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Characterization of the interaction between chitosan and inorganic sodium phosphates by means of rheological and optical microscopy studies

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ABSTRACT

The physicochemical and rheological properties of chitosan and two different inorganic sodium phosphate dispersions (NaH_2PO_4 and Na_3PO_4) were investigated in order to elucidate the role of different factors, such as ratios between polymer and sodium inorganic phosphates, different pHs and storage stability, on the gelling properties of chitosan. This was deemed opportune since physico-chemical characterizations of chitosan in the literature often appear incomplete and questionable. We also compared the elastic modulus values of the different chitosan/inorganic phosphate systems and examined their behaviour through optical microscopy analyses.

The most efficient formulations that showed a thermogelling capacity with a significant gel transition behaviour after 24 h were the NaH_2PO_4/chitosan and Na_3PO_4/chitosan systems at ratio 2 and pH 7.0.

These results confirmed the importance of the pH value and ratio selection for the final systems.

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1. Introduction

Stimuli-responsive polymers have been proposed for a wide range of applications (Bajpai, Shukla, Bhanu, & Kankane, 2008; Jeong & Gutowska, 2002; Osada & Gong, 1993) and in the last decade a great interest was deserved to those polymers able to modify their physical state from sol to gel as the temperature changes (Ruel-Gariépy & Leroux, 2004; Schmaljohann, 2006).

Chitosan, an amino polysaccharide obtained by alkaline deacetylation of chitin, is a biocompatible and biodegradable pH-dependent cationic polymer that has been proposed as suitable material for many purposes in a wide range of medical, pharmaceutical and food applications (Dash, Chiellini, Ottenbrite, & Chiellini, 2011; Illum, 1998; Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Ravi Kumar, 2000; Shahidi, Arachchi, & Jeon, 1999).

Solubility of chitosan in aqueous solutions is attained via protonation of its amine groups in acidic environments. Its limited water solubility is well known (pK_a 6.3); moreover, the neutralization of its amino groups leads to the formation of a hydrated gel-like chitosan precipitate. In fact, the deprotonation of the amino groups present in its backbone leads to the removal of repulsive inter-chain electrostatic forces, allowing hydrogen bonding and hydrophobic interactions between chains (Goycoolea et al., 2007).

In addition, its ability to interact with different salts, anionic molecules or crosslinkers to form hydrogel for pharmaceutical and biomedical applications has been well reviewed by various authors (Berger et al., 2004; Bhattarai, Gunn, & Zhang, 2010; Liu, Tang, Wang, & Guo, 2011; Ruel-Gariépy, Chenite, Chaput, Guirguis, & Leroux, 2000).

In the last decade, from the first works reported by Chenite and colleagues (Chenite et al., 2000; Chenite, Buschmann, Wang, Chaput, & Kandani, 2001; Ruel-Gariépy et al., 2000), the use of chitosan as a hydrogel able to undergo sol–gel transition has attracted the attention of many research groups (Cho, Heuzey, Bégin, & Carreau, 2005; Chung, Bae, Park, Lee, & Park, 2005; Crompton et al., 2005; Kempe et al., 2008; Zan, Chen, Jiang, Lin, & Ding, 2006; Zhou et al., 2008).

Different strategies were developed to obtain chitosan hydrogels and rheological characterization has been proposed to elucidate the influence of the different parameters involved. Various organic and inorganic phosphates, for example β-glycerophosphate (β-GP), potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2PO_4), tripotassium phosphate (K_3PO_4), ammonium hydrogen phosphate (AHP), sodium tripolyphosphate (TPP) and dibasic sodium phosphate (Na_2HPO_4), have been employed as gelling agents (Chenite et al., 2000, 2001; Cho et al., 2005; Li & Huang, 2012; Li et al., 2010, 2011; Nair, Starnes, Ko, & Laurencin, 2007; Ta, Han, Larson, Dass, & Dunstan, 2009).

