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*Article*

# Thermosensitive Chitosan Hydrogels as a Vehicle for Iron Dextran as a Potential Strategy for Parenteral Nutrient Supplementation

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**Abstract:** Iron deficiency anemia (IDA) is a world health problem for humans and other mammals; affecting critical stages of development. Pigs have been used as an experimental model for the study and prevention of anemia because of physiological and metabolic similarities with humans. Iron dextran particles (IDP) are used for intramuscular (IM) IDA treatments in pigs, but are insufficient for prevention of anemia due to quick metabolization. Therefore, the objective of this study was to develop chitosan thermosensitive hydrogels (CTH) formulations and to study their potential as a mammalian parenteral iron dextran supplementation strategy. CTH were loaded with IDP at increasing iron concentrations (0.1, 0.2 and 0.4 g of theorical iron/g of chitosan) and characterized as an IM iron supplement. All the CTH-IDP formulations were thermosensitive and can be injected IM at ~4 °C, with a significant rise in viscosity between 25-37 °C. IDP content was physically trapped in the chitosan hydrophobic gel at 37 °C, without evidence of chemical bonding. We conclude that CTHs are a promising strategy for IM delivery strategy of IDP, and these results may be useful for future iron supplementation studies in pigs, humans and other mammals.

**Keywords:** chitosan; thermosensitive hydrogel; iron deficiency; pig

## 1. Introduction

Iron deficiency anemia (IDA) is the leading nutritional deficiency in the world, affecting one-third of the global population, especially during critical developmental stages, such as childhood, pregnancy, and lactation [1,2]. This deficiency is caused by the high demand for iron during these stages and the low intake of iron in forms of higher bioavailability, such as those found in animal-sourced foods, which are expensive [3]. Multiple iron supplementation alternatives have been studied in humans and other mammalians to try and prevent anemia, with unsatisfactory results. In fact, iron deficiency has increased in the world. The use of low-bioavailability sources of non-heme iron (such as ferrous sulfate) in oral supplementation strategies and food fortification is the reason strategies have failed. Non-heme iron also causes adverse side effects like gastrointestinal disorders and have unpleasant sensory properties [2,4,5].

In humans, there are few options for parenteral iron supplementation formulations, mainly sodium ferric gluconate and intravenous (IV) iron sucrose. However, their use is not common due to the invasive nature of the procedure, risk of anaphylaxis and high cost, as these procedures require

hospitalization of the patient [6]. IV supplementation is highly effective in iron supplementation, achieving a more efficient increase in hemoglobin compared to oral supplementation mainly due to its high bioavailability. However, there is risk of infections, anaphylactic reactions, endothelial damage, oxidative stress and hepcidin overexpression [7].

In pigs, IM supplementation of 150-200 mg of a commercial formulation of iron dextran (CIDF) in a single dose is used for the prevention of iron deficiency anemia [8], as the condition is highly prevalent in pigs raised in an intensive production system [9,10]. Pigs are considered a relevant and valid animal model to investigate iron deficiency and supplementation due to the physiological and metabolic similarities they share with humans and other omnivorous mammals [11,12]. CIDF is an aqueous dispersion of iron dextran particles (IDP) with a ferric hydroxide core covered by a dextran shell, which imparts high hydrophilicity, low reactivity, and a nanometric particle size of ~ 11.5 nm [13]. When CIDF is injected into the muscular tissue, IDP are rapidly dispersed across the surrounding surface through simple diffusion mechanisms within muscle fibers and transported by the lymphatic system into the bloodstream, captured by macrophages, which are responsible for extracting iron and binding it to transferrin for transport to the site of utilization or storage [8]. The preventive administration of CIDF to piglets has been employed for over 70 years. However, it exhibits several disadvantages linked to the rapid metabolism and high amount of injected iron, because a high iron load leads to a substantial increase in blood iron concentration within the first 10 hours post-injection [14]. This triggers the overexpression of hepcidin, a hormone that promotes iron efflux from the body and reduces iron absorption, resulting in inefficient utilization of the supplemented iron [15,16]. Furthermore, the accumulation of such a substantial quantity of iron in storage sites induces toxic effects on adjacent tissues due to its high reactivity potential [16,17].

