

# Time-domain NMR relaxometry as an alternative method for analysis of chitosan-paramagnetic ion interactions in solution



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## ARTICLE INFO

### Article history:

Received 22 November 2016

Received in revised form 4 January 2017

Accepted 18 January 2017

Available online 21 January 2017

### Keywords:

Relaxometry

Chitosan

Complexation

Paramagnetic ions

NMR

## ABSTRACT

Chitosan (CHI)-paramagnetic ion interactions in aqueous solution as affected by pH were studied using time-domain NMR (TD-NMR) relaxometry. Longitudinal ( $T_1$ ) and transverse ( $T_2$ ) relaxation times of CHI solutions with  $Fe^{3+}$ ,  $Cu^{2+}$ , and  $Mn^{2+}$  were measured using a single-shot pulse sequence named CP-CWFP<sub>x-x</sub>. The results indicate that CHI interacted with  $Fe^{3+}$  ions or iron oxide nanoparticles, maintaining the metal ion in solution at pH ranging from 3 to 5. Above this pH, CHI coagulated and removed Fe ions or nanoparticles, resulting in a clear supernatant. CHI probably interacted with Cu ions at pH 6.2 through the deprotonated CHI-NH<sub>2</sub> groups maintaining the relaxation effect in alkaline solution, whereas  $Mn^{2+}$  did not interact with CHI strongly enough to produce a complex of high stability. The proposed method can also be further exploited in the study of interactions paramagnetic ions with others CHI and CHI derivatives as well as of other water-soluble polysaccharides.

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## 1. Introduction

Due to their polyfunctional metal-binding properties, chitosan (CHI) and CHI derivatives have been widely studied as ligands for the recovery, separation, and concentration of metal ions as well as for the fabrication of materials intended for optical, catalytic, sorption, and biological applications [1,2]. Therefore, knowing the precise mechanisms involved in CHI-metal ion interactions and in the stability of such complexes in solution is of outmost importance to understand these applications [3].

Although CHI-metal ion interactions in solution have been widely studied, the precise mechanisms participating in this process remain still unclear [4,5]. The most accepted mechanisms involve adsorption [6], coagulation/flocculation [7], and chelation [3,8]. Experimental and theoretical [9] studies have indicated that one (pedant model) or more (bridge model) amino groups are comprised in the binding process [3,4].

CHI-metal binding properties and stability in solution have been investigated using several analytical methods [10], including UV-vis spectrophotometry [11], atomic absorption spectrometry [12,13], zeta potential [13], potentiometric titration [1] and inductively couple plasma-optical emission spectroscopy [14].

To the best of our knowledge, no studies involving CHI-metal ions have been performed using low-field, time-domain nuclear magnetic resonance (TD-NMR) relaxometry. We have recently demonstrated that TD-NMR relaxometry can be a simple, rapid method to measure CHI coagulation [15] and solubility product constant [16] as well as to monitor the complexation of paramagnetic ions in solution [17], even in cases where the equipment has instrumental limitation, as long dead time [18]. All these relaxometric studies have been performed as functions of solution pH.

In this paper, we report the use of TD-NMR relaxometry as an alternative method to study CHI-paramagnetic ion interactions and their stability in solution as functions of pH. The method proposed here has measured the influence of water longitudinal ( $T_1$ ) and transverse ( $T_2$ ) relaxation times, depend upon CHI-paramagnetic ion interactions. These two relaxation times have been measured simultaneously using a new continuous wave free precession pulse sequence, named CP-CWFP<sub>x-x</sub> [19].

## 2. Experimental

### 2.1. Reagents and sample preparation

CHI was purchased from SIGMA-ALDRICH. The average viscosimetric molecular weight (MW) was determined dissolving the polymer in 0.3 mol L<sup>-1</sup> acetic acid/0.2 mol L<sup>-1</sup> sodium acetate buffer (pH 4.5). The AVS-360 viscometer and the AVS-20 automatic