Studies indicate that the most used phosphate, β-glycerophosphate, allows better solvation of chitosan at physiological pH (from 6.3 to 7.4) and improves thermogelling (Berger et al., 2005; Chenite et al., 2001; Cho, Heuzey, Bégin & Carreau, 2006).

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Moreover, tests with the different inorganic phosphate salts showed that the proper pH value was fundamental for achieving thermogelification of the chitosan solution (Li et al., 2010; Nair et al., 2007; Ta et al., 2009). The use of weak base phosphates is decisive for avoiding chitosan precipitation during the initial phase of sample preparation, and improves the gelling ability of the final systems.

The aim of this study was to definitively clarify the role of different physiochemical parameters in chitosan thermo-hydrogel systems. We used two different sodium inorganic phosphate salts (NaH_2PO_4, a weak base with a pK_a of 7.2, and Na_3PO_4, a stronger base with a pK_b of 2.23) as the gelling agent. Moreover, we evaluated the influence of different pHs (from 5.0 to 7.0) and phosphate/chitosan ratios (0.5, 1.25 and 2), as well as the storage stability of the hydrogel systems.

The rheological data obtained was corroborated by optical microscope analyses, which made it possible to confirm and clarify the thermogelling ability of the system.

2. Experimental

2.1. Materials

Chitosan in its free amine form (cat. no. 448869, molecular weight 50–190 kDa, viscosity 20–300 cps determined by Brookfield method, 1% solution in 1% acetic acid, and degree of deacetylation of 75–85%), monosodium phosphate (NaH_2PO_4), and trisodium phosphate (Na_3PO_4) were purchased from Sigma Aldrich. Deionized water was produced with a laboratory deionizer (Osmo Lab UPW 2, Gamma 3, Italy).

2.2. Preparation of chitosan solution

2% (w/w) chitosan stock solution was prepared in a 0.1 M hydrochloride acid water solution, under constant magnetic stirring overnight at room temperature. The resultant solution was refrigerated at 4 °C until investigation.

2.3. Preparation of chitosan/inorganic phosphate dispersions

Depending on the chitosan/inorganic phosphate ratio, a certain amount of inorganic phosphate (NaH_2PO_4 [0.7 M] or Na_3PO_4 [0.7 M]) solution was added dropwise to chitosan stock solution under magnetic stirring. The chitosan/inorganic phosphate systems were prepared at three different pHs (5.0, 6.0 and 7.0) by adding HCl (0.1 M) or NaOH (0.3 M) aqueous solutions. Before to start the analyses the pH values were well determined, however we found a decreasing in the pH values when the temperature were increased during the rheological tests. So far we decide to prepared the sample at a slightly higher pH values before to run the rheological tests.

The final chitosan concentration was 1.4% (w/w), while the inorganic phosphate/chitosan ratios were 0.5, 1.25 and 2. These ratios refer to equivalent amino groups estimated on the chitosan backbone.

The resultant dispersion systems were stored under refrigeration at 4 °C for later use.

2.4. Rheological characterization

Rheological analyses were performed in triplicate using a stress control rheometer (Stress-Tech, Reologica) equipped with a 4/40 cone-plate geometry (cone diameter was 40 mm, cone angle was 4°) operating in the oscillation mode. The gap was 150 μm. Samples were analysed in triplicate at 1 day, 3 and 7 days from the preparation.

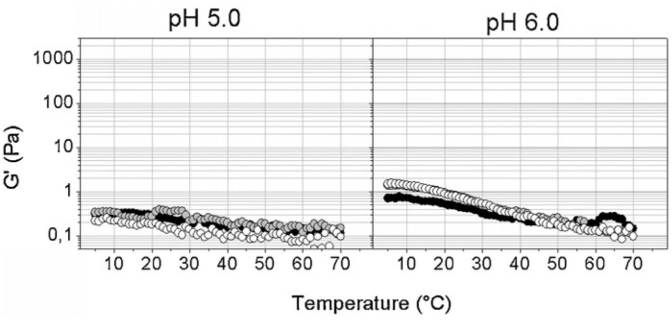

Fig. 1. Temperature sweep of chitosan dispersions (1.4%, w/w) at pH 5.0 and 6.0 ((●) 1 day, (●) 3 days, (○) 7 days).