To mitigate these adverse effects and enhance the effectiveness of injected IDP, the use of chitosan thermosensitive hydrogels (CTH) is proposed as a vehicle for sustained release of IDP. Chitosan is a biocompatible and biodegradable biopolymer that has been widely utilized to develop sustained release systems in various formats, including IM supplementation [18]. CTH offer the advantage of being injected as a liquid (sol state) at room temperature (~ 4-25 °C) but then has a significant increase in viscosity, achieving a gel state when in contact with muscular tissue at ~ 36-37 °C [19]. Several studies have demonstrated the various benefits of CTH for controlled and/or prolonged release of active agents, which enhances drug retention *in situ*, thereby extends the duration of release from the injection site [20,21].

Various molecules have been used to achieve thermosensitive behavior with chitosan and this study focuses on the use of glycerophosphate (GP) due to its low toxicity and favorable outcomes [18]. At room temperature (~ 4-25 °C), GP functions as an intermediary agent in the chitosan-GP-water system, enabling the maintenance of the sol state (with chitosan suspended) when the formulation is at a neutral pH. This interaction prevents chitosan from losing its cationic potential and settling due to its non-interaction with water [18]. However, as the temperature of the formulation is raised to approximately 30 °C, the electrostatic chitosan-GP binding is disrupted, leading to chitosan-chitosan union through hydrogen bonding, resulting in the formation of a highly viscous hydrophobic three-dimensional network that impedes the outflow of content from the injection site, achieving the gel state [18].

For these reasons, it is proposed to develop and study CTH-IDP formulations that maintain the sol state at room temperature (~ 4-25 °C) and undergo a transition to the gel state at a temperature close to the intramuscular (IM) temperature in mammals, such as humans and pigs (~ 37 °C). The loading and entrapment of IDP for prolonged iron supplementation through CTH will be investigated as a nanometric and hydrophilic active agent and the outcomes of this study may serve as a model for the development of release studies involving IDP or similar active agents. Therefore, the objective of this study was to develop CTH-IDP formulations and to study their potential as a mammalian parenteral iron dextran supplementation strategy.

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## 2. Materials and Methods

### *2.1. Materials*

Iron dextran particles (IDP) dispersed from a commercial iron dextran formulation (CIDF, 20 % w/v of iron) were used as the iron source and were obtained from the same manufacturing batch (Veterquímica S.A., Chile). For the development of chitosan thermosensitive hydrogels (CTH), chitosan derived from crab shell (300-350 KDa) with a degree of deacetylation >80 % (Sigma-Aldrich, USA) and hydrated disodium glycerophosphate (GP, Sigma-Aldrich, USA) were used. All other reagents were of analytical grade and procured from Merck S.A.

### *2.2. Preparation of CTHs*

The CTHs were developed following the procedure described by Sun *et al*. [22] with some modifications. A 0.2 % v/v acetic acid solution in Milli-Q water at 4 °C was prepared, and 1 % w/v chitosan was added, which was maintained at 4 °C for 12 hours until a transparent, viscous solution with a pH of 5-5.5 was obtained. Simultaneously, GP was prepared at 50 % w/v in Milli-Q water and added to the chitosan solution at a rate of 0.2 mL/min, while constantly monitoring the pH until it reached a neutral pH value (7 ± 0.5) through continuous magnetic stirring at 4 °C, resulting in a final GP concentration of ~6-8 % v/v. In this manner, the CTHs were obtained and refrigerated until use.

After the CTH was formed, IDP were added directly as CIDF, using a syringe at a rate of 1 mL/min and continued to be homogenized using a paddle stirrer (OS40-PRO, D-LAB, China) at 1000 rpm for 1 hour. Thus, three formulations with increasing iron concentrations (0.1, 0.2, and 0.4 g of theoretical iron/g of chitosan) were developed, referred to as CTH0.1, CTH0.2, and CTH0.4, respectively (CTH0, without iron, is the control).

### *2.3. CIDF Characterization*

CIDF was characterized in terms of pH (AD1020, Adwa, Hungary), particle size using dynamic light scattering (DLS, Zetasizer Nano-ZS90, Malvern Instruments, United Kingdom), Zeta potential by laser Doppler anemometry (Zetasizer Nano-ZS90, Malvern Instruments, United Kingdom), and viscosity using a rotational viscometer with a N° 1 needle (NDJ-8S, Nirun, China).