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burette, both from Schott-Geräte, were used for the viscosity measurements. A glass capillary ( $\phi = 0.53$  mm) containing 15 mL of the polymer solution was immersed in a water bath maintained at  $25 \text{ }^\circ\text{C} \pm 0.01 \text{ }^\circ\text{C}$  and previously programmed volumes of the solvent, were sequentially added to provide the desired dilution. The intrinsic viscosity ( $\eta$ ) was determined from the extrapolation of the plot  $\eta_{sp}/C$  versus  $C$  to infinite dilution, where  $\eta_{sp}$  is the specific viscosity and  $C$  is the concentration for the polysaccharide solution. The estimated value ( $1.75 \times 10^5 \text{ g mol}^{-1}$ ) for the molecular weight (MW) was determined from the corresponding intrinsic viscosities ( $[\eta] = 716 \text{ mL g}^{-1}$ ) and using the Mark–Houwink–Sakurada parameters ( $K = 0.074$  and  $\alpha = 0.76$ ) in agreement to polymer nature, solvent and temperature, in consonance with [20,21].

The degree of deacetylation (DD) was estimated from  $^1\text{H}$  NMR measurements [21,22]. For these experiments, an appropriate weight of CHI was dissolved in 1% (v/v) HCl/D<sub>2</sub>O solution, performing a concentration of 10 mg/mL upon constant magnetic stirring at room temperature for 24 h to result in a clear solution. The DD (75.50%) was determined from the corresponding  $^1\text{H}$  NMR spectra, considering the intensity of the signals due to methyl hydrogens from acetamido groups ( $\approx 2.0$  ppm) and due to H<sub>2</sub>–H<sub>6</sub> (4.1–3.4 ppm) [22]. The  $^1\text{H}$  NMR spectra were acquired at  $80 \text{ }^\circ\text{C}$  using a Bruker AVANCE II spectrometer (600.00 MHz for  $^1\text{H}$ ), without spinning and employing 3-(Trimethylsilyl) propionic-2,2,3,3-d<sub>4</sub> acid sodium salt (TMSP) as external reference.

For the experiments about the interaction between CHI and paramagnetic ions, CHI solution ( $1.5 \text{ g L}^{-1}$ ) was prepared dissolving undergoing stirring for 48 h, an appropriate amount of this polysaccharide in acetic acid  $0.60 \text{ mol L}^{-1}$  (pH 2.42) at room temperature. For the CHI-paramagnetic ion solutions, we employed CuSO<sub>4</sub>·5H<sub>2</sub>O (SIGMA-ALDRICH, 98%), MnSO<sub>4</sub>·5H<sub>2</sub>O (Synth, 98%), and FeCl<sub>3</sub>·6H<sub>2</sub>O (Mallinckrodt Chemical, 97%). The concentrations of these solutions were 0.82 g/L, 0.79 g/L and 0.89 g/L, respectively. To carry out the measurements in the absence of precipitate, samples were centrifuged at 10,000 rpm for 3 min.

## 2.2. Methods

### 2.2.1. pH measurements

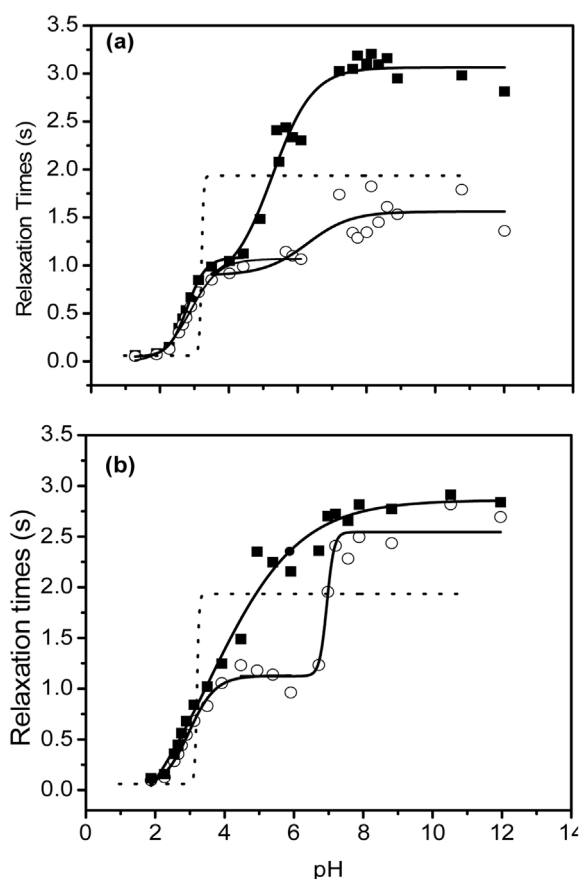
Sample pH was measured in a pH meter, model PG1800 (GEHAKA), that was calibrated prior to each run using buffer solutions. For this, Falcon tubes were filled with 5.00 mL of sample. Once pH had been adjusted for the desired value with  $1.0 \text{ mol L}^{-1}$  NaOH and HCl solutions, the tube volume was completed to 10.00 mL, to reach ion-CHI final concentrations of  $3.30 \times 10^{-3}$  and  $6.60 \times 10^{-3} \text{ mol L}^{-1}$ . Then, pH was checked again.