The analyses were carried out as follows: in the oscillation stress sweep test, the samples were exposed at 25 °C and stress was increased at a constant frequency (1 Hz) in the range of stresses between 0.05 and 10 Pa. This test determines the linear viscoelastic regime (LVR) of the sample, and therefore the consequent choice of the stress value to use in the other oscillation tests.

In the temperature sweep test, the chitosan and chitosan/inorganic phosphate systems were analysed at constant frequency (1 Hz), at a stress of 1 Pa and in the range of temperature from 0 to 70 °C at a rate of 1 °C/min. Evaporation of samples was prevented by using a silicon oil sealing.

2.5. Optical microscope analyses

An optical microscope (MT9000 polarizing microscope, Meiji Techno Co. Ltd., Japan) equipped with a 3 megapixels Cmos camera (Invenio 3S, DeltaPix) was used to collect images of chitosan/inorganic phosphate systems at different stages.

3. Results

3.1. Rheological data analyses

Preliminary rheological analyses were performed on chitosan solutions prepared at different pHs. Both chitosan samples (pH 5.0 and 6.0) showed the same appearance and rheological properties (Fig. 1). On the contrary, it was impossible to prepare a chitosan solution at pH 7.0 because after pH 6.0 the chitosan solution started to aggregate inducing a phase separation.

No evident phenomena were observed when the temperature was increased for both samples, even if pH 6.0 has been reported to be more sensitive to temperature variation, as confirmed by a slight decrease of the G' modulus in our study. Moreover, during the seven day stability tests, both chitosan solutions appeared stable and did not show any significant change in the rheological properties.

The chitosan/NaH_2PO_4 systems at pH 5.0, independently of the ratio, showed a behaviour similar to that of chitosan solution at the same pH (Fig. 2). In fact, they were stable and their behaviour was independent of the different temperatures applied. Moreover, the G' value was practically the same as that of the chitosan solution.

On the contrary, increasing the pH to 6.0 caused the systems to appear more opalescent, though without evident formation of aggregates, and modified the rheological behaviour for all the three ratios analysed. This was accompanied by an increase in the starting value of G' compared to the value at pH 5.0. However, at pH 6.0, a different G'/temperature trend was observed for the three inorganic phosphate/chitosan ratios. At a ratio of 0.5, a decrease in G' values at increased temperature were observed, confirmed for all seven days of the stability test (the three curves are superimposed). However, at a ratio of 1.25, we had a slight increase in G' at about 65 °C for the first and third days, a trend that disappeared after seven days,

