	78.5	66.82
10	74.1	65.66	72.8	64.53	71.8	63.6	71.4	63.12	70.0	61.84	75.7	63.98
30	64.3	56.59	61.1	53.79	57.7	50.7	56.9	50.18	52.6	45.44	70.0	58.97
50	54.9	47.82	49.0	42.92	43.0	37.8	42.5	37.03	35.4	29.80	64.0	53.36
70	45.0	38.81	38.0	32.86	30.7	26.4	28.7	24.85	21.4	17.41	55.6	46.33
90	35.7	30.67	28.1	24.08	22.7	19.3	20.3	17.11	12.4	10.00	45.5	38.19
100	31.5	27.44	24.3	20.87	20.1	17.1	18.0	15.06	9.9	7.67	40.1	33.82

chitosan and gelatin.¹⁵ Conventional chitosan cross-linking agents have also involved poly(acrylic acid) derivatives using UV irradiation¹⁶ or a self-curing method of chitosan with acrylic acid.⁷ For biomedical applications, a great effort was made to obtain hydrogels formed thanks to chitosan and other natural polymers. Most of them were also chemically cross-linked. Recently Mi et al.¹⁷ synthesized a chitosan/genepin network by means of bifunctional linkages of genepin with the amino groups of chitosan.

Beside chemical gels, some physical hydrogels based on chitosan were produced according to two different ways: physical hydrogels obtained thanks to permanent physical cross-linking agents and physical hydrogels formed without residual cross-linking agent.^{18–22}

The physical cross-linking can involve different types of physical junctions. Thus, an ionic cross-linking can result in the formation of a polyelectrolyte complex between chitosan, as polycation and chondroitin sulfate as polyanion, an important glycosaminoglycan found in the Hyaline cartilage.²³ Dimitriu et al. showed that it was possible to form hydrogels thanks to the ionic bonding between the NH_3^+ groups of chitosan and the carboxylate sites of xanthan, in addition to van der Waals interactions arising between the two biopolymers.²⁴ Li et al.²⁵ formed physical hydrogels of chitosan/glycerol phosphate/hydroxycellulose. Chitosan and β -glycerol phosphate interacted via ionic interactions, but the system was unable to form a stable three-dimensional network. Then, hydroxy-cellulose was added to induce a cross-linking through hydrogen bonding with chitosan molecules.

Vachoud et al. studied in detail the possibility to form physical hydrogels of chitin through the *N*-acetylation of a highly deacetylated chitosan in a hydroalcoholic solution.^{26,27}

The formation of true physical hydrogels of chitosan with no external cross-linking agent was reported recently for the first time by Montembault et al. Thus, two different ways to form true physical hydrogels of chitosan without any residual cross-linking agent were proposed. In a first case, the gelation of chitosan of various DAs was performed by evaporating a solution of a chitosan acetate salt in a hydroalcoholic medium.^{28,29} After a full neutralization and washings in water, the gels only contained chitosan and a large amount of water. In a second process, physical hydrogels were formed from aqueous solutions of the polymer simply subjected to gaseous ammonia.²² Montembault et al.^{22,28,29} investigated the influence of the degree of acetylation, temperature, the composition of the initial solvent on the time to reach the gel point and the properties of the gels obtained from the acetate form

of chitosan in solution. It has been shown that hydrophobic interactions involving *N*-acetyl groups played a major role during gelation and that hydrogen bonding was also involved in the gelation mechanism.

In the present work, we also investigated the formation of physical hydrogels of chitosan in hydroalcoholic medium. However, we were interested to show that this was not limited to the acetate form and was possible with another salt of chitosan such as the hydrochloride. We were also interested to confirm, in this case, the three major conditions necessary to observe the gelation: (i) an initial concentration in polymer over C^* , (ii) a critical value of the hydrophobic/hydrophilic balance, and (iii) the formation of a sol–gel transition interphase corresponding to uniform percolating conditions.

The aim of the present work was to characterize and quantify the evaporation step as a function of the physico-chemical parameters of the medium: the associated acid, the alcohol structure, water, and the initial polymer concentration. We particularly pointed out the influence of the nature of the counterion on the kinetics of gel formation. This was particularly important since, from a unique polymer, it could be possible to obtain physical hydrogels with different molecular organizations and then, with different properties.

The ultimate goal was to propose a mechanism of formation of physical hydrogels of chitosan in a mixture of water and alcohol, during the evaporation of the solution.

Experimental Section

Purification of Chitosan. We used a chitosan from squid pens, purchased by Mahtani Chitosan (batch number 114, 15/11/02). For purification, chitosan was dissolved at 0.5% (w/w) in acetic acid to achieve the stoichiometric protonation of the NH_2 sites. The mixture was filtered successively on 3, 1.2, 0.8, and 0.45 μm Millipore membranes. Then, the polymer was precipitated by means of concentrated ammonia (28% (w/w)). After several washings and centrifugation steps in deionized and distilled water, until a neutral pH was measured, the precipitate was lyophilized. Then, a chitosan of low DA (2.6%) and very high weight-average molecular weight (close to 540 000 g/mol) was obtained, with a polydispersity index (I_p) of 1.6 ± 0.2 , and a water content of $8 \pm 1\%$.

Determination of the DA. The DA of the different samples was deduced from ^1H NMR spectroscopy. Spectra were recorded on a Bruker 250 spectrometer (250 MHz) at 25 °C. A total of 10 mg of chitosan was dissolved in 1 g of

D₂O in the presence of HCl. The DA was calculated thanks to the method proposed by Hirai et al.,³⁰ from the ratio of the area of the methyl protons of the *N*-acetylglucosamine residues to that of the H₂ to H₆ protons of both glucosamine and *N*-acetyl glucosamine residues.

Determination of M_w and Ip. The weight-average molecular weight M_w was evaluated by size exclusion chromatography (SEC). SEC was performed by means of an IsoChrom LC pump (Spectra-Physics) connected with a Protein Pack glass 200 SW column and a TSK gel 6000PW. A Waters 410 differential refractometer and a multiangle laser-light scattering detector operating at 632.8 nm (Wyatt Dawn DSP) were connected on line. A 0.15 M ammonium acetate/0.2 M acetic acid buffer (pH 4.5) was used as eluent. Chitosan was dissolved in the buffer (1 mg/mL), filtered on a 0.45 μ m Millipore membrane, and then injected (100 μ L). In our case, the refractive index increment dn/dc was evaluated at 0.196 cm³/g.³¹ Ip was deduced from the ratio of M_w/M_n , with M_n , the number-average molecular weight.