### *2.4. CTH Characterization*

#### 2.4.1. Macroscopic Appearance

Digital images were obtained for macroscopic evaluation of the CTH0, CTH0.1, CTH0.2, and CTH0.4 formulations. The images were captured with the CTH at room temperature (~22 °C) in test tubes from a focal point at 10 cm, focusing on the lower section of the tubes to visualize precipitates and aggregates.

#### 2.4.2. Electron Microscopy

Images of the CTH0, CTH0.1, CTH0.2, and CTH0.4 formulations in a gel state were obtained using scanning electron microscopy (SEM) to provide a better structural appreciation of the gel-state. 1 mL of each gelled formulation (previously incubated at 37 °C for 30 minutes) were extracted, frozen at -80 °C, and lyophilized for 24 hours in Eppendorf tubes in a lyophilizer (L101, Liotop, Brazil) to dehydrate the samples. Subsequently, the samples were coated with a 10 nm gold film using a Sputter Coater (Cressington model 108, Ted Pella Inc., USA), and microscopic images were captured using a scanning electron microscope (FEI inspect F50, Thermo Fisher Scientific, USA) equipped with an energy dispersive detector (Ultradry Pathfinder Alpine 129 eV, Thermo Fisher Scientific, USA).

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2.4.3. pH

The pH of the CTH0, CTH0.1, CTH0.2, CTH0.4, and CIDF formulations was studied to assess compatibility with muscle tissue. The analysis was conducted on 100 mL samples using a standard pH meter (AD1020, Adwa, Hungary).

2.4.4. IDP Content

The concentration of IDP in the CTH0, CTH0.1, CTH0.2, CTH0.4, and CIDF formulations was measured using a UV spectrophotometer (UV-5100, Metash, China) to measure the IDP content. A calibration curve was developed based on the concentration of IDP at 486 nm, yielding a specific molar extinction coefficient in water of 2.9507 mL/mg·cm (slope of the curve). Based on this, the necessary dilutions were made for each formulation to achieve a theoretical concentration of 1 mg/mL of iron. Results were presented as mg of IDP/mL of formulation.

2.4.5. Viscosity and Sol-Gel Transition Time

To confirm and analyze the thermo-sensitivity of the CTH0, CTH0.1, CTH0.2, and CTH0.4 formulations, the viscosity was determined in 200 mL samples at 4, 25, and 37 °C using a rotational viscometer (NDJ-8S, Nirun, China). All measurements were carried out with a N° 1 needle, and the unit of measurement used was milliPascal-second (mPa·s). The formulations were incubated (BJPX-200B, Biobase, China) for 30 minutes prior to each experiment, which was conducted at room temperature (~ 20 °C) immediately following incubation.

To determine the time necessary for the sol-gel transition of these formulations at 37 °C, the tube inversion method described by Wang *et al*. [23] was used. 5 mL of sample was transferred to a sealed glass test tube (13 mL capacity, 1.5 mm diameter) and kept at 4 °C. Simultaneously, a beaker containing distilled water was incubated at 37 °C. The experiment consisted of submerging three-quarters of the test tube into the beaker and measuring the time it takes for the formulation to reach the gel state. The sample was considered to be in a gel state when, upon rotating the tube 180°, the formulation did not flow. The sol-gel transition was monitored every 30 seconds.

2.4.6. Water-Gel Phase Separation

In order to determine if IDP is entrapped within the CTHs, CTH0.1, CTH0.2, and CTH0.4 formulations were centrifuged in the gel state to induce separation between the hydrophobic gel and the aqueous phase of the formulation, thereby revealing the position of the IDP through macroscopic digital images. Additionally, the same experiment was carried out on the CTH0 and CIDF formulations. 30 mL of the formulations were incubated in Falcon tubes at 37 °C for 30 minutes and then centrifuged at 3000 x g for 30 minutes using a Thermo Scientific Heraeus Megafuge 16R centrifuge (USA). After centrifugation, the macroscopic appearance of the formulations was assessed in the same manner described in the section on macroscopic appearance.

