

Rheometric Study of Chitosan/Activated Carbon Composite Hydrogels for Medical Applications Using an Experimental Design

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ABSTRACT: Composite chitosan/activated carbon hydrogels were prepared with the vapor-induced phase separation process. A rheometric study was performed with a factorial fractional design to determine the formulation and process parameters significantly influencing the mechanical properties of the gels. The results revealed that three factors played a key role in the storage modulus of the gels. According to the model, these factors could be classified with respect to their relative influence on the storage modulus in the following descending order: chitosan concentration > gel time of exposure to ammonia vapors > temperature of the reactor. Increasing these parameters led to an increase in the physical crosslinking density within the matrices and resulted in a reinforcement of the mechanical properties of the hydrogels. Two interactions were also shown to be significant and promoted the formation of supplementary junction zones within the matrix: the first one

corresponded to the interaction between the chitosan concentration and the exposure time to ammonia vapors, and the second one concerned the interaction between the chitosan concentration and the temperature of the reactor. A second-order model was obtained from statistical analysis. Because of the determination coefficient (89.4%) and the *P* value related to the lack of adjustment of the model (0.043), which was associated with a 95% confidence level, this model could be considered to be of good quality. Three gels were used to validate the model, and good accuracy was obtained. The maximum elastic modulus was obtained with the highest chitosan concentration [4% (w/v)], the highest temperature in the gelation chamber (50°C), and the longest time of exposure to ammonia vapors (24 h). © 2010 Wiley Periodicals, Inc. *J Appl Polym Sci* 120: 808–820, 2011

Key words: biopolymers; composites; mechanical properties

INTRODUCTION

Chitosan mostly comes from chitin, a natural biopolymer extracted from the shells of crustaceans. Because of its numerous biological properties, chitosan in membrane and gel forms, alone and in association with other polysaccharides, and in natural and crosslinked forms has been extensively studied, especially in the field of wound dressings.^{1–5}

Activated carbon (AC) is a porous carbon material produced from raw vegetal or mineral materials by chemical or physical activation.^{6–8} Because of its very high specific area (up to 2000 m²/g), AC can be used as an adsorbent for various applications such

as water and gas treatments.^{9,10} In addition, molecules belonging to different classes, such as heavy metals and organics, can be removed with AC.^{11,12}

Many works have mentioned the use of composites made of chitosan and a mineral product^{13,14} or AC and an organic product,^{15,16} and most concern environmental applications. However, only a few studies have reported the use of both materials to produce composites for medical applications.^{17,18} Because of its numerous biological properties, chitosan could be used as a bioactive product leading to the healing of pathologies such as bedsores and burns. Because of its effective adsorption properties, AC could be used to remove inappropriate odors caused by wounds. It is also believed that AC could enhance the mechanical properties of matrices.^{19,20}

The vapor-induced phase separation (VIPS) process is currently used to prepare organic films and membranes.^{21–28} Briefly, a polymer is solubilized in an appropriate solvent. The second step corresponds to the penetration of nonsolvent vapors into the binary system, which leads to unique and controlled

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membrane morphologies. This process involves slow solvent and nonsolvent exchanges because of the gaseous physical state of the nonsolvent.

The ternary systems mentioned in the literature have two common points: (1) the polymer is synthetic, and (2) the nonsolvent is water. In addition, according to previous studies, different parameters influence the final porous structure and thus the final mechanical properties of matrices prepared with the VIPS process. They correspond to process parameters such as the nonsolvent partial pressure²⁵ and the exposure time to vapors.²⁶ A formulation parameter, the polymer concentration, is also known to play a key role in the final structure.^{27,28}

Physicochemical studies concerning the gelation of chitosan from aqueous or hydroalcoholic solutions have been successfully conducted.^{29,30} In the specific case of aqueous solutions,²⁹ chitosan hydrogels were prepared with ammonia vapors produced from a dilute ammonia solution, but no process parameter was controlled. The aim was to study the influence of several formulation parameters on gelation, such as the polymer concentration, the deacetylation degree of chitosan, and the composition of the initial solution. An investigation of the rheological properties was performed to optimize the physical properties of the hydrogels. Then, the optimal gelation conditions with respect to the application, cartilage tissue engineering, were determined. Moreover, García et al.³¹ in 2004 reported a significant influence of the chitosan concentration on the storage (or elastic) modulus (G'). This parameter increased from 2.234 ± 0.507 to 4.923 ± 0.636 N/mm when the polymer concentration was doubled from 1 to 2%. Later, Díez-Sales et al.³² in 2007 performed a rheological characterization of chitosan matrices. They concluded that the addition of poly(ethylene glycol) induced a complementary reinforcement of the mechanical properties of the system. Their work also highlighted the role of the polymer concentration. Thus, when a constant stress was applied in the linear region, matrices with initial polymer concentrations ranging from 1.25 to 1.67% fully recovered; this meant that the initial elastic properties were maintained, and this made them suitable for the development of films for drug delivery. Another rheological study of chitosan films was performed by Lopes Da Silva and Santos³³ in 2007. They discussed the effects of the degree of acetylation and molecular weight of the biopolymer on the linear stress–relaxation behavior of the films. In their work, it was shown that the molecular weight influenced the cohesiveness of the network and thus the elastic character of the final films. With respect to the degree of acetylation, no remarkable influence on the viscoelastic behavior of the films was noticed in the analyzed range (0–27%).

When several factors are involved in or are supposed to influence a specific characteristic (so-called response), it is very convenient to study their effect with an experimental design. This enables (1) the evaluation of the effects of interactions between parameters and (2) the reduction of the number of experiments to be performed in comparison with a classical approach for the same number of tested parameters.³⁴

The use of the VIPS process for the preparation of chitosan–AC composite hydrogels, which involved ammonia vapors as a nonsolvent and was coupled to a model of mass transfers, was presented in a recent work.³⁵ Nevertheless, there are still many questions to be answered. Thus, it would be useful to study the influence of the process and the formulation parameters on the mechanical properties of the final matrix, which are essential because of the final targeted application. Therefore, chitosan–AC composite hydrogels were prepared with the VIPS process. Parameters, including the temperature of the reactor and the nonsolvent concentration, were fully controlled. A rheometric study was carried out to evaluate the influence of five specific parameters on G' of the gels. These parameters were believed to play a key role in the crystalline morphology of the gels and, as a result, on the mechanical properties of the gels. They corresponded to (1) the chitosan concentration in the initial polymeric solution (X_1), (2) the AC concentration in the initial polymeric solution (X_2), (3) the time of exposure to the nonsolvent vapors (X_3), (4) the temperature of the atmosphere inside the reactor (X_4), and (5) the ammonia concentration in the liquid solution from which nonsolvent vapors are produced (X_5). In this study, a factorial fractional design (2^{5-1}) and a response surface methodology were used to determine the variables and the interactions between the variables significantly affecting the mechanical behavior of the final gels. The aim was a better understanding of the impact of the formulation and elaboration parameters on the mechanical properties of the gels. These significant variables were thus involved in a second-order model from which the response could be evaluated and optimized in the whole experimental region.