Determination of the Water Content. The water content was determined by means of a thermogravimetric analyzer, DuPont Instrument 2950. About 10 mg of chitosan was analyzed under a helium flow, operating at a ramp of temperature of 2 °C/min, from 30 to 150 °C.

Preparation of Solutions. Chitosan was dispersed in distilled and deionized water and pure acetic acid or a hydrochloric acid aqueous solution (37% w/w) was added to achieve the stoichiometric protonation of the NH₂ sites. The mixture was stirred overnight. All of the polymer concentrations considered in this paper were the concentrations of chitosan in water at this stage of preparation. Then, 1,2-propanediol was added (50% w/w), and the mixture was stirred for 1 h.

Gas Chromatography. A gas chromatography method was used to evaluate the proportion of alcohol all along the evaporation step. The chromatograph HP5890 was equipped with a Poraplot Q column of 0.32 mm diameter and 25 m long with a porous polymer as stationary phase. The injection and column temperatures were 240 °C. The integrator was an HP3396. We used an ionization flame detector and helium as gas vector, a total flow of 18 psi, with a split of 1% and 1 μ L injections. We prepared series of 40 samples. At each time of measurement two samples were recovered and weighted. For each of them, we processed rheological measurements and an evaluation of the 1,2-propanediol content. In the latter case, chitosan solutions were precipitated in an excess of NaOH 0.1 mol/L. The mixture was stirred and a part of the supernatant was then diluted in water. A same amount of ethanol was added to each sample to constitute a standard. Three injections of this solution of exactly 1 μ L for each sample were performed.

Ionic Chromatography. The study of the variation of the content in chloride ions in the gelation media was performed with an anion-exchange chromatography Dionex DX 100 constituted of a Dionex AG9HC (250 \times 4) column of 3 mm as internal diameter and 7.5 cm long. This column was filled with a polymeric matrix grafted with ammonium groups and constituted of spherical particles of 10 μ m. The eluent was a 12/5 molar mixture of Na₂CO₃/NaHCO₃ in ultrapure water

with a flow of 110 mL/min. A chemical suppressor Dionex SRS controller was used with a H⁺ ion-exchange resin. An injection loop of 33 μ L was used, and the apparatus was equipped with a conductometric detector.

After precipitation of the gelation media in NaOH 0.1 mol/L, a part of the supernatant was diluted with ultrapure water. Three injections of the latter solution were then processed for each studied evaporation time.

Weight Measurements. The samples were weighed during gelation on a PRECISA 125 A balance, with a precision of 0.1 mg.

Rheological Measurements. Rheological measurements were performed at room temperature with a RMS 800 rheometer operating with a plate–plate geometry (diameter 25 mm). The rheological properties were investigated with dynamic-mechanical experiments. The values of the strain amplitude were verified in order to ensure that all of the measurements were performed within the linear viscoelastic region, to have a storage modulus (G') and a loss modulus (G'') independent of the strain amplitude. Samples of 1 mm thickness were introduced between the plates.

Static Laser Light Scattering (SLLS). The gel point was determined by static laser light scattering during the evaporation of the solution. A Dawn DSP-EOS (Wyatt) spectrometer was used to determine the variation of the scattered light intensity at 632.8 nm and an angle of 90°.

The hydroalcoholic solution was first filtered throughout a 0.8 μ m PTFE Millipore filter and then displayed in a quartz cell. We always introduced the same height of liquid corresponding to 1 cm, for each sample studied. The mixture rested at ambient temperature for 30 min for degassing and then put in an oven at 50 °C to process the evaporation. The scattered intensity was measured at ambient temperature.

Results and Discussion

1. Conditions of Gelation of the Hydrochloride Form of Chitosan Dissolved in Hydroalcoholic Medium. In a previous work, we showed that, whatever the DA, it was always possible to form a physical hydrogel from a solution of chitosan, without any use of either a chemical reaction or a permanent physical cross-linker.²⁹ For that, it is necessary to observe three major conditions. (i) the initial polymer concentration must be initially above C^* , (ii) a change of the balance between hydrophobic and hydrophilic interactions must occur in order to favor hydrophobic interactions and hydrogen bonding up to a critical value corresponding to the gel point, and (iii) gelation must be performed homogeneously throughout the moving of a thick sol–gel transition layer corresponding to uniform percolating conditions.

For flexible chains, Terbojevich³² defined the critical concentration of chain entanglement C^* as the reverse of the intrinsic viscosity. In our case, assuming in first approximation that the conformation of chitosan chains in solution corresponds to this model, C^* was found as close to 0.06% (w/w). For all of the gel systems studied hereafter, the polymer concentration was always largely over C^* .

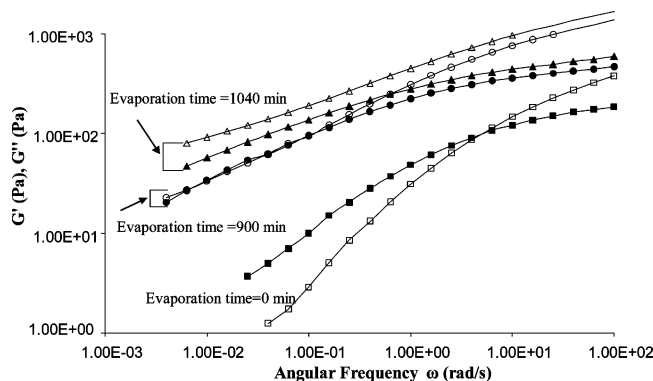


Figure 1. Evolution of the viscous, G'' (■; ●; ▲), and elastic modulus, G' (□; ○; △), as a function of ω , the angular frequency, during the formation of a physical hydrogel of chitosan in the hydrochloride form. The initial concentration was $C_{\text{polymer}} = 3\%$ (w/w), and a plate-plate geometry with disks of diameter = 25 mm was used. Measurements were performed at 25 °C, at a shear rate of 50 s^{-1} . Initial gel samples were in the form of disks with a diameter of 30 mm and a thickness of 10 mm. They were processed from an initial weight of solution of $6.00 \pm 0.02 \text{ g}$, at 50 °C.

When the formation of a physical hydrogel of chitosan occurs in hydroalcoholic medium, alcohol molecules play an important role in the formation of hydrophobic interactions.²⁹ Indeed, in addition to their influence on the charge density of chitosan, their amphiphilic structure certainly allows the reinforcement of the formation of hydrophobic domains and then, hydrophobic junctions between polymer chain segments.