2.4.7. Fourier Transform Infrared Spectroscopy

An analysis of the infrared spectrogram was performed on CTH0, CTH0.1, CTH0.2, and CTH0.4 formulations, as well as the chitosan polymer and CIDF (dried at 50 °C for 48 hours in an oven) to understand the predominant bonds in the formulations/precursor materials and how IDP content influences the bonds. Fourier-transform infrared spectroscopy (FTIR) analysis was conducted using an ATR/FTIR instrument (Interspec 200-X spectrometer, Estonia). The formulations were incubated at 37 °C for 30 minutes before measuring to ensure they were in a gel state. Spectra were obtained by averaging 20 scans in the spectral range of 600-4000 $cm^{-1}$.

2.4.8. Injectability in Pork Meat

The selected formulations (CTH0.4, due to its higher IDP content, and CIDF as a control) were injected *ex vivo* to confirm injectability. Digital images were obtained to evaluate and compare the

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distribution of each formulation after injection. The pork "posta negra" cut according to Chilean typification was used, corresponding to the semitendinosus muscle of the hind limb (usual CIDF IM injection site in pigs), of an approximate size of 5 (width) x 5 (length) x 3 (height) cm3. The meat was purchased from a supermarket, then sized and kept refrigerated, to be used within the next 12 hours. Meat samples were injected to a depth of 1 cm (using a 3 mL plastic syringe attached to a G21 needle, with an internal diameter of 0.60 mm) with 1 mL of each formulation. The injected meat samples were then incubated at 37 °C for approximately 1 hr, to simulate the temperature of a mammalian *in vivo* muscle tissue. The image was captured after injection and incubation from a focal point at 10 cm, focusing on the injection site, which was cut transversely to visualize the distribution of the formulation in the piece of meat.

#### 2.4.9. Statistical Analysis

Characterizations of pH, sol-gel transition time, viscosity, IDP content, particle size, and Zeta potential generated results with continuous and normal data (Shapiro-Wilk test, $p>0.05$). Characterizations were carried out in triplicate. To determine significant differences, ANOVA ($p<0.05$) and the Tukey test ($p<0.05$) were used. Calculations were performed using R software version 4.3.1 (R package, USA). FTIR analysis was conducted through graphical representation for better visualization and comparison. Macroscopic appearance, electron microscopy, water-gel separation, and injectability analyses were completed using the obtained images.

## 3. Results and Discussion

### *3.1. CIDF Characterization*

CIDF was characterized by pH and viscosity; which are properties of interest for understanding the use of CIDF as an IM supplement. The pH was 6.38 ± 0.03, which is considered suitable for IM use in mammals and humans, as these have a physiological pH close to 7. Therefore, CIDF is unlikely to cause pain upon injection due to activation of pH-sensitive receptors [24,25]. The viscosity of CIDF decreased significantly with temperature; 23.8 ± 0.2 mPa-s at 4 °C, 12.9 ± 0.2 mPa-s at 25 °C and 10.2 ± 0.5 mPa-s at 37 °C. This change can be explained by the progressive breaking of hydrogen bonds between the IDP and the water in the formulation as the energy in the system increases due to the effect of temperature. Therefore, when injected, the temperature of the muscle decreases the viscosity of the CIDF, facilitating its dispersion in the adjacent tissues. The viscosity obtained is in line with the values obtained by other authors (10-25 mPa-s) and is considered suitable for extrusion using a needle with a 21-25 G lumen, commonly used in IM injection in pigs, humans and other mammals [26].

The average particle size of IDP in CIDF was 81.9 ± 0.2 nm, which differs from what was described [27], where a particle size of 11.5 nm was obtained using the same method employed in the present study (DLS). This size of IDP particles prevents diffusion directly into the bloodstream and are metabolized through the lymphatic system, acting as a barrier that provides a delayed iron absorption a priori [8]. However, IDP use pospone the iron absorption moment but does not add iron sustained and prolonged release, resulting in elevated serum iron concentrations within the first 1-10 hours post-injection [14]. The zeta potential of IDP was -0.15 ± 0.56 mV, representing a neutral surface charge value, implying low potential for interaction with membranes and molecules bearing more significant electrical charges [28]. A neutral charge increases the potential for particle aggregation as they do not electrostatically repel each other [29], which is advantageous for retention within a CTH, promoting accumulation and stability at the injection site.