### 2.2.2. TD-NMR measurements

The  $T_1$  and  $T_2$  relaxation times were measured in a benchtop time-domain NMR spectrometer, Minispec, model BRUKER mq-20 (Germany) equipped with a 0.47-T (20.00 MHz for  $^1\text{H}$ ) permanent magnet as well as a 32.00-mm-diameter probe. The minispec software was used for data acquisition. All samples were placed into a temperature-controlled spectrometer at  $28.0 \pm 0.1 \text{ }^\circ\text{C}$ .

For the TD-NMR measurements, a rapid pulse sequence named Carr-Purcell continuous wave free precession with phase alternance (CP-CWFP<sub>x-x</sub>) was employed. This sequence is able to simultaneously determine both the longitudinal ( $T_1$ ) and transverse ( $T_2$ ) relaxation times [19].

The values were obtained by applying a sequence with the following parameters: a train of 2.34- $\mu\text{s}$ -long  $90^\circ$  pulses, a time interval between pulses ( $t_p$ ) of 300  $\mu\text{s}$ , a recycle delay of 5 s, 4 scans, and 45,000 echoes. In each measurement, 500  $\mu\text{L}$  of each solution was used.  $T_1$  and  $T_2$  were obtained by the intensity of the NMR signal after the first pulse ( $M_0$ ) and in the steady state ( $M_{ss}$ ), whereas the time constant  $T^*$  was obtained by the monoex-



**Fig. 1.**  $T_1$  (■) and  $T_2$  (○) relaxometric profiles of chitosan (CHI)-ion samples and supernatant, obtained with CP-CWFP<sub>x-x</sub> pulse sequence. a) CHI-Fe<sup>3+</sup> and its respective supernatant. b) Solid and dotted lines are the sigmoidal fitting of experimental data acquired from CHI-Fe<sup>3+</sup> and Fe<sup>3+</sup> aqueous solutions, respectively.

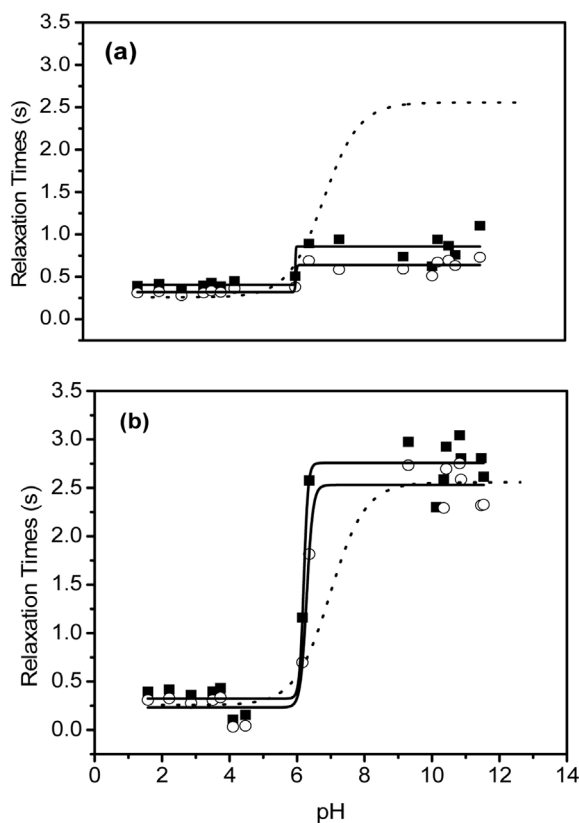
ponential fitting of signal decay [18]. Fittings were performed in the Origin<sup>®</sup> software, version 9.0. All data obtained as affected by pH were fitted by a Boltzmann sigmoidal curve and corresponded to the average of three measurements. The uncertainty of relaxometric measurements was estimated at  $\pm 10$  ms considering the standard deviations of triplicate runs.