In the present work, chitosan was initially dissolved in aqueous media, in the presence of hydrochloric acid. After complete dissolution, 1,2-propanediol was added. The mixture was then stirred, and samples were transferred into several Petri dishes. To be comparable, all of them had the same weight and the same geometry. Indeed, as shown below, the geometry of the initial solution has an influence on the kinetics of gelation.

Evolution of the Moduli G' and G'' during Gelation.

To confirm that we also formed a gel starting with the hydrochloride form of chitosan, we studied the evolution of the rheological behavior of the gelation medium with time. As shown in Figure 1, we observed the variation of the elastic (G') and viscous (G'') parts of the complex shear modulus as a function of the frequency, at different evaporation times. It is generally considered that when $G' < G''$, the behavior is of liquid type, although when G' becomes largely over G'' , in a large frequency range, the material behaves like a gel. This transition is clearly shown in Figure 1 for an initial polymer concentration of 3% (w/w).

Then, for the solution, in the initial state (time of evaporation = 0 min), G' was below G'' , and after 900 min of evaporation, G'' became larger than G' . This kind of behavior is related to the structural change arising from the formation of a cross-linked polymer network. We also observed that the crossing point between the curves representing the variations of G' and G'' (not shown) shifted from high to low frequencies, when the gelation time increased, i.e., when the amount of water in the medium decreased. The shift of the crossing point could be interpreted in terms of an increase of the time of disentanglement of the polymer

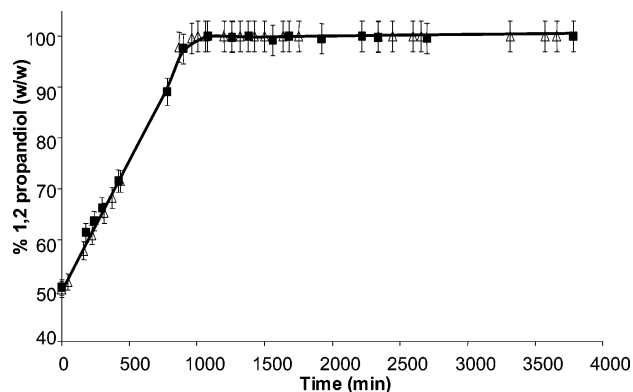


Figure 2. Evolution of the % of 1,2-propanediol in the hydroalcoholic medium (initially at 50% w/w) as a function of the evaporation time: (■) obtained from gas chromatography, and (△) weight measurements. The hydrochloride form of chitosan was initially at a concentration $C_{\text{polymer}} = 3\%$ (w/w), in water. Samples were in the form of disks with a diameter of 30 mm and a thickness of 10 mm. They were processed from an initial weight of solution of $6.00 \text{ g} \pm 0.02 \text{ g}$, at 50 °C.

chains with the time of evaporation and, thus, as an increase of the number of chain entanglements with time. On the gel point, due to physical cross-links, this time became infinite.

In relation to the viscoelastic behavior, Winter and Chambon³³ proposed the following empirical relation on the gel point: $G' \cong G'' \cong \omega^{0.5}$, with ω the angular frequency. In our case, as shown in Figure 1, at the gel point corresponding to a time close to 900 min where $G' \cong G''$, the relaxation exponent was 0.47 ± 0.02 , in good agreement with their predictions, confirming thus a real change from a solution to a gel state.

Depending on their behavior during a strain stress, physical gels are divided into strong and soft gels.³⁴ A strong gel exhibits a solid-state behavior even under large deformations, although a soft gel behaves like an organized liquid within a large range of deformation. Rheological measurements also showed that we formed “strong physical hydrogels” in relation with their stability on a large range of deformation showing their good mechanical properties (to be discussed in a next paper).

Then, from a hydrochloride form of chitosan in solution in a hydroalcoholic medium, it was possible to form true and strong physical hydrogels, without any external cross-linking agent. As mentioned above, this was possible due to the observation of the three conditions necessary to induce a percolating gelation reminded above. Thanks to the previous works of Montembault et al. with the acetate form, we could also expect that, for the hydrochloride form of chitosan, gelation would be observed whatever the degree of acetylation.²⁹ The gels obtained in these conditions were also more transparent than with the acetate form.

Evaluation of the Solvent Composition during the Gelation Process. To have a better understanding of the mechanism of gelation, chromatographic titrations were used to evaluate both the water/alcohol (Figure 2) proportion and the amount of chloride anions (Figure 3) in the gelation medium during evaporation.

Considering data found in the literature,^{29,35–37} as there is no known azeotropic composition between water and the chosen alcohol, and due to its high boiling point (187.4 °C),

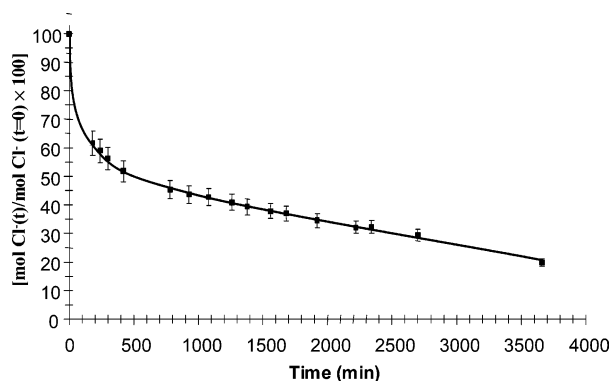


Figure 3. Variation, as a function of the evaporation time, of the ratio $\times 100$ of the number of moles of chloride anions at a time t over the initial number of moles of chloride anions ($t = 0$ min), in the hydroalcoholic medium. Results were obtained by ionic chromatography; the initial samples of chitosan were as described in Figure 2.

the previous work with the acetate form of chitosan²¹ suggested that the elimination of 1,2-propanediol during evaporation of the solution was negligible. In the present paper, the evaporation of the solvent was experimentally characterized. Figure 2 shows the variation of the proportion of 1,2-propanediol as a function of the evaporation time, for an initial concentration in polymer in water of 3% (w/w).

Two techniques were compared: the measurement of the weight loss of the sample and the titration of 1,2-propanediol by gas chromatography. The very good agreement between the results obtained from both methods allowed us to confirm that the weight loss measured during the evaporation process was only due to the elimination of water. This kind of result was obtained whatever the initial polymer concentration. Therefore, after complete elimination of water, the composition of the medium remained constant and only contained chitosan and 1,2-propanediol.