### *3.2. CTH Characterization*

#### 3.2.1. Macroscopic Appearance

All proposed and developed formulations are shown in Figure 1a. Those containing IDP exhibit a brown/orange coloration, which is attributed to the presence of iron hydroxide in the IDP cores. At

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room temperature (~ 22 °C), it is evident that all formulations have a heterogeneous appearance. Moreover, the formation of small clusters can be observed by the naked eye, which may result from the premature gelation of CTH mediated by nucleation-aggregation processes, where chitosan-chitosan bonds, established via hydrogen bridges at multiple points that grows simultaneously, forming small hydrophobic aggregates that increase the formulation's viscosity [18]. The presence of aggregates is initially considered a disadvantage for extrusion through a syringe with a 21-25 G needle due to potential obstructions. Additionally, if this gelation process is triggered at lower temperatures than 20 °C, it also poses a potential disadvantage due to the non-uniform distribution of the active ingredient, which could impede dose uniformity, therefore the formulations should be injected at lower temperatures.

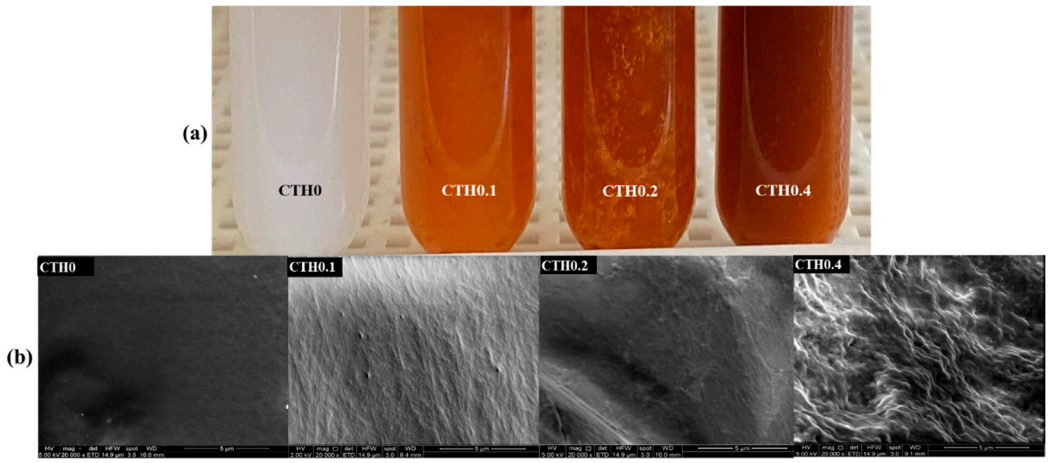


**Figure 1.** Macroscopic appearance (a) and electron microscopy (b) images of the thermosensitive chitosan/iron dextran hydrogel formulations (CTH) with increasing iron concentrations (0.1, 0.2 and 0.4 g of theoretical iron/g of chitosan).

### 3.2.2. Electron Microscopy

SEM images of the lyophilized formulations are presented in Figure 1b. The surfaces of the formulations containing IDP appear more heterogeneous than without it (CTH0), which is most noticeable in the formulatio.n with a higher IDP content (CTH0.4), which also has a rougher surface. This could be attributed to the presence of IDP interrupting the spatial distribution of CTH, leading to more discontinuous materials. In an interesting study, Zhao et al. [30] obtained images of CTH in the gel state (without active principles added), prepared with different types of acid to protonate the amino groups of chitosan, including the same acid used in the present study (acetic acid). They observed highly heterogeneous structures, which align with the aggregation of chitosan-chitosan in multiple cores simultaneously as the temperature increases in CTH [18]. EDS analysis revealed that the lyophilized CTH0, CTH0.1, CTH0.2, and CTH0.4 formulations exhibit a high presence of oxygen, phosphorus, carbon, and sodium, which are characteristic elements of chitosan and GP. With this technique, the presence of iron is also observed in the CTH0.1, CTH0.2, and CTH0.4 formulations (~ 10-20 % w/w), indicating the retention of this element in the lyophilized CTH. This could occur through the physical entrapment of IDP within the CTH, allowing retention even when water is extracted. Therefore, the three-dimensional networks formed in the gel state of CTH are heterogeneous due to gelation of CTH mediated by nucleation-aggregation process and the presence of IDP, being capable of retaining iron content after water extraction.