EXPERIMENTAL

Materials: Chitosan and AC

Chitosan produced from shrimp shell waste (batch no. 342) was supplied by France Chitine Co. (France). The degree of acetylation was measured by ¹H-NMR with a Bruker 300-MHz spectrometer (Bruker, Wissembourg, France). The ¹H-NMR spectra were recorded at 303 K with a deuterium oxide solution of chitosan, which was prepared according

TABLE I
Characteristics of Chitosan

Producer	Degree of acetylation (%)	Viscosity at 25°C (cps) ^a	Weight-average molecular weight (g/mol)
France Chitine	20	100	180,000

^a Data producer.

to the following procedure. A chitosan powder was first dissolved at a concentration of 33 mg/mL in deuterium oxide (hydrochloric acid was used to achieve the stoichiometric protonation of the $-\text{NH}_2$ sites). Then, this solution was freeze-dried three times to exchange labile protons with deuterium atoms. Molecular weights were assessed by size exclusion chromatography and multi-angle laser light scattering with TSK-GEL G-4000 PWXL and G-6000 PWXL columns and an IsoChrom LC pump (Spectra Physics, Evry, France). The columns were connected to a Waters 410 differential refractometer (Millipore, Molsheim, France). An acetic acid/ammonium acetate buffer at pH 4.3 was used as the eluent. Multi-angle laser light scattering detection was performed with a Wyatt Dawn F detector (Wyatt Technology, Toulouse, France) online at 632.8 nm. The polymer solutions [0.1% (w/v)] were filtered on a 0.22- μm -pore cellulose acetate membrane (Millipore) before injection by means of an injection loop (50 μm). The chitosan characteristics are reported in Table I.

The powdered AC used in this study was a commercial product from Pica Co. (St Maurice, France). Granulometric measurements allowed the estimation of the mean diameter of the AC particles. They were performed with a Mastersizer/E apparatus (Malvern Instruments, Orsay, France). The pore characteristics of AC were determined from N_2 adsorption isotherms at 77 K, which were performed with a Micromeritics 2010 analyzer (Verneuil-en-Halatte, France) after degassing for 48 h at 300°C. The theories of Brunauer et al.,³⁶ Horvath and Kawazoe,³⁷ and Barrett et al.³⁸ were used to calculate the specific surface area, the micropore volume, and the mesopore size distribution, respectively. The pH at which the AC surface has no charge [i.e., the pH at the point of zero charge (pH_{PZC})] is another important feature. Its determination was carried out according to the so-called pH drift method described by Kadirvelu et al.³⁹ in 2000: 100 cm^3 of a distilled water solution was placed in Erlenmeyer flasks, and the pH was adjusted to a value between 1 and 13 through the addition of hydrochloric acid or sodium hydroxide (0.1M). Then, 0.1 g of AC was added, and the final pH (pH_{final}) was measured after 24 h of stirring at $20 \pm 2^\circ\text{C}$. pH_{PZC} is the point at which the curve of

pH_{final} versus the initial pH ($\text{pH}_{\text{initial}}$) crosses the $\text{pH}_{\text{final}} = \text{pH}_{\text{initial}}$ line. Finally, scanning electronic microscopy (SEM) observations of AC particles were carried out with a Hitachi S-4800 apparatus (Hitachi, Verrières-le-Buisson, France) (Fig. 1). Characteristics of the AC particles are presented in Table II.

Methods

Preparation of the solutions

The methodology used to prepare the composite solutions was described in detail in a previous work.⁴⁰ Briefly, chitosan solutions were obtained by the dissolution of a chitosan powder (mixed with AC powder) in a diluted acetic acid solution to achieve stoichiometric protonation of the polymer. The final chitosan concentration ranged from 3.00 to $4.00 \pm 0.01\%$ (w/v), whereas the AC concentration was lower [$0\text{--}1.300 \pm 0.001\%$ (w/v)] and depended on the test. Each compound was weighed with accuracy with a Sartorius BP221 S balance (Balco, St Mathieu de Trévier, France). The mixture was then stirred for 24 h and stored at 4°C.

Preparation of the hydrogels with the VIPS gelation process

The VIPS process was carried out in a reactor composed of various elements (Fig. 2). Gels were prepared in a dedicated double-walled chamber (Legal-lais). An external water bath, heated or cooled at a set temperature, maintained a constant temperature inside this chamber, which contained an aqueous solution of ammonia. The composition of the gaseous phase above the gels was kept constant because the temperature was well controlled and the vessel was closed. The initial relative humidity was measured for each experiment and ranged from 96 to $98 \pm 1\%$.

The chitosan solution (5 ± 0.1 g) was cast onto a Petri dish with a 5-cm inner diameter and then exposed to ammonia vapors. Gelation was induced by the contact between this chitosan solution and the ammonia vapors produced from the heated aqueous ammonia solution. Weight variation during gel formation was monitored with a balance (Precisa XB 320 M, Balco) placed below the chamber. A data acquisition system (Balint, a free software) recorded the mass variation of the sample as a function of time.

The time of exposure to ammonia vapors ranged from 0.5 to 24 h and depended on the experiment. The final thickness was approximately 2 mm. Then, the gels were rinsed with deionized water and stored under the same conditions until use.