In our gelation process, the first role of the alcohol was to initially reduce the dielectric constant of the medium and then to induce its continuous decrease by a progressive concentration of the less polar solvent during the evaporation. This change of solvent properties was described in the literature by Akerlöf³⁸ for various mixtures water/alcohol, at different temperatures with different alcohols. Their results reported in Table 1 show that the dielectric constant decreases on increasing the alcohol content, the temperature, and the size of the alkyl chain associated with the OH function. The same variation arises when changing a primary by a secondary alcohol. On the contrary, as expected, it increases on increasing the number of alcohol functions present in the structure, as illustrated for the only case of glycerol.

As a conclusion, from our first set of experiments (Figure 2) and those of Table 1, we could confirm that the linear increase of the content of alcohol during the evaporation of the solution really contributed to a displacement of the hydrophilic/hydrophobic balance of the medium in favor of an important increase of the hydrophobicity of the environment of the polymer chains. As a consequence, the alcohol played two first major roles. Thus, 1,2-propanediol could be directly involved in the formation of physical links thanks to hydrophobic junctions between polymer chain segments belonging to different chains. Second, due to the decrease of the dielectric constant, it also disfavored the ionization,

hence reducing the charge density carried out by the polycationic structure of chitosan, thus contributing in another manner to reinforce the formation of hydrophobic junctions and hydrogen bonding. The linear variation of the alcohol (or water) concentration was observed whatever the initial concentration in polymer studied. This behavior justifies that, for a given initial concentration, the mechanism of evaporation occurred probably without important transition and corresponded to a first-order transition with regard to the water molar concentration.

The second step of this study was to identify the role of ionic sites on the gel formation. To verify that the conditions of gelation effectively influenced the ionization of the amine groups of glucosamine residues, we evaluated the variation of the concentration of Cl^- ions during the evaporation, by anion-exchange chromatography. Figure 3 represents the evolution of the relative proportion of chloride anions as a function of time.

We can clearly evidence a decrease of Cl^- illustrating two behaviors. In a first step, corresponding to a time below 100 min, the elimination of the acid was very fast. This must be related to the fact that in a such medium, HCl would become a weak acid, only partially dissociated. Moreover, its solubility should be lower than in pure water. For both reasons, the free acid should be easily eliminated by evaporation. For similar reasons, the amine functions of glucosamine residues should be less protonated thus contributing to consume less HCl than in pure water and then reinforcing the presence of free HCl. Beyond this period, its elimination was progressively reduced thus illustrating the role of several equilibria corresponding to the evolution of the dissociation and solubility of this acid as well as the ionization of the amine functions of chitosan.

As a conclusion, during the solvent evaporation, both water and HCl were eliminated. This phenomenon was responsible for an important decrease of the apparent charge density of chitosan chains and effectively contributed to change the balance between hydrophilic and hydrophobic interactions up to the gel point. However, HCl was not fully eliminated on the gel point (near 900 min) and even after, in agreement with the fact that this acid was soluble in the pure alcohol. Thus, we noticed that after complete elimination of water it still remained almost 20% of the initial concentration of chloride ions. The presence of a given number of ionic sites still present on polymer chains on the gel point also agreed with the necessary maintaining of sufficient electrostatic repulsions to preclude a full depletion of the polymer chains thus avoiding a precipitation instead of a gel formation.

2. Parameters Influencing the Gel Formation and the Mechanism of Gelation. *Influence of the Structure of the Alcohol on the Formation of Physical Gels of Chitosan.* Apart from the contributions discussed above, the direct participation of the alcohol to the formation of hydrophobic junctions and hydrogen bonding is supposed but not really demonstrated. Another aspect of this role concerns the influence of the structure of the alcohol. This kind of study was performed in the case of the gelation of chitin.^{39–41} In the case of chitosan gels, only the 1,2-propanediol was considered in previous works.^{28,29} Table 2 presents some of the

Table 2. Chemical Structures and Physical Constants of the Different Alcohols Tested and Their Involvement or Not in the Formation of Gels for a Water/Alcohol Proportion of 50/50 (w/w), $C_{\text{polymer}} = 3\%$ (w/w)

Alcohol	Chemical structure	Bp (°C)	density	Dielectric constant	Observations
1,2 propanediol		187	1.03	32 (20°C)	Transparent rigid hydrogels
1,3 propanediol		214	1.052	35 (20°C)	Transparent rigid hydrogels
1,2 butanediol		192.4	1.01	30 (17°C)	White and very rigid hydrogels
1,4 butanediol		235	1.02	31.4 (25°C)	White and very rigid hydrogels
1,2 pentanediol		210	0.98	?	No macroscopic gelation, presence of particles of gel dispersed in a viscous media
1,4 pentanediol		220	0.988	?	idem
1,5 pentanediol		260	0.994	?	idem
2,4 pentanediol		202	0.95	?	idem
1,2 hexanediol		224	0.952	?	No gelation
1,6 hexanediol		250		?	No gelation
glycerol		290	1.26	42.5 (20°C)	Very rigid hydrogel

different alcohols tested in this study with their chemical structures and physical characteristics. We also give the results obtained in their presence during a gelation process. The different alcohols were used in the same manner as above for the 1,2-propanediol, especially at a ratio water/alcohol of 50% (w/w).

Results obtained with primary mono alcohols such as methanol, ethanol, propanol, or butanol are not reported. In fact, several problems were encountered during their use. (1) Due to the great difference of viscosity between these alcohols and our mixture, the homogenization was very difficult to achieve. (2) The boiling point of these alcohols is low or close to that of water (methanol, 64.7 °C; ethanol, 78.5 °C; propan-1-ol, 97 °C; butan-1-ol, 118 °C) such as their evaporation occurred before or with that of water. For some of them, the situation was complicated by the presence of an azeotropic composition. In any case, the evaporation led to a film forming instead of a gelation.