## 3. Results and discussion

The interactions between CHI and the paramagnetic ions Fe<sup>3+</sup>, Cu<sup>2+</sup>, and Mn<sup>2+</sup> were studied as functions of pH using TD-NMR relaxometry. These paramagnetic ions were chosen because they precipitate as hydroxides in aqueous solutions at pH values 3.2, 7.0, and 9.3, respectively [16]. Therefore, their interactions with CHI may be studied in a broad pH range. The CHI concentration ( $1.5 \text{ g L}^{-1}$ ) was selected because it is within the ideal range for TD-NMR relaxometry runs [15].

Figs. 1a, 2a, and 3a show the  $T_1$  (■) and  $T_2$  (○) relaxometric profiles of samples comprising CHI and Fe<sup>3+</sup>, Cu<sup>2+</sup>, and Mn<sup>2+</sup> as functions of pH. Figs. 1b, 2b, and 3b present the  $T_1$  and  $T_2$  relaxometric profiles for the same samples, but after being centrifuged. The analyzed portion was the supernatant. Solid lines indicate the sigmoidal fitting of the experimental data for the CHI-ion samples. Dotted lines correspond to the fitting of the respective hydroxide in agreement with Cobra et al. [16]. The experimental data for each ion were not plotted in order to simplify the figure.

These images show the strong effect of pH in the relaxation profiles of aqueous solution comprising CHI and paramagnetic ions. Such relaxation profiles were remarkably different from those



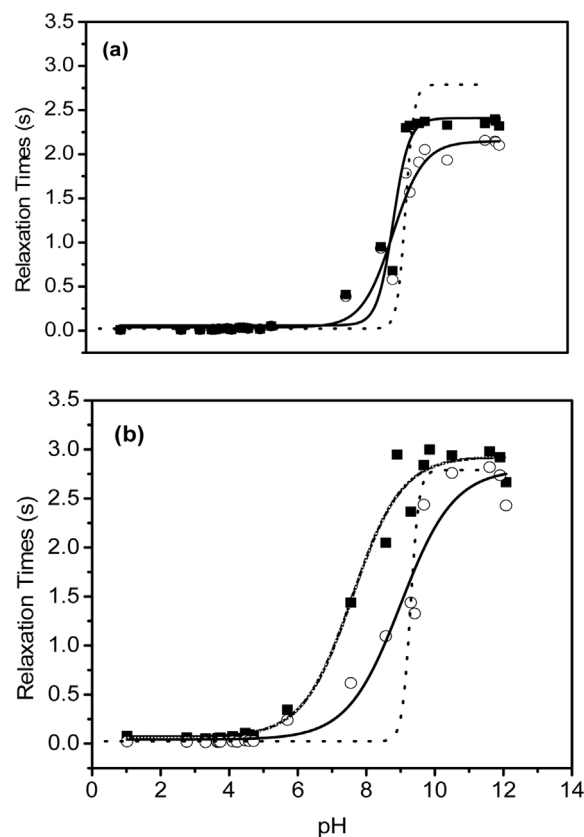
**Fig. 2.** T<sub>1</sub> (■) and T<sub>2</sub> (○) relaxometric profiles of chitosan (CHI)-ion samples and supernatant, obtained with CP-CWFP<sub>x-x</sub> pulse sequence. a) CHI-Cu<sup>2+</sup> and its respective supernatant. b) Solid and dotted lines are the sigmoidal fitting of experimental data acquired from CHI-Cu<sup>2+</sup> and Cu<sup>2+</sup> aqueous solutions, respectively.

observed for the same CHI type and concentration in aqueous solution, but in the absence of paramagnetic ions [15]. T<sub>1</sub> and T<sub>2</sub> values of CHI aqueous solution showed two plateaus, from pH 1 to 7 and pH 7 to 10, with the transitions at approximately pH 7 being related to coagulation [15]. T<sub>1</sub> values in the two plateaus were 2.80 and 3.15 s, respectively. T<sub>2</sub> values in the two plateaus, from pH 1 to 6 and pH above 8, were equal to 2.50 and 1.20 s, respectively. Therefore, coagulation played an opposite role in relaxation time, *i.e.*, it increased T<sub>1</sub> while reduced T<sub>2</sub> [15].