Rheological measurements

Rheological experiments were performed with a rotational controlled stress rheometer (Haake

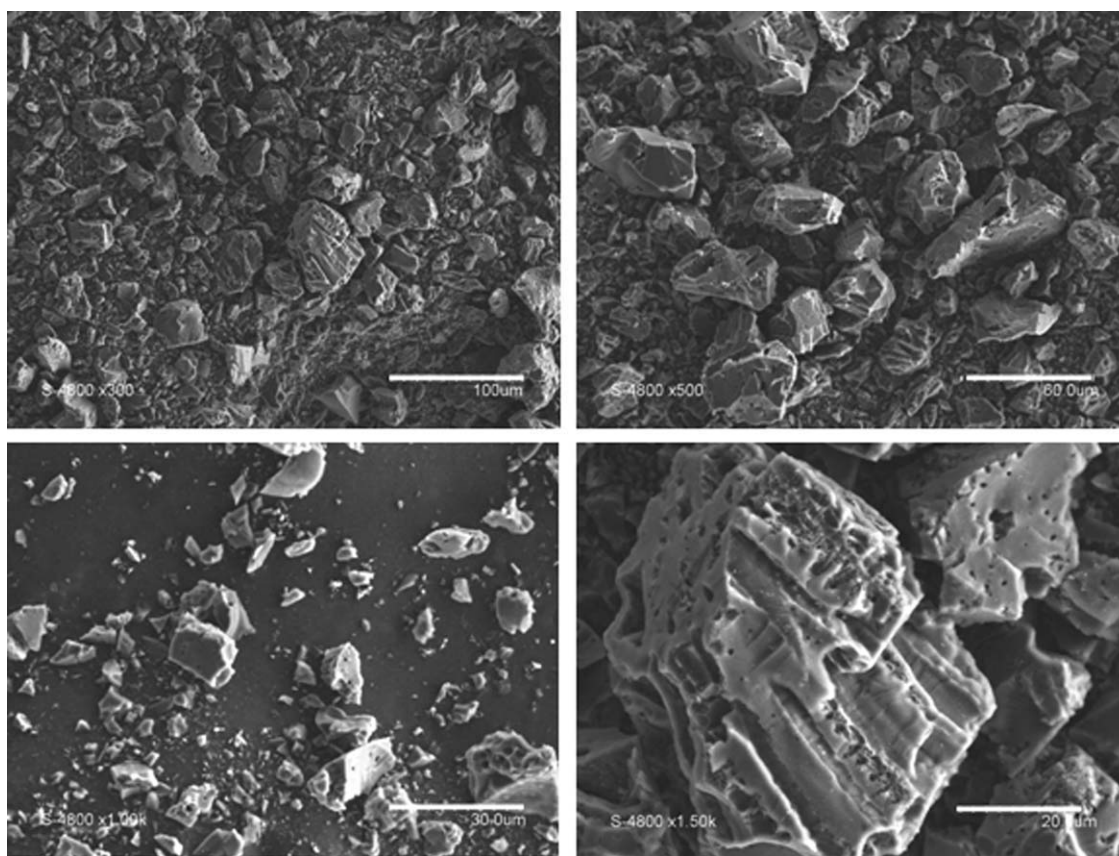


Figure 1 SEM images of AC particles.

Rheostress RS 100, Thermo Fisher Scientific, Illkirch, France) coupled to Rheowin software. A dynamic mode was used for the rheological characterization of the gels: it consisted of applying an oscillatory stress to the sample and then measuring the resulting strain. A parallel-plate geometry with a 20-mm diameter was used. The gap between the plates varied from 1 to 2 mm and depended on the gel thickness. Stress sweep tests were first performed at a frequency of 0.316 Hz and at a temperature of 20°C to determine the linear viscoelastic region in which G' and the loss (or viscous) modulus (G'') were independent of the stress.

Then, from this linear region, an appropriate stress as high as possible was selected to prevent too little torque. With this suitable stress, frequency sweep

tests were carried out at 20°C with frequencies ranging from 0.0079 to 6.37 Hz. The measuring device was equipped with a temperature unit (Peltier element) to ensure temperature control over an extended time.

Experimental design

From preliminary works and a literature survey, five factors (X_1 – X_5) were identified as the most influential parameters with respect to the final characteristics of composite materials using a single polymer and a single AC. A literature survey of the

TABLE II
Physicochemical Properties of AC

Producer	Mean diameter (µm)	Specific surface area (m ² /g)	Pore volume (cm ³ /g)	Micropore volume (%)	pH _{PZC}
Pica	29.16	1,744	1.13	25	9.8

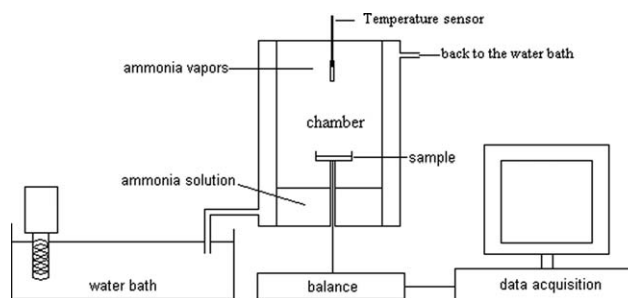


Figure 2 Schematic of the VIPS reactor.

TABLE III
Experimental Design

	Run	X ₁ [% (w/v)]	X ₂ [% (w/v)]	X ₃ (h)	X ₄ (°C)	X ₅ [% (w/w)]	G' (Pa)
2 ⁵⁻¹ experimental design	1	3.25	0.325	6.375	20	1.464	1364
	2	3.25	0.325	6.375	40	0.788	1161
	3	3.25	0.325	18.125	20	0.788	1290
	4	3.25	0.325	18.125	40	1.464	962
	5	3.25	0.975	6.375	20	0.788	1159
	6	3.25	0.975	6.375	40	1.464	1084
	7	3.25	0.975	18.125	20	1.464	1082
	8	3.25	0.975	18.125	40	0.788	1119
	9	3.75	0.325	6.375	20	0.788	1229
	10	3.75	0.325	6.375	40	1.464	1457
	11	3.75	0.325	18.125	20	1.464	1935
	12	3.75	0.325	18.125	40	0.788	2239
	13	3.75	0.975	6.375	20	1.464	1585
	14	3.75	0.975	6.375	40	0.788	1506
	15	3.75	0.975	18.125	20	0.788	2003
	16	3.75	0.975	18.125	40	1.464	7068
Central runs	17	3.50	0.650	12.250	30	1.126	1527
	18	3.50	0.650	12.250	30	1.126	1121
	19	3.50	0.650	12.250	30	1.126	1711
	20	3.50	0.650	12.250	30	1.126	1554
Star point runs	21	3.00	0.650	12.250	30	1.126	1473
	22	4.00	0.650	12.250	30	1.126	2625
	23	3.50	0.000	12.250	30	1.126	2527
	24	3.50	1.300	12.250	30	1.126	1603
	25	3.50	0.650	0.500	30	1.126	1308
	26	3.50	0.650	24.000	30	1.126	1782
	27	3.50	0.650	12.250	10	1.126	2201
	28	3.50	0.650	12.250	50	1.126	3494
	29	3.50	0.650	12.250	30	0.451	1524
	30	3.50	0.650	12.250	30	1.802	1564

elaboration of synthetic polymeric matrices via the VIPS process showed that the polymer concentration,^{27,28} the exposure time to a nonsolvent vapor,^{26,41} and the relative humidity^{25,42} were the most influential parameters with respect to the final film morphologies. X₂ was also selected because of its potential role in the mechanical properties of composites, as mentioned before. The ammonia partial pressure (corresponding to the relative humidity for systems in which the nonsolvent is water) was replaced by two factors easier to control: X₄ and X₅.