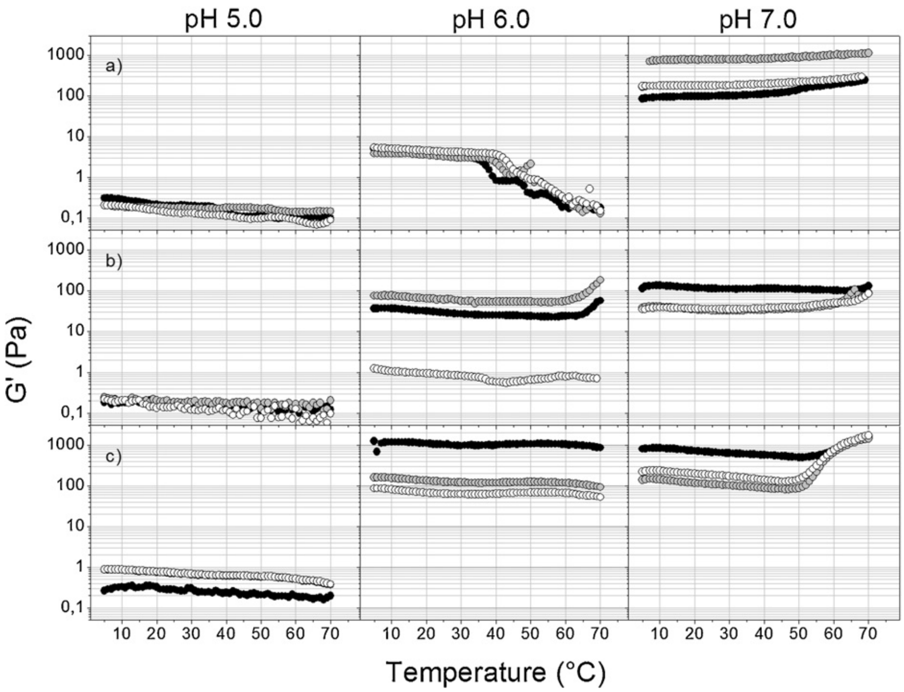

Fig. 2. Temperature sweep of NaH_2PO_4/chitosan systems at different ratio ($a = 0.5$, $b = 1.25$ and $c = 2$) and pHs (5.0, 6.0 and 7.0) ((●) 1 day, (◍) 3 days, (○) 7 days).

together with a lowering of the elastic component. Finally at ratio 2, no temperature dependency was detected and the lowering of G' modulus already appeared after the three days of storage, in comparison with the ratio 1.25, where this phenomenon appeared after seven days.

At pH 7.0, for all the ratios, the increase of the starting G' value was confirmed. Particularly interesting is ratio 2 (Fig. 2). In this case, a gradual increase of the G' value indicated the start of the gelation process at 55 °C, confirming the ability of the chitosan/NaH_2PO_4 system to form pseudo thermogels.

The gelation process of chitosan/phosphate systems appeared to be influenced mainly by the pH value of the dispersions and the phosphate/chitosan ratio.

The chitosan/Na_3PO_4 systems at pH 5.0 showed the same rheological behaviour of the previous inorganic phosphate (Fig. 3).

At pH 6.0, the temperature dependence of the modulus G' was still present for all the ratios, but while at ratio 0.5 the curves are superimposed, at 1.25 and 2 the trend is different. The ratio 1.25, after 3 and 7 days of storage, showed a G' increase compared to the sample analysed one day after preparation. On the other hand, for ratio 2 an increase of G' was observed only after 7 days. This result highlights a certain time-dependency in the rheological behaviour of the samples. At pH 7.0, the chitosan/phosphate ratio appeared to be fundamental for the mechanical properties of the final systems. In fact, the G' value of the 0.5 ratio was definitely higher compared to those observed at pH 5.0 and 6.0, but there was no significant temperature dependence, and good sample stability was observed during the 7 days storage. At ratio 1.25 at 1 and 3 days, no dependence to temperature was detected, while G' values increased slightly. For this ratio at 7 days, the initial part of the G' curve appeared superimposed on the three day storage plot until 52 °C, and then it increased slightly until reaching the values observed 1 day after preparation. At ratio 2, we observed that the elastic modulus values at 3 and 7 days were higher than those of the system analysed after 24 h and were nearly superimposed until the temperature of 45 °C. After this temperature, all the samples showed a gradual increase in the G', demonstrating pseudo-thermogelling behaviour already underway 24 h after preparation. Increased storage time lead to greater consistency in the samples.

Comparison of the two inorganic phosphate salts highlighted significant differences at only pH 6.0 and 7.0. Generally, the lowest elastic properties were observed using Na_3PO_4 salt. On the other hand, for the ratio 2 at pH 7, both salts were able to confer thermogelling properties to the chitosan dispersions.