On the other hand, the relaxation curves of Figs. 1–3 showed trends similar to those observed using the same paramagnetic ion in aqueous solution (dotted lines). In aqueous solution, both relaxation times increased with pH at approximately 3, 7, and 9, for Fe<sup>3+</sup>, Cu<sup>2+</sup>, and Mn<sup>2+</sup>, respectively [16].

The major differences in the relaxations between CHI-ions and the respective ion in aqueous solution were observed with Fe<sup>3+</sup> (Fig. 1a). The relaxation profiles of CHI-Fe<sup>3+</sup> solutions showed two turning points, at pH 2.9 and 6.2. At pH values lower and higher than the first turning point, the relaxation times were 0.05 and 1.00 s, respectively. However, T<sub>1</sub> stayed at 1 s up to pH 4 whereas T<sub>2</sub> remained constant until pH 6. T<sub>1</sub> increased again from pH 4 to 7 and reached a plateau at pH 2.8 s. T<sub>2</sub> also increased from pH 6 to 8 as well as reached a plateau at 1.5 s.

When the CHI-Fe<sup>3+</sup> solutions (Fig. 1a) were centrifuged, T<sub>1</sub> (■) profile showed a single turning point at pH 3.5. T<sub>1</sub> values progressively increased from 0.11 (pH 2) to 2.8 s (pH 8–12). On the other hand, T<sub>2</sub> (○) profile presented two turning points at pH 2.9 and 6.9. T<sub>2</sub> profile of the supernatant (Fig. 1b) was similar to that of the same sample prior to centrifugation (Fig. 1a). However, the first plateau at 1.2 s, at pH values between 4 and 6.5, was more defined in the T<sub>2</sub> profile of the supernatant than in that of the solution. T<sub>2</sub> profile



**Fig. 3.** T<sub>1</sub> (■) and T<sub>2</sub> (○) relaxometric profiles of CHI-ion samples and supernatant, obtained with CP-CWFP<sub>x-x</sub> pulse sequence. a) CHI-Mn<sup>2+</sup> and its respective supernatant. b) Solid and dotted lines are the sigmoidal fitting of experimental data acquired from CHI-Mn<sup>2+</sup> and Mn<sup>2+</sup> aqueous solutions, respectively.

also showed a sharp increase between pH 6.7 (1.3 s) and 7.5 (2.5 s) and then stayed almost constant until pH 12. The T<sub>2</sub> value in this plateau was higher than those observed in CHI-ion (Fig. 1a) and aqueous ion solutions (dotted line).

Samples having pH within this range showed the same color changes and precipitations. At lower pH values, the solution was pale yellow and color intensity increased up to approximately pH 4.7. From pH 4.9 to pH 7, precipitation took place but the supernatant remained yellow. Above pH 7, the supernatant became colorless.

The first turning point at ca. pH 3 observed in the T<sub>1</sub> profile of Fig. 1a and T<sub>2</sub> profiles of Fig. 1a and b was remarkably close to that observed in CHI-free aqueous solution (pH 3.2, dotted line). This indicates that such turning was associated to the formation of insoluble iron hydroxides. However, no precipitation was observed in this pH range. Therefore, the relaxation profiles, color enhancement, and absence of precipitate indicate that some interaction occurred among CHI and Fe<sup>3+</sup>. The CHI-OH groups must be involved in such interaction because amino groups are protonated at low pH values. This result agrees with other measurements performed elsewhere [1]. Therefore, the increase in relaxation times up to pH 4.9 can be related to the reduction of water accessibility to Fe<sup>3+</sup> – which is partially protected by CHI – or to the formation of tiny FeOOH nanoparticles in the order of 5–10 nm – covered by a loose layer of adsorbed CHI, as proposed by Sipos et al. [2].