To evaluate the influence of these five variables and the impact of their eventual interactions, a 2⁵⁻¹ factorial fractional design was used; this was composed of 16 experiments, to which 2 × 5 star points [$\alpha = (2)^{(5-1)/4} = 2$, representing the distance between star points and the center of the experimental domain] were added. Four replicates of the central run allowed the determination of the experimental error.

Experimental conditions used for the development of this design are defined in Table III. The levels chosen for each parameter ($-\alpha$, -1 , 0 , $+1$, and $+\alpha$) are also presented in this table.

A first set of experiments allowed the determination of the lower and upper limits of X₁ for this spe-

cific chitosan (batch 342). They were found to be 3.00 and 4% (w/v), respectively. Gels prepared with X₁ values lower than 3.00% (w/v) were very weak and too brittle to be handled or characterized. When the initial concentration was higher than 4.00% (w/v), the solution became too viscous to be properly cast in a Petri dish, and the thickness of the resulting gel was not homogeneous.

To choose the maximum level of X₂ (+2), X₁ was set to 3.50% (w/v). Preliminary tests showed the presence of breaking points in the gel matrix for X₂ values higher than 1.300% (w/v), even if a very low stress was applied to the sample. In this case, it was impossible to characterize the gel. As a result, the maximum level of X₂ (+2) was set to 1.300% (w/v). Moreover, to obtain the maximum range for X₂, the lower limit was chosen to be 0% (w/v). Thus, the level of -2 corresponded to a chitosan gel without AC. The minimum level of X₃ was chosen with all parameters set to their central value. Only X₂ was fixed to the level of -2 (a gel without AC) to enable the visual observation of gelation. With an ammonia vapor exposure time of 15 min, the full thickness of the initial system changed from being transparent to being opaque, and this indicated that gelation had

TABLE IV
Results Related to the Factorial Fractional Design

Factor	Regression coefficient	Standard error	<i>t</i> value	<i>P</i> value
Average value	1579.02	320.3	4.930	0.001
X_1	504.38	135.3	3.727	0.005
X_2	130.04	135.3	0.961	0.362
X_3	337.54	135.3	2.494	0.034
X_4	313.96	135.3	2.320	0.046
X_5	204.63	135.3	1.512	0.165
$X_1 \times X_1$	67.11	128.4	0.523	0.614
$X_1 \times X_2$	352.19	165.8	2.125	0.063
$X_1 \times X_3$	486.40	165.8	2.935	0.017
$X_1 \times X_4$	380.44	165.8	2.295	0.047
$X_1 \times X_5$	331.56	165.8	2.000	0.077
$X_2 \times X_2$	71.11	128.4	0.554	0.593
$X_2 \times X_3$	295.19	165.8	1.781	0.109
$X_2 \times X_4$	309.19	165.8	1.865	0.095
$X_2 \times X_5$	327.06	165.8	1.973	0.080
$X_3 \times X_3$	-58.89	128.4	-0.459	0.657
$X_3 \times X_4$	325.44	165.8	1.963	0.081
$X_3 \times X_5$	247.56	165.8	1.494	0.170
$X_4 \times X_4$	266.74	128.4	2.077	0.068
$X_4 \times X_5$	266.31	165.8	1.607	0.143
$X_5 \times X_5$	-59.14	128.4	-0.461	0.656
$R^2 = 89.42\%$	Standard deviation = 663.033		P value (lack of adjustment) = 0.043	

$X_i \times X_i$ = quadratic effect of the X_i factor.

occurred in the whole thickness of the solution. Thus, to ensure complete gelation even with the minimum value of the nonsolvent partial pressure, the -2 level of X_3 was set to 30 min. The maximum value of X_3 was fixed to 24 h according to a previous study dealing with the modeling of the mass transfers occurring during the elaboration of chitosan/AC matrices.³⁵ In this work, modeling and experimental data showed that once the gelation of the whole system thickness was achieved, water outflow occurred. Thus, it was decided to limit X_3 to 24 h to maintain the hydrogel state and to avoid the drying of the sample (film formation).

The two last parameters, X_4 and X_5 , are related to the nonsolvent partial pressure. Experimentally, the ammonia partial pressure can be controlled via the setting of X_4 and the initial value of X_5 .⁴³ The highest level of X_4 was set to 50°C because depolymerization of chitosan may occur with X_4 values that are too high and lead to modification of the mechanical behavior of the gels.⁴⁴ The minimum level was set to 10°C so that the study of the mechanical properties of the gels could be conducted over a wide X_4 range.

Finally, the minimum level of X_5 was set to 0.451% (w/w) on the basis of previous experiments to avoid a long gelation time and experiments that were too time-consuming. The highest level was chosen on the basis of a previous work.²⁹

A second-order model enabling the prediction of each response (Y) in the experimental region was used:

$$Y(X_{i,j=1-5}) = \sum_{i=1}^5 \beta_i X_i + \sum_{i=1}^5 \beta_{ij} X_i^2 + \sum_{i=2}^5 \sum_{j=1}^{i-1} \beta_{ij} X_i X_j \quad (1)$$

where X_i and X_j are individual factors and $X_i X_j$ is their interaction. Y of the experimental design corresponds to G' (Pa). For each value of Y , parameters β_i and β_{ij} of the model were estimated from experiments by least-square regression with Minitab software. In addition, only the second-order interactions ($X_i \times X_j$) were considered to be potentially significant.

RESULTS AND DISCUSSION

Regression coefficients for G' of the gels are gathered in Table IV. The determination coefficient (R^2) was found to be 89.42%, and this indicates rather good precision of the fitting of the experimental data by the model. From the t tests and P values, it is possible to assess the significance of factors at a 95% confidence level ($P < 0.05$) and thus to discriminate factors influencing the mechanical properties of the hydrogels. As highlighted in Table IV, three were found to significantly influence G' of the gels: X_1 , X_3 , and X_4 . Before a detailed analysis of the effect of each parameter, the significant variables can be classified with respect to their relative influence on G' in

the following descending order: X_1 ($P = 0.005$) > X_3 ($P = 0.034$) > X_4 ($P = 0.046$). The polymer concentration, a formulation parameter, is the predominant parameter; this means that the mechanical behavior of the gels depends mainly on their composition. Then, among the two process parameters, X_3 is predominant in comparison with X_4 because it directly affects the charge density of chitosan chains, as explained later.

On the contrary, X_2 and X_5 were not found to significantly affect the mechanical properties of the gels in the tested range of values.