The NaH_2PO_4 system showed a significantly increased G' value from 220 to 1700 Pa G' at 50 °C and a phase degree very close to zero.

Also, the corresponding Na_3PO_4 sample showed a great G' increase (from 143 to 1061 Pa) at around 45 °C, with a final phase degree value typical of a well structured system (3.5°).

3.2. *Optical microscope data*

The differences between the pH values and the kind of inorganic phosphate used were evident even from the optical microscopy images.

All the systems were analysed after 24 h of preparation. First, we analysed each system at room temperature ($t_{0\,\min}$), then after 10 min at 70 °C ($t_{10\,\min}$) and finally the heated sample was left for 15 min at room temperature and then analysed again ($t_{15\,\min}$).

In the case of the chitosan/NaH_2PO_4 system (phosphate/chitosan ratio 2), different behaviour was evident at the two pH values (Fig. 4). At pH 7.0, time zero, the system appeared quite inhomogeneous ($t_{0\,\min}$), while after 10 min at 70 °C its appearance changed, looking more homogenous ($t_{10\,\min}$), indicating greater polymer solvation. Finally, after cooling, there was a sort of phase separation with a gel phase section and a more pronounced sol homogenous phase ($t_{15\,\min}$). This visual examination confirmed the thermogelling behaviour of the pH 7.0 system observed by rheological analyses. On the contrary, at pH 6.0, the solvation process was more pronounced but without reaching a gellified system.

The chitosan/Na_3PO_4 system (phosphate/chitosan ratio 2) at pH 7.0 showed behaviour similar to that of the corresponding chitosan/NaH_2PO_4 sample (Fig. 5). Even in this case, significant

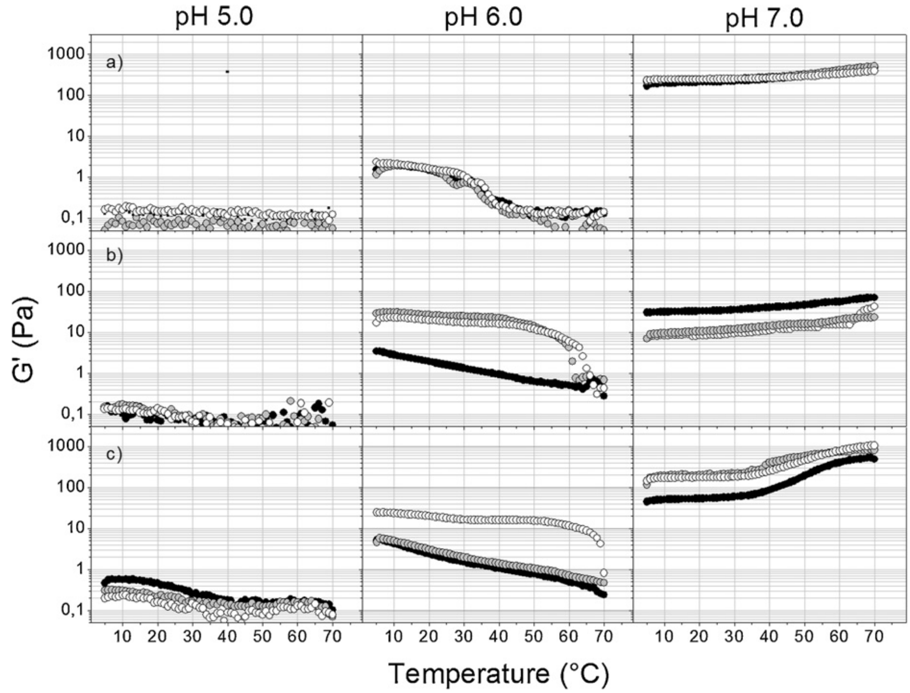

Fig. 3. Temperature sweep of Na_3PO_4/chitosan systems at different ratio ($a = 0.5$, $b = 1.25$ and $c = 2$) and pHs (5.0, 6.0 and 7.0) ((●) 1 day, (●) 3 days, (○) 7 days).

differences could be observed among the three different systems at t_0, t_{10} and t_{15}. The aspect of the t_{10} sample highlighted an important polymer solvation, with initial formation of gel-like zones surrounded by a more homogeneous phase. After cooling (t_{15}), the system looked less homogeneous, with a more pronounced gel-like character, confirming the thermogelling properties of this sample.