The alcohols reported in Table 2 differ by the length of their alkyl chain and the position and the number of hydroxyl groups. The consideration of the whole parameters presented in this table did not allow us to define a precise tendency. Nevertheless, we may notice that among di-alcohols the distribution of OH groups had no influence on the gelation. If we consider the di-alcohol tested, the major parameter seemed to be the ratio between the number of OH groups and the number of carbon atoms. Indeed, whatever the

position of hydroxyl groups, we noticed that the strength of the gels increased when the number of carbon atoms in the alcohol chain increased from three to four. On the contrary, for five carbons, only particles of gel were present in a continuous liquid phase and for six carbons, no gel could be observed. As a consequence, a particular value of the balance between hydrophobic and hydrophilic parts of the alcohol had to be maintained. Thus, in the formation of possible hydrophobic junctions between chitosan chain segments involving the alcohol, a certain hydrophilicity had to be preserved to induce gelation. Therefore, the alcohol could be involved in the formation of a ternary complex between two polymer chain segments. This study also confirmed the important role played by the boiling temperature of the alcohol compared to that of water. We also observed the formation of very strong and rigid gels with glycerol, the only tri-alcohol we tested. The role of the complex mentioned above could be emphasized in this case. The conclusion of this study was that in addition to the role played by the used acid, the nature of the alcohol also contributed to influence both the gelation and the physical properties of the gel.

Determination of the Kinetics Parameters of the Solvent Evaporation. As shown in Figure 2, for a polymer concentration of 3% in the initial solution, we first observed a continuous evaporation of the whole water, and when we were in the gel form, the amount of alcohol in the medium remained constant. The step of water elimination appeared

Table 3. Comparison of the Rates of Evaporation of Water Measured in Various Media^a

medium	a_g (min ⁻¹)
pure water	0.1437 ± 0.003
water/1,2-propanediol(50/50 w/w)	0.0780 ± 0.003
water/1,2-propanediol(50/50 w/w)/ HCl (0.54%w/w)	0.0763 ± 0.003
water/1,2-propanediol/chitosan hydrochloride 3% (w/w)	0.0455 ± 0.003

^a a_g corresponds to the slope of the variation of the percentage of alcohol in the medium with time.

to be kinetically determining for the gel formation. Therefore, we decided to study the variation of the kinetics of evaporation of water. Then, from the variation of the concentration of alcohol or water as a function of time, $d[\text{alcohol}]/dt = -d[\text{H}_2\text{O}]/dt$, we could deduce a_g , the slope of the first part of the curve illustrating the rate of variation of the percentage of 1,2-propanediol or that of water in the medium. We thus examined the values of a_g measured for pure water, in a mixture water/1,2-propanediol, in the presence of HCl, and in the presence of chitosan hydrochloride. As reported in Table 3, we observed a very great difference between the values measured in each case, signifying that a_g was an important and very sensitive kinetics parameter in the process of evaporation of water and therefore of gelation. These changes were necessarily attributed to differences in the nature of hydrogen bonding between water molecules in pure water and in the other circumstances, to changes of the activity of water molecules. In a similar manner, the presence of polymer chains at a concentration over C^* also contributed to a significant decrease of the rate of evaporation. According to the results of this table, two important contributions to the decrease of a_g were brought about by the introduction of the alcohol and then by the presence of polymer chains. If we consider the weight proportions, the presence of polymer chains could be considered as playing the major role. On the other hand, for an initial concentration in polymer of 3%(w/w), i.e., of 1.5% (w/w) in the hydroalcoholic media, we could deduce the following information on the molar ratios: $[\text{H}_2\text{O}]/[\text{NH}_2] \approx 325$, $[\text{alcohol}]/[\text{NH}_2] \approx 77$, and then $[\text{H}_2\text{O}]/[\text{alcohol}] \approx 4.22$.

Influence of the Polymer Concentration on the Gelation Kinetics. The influence of the polymer concentration, considering the concentration before addition of the alcohol, on the gelation kinetics was evaluated. In this set of experiments, all of the samples had the same geometry and the same weight at the initial time.

As shown in Figure 4, we first compared the variation of a_g as a function of the initial polymer concentration for the acetate and hydrochloride forms of chitosan.

We observed a continuous decrease of a_g as the initial concentration of the polymer increased. This behavior was attributed to several interdependent parameters. The most important probably corresponds to the increase of the involvement of water molecules both in the formation of hydrogen bonding with the polymer structure and the hydration of ionic sites. The second is related to an increase of the viscosity of the medium, in agreement with an increase of the density of chain entanglements.

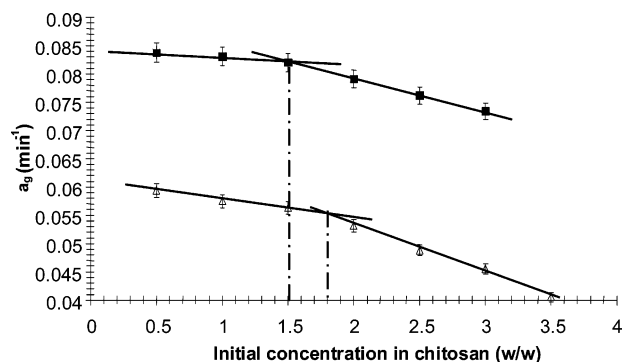


Figure 4. Variation of a_g , the rate of evaporation of water as a function of the chitosan concentration in the initial solution, for (■) acetate and (△) hydrochloride forms, respectively. The initial samples of chitosan were as described in Figure 2.

It was also particularly interesting to notice the presence of a law of behavior exhibiting two different kinetics domains, with a change of slope of the variation of a_g with C_p . In the case of hydrochloric acid, this change appeared for an initial C_p close to 1.8% (Figure 4). Thus, over this value, the rate of water evaporation decreased rapidly and the slope of the variation of a_g was approximately three times that observed below 1.8%. We could thus conclude the existence of a second critical concentration C^{**} . This concentration was first evidenced by Montembault et al.²² during the gelation process consisting of placing a pure aqueous solution of chitosan acetate in contact with gaseous ammonia. In the latter case, the situation was quite different, but the variation of the time to reach the gel point (without any solvent evaporation), as a function of the initial polymer concentration, exhibited a similar law of behavior. Although the conditions were very different in both experiments, the initial polymer concentration was a common parameter. Thus, over C^{**} , in one case, the water evaporation was much more difficult than below although, in the second case, the time to reach the gel point became much shorter. In our conditions, with the hydrochloride form of chitosan, during the evaporation of water in the hydroalcoholic medium, we also found a much shorter time to reach the gel point over C^{**} (not shown). To explain these behaviors, necessarily related to the interaction parameters between chain segments and the two components of the solvent as well as to the entropy of the mixture, we propose that the following mechanism be considered.