In addition to these three pure factors, two interactions, $X_1 \times X_3$ and $X_1 \times X_4$, were also considered to significantly influence the response ($P < 0.05$). Because the coefficients related to each variable or interaction are positive, an increase in any parameter will lead to an increase in G' (synergetic effect).

Before we discuss the results, it is important to remember the different steps of gel formation²⁹:

1. An acidic chitosan solution is exposed to an atmosphere saturated with ammonia vapors.
2. Ammonia reacts with protonated functions along the chitosan chains (Chit-NH_3^+). Thus, the charge density of the polymer continues to decrease during the process, and this leads to the insoluble form of chitosan (Chit-NH_2).
3. When a critical value of the charge density is reached, the sol–gel transition occurs. The system behaves as a gel rather than a solution. This gel behavior is evidenced from rheological measurements by a G' value higher than G'' and by a relatively constant G' value over a wide range of frequencies.
4. The gels are then stabilized by the formation of physical crosslinks (hydrogen bonds and hydrophobic interactions) between polymer chains. These zones of junctions are responsible for the final mechanical properties of the matrices.

Influence of X_1

According to the t test and the associated P value (0.005), X_1 strongly influences the mechanical properties of the gels in the tested range of concentrations. Moreover, the positive sign of its regression coefficient (+504.38) confirms the synergetic effect of X_1 on G' . An increase in X_1 leads to an enhancement of G' of the gels. This result is also highlighted by Figure 3, which presents G'' and G' for two gels (nos. 5 and 13) prepared at levels -1 and $+1$ of X_1 [uncoded values: 3.25 and 3.75% (w/v)]. The other influential parameters, X_4 and X_3 , were kept constant and set to 20°C and 6.375 h, respectively.

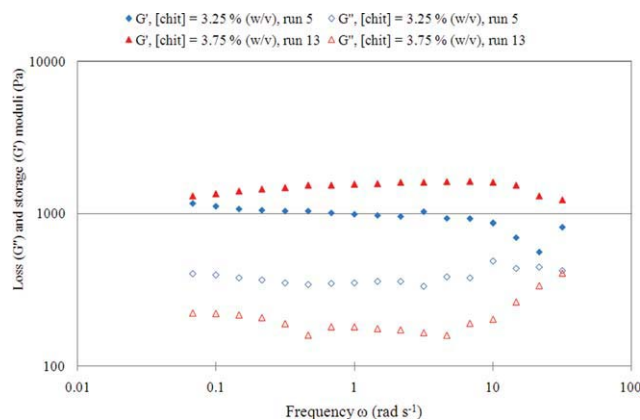


Figure 3 Influence of X_1 on G'' and G' of the gels (temperature = 20°C, VIPS time = 6.375 h). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

This result may be explained by the role of chitosan chains, which constitute the three-dimensional network structure of the composite gel and are responsible for the elastic component of the material.

An improvement in the mechanical properties of the gels with the polymer concentration has been observed for other systems.^{45,46} Kong et al.⁴⁷ in 2003 studied the formation of alginate hydrogels and observed that increasing the polymer concentration led to an increase in G' of the gels. In a recent study, Buckley et al.⁴⁸ (2009) showed that the global mechanical properties of agarose hydrogels were enhanced when the initial polymer concentration was increased.

For chitosan gels, Montembault et al.²⁹ in 2005 studied the evolution of the storage modulus corresponding to the value of G' at low frequencies (G_e) as a function of the polymer concentration in the sample. They clearly measured an increase in G_e with the polymer content, and they attributed this result to the presence of more entangled junctions between chitosan chains in the polymer solution. In addition, with a degree of acetylation of 20%, G_e was found to be close to 1250 Pa, which is of the same order of magnitude as the value obtained in this work (average = 1579 Pa). More recently, for a given frequency range, G' was shown to increase with the polymer concentration in chitosan–xanthan hydrogels.⁴⁵ In a previous work concerning chitosan–AC composite hydrogels prepared via the immersion process, it was also found that G' increased with the polymer concentration.⁴⁰ These works point out the importance of formulation parameters with respect to the gel strength, whatever process is used. In our study, X_1 was the factor with the most significant influence on G' (the smallest P value). Thus, this result is in good agreement with the literature.

Role of AC particles in the mechanical behavior of the composite gels: X_2

The statistical results presented in Table IV show a nonsignificant influence of X_2 on the mechanical properties of the gels in the studied concentration range ($P = 0.362$). It is quite surprising because of works reported in the literature. Therefore, it seems relevant to discuss this result.

The literature mentions the use of AC to enhance the mechanical properties of matrices; this was highlighted by Zhao et al.⁴⁶ in their 2008 study of the elaboration of carboxymethylated chitosan/AC hybrid gel films. They showed that films containing AC had upgraded mechanical properties in comparison with gel films of pure carboxymethylated chitosan. More specifically, the tensile strength was increased. However, to be fully effective, AC particles have to be perfectly distributed within the polymer to ensure a homogeneous matrix and to avoid the formation of breaking points in the gel structure. This homogeneous distribution can be achieved via ultrasonic homogenization,⁴⁹ which was not performed in our study because ultrasonic radiation could promote polymer degradation^{50–52} and lead to lower mechanical properties. Consequently, magnetic stirring was used.

In addition, Figure 1, which presents SEM images of AC particles at different magnifications, clearly shows that the AC powder used in this work was composed of particles of various sizes and shapes. This inhomogeneous distribution of particles might be a supplementary factor explaining why the mechanical properties of the composite gels were not enhanced by AC.

Finally, it could also be assumed that X_2 was low [0–1.3% (w/v)] in comparison with X_1 [3–4% (w/v)] to measure a significant influence of AC on the mechanical properties of the gels. X_2 could not be increased above 1.3% because a high value of X_2 caused the presence of breaking points in the matrix.