The second turning points observed in T<sub>1</sub> and T<sub>2</sub> profiles of Fig. 1a and in T<sub>2</sub> profile of Fig. 1b were similar to that of the solution containing only CHI (pH ≅ 7) [15]. These results suggest that such turning point occurred because of CHI coagulation/flocculation from the bulk. This hypothesis may be confirmed experimentally

through the precipitation that occurred from pH 4.9 to pH 7. Additionally, the low  $T_2$  values ( $\cong 1.50$  s) observed in Fig. 1a might be attributed to presence of free  $\text{Fe}^{3+}$  species in the bulk after complexation with CHI, which is consistent with the yellow color observed in the supernatant within this pH range. Furthermore, the removal of  $\text{Fe}^{3+}$  species through CHI coagulation (pH 7) eliminated the yellow color of the supernatant and increased the relaxation times towards values close to the pure solvent ( $T_1 \cong T_2 \cong 3.0$  s) (Fig. 1b). Therefore, it is possible to one may infer that deprotonation of CHI-NH<sub>2</sub> groups occurred in this region. These groups are able to complex such ions as well as improve complexation efficiency, which justifies the increased relaxation times. These results are in accordance with the models proposed by Hernández et al. [1], who suggested the participation of deprotonated amino groups on  $\text{Fe}^{3+}$  complexation within this pH range, with additional water molecules or chloride ions completing the coordination sphere for such ion.

Conversely, the relaxation profiles of CHI aqueous solution in the presence of  $\text{Cu}^{2+}$  species (Fig. 2a) showed two plateaus from low to high  $T_1$  and  $T_2$  values with a transition at pH 6.2, which was ca. 0.8 lower than the turning point observed for  $\text{Cu}^{2+}$  in aqueous solution (dotted line). For CHI- $\text{Cu}^{2+}$  and its respective supernatant (Fig. 2b), relaxation times were close to 0.46 ( $T_1$ ) and 0.3 s ( $T_2$ ) up to pH 6. Above the turning point of CHI- $\text{Cu}^{2+}$  (Fig. 2a),  $T_1$  and  $T_2$  values reached a plateau at 1.00 and 0.50 s, respectively. On the other hand, relaxation times for the supernatant were higher ( $T_1 = 2.96$  and  $T_2 = 2.60$  s), as observed in Fig. 2b. These were also similar to the relaxation times of pure water and of CHI-free  $\text{Cu}^{2+}$  solution (dotted lines).

When the effects of  $\text{Fe}^{3+}$  (Fig. 1a) and  $\text{Cu}^{2+}$  (Fig. 2a) on CHI solutions are compared, it is observed that, after the turning point, the copper sample presented lower relaxation times than those of  $\text{Fe}^{3+}$  and  $\text{Cu}^{2+}$  aqueous solution (dotted line). Therefore,  $\text{Cu}^{2+}$  still affected water relaxation times in the presence of CHI at pH values higher than 7. This result suggests that a CHI- $\text{Cu}^{2+}$  complex was formed and that there were water molecules directly coordinated to metal ions. This hypothesis agrees with Modrzejewska [23] and Pestov and Bratskaya [3], who proposed that interactions among CHI-NH<sub>2</sub> groups and  $\text{Cu}^{2+}$  occur in two arrangements – known as pedant and bridge models – containing water molecules directly bonded to copper, which justifies the low relaxation times observed in Fig. 2a.

For the supernatant (Fig. 2b), the major difference occurred above the turning point, indicating that relaxation times were close to those of pure solvent as well as that such CHI- $\text{Cu}^{2+}$  complex was completely removed by an alkaline solution. This result is in agreement with experimental observations, wherein increased amounts of gelatinous blue precipitate as well as the presence of colorless supernatant were noticed. Similar results were reported by Cobra et al. [16]. According to these authors, metallic species precipitated as hydroxides at high pH values, reaching relaxation times close to those of pure solvents. Taking this observation into account, one may infer that, for a system containing the same ions but in the presence of CHI, high hydroxyl contents can hydrolyze the complex and precipitate it, increasing  $T_1$  and  $T_2$  values.