Below C^{**} , we are in a situation where the polymer chains are entangled according to a relatively isotropic molecular organization in a homogeneous solvent phase. Nevertheless, as shown in previous studies, these solutions contain precursors corresponding to nanoaggregates of polymer chains.^{42,43}

At C^{**} and over, we have a critical phenomenon corresponding to a phase separation between a homogeneous phase containing isolated chains at a low concentration, possibly below C^* , in a mixed solvent poorer in water and, a dispersion of nanoparticles constituted of aggregates of polymer chains entrapped in a solvent rich in water. In both phases, water molecules are much more strongly involved in different kinds of hydrogen bonding than below C^{**} with either the alcohol molecules or the polymer chains and, possibly between each other.

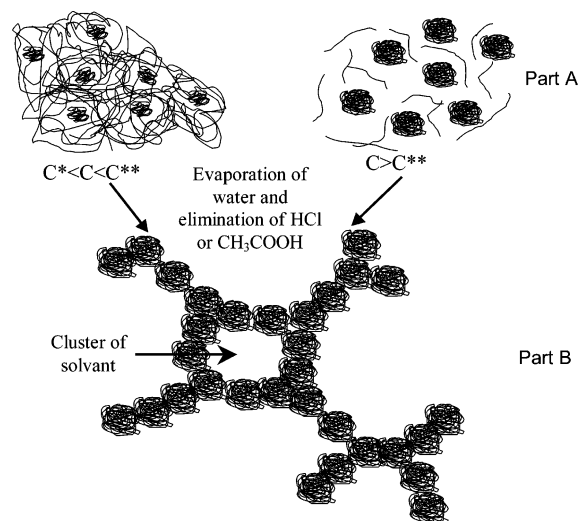


Figure 5. Schematic representation of the formation of a physical hydrogel of chitosan, in the hydroalcoholic medium. (A) Solution state before the gel point, considering two cases: $C^* < C < C^{**}$ with a particular molecular organization, in a homogeneous solvent phase; $C > C^{**}$ with a phase separation between a continuous phase containing isolated chains, at low concentration, in a mixed solvent poor in water, and a dispersion of nanoparticles constituted of nanoaggregates of polymer chains in a solvent rich in water. (B) Representation the physical network at the gel point, after evaporation of water and partial elimination of acid.

Thus, we have a partition coefficient of water molecules between the alcohol and polymer chains that changes drastically in favor of polymer chains when $C > C^{**}$. In addition, polymer/polymer interactions become also more favored. This change should be due to a progressive condensation of entangled chains on the precursors, up to a full macroscopic disentanglement at the origin of the phase separation proposed above. This behavior is certainly accelerated during the first minutes of evaporation in relation with an important elimination of acid.

This mechanism can be illustrated by the scheme presented in Figure 5, part A.

Nevertheless, the results obtained in the present work did not allow us to give a more precise description of the structure of the network junctions and of the nanoaggregates.

Influence of the Counterion on the Gelation Kinetics. In the works of Montembault et al.,^{28,29} referred to above, it was shown that physical hydrogels of chitosan could be formed from different initial conditions. Thus, we decided to compare the formation of gels in hydroalcoholic medium from acetate and hydrochloride forms. As for the hydrochloride form, we also observed that, for the acetate form, the mechanism of gelation was initiated by the only evaporation of water and the elimination of the associate acid. Thus, in Figure 4, we also plotted the evolution of a_g for the acetate form as a function of the chitosan concentration in the initial solution, in the same conditions as for the hydrochloride form. In a similar manner, the slope of the curve showing the variation of the rate of evaporation of water continuously decreased with the increase of the initial chitosan concentration. The law of behavior was similar, with a value of C^{**} observed in this case at $C_p = 1.5\%$.

Three major differences could be noticed when comparing the two curves presented in Figure 4. (1) The initial rate of

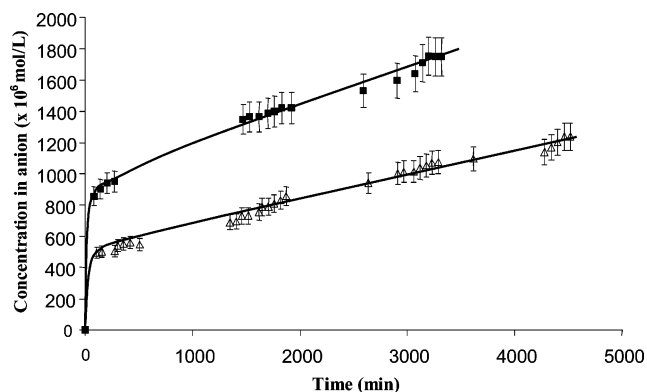


Figure 6. Evolution of the concentration $\times 10^6$ of liberated acid, collected in 50 mL of water for (■) CH_3COOH and (△) HCl , as a function of the time of the evaporation. Results obtained by conductivity, headspace analysis of 15 g of hydroalcoholic solution at an initial concentration $C_{\text{polymer}} = 3\%$ (w/w) in water, at 50°C .

evaporation of water was much higher in the case of the acetate form of chitosan. (2) the slopes of the first and second parts of the curves were lower in the acetate form. (3) In contrast, the value of C^{**} in the acetate form was lower. These differences are necessarily related to a particular situation although the total amount of water and alcohol are the same in both cases as well as the polymer concentration. The parameters varying from one case to the other are as follows: the better solubility of HCl compared to CH_3COOH in the considered solvent, the better solubility of the acetate form of chitosan compared to the hydrochloride form, and a lower ionization of the NH_2 groups in the presence of acetic acid. These assumptions are often conflicting to allow an easy interpretation. Nevertheless, the hydration of chitosan should be favored in the acetate form, a behavior emphasized by the formation of a complex between glucosamine residues and the acetate anion, although the hydrochloride corresponds more to the formation of ion pairs, as shown by molecular modeling (to be published). Then, the role of a partition coefficient of water in favor of the polymer phase in the case of the acetate has to be considered and could be used to explain a lower value of C^{**} .