Actually, neither mechanical improvement nor mechanical degradation was observed because X_2 was not significant to the response ($P = 0.362$). Thus, although the homogenization was not perfect and the size distribution of AC was not monomodal, they were good enough to avoid the formation of too many breaking points in the matrix, which could lead to a decrease in G' of the gels.⁴⁰

Influence of X_3

X_3 is the second most significant variable; this was confirmed by the value of the coefficient in the second-order model (+337.54) and the P value (0.034). The positive value of the coefficient demonstrates a synergetic effect on G' , which is illustrated in Figure

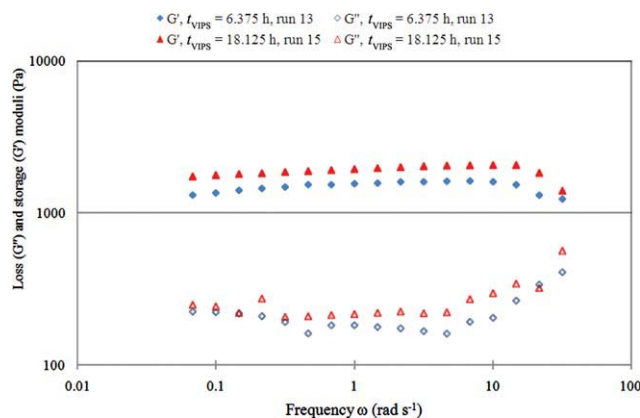


Figure 4 Influence of X_3 on G'' and G' of the gels [$X_1 = 3.75\%$ (w/v), temperature = 20°C, $t_{VIPS} = VIPS$ time]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

4. This figure presents G'' and G' for two gels (nos. 13 and 15) prepared at levels -1 and $+1$ of X_3 (uncoded values: 6.375 and 18.125 h). The other influential parameters, X_4 and X_1 , were set to 20°C and 3.75% (w/v), respectively. When X_3 of the gels increased, G' was enhanced (20%).

Gels prepared from chitosan solutions without any chemical modifications are physical gels.⁵³ The network is stabilized because of hydrogen bonding and hydrophobic interactions between chitosan chains.²⁹ As shown by Vachoud and Domard⁵⁴ in their 2001 study of the properties of physical chitin gels, a syneresis phenomenon produced by a thermodynamic imbalance between the polymer and the solvent generated from the gel point may occur. This imbalance leads to a progressive decrease in the gel volume with respect to the release of the solvent. The syneresis is associated with a change in the mechanical properties of the gels because the polymer concentration increases.

In our study, X_3 ranged from 0.5 to 24 h. At 0.5 h, the samples behaved as gels: $G'(\omega) > G''(\omega)$ (where ω is the angular frequency). For longer times, various phenomena linked to the physical nature of the gels can occur. The variation of the polymer concentration caused by these phenomena may induce a continuous change in the gel mechanical behavior.

Once the gel point has been reached, there are still protonated amino functions remaining on chitosan chains.^{55,56} If the gelled sample is kept in the reactor, the charge density of the polymer chain continues to decrease because of the continuous penetration of ammonia vapors. Thus, increasing X_3 of the gel reduces repulsive electrostatic interactions between chitosan chains and favors polymer–polymer interactions. Consequently, the mechanical properties will be increased.

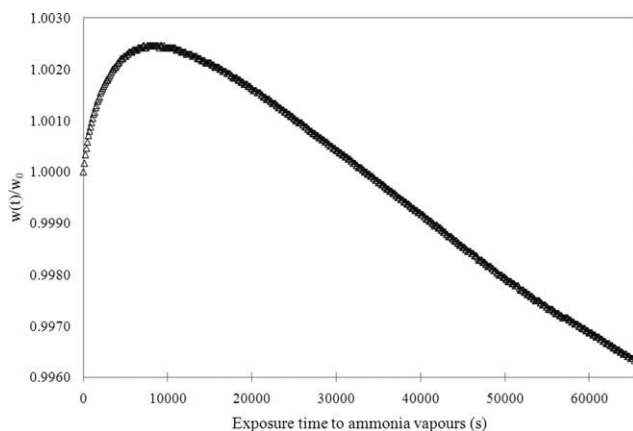


Figure 5 Gravimetric kinetics related to run 7 of the experimental design [$w(t)$ = weight of the system at time t , w_0 = initial weight of the system at time 0].

In addition, because of a slight water chemical potential gradient between the gaseous phase and the sample, water outflow from the gel was responsible for sample weight variation during the process. This water outflow occurred simultaneously with the ammonia inflow (responsible for gelation) but lasted longer, and this could be globally attributed to a slow drying of the gel under smooth conditions.³⁵ This water loss could be easily highlighted because of online weight measurements: the gravimetric kinetics corresponding to the weight evolution of the gels as a function of X_3 of the gels is reported in Figure 5. After approximately 3 h, a weight decrease was recorded, and this was attributed to the evaporating flux of water. Therefore, the polymer concentration in the gels continuously increased, and this led to an increase in the mechanical properties of the matrices.

Influence of X_4

Concerning the third and last significant variable, that is, X_4 (coefficient = +313.96, $P = 0.046$), Figure 6 presents its synergetic effect on G'' and G' for two gels (nos. 15 and 16) prepared at minimum and maximum temperatures (20 and 40°C). The other influential parameters, X_1 and X_3 , were set to 3.75% (w/v) and 18.125 h, respectively.

If we first focus on the VIPS process rather than the physicochemical properties of the gels, we find that X_4 influences the nonsolvent partial pressure. Increasing this parameter leads to an increase in the ammonia saturating vapor pressure and, as a result, an enhancement of the ammonia chemical potential gradient between the gaseous atmosphere surrounding the gel and the polymer solution. Thus, a larger amount of ammonia should penetrate the polymeric solution. On the basis of these considerations, the

influence of the nonsolvent vapor pressure on the mechanical strength of the gels also has to be discussed in this section. However, we also found that X_5 had no significant influence on G' of the samples ($P = 0.165$). Because of the variation ranges of the two parameters [10–50°C and 0.451–1.802% (w/v) for X_4 and X_5 , respectively], their impact on the ammonia partial pressure was almost equivalent. Consequently, the temperature does not affect G' through the ammonia partial pressure.

G' may be influenced by the temperature for other reasons. The variation of the temperature affects both the mass-transfer kinetics from the gas phase to the air/solution interface and the diffusion kinetics of ammonia within the chitosan solution. Thus, the ammonia intake rate in the chitosan solution increases when the temperature is raised. In other words, the ammonia content in the polymer solution is enhanced for a given sample exposure time in the reactor. As a result, the charge density along the polymer chains is lowered, and then the electrostatic repulsive interactions between chitosan chains are reduced; this leads to a higher G' value.

The effect of temperature also has to be analyzed because of its direct role in the polymer properties. An increase in the temperature promotes the molecular mobility and then favors the probability of connections between polymer chains; this allows the chitosan macromolecules to form physical junction zones more easily.