The relaxation profiles of CHI- $\text{Mn}^{2+}$  sample (Fig. 3a) and its respective supernatant (Fig. 3b) exhibited low relaxation times (0.06 s for  $T_1$  and 0.02 s for  $T_2$ ) before the turning points. These values were similar to those observed in a  $\text{Mn}^{2+}$  aqueous solution [16]. These low values are attributed to the high relaxivity of  $\text{Mn}^{2+}$  when compared to  $\text{Cu}^{2+}$  and  $\text{Fe}^{3+}$  [16]. The relaxation times in the presence of CHI showed a turning point at pH 8.90. This value was almost 0.4 lower than that of  $\text{Mn}^{2+}$  in water. Above the turning points of CHI- $\text{Mn}^{2+}$  solution,  $T_1$  was longer than  $T_2$ , but smaller than the  $T_1 = T_2$  (dotted line) observed in aqueous solution.  $T_1$  values of the supernatant (Fig. 3b) showed a lower turning point (pH

7.6) than those of  $T_2$  (pH 9.0) as well as  $T_1$  and  $T_2$  of CHI- $\text{Mn}^{2+}$  (Fig. 3a). Another difference between CHI- $\text{Mn}^{2+}$  and its respective supernatant is the pH at which  $T_1$  begins to increase. In the supernatant, the variation started at pH values lower than 6 (Fig. 3b), whereas in the CHI- $\text{Mn}^{2+}$  sample (Fig. 3a) it started at pH 7.

CHI- $\text{Mn}^{2+}$  had a minor effect on water relaxation times when compared to the values measured in an aqueous solution of the same ions. Most of the observed differences could be explained by CHI coagulation at pH higher than 6. This process removes  $\text{Mn}^{2+}$  by flocculation, which justifies the similarity between the obtained relaxometric profiles. These results indicate that the CHI- $\text{Mn}^{2+}$  complex had a low stability constant and that these species are mostly removed by the action of pH. This hypothesis is in line with results reported elsewhere [24,25] and may be corroborated experimentally through the emergence of a gelatinous, dark brown precipitate upon increased alkalinity. This is attributed to  $\text{Mn}(\text{OH})_2$  and CHI flocculation.

Finally, the high relaxation times ( $\cong 2.70$  s) of supernatant samples (Figs. 1b, 2b, and 3b) demonstrated the high efficiency of CHI in cleaning the solution by removing  $\text{Fe}^{3+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Mn}^{2+}$  ions. This observation agrees well with data previously reported in the literature [1,23–25]. It also corroborates the ability of TD-NMR relaxometry in monitoring chitosan-paramagnetic ion interactions in solution.

#### 4. Conclusions

Based on the results presented here, one may conclude that simultaneous measurements of  $T_1$  and  $T_2$  relaxation times by CP-CWFP<sub>x-x</sub> pulse sequence in a benchtop TD-NMR relaxometer stands out as a fast, efficient method to study CHI-paramagnetic ion interactions in aqueous solution. The relaxation profiles of CHI- $\text{Fe}^{3+}$  aqueous solutions demonstrated that CHI interaction with these ions are affected by pH. At pH values lower than 5, CHI interacted with  $\text{Fe}^{3+}$  ions or iron oxide nanoparticles and maintained the metal ion in solution. At pH values higher than 7, CHI coagulated and removed Fe ions or nanoparticles, resulting in a clear supernatant. On the other hand, CHI- $\text{Cu}^{2+}$  relaxation profiles indicated the direct formation of a stable complex with water molecules directly linked to the paramagnetic ion. As for CHI- $\text{Mn}^{2+}$ , the results suggested the formation of an unstable complex in solution, which was removed through coagulation at alkaline pH. Although the measurements were performed with  $\text{Fe}^{3+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Mn}^{2+}$  here, these can also be used to study CHI interactions with other paramagnetic ions, including  $\text{Cr}^{3+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Mo}^{2+}$ , and  $\text{V}^{3+}$  ions that are dangerous pollutants. Furthermore, TD-NMR relaxometry can be used to study interactions among paramagnetic ions and others CHI molecules of different molecular weights, acetylation degrees, and derivatives as well as between other water-soluble polysaccharides and paramagnetic ions.

#### Acknowledgements

The authors would like to thank the Brazilian agencies FAPESP (grants 2012/23169-8 and 2014/22126-9) and CNPq (grants 303837/2013-6 and 403075/2013-0), for their financial support.

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