On the contrary, the visual appearance of the pH 6.0 system indicated gradual polymer solvation until formation of a homogenous system.

4. Discussion

Our work confirmed previously reported data on behaviours related to the addition of inorganic phosphate salts or pH values. First of all, the phosphate gradually avoids aggregation phenomena of chitosan, facilitating the solvation process of the polymer at a pH higher than 6.0.

A previous work (Ta et al., 2009) compared different potassium phosphate salts (KH_2PO_4, K_2HPO_4 and K_3PO_4) at different molar ratios versus chitosan, but the authors did not compare the different salts or chitosan/phosphate ratios at the same pH value. Thus, only the K_2HPO_4 was able to gellify the chitosan, being the only dispersion at pH 7.1/7.6.

In the same direction, Li et al. (2010, 2011), using the disodium phosphate salt (Na_2HPO_4) as gelling agent, reported its use only at pH close to neutrality, without analysing the chitosan/phosphate system behaviour at different pHs.

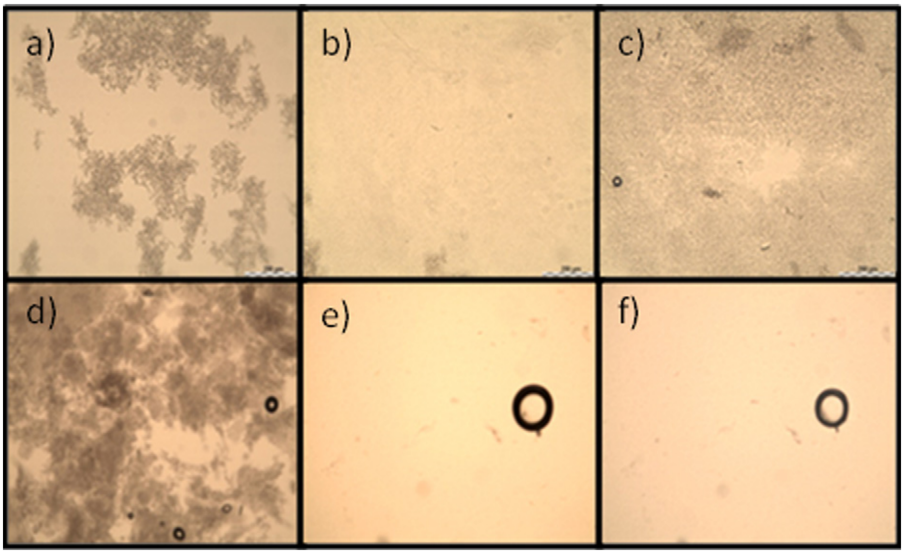

Fig. 4. Optical microscope images of chitosan/NaH_2PO_4 systems at ratio 2 and pH 7.0 (a: $t_{0\,\mathrm{min}}$ at RT; b: $t_{10\,\mathrm{min}}$ at 70 °C; c: $t_{15\,\mathrm{min}}$ at RT) and pH 6.0 (d: $t_{0\,\mathrm{min}}$ at RT; e: $t_{10\,\mathrm{min}}$ at 70 °C; f: $t_{15\,\mathrm{min}}$ at RT).