The temperature also plays a particular role in low-energy interactions involved in the formation of physical polymer networks, as evidenced by the 2007 study of a chitosan-based hydrogel by Ganji et al.⁵⁷ The authors investigated the effect of temperature on the gelation time of a chitosan/glycero-phosphate disodium salt solution. Their results showed the gelation kinetics to be strongly

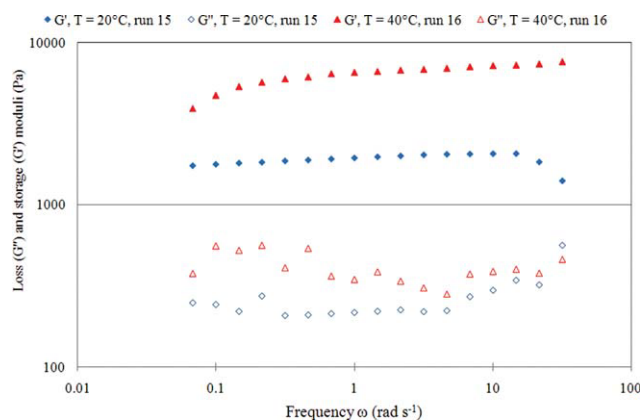


Figure 6 Influence of the temperature (T) on G'' and G' of the gels [$X_1 = 3.75\%$ (w/v), VIPS time = 18.125 h]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

influenced by the temperature increasing. Thus, for a solution composed of 2% (w/v) chitosan and 0.56M glycerophosphate disodium, the gelation time decreased from 4 min at 37°C to 21 s at 60°C. Their explanation was based on chitosan–water interactions, which are strong at low temperatures and prevent chitosan chains from aggregation. However, at higher temperatures, sheaths of water molecules are removed, and chitosan–chitosan hydrophobic interactions are then strengthened and lead to faster gelation. Although the temperature directly controls the gelation time, it may also influence the final mechanical properties of gels because it enhances interactions between chitosan chains. The 2007 study by Goycoolea et al.⁵⁸ also illustrates the effect of temperature on polymer–polymer interactions. These authors studied the effect of temperature on the mechanical behavior of chitosan hydrogels. One of their results concerned the response of the gels to oscillating changes in temperature. They observed an increase in G' with the temperature (range = 5–40°C), whereas upon cooling to 0°C, G' values decreased. Nevertheless, this result was less pronounced when the equilibration pH of the gels increased (range = 7.3–12), and no changes were observed at pH 12.

Finally, our results, coupled to those in the literature, point out the importance of the elaboration temperature on the mechanical properties of hydrogels. Its role has to be explained through the physicochemical properties of the polymer and the mass-transfer kinetics of the nonsolvent.

In addition to this linear effect, a quadratic effect of X_4 (X_4^2) on the studied response can be considered (coefficient = +266.74, $P = 0.068$). This quadratic effect, although slight ($0.05 < P < 0.10$), justifies the use of a second-order model to describe the effects of the various parameters on G' of the gels.

Influence of interactions

Two interactions are considered to extensively influence the mechanical response for a 95% significance level: the $X_1 \times X_3$ interaction on the one hand ($P = 0.017$) and the $X_1 \times X_4$ interaction on the other hand ($P = 0.047$).

The polymer concentration is involved in both interactions, and this reinforces the fact that this parameter is the most significant parameter for the mechanical strength of the gels ($P = 0.005$). In this section, we discuss how the variation of X_1 affects the influence of the two other parameters (X_3 and X_4) on G' .

$X_1 \times X_3$ interaction

Although the gelation mechanism is not completely identified, it is currently admitted that it occurs

when a critical number of protonated functions are neutralized by basic media.^{55,56,59} Once the gel point has been reached, exposing the physical gel for a longer time to basic vapors favors the formation of supplementary hydrogen bonds and hydrophobic interactions between polymer chains because additional ammonia comes into contact with the protonated functions of the polymer and produces free amino groups (Chit-NH₂). In the gel state, some amino functions along the polymer chains remain in their protonated form, and the neutralization of these functions lasts if the gelled sample is stored in the reactor. The mechanical properties of the final gels depend on the degree of neutralization of amino groups and, as a result, on the exposure time to ammonia.

The positive value of the regression coefficient for $X_1 \times X_3$ (+486.4) indicates the synergetic effect of the $X_1 \times X_3$ interaction on G' . This result is highlighted by the response surface plot [Fig. 7(b)], which reveals that higher values of G' are reached with simultaneously high values of X_1 and X_3 .

For low chitosan contents, the concentration of protonated amino functions reacting with ammonia and the number of possible physical junctions in the chitosan matrix are low. Thus, even a high X_3 value will not lead to an improvement in G' . When the polymer concentration increases, the concentration of functions to be deprotonated (Chit-NH₃⁺) in the sample rises, and X_3 becomes a critical parameter that will strongly influence the physicochemical properties of the gels, including their mechanical properties.

$X_1 \times X_4$ interaction

The influence of the $X_1 \times X_4$ interaction is represented by the response surface plot in Figure 7(a), which suggests that the influence of X_4 on G' is enhanced at high polymer concentrations. Actually, increasing X_1 leads to an increase in the number of physical junctions stabilizing the three-dimensional network of polymer chains.⁶⁰ Thus, the role of X_4 in the formation of supplementary polymer–polymer interactions is more important when X_1 is increased.

In the previous section concerning the influence of X_4 (as an individual factor) on G' , results are discussed with respect to the low-energy interchain interactions, which may be favored at higher X_4 values and lead to an increase in the elastic component of the material. The effect of X_4 is also discussed in terms of mass-transfer kinetics. However, X_1 does not significantly affect the internal diffusion of ammonia within a sample.⁶¹ Consequently, the effect of the $X_1 \times X_4$ interaction on G' cannot be explained by a change in the diffusional phenomena within the chitosan solution.