To dispose of other arguments on the differences between the two salt forms of chitosan, we evaluated the kinetics of elimination of HCl and CH_3COOH during the gelation process. For that, in both cases, we collected the vapors formed at 50°C at the surface of the gelation medium, in 50 mL of water, which variation of conductivity with time was recorded. Taking into account the values of the equivalent limit conductivity of both anions ($\lambda(\text{Cl}^-) = 7.63 \times 10^{-3} \text{ S}\cdot\text{m}\cdot\text{mol}^{-1}$, $\lambda(\text{CH}_3\text{COO}^-) = 4.09 \times 10^{-3} \text{ S}\cdot\text{m}\cdot\text{mol}^{-1}$), we could deduce the variations of the anion concentration. As for the results reported in Figure 3, the variation of the concentration in eliminated anion with time (Figure 6) shows that for both salt forms, two successive domains are observed. Thus, in a first part, up to about 100 min, we notice a sharp domain with a fast increase of eliminated anion, related both to an easy elimination of the un-dissociated part of the acids present in the hydroalcoholic medium and an easy displacement of the ionized to a deprotonated form of amine functions. We also observed that acetic acid was more rapidly eliminated than hydrochloric acid. The latter behavior must

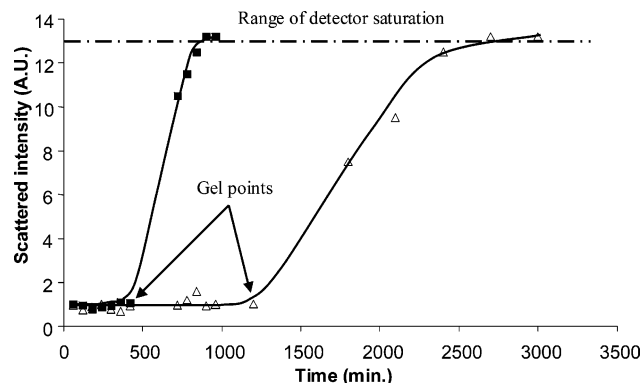


Figure 7. Evolution of the scattered intensity as a function of the time of evaporation of (■) the acetate, and (△) hydrochloride forms of chitosan. The initial concentration was $C_{\text{polymer}} = 0.5\%$ (w/w) in water, at 50 °C.

be necessarily interpreted in relation with its lower acidity and solubility compared to HCl in the solvent studied.

The time to reach the gel point as a function of the salt form of chitosan became an interesting parameter to study. As reported in previous papers, the gel point can be determined thanks to various methods such as light scattering and rheological measurements.^{33,44} In the current case, this time, termed t_{gel} , was determined by static laser light scattering by measuring the variation of the intensity of scattered light through the solution at an angle of 90°, during the evaporation process. For a polymer solution, the scattered intensity is known to depend on the polymer concentration, the molecular weight, the second virial coefficient, the gyration radius, and the detection angle. On the gel point, the intensity increases rapidly in relation with molecular dimensions becoming infinite.

Figure 7 shows the variation of the scattered light intensity as a function of the evaporation time for the two different salt forms of chitosan studied. Due to the very high weight-average molecular weight of our sample, we could not measure t_{gel} for all the range of concentrations studied in this work and we were limited at a maximum value of C_p close to 0.5%. Indeed, for highly concentrated systems, the saturation of the detectors was achieved at the initial stage of the experiment. On Figure 7, we observe that the gel point was reached much more rapidly for the acetate than for the hydrochloride form. If we compare the ratios of the values of a_g to that of t_{gel} (measured at $C_p = 0.5\%$) for the acetate and hydrochloride forms, this ratio close to 1.6×10^{-4} and $0.5 \times 10^{-5} \text{ min}^{-2}$, respectively, reflects the same tendency. Nevertheless, the much lower value in the second case signifies that although a_g plays a major role, other parameters than the content of water should be involved in the kinetics of gelation.

Moreover as already mentioned by Montembault et al.,²⁹ we verified that on the gel point, in the case of the acetate form, it remained almost 10% of water in the medium, contrary to the case of the hydrochloride for which water was fully eliminated. We know that the lower acidity constant of acetic acid, the higher hydrolysis, and lower solubility of its salts are important parameters allowing us to achieve early the gel point by reaching earlier the critical value of the initial charge density of gelation. In addition, the consideration of

the formation of a complex between acetate ions and glucosamine residues could also contribute to favor and then accelerate the gelation thanks to the faster collapse of larger nanoparticles due to their higher hydration. This assumption is supported by the fact that gels formed with the acetate form are softer gels than those processed from the chitosan hydrochloride.

The whole results discussed above confirm the concept of a percolating gelation of chitosan in which a sol/gel transition arises from the evaporation of a hydroalcoholic solution of chitosan. Depending on the initial concentration in polymer, during this evaporation, we contribute to achieve a critical condition where the solution undergoes a molecular reorganization due to changes of the thermodynamic parameters allowing a decrease of the energy of the system constituting the solution. Then, the medium evolves up to a percolating point corresponding to the gel point. This situation, which depends on the nature of the counterion, can be illustrated as in Figure 5.

Thus, initially, when the concentration is located between C^* and C^{**} , the polymer molecules are involved in a three-dimensional network of entangled chains in which are dispersed nanoaggregates, precursors of the reorganization of the solution. On evaporating water molecules, we decrease the dielectric constant of the medium, thus favoring the elimination of the associated acid and, as a consequence, the decrease of the apparent charge density of chitosan chains. This situation, as already mentioned, is initially reinforced by the fast elimination of an important amount of free acid not involved in a process of ionization and weakly soluble in the medium. This context induces the condensation of the entangled chains on the nanoaggregates initially present. These domains progressively loose their water thus reinforcing the interactions between chain segments thanks to hydrophobic interactions and hydrogen bonding. The situation becomes progressively similar to that described above when the solution is initially over C^{**} . If we continue to evaporate the medium, the isolated chains continue to be condensed on the nanoaggregates, which number should be constant but with a size increasing with time. We finally achieve the gel point when these nanoaggregates are collapsing all together to form a gel corresponding to the juxtaposition of clusters of solvent. The surface of these cells filled with alcohol is formed of a membrane constituted of collapsed nanoaggregates of polymer chains. If, initially, the concentration is over C^{**} , the initial molecular organization is different, but the mechanism of progressive condensation of independent chains on the aggregates should be relatively similar.

These schemes should also be similar whatever the salt form of chitosan or the DA. Nevertheless, in each case, we should have some differences of local molecular organization at the origin of some differences we may observe between the gels obtained from the two salt forms. Indeed, gels processed from the hydrochloride form are much more rigid, elastic, and transparent than those obtained with the acetate salt which are also more brittle.