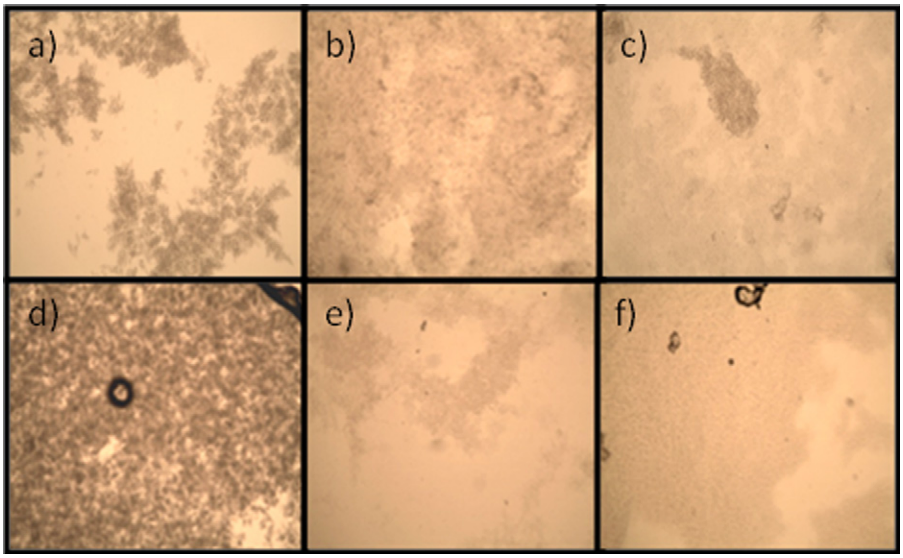

Fig. 5. Optical microscope images of chitosan/Na_3PO_4 systems at ratio 2 and pH 7.0 (a: $t_{0\,min}$ at RT; b: $t_{10\,min}$ at 70 °C; c: $t_{15\,min}$ at RT) and pH 6.0 (d: $t_{0\,min}$ at RT; e: $t_{10\,min}$ at 70 °C; f: $t_{15\,in}$ at RT).

Our results explain why only at pH 7.0 a pseudo thermo gelling system was achieved. Furthermore, it is evident that not only the pH but also the salt/chitosan ratio are fundamental for the thermogelation process.

Probably, different molecular mechanisms (e.g. hydrogen-bonding, electrostatic and hydrophobic interactions), are involved in the rheological behaviour of these kinds of systems. The importance of these macromolecule interactions was also evident from the optical microscopy analyses, which highlighted a gradual polymer solvation with consequent formation of gel-like regions.The results obtained with this technique suggested the role of temperature and pH in aiding polymer solvation (systems are transparent at pH 6.0) and gelation (pH 7.0). In fact, particularly at pH 7.0 and at the ratio 2 of phosphate/chitosan and in presence of both salts, systems always showed increased polymer solvation after heating, and even the formation of a gel like precipitate (at $t_{15\,min}$).As for the mechanism proposed for the chitosan/β-GP system (Chenite et al., 2000, 2001), after an initial solvation, the system required reduced chitosan chain polarity and increased hydrophobicity upon heating, together with thermally induced transfer of protons from chitosan amine groups to the phosphate moieties.

Regarding the type of salt, there were no relevant differences on the thermogelling ability of the two selected phosphates. This fact pointed out that pH and phosphate/chitosan ratio remained the two key factors involved in the gelling phenomenon.

5. Conclusions

This work confirmed a clear interdependence of temperature, pH, and phosphate concentration on the chitosan dispersion gelling properties. Particularly, it highlighted how previous works have in fact overlooked some key issues in analysing the role of the type of salt used as well as the fundamental importance of pH and chitosan/salt ratio, giving rise to often questionable results.

The final pH value of the prepared chitosan/inorganic phosphate systems strongly affected the behaviour of rheological samples, conferring pseudo thermogelling properties. Thus, in line with the literature, our data confirm that a pH value close to 7.0 is of primary importance. Moreover, the ratio between the chitosan and the salts, more than the type of inorganic phosphate, is key in determining the equilibrium state between the global positive charge of the polysaccharide and the negative charge of the salt.

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