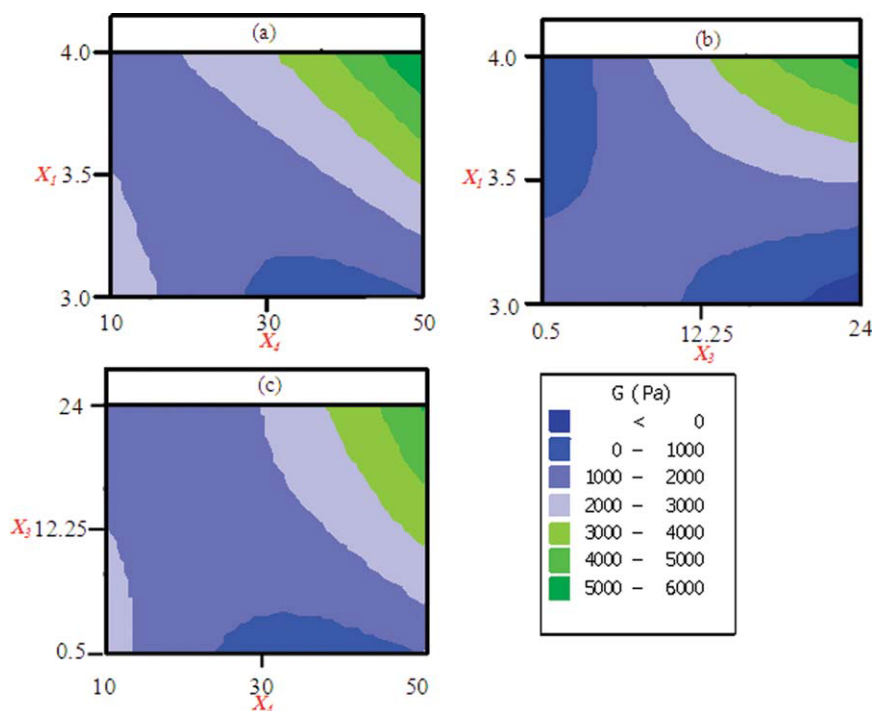


Figure 7 Response surface plots of G' as a function of (a) X_1 and X_4 , (b) X_1 and X_3 , and (c) X_3 and X_4 . For each plot, the parameters were set to their central values when they were not variables on the axis [$X_1 = 3.50\%$ (w/v), $X_2 = 0.065\%$ (w/v), $X_3 = 12.25$ h, $X_4 = 30^\circ\text{C}$, $X_5 = 1.126\%$ (w/w)]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Interactions at a 90% significant level

Other interactions significant at a lower level ($P < 0.10$) can be mentioned. They correspond to the interactions between (1) X_1 and X_2 (coefficient = +352.19, $P = 0.063$), (2) X_1 and X_5 (coefficient = +331.56, $P = 0.077$), (3) X_2 and X_4 (coefficient = +309.19, $P = 0.095$), (4) X_2 and X_5 (coefficient = +327.06, $P = 0.08$), and (5) X_3 and X_4 (coefficient = +355.44, $P = 0.081$). This result points out the multitude and complexity of physicochemical phenomena involved in the gelation mechanism and, therefore, in the final rheological characteristics of the gels.

Validation of the second-order model

By taking into account the significant variables and interactions for which the P value is lower than 0.05, we can establish the second-order model for G' of the gels as follows:

$$Y = 1579.02 + 504.38X_1 + 337.54X_3 + 313.96X_4 + 486.4X_1X_3 + 380.44X_1X_4 \quad (2)$$

Y corresponds to the response, that is, G' of the gel. The R^2 value of 89.42% means that 10 to 11% of the result variability is not explained, and this may be due to either experimental errors or the model's lack of fit. Nevertheless, with the P value of the lack of

fit being 0.043 (<5%), it can be concluded that the model is well adjusted and that the percentage of unexplained results is due to experimental errors.

The final part of this study concerns the validation of the model. Three gels were prepared under conditions different from those used to develop the experimental design. However, the levels of the process and formulation parameters (X_1 , X_3 , and X_4) all were within the -2 to $+2$ interval (Table V). The values of the experimental elastic modulus (G'_{exp}) and theoretical elastic modulus (G'_{th}), which was calculated with the second-order model, are also reported in Table V. Because the variation coefficient is lower than 7% in each case, the second-order model can be considered reliable and is consequently validated. As a result, eq. (2) can be used to determine with good accuracy G' of the gels if all

TABLE V
Validation of the Second-Order Model

Gel	X_1	X_3	X_4	G'_{exp} (Pa)	G'_{th} (Pa) ^a	Δ (%)
A	-1.6	-1.9	0.5	1402	1462	4.3
B	-0.4	1.8	-0.2	1501	1602	6.3
C	-0.8	-1.75	1	1364	1275	7.0

^a Calculated from the second-order model. ^b Variation coefficient: $\Delta = |G'_{\text{exp}} - G'_{\text{th}}| / (G'_{\text{exp}}) \times 100$.

formulation and process parameters are within the -2 to $+2$ interval.

In conclusion, optimal operating conditions for obtaining the best mechanical properties can be determined. Because the coefficients related to significant factors are all positive, G' will be optimal ($G' = 7358.12$ Pa) at the highest X_1 value [4% (w/v)], the highest X_4 value (50°C), and the highest X_3 value (24 h). However, exceeding these values is not recommended. Indeed, if X_1 is too high, the initial solution will be too viscous to be cast, and it will be impossible to obtain a material with a homogeneous thickness, as explained before. In addition, a higher X_4 value could enhance chitosan depolymerization.⁴⁴ Finally, an X_3 value that is too high will lead to an important weight loss, and then the final composite hydrogel will be closer to the dried form (film) than the gel state.

CONCLUSIONS

Composite hydrogels made of chitosan and AC were prepared with the VIPS process. A factorial central composite design was developed to study the effects of various factors on G' of the gels. The individual formulation and elaboration parameters statistically identified as prevailing factors for the mechanical properties of the gels are X_1 , X_3 , and X_4 .

The role of X_1 in the reinforcement of mechanical properties has been explained by more entangled polymer chains in the solution, which lead to more favorable conditions for the formation of the physical junctions stabilizing the network. The effect of X_3 on G' of the gels has also been shown to be significant for two main reasons: a decrease in repulsive electrostatic interactions between chitosan chains and water outflow from the gels highlighted by gravimetric measurements. G' is also influenced by X_4 because this parameter affects the mobility of chitosan chains, the polymer-polymer interactions, and the mass-transfer kinetics of ammonia. Moreover, according to the model, these individual factors can be classified with respect to their relative influence on G' in the following descending order: $X_1 > X_3 > X_4$.

Two interactions, both involving X_1 , have also been considered in the final model because of their significant influence on G' of the gels. The first one corresponds to the interaction between X_1 and X_3 . The effect of X_3 on G' is enhanced by an increase in X_1 . This has been attributed to a larger number of protonated functions in the initial chitosan solution. The second significant two-factor interaction is that between X_1 and X_4 . Indeed, the role of X_4 in the formation of supplementary polymer-polymer interactions will be more pronounced for higher X_1 values.

A second-order model has been established and allows the forecasting of G' of the gels with accuracy. It is in good agreement with the experimental data. This model has been validated by three gels elaborated with formulation and process parameters included in intervals limited by star points. The G' values, deduced from the equation of the model or experimentally, are in good agreement with a variation coefficient lower than 7%.

The physical properties of gels, such as mechanical properties, are largely influenced by the morphology at various scales. Consequently, the characterization of gel morphology is under investigation. A combination of light and X-ray scattering techniques will be used to provide a description of the structure from the nanometer range to the micrometer range (e.g., particles of polymer aggregates of different sizes, crystallinity, crystallite sizes, and characteristic distances between such entities). The aim will be to establish relationships between the mechanical characteristics of the gels and their internal structure; this is essential for the final applications (wound dressings) of these composites.

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