Influence of the Geometry of the Sample on the Gelation Kinetics. Since the sol/gel transition took place in a percolat-

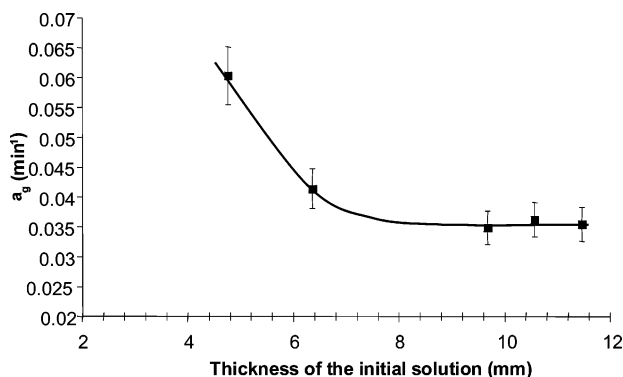


Figure 8. Influence of the thickness of the initial solution on a_g , the rate of evaporation of water (expressed from the variation of the percentage of alcohol with time, see Figure 2), for an initial concentration $C_{\text{polymer}} = 3\%$ (w/w) in water, in the hydrochloride form. The diameter of the gelation reactor was 30 mm, at 50 °C.

ing manner inside a layer moving from the top to the bottom of the reactor, the geometry of the initial solution necessarily played an important role on the kinetics of gelation.

To only vary one parameter, we did our study with a chitosan solution in the hydrochloride form, initially over C^{**} , at 3% (w/w). Then, the solution was poured in Petri dishes of different diameters, but the thickness of the solution was always the same (5 ± 0.4 mm). In the range studied (within 452 mm and 2123 mm), the variation of a_g was directly proportional to the surface of exchange with the atmosphere (not shown), with a slope of $4 \times 10^{-5} \pm 0.3 \times 10^{-5} \text{ mm}^{-2} \cdot \text{min}^{-1}$. Then, as we could predict, the surface was an important parameter, whose variation influenced the rate of gelation. We also verified that the increase of the ventilation speed of the oven contributed to the displacement of the thermodynamic equilibrium of evaporation in favor of a faster elimination of water.

In a second step, we kept the surface of the Petri dish constant and the thickness of the solution was varied.

Figure 8 shows the evolution of the rate of water evaporation as a function of the thickness of the initial solution, for the same sample diameter. We may consider a variation in two parts with a transition range corresponding to two different kinetics modes. In the first part, we notice a progressive decrease of the rate of evaporation in relation to a gradient of concentration in the medium (during evaporation) that increases as the height of the liquid increases. This is responsible for a progressive decrease of the water diffusion in the medium and then the evaporation rate. In the second part, for a height of liquid over 6 mm, the rate becomes almost independent of the thickness. In this case, we form a biphasic system, which can be visually observed, with a gel already formed on the top and a solution below. The sol/gel transition becomes very clear and thin. Therefore, the diffusion of water through the gel becomes the limiting factor and the rate is constant.

Influence of the Temperature on the Gelation Kinetics. Temperature is an important thermodynamic parameter to be considered in the formation of physical gels. It increases the molecular mobility of polymer chains and influences interactions of low energy. Hirano et al.^{39,45} found that the reaction of acylation and the formation of chitin gels were

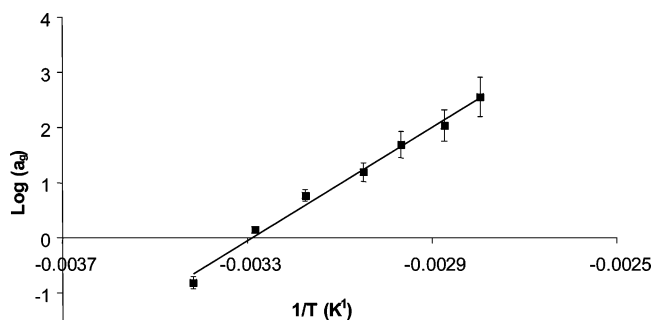


Figure 9. Evolution of $\log(a_g)$ as a function of $1/T$ (K⁻¹) for an initial concentration $C_{\text{polymer}} = 3\%$ (w/w) in water, in the hydrochloride form. Initial samples were as described in Figure 2.

accelerated by an increase of temperature. Moore and Roberts⁴⁰ showed that the time to form chitin gels decreased when the temperature of the reaction of N-acylation increased. Montembault et al. observed that the time to reach the gel point decreased significantly on increasing temperature.²⁹

Figure 9 represents the variation of $\log(a_g)$ as a function of $1/T$.

An increase of temperature should favor an increase of the water diffusion and the reduction of hydrogen bonding and then of an increase of the rate of water evaporation, which is the case for the results shown in Figure 9. We also observed that, in the range of temperatures studied (23–89 °C), we only noticed one activation energy. In agreement with Figure 5, this signifies that for our experimental conditions (range of temperatures and C over C^{**}), we had only one mode of evaporation for this concentration and certainly only one mode of gelation. From the Arrhenius equation we calculated E_a , the activation energy. For our conditions, we obtained $E_a = 42.7 \pm 0.3 \text{ kJ} \cdot \text{mol}^{-1}$. This value is of the same order as those found in the literature: 43 kJ/mol for sceraldehyde-chitosan co-gels,⁴⁶ 67.3 kJ/mol for chitosan physical hydrogels obtained from the acetate form of chitosan, in a hydroalcoholic medium and a DA = 5.2%.²⁹ Our value of E_a was lower compared to that of Montembault et al. This difference could be attributed to the fact that the results of Montembault were made at a concentration below C^{**} , although, in the present case, we were largely over. Indeed, in the concentrated regime, the organization (see Figure 5) was much more favorable to gelation than below.

Conclusion

This paper presents new aspects of the formation of physical hydrogels of chitosan in hydroalcoholic medium. We showed that it was possible to form a true physical hydrogel, without any cross-linking agent, from a water/alcohol/chitosan hydrochloride mixture, thanks to the evaporation of water in association with the partial elimination of the associated acid. The step of evaporation has been particularly investigated and appears to be the important kinetics parameter. It depends on the concentration in polymer in the initial solution, the nature of the acid used, the geometry of the sample, and temperature.

This study also confirmed the existence of the critical concentration C^{**} , first observed by Montembault et al.²²

C^{**} is proposed to be the limit between two organizations of the initial solution. Over or below C^{**} , the kinetics of gel formation was not the same, but whatever the concentration in polymer, a unique mechanism of formation of hydrogels has been proposed.

Finally, it was interesting to notice that mechanical properties of physical hydrogels of chitosan could be adapted to the application, by varying the acid or the alcohol used. For an application of these gels to burn injury treatment, we needed both rigid and elastic hydrogels, with good mechanical properties. Chitosan physical hydrogels presented above seem to be particularly adapted. In a paper in preparation, we will present the relation between morphologies of physical hydrogels of chitosan and their mechanical properties.

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