### 3.2.3. pH

The pH is an important property for the biocompatibility of CTH, since a change in proton concentration at the injection site can cause pain in the animal due to the activation of pH-sensitive receptors [24,25]. As shown in Table 1, all CTH formulations has pH values similar to each other and close to neutral. These results demonstrate that the addition of IDP does not have an acidifying effect

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on the formulations, obtaining pH values close to the physiological range for pigs, humans or other mammals (~ 7-7.4); due to the neutralizing action of GP [31]. Therefore, the CTH obtained has a pH suitable for use as an IM supplement for pigs and other mammals and there was no need for pH rectification.

**Table 1.** pH, sol-gel transition time, iron dextran particles (IDP) content, and viscosity at 4, 25, and 37 °C of the chitosan thermosensitive hydrogel (CTH) formulations with increasing iron concentrations (0.1, 0.2 and 0.4 g of theoretical iron/g of chitosan) and CIDF.

| Formulation | pH | Sol-gel transition time (s) | IDP content (mg/mL) | Viscosity (MPa·s) | | |
|---|---|---|---|---|---|---|
| | | | | 4 °C | 25 °C | 37 °C |
| **CTH0** | 6.88 ± 0.07 | 90 [a] | - | 45 ± 10 [a] | 65 ± 13 [a] | 2925 ± 108 [b] |
| **CTH0.1** | 6.83 ± 0.07 | 120 [b] | 2.0 ± 0.3 [a] | 72 ± 15 [a] | 383 ± 33 [a] | 2807 ± 284 [b] |
| **CTH0.2** | 6.62 ± 0.01 | 120 [b] | 4.0 ± 0.8 [a] | 74 ± 9 [a] | 269 ± 40 [a] | 3052 ± 421 [b] |
| **CTH0.4** | 6.68 ± 0.07 | 300 [c] | 13 ± 2 [a] | 134 ± 14 [a] | 447 ± 13 [a] | 3060 ± 151 [b] |
| **CIDF** | 6.38 ± 0.03 | - | 186.7 ± 2.5 [b] | 23.8 ± 0.2 [a] | 12.9 ± 0.2 [a] | 10.2 ± 0.5 [a] |

* Different letters indicate significant differences in each characterization ($p<0,05$).

3.2.4. IDP Content

IDP content of the CTH formulations is presented in Table 1 and is a direct consequence of the initially added CIDF concentrations, which were chosen based on chitosan content (0.1, 0.2, and 0.4 g of theoretical iron/g of chitosan). The IDP content in CTH0.4 is significantly higher than in the other CTH formulations but lower than CIDF. The iron content in the formulations (in the form of IDP) could be increased to approach CIDF value, however, this would result in an excessive increase in viscosity in the formulations, possibly due to the interference of IDP particles and the consequent decrease in the concentration of thermosensitive molecules in the formulation.

3.2.5. Viscosity and Sol-Gel Transition Time

The thermosensitivity of CTH is the most relevant characteristic for IM use, as it enables the smooth injection of the formulation in a sol state and subsequent transition to the gel state within the muscular tissue. As shown in Table 1, the viscosity values obtained demonstrate thermosensitivity in all developed formulations, with similar values at 4 and 25 °C across all groups. However, as the temperature increases to 37 °C, all CTH exhibit significantly higher and similar viscosity values. The transition to the gel state occurs independently of the added IDP content.

The thermosensitivity of these formulations is explained by a series of chemical reactions triggered by the increase in the formulation temperature. At neutral pH, it is not possible to solubilize chitosan in water because it is necessary to protonate its amino groups for the molecule to acquire a polar character and interact with water. However, the addition of GP allows chitosan to not precipitate at neutral pH and remain in a sol state at room temperature (~ 4-25 °C). This is because GP molecules act as intermediaries in the chitosan-GP-water interaction at neutral pH, allowing chitosan to remain suspended as long as the GP-chitosan interaction between the phosphate and amino groups is maintained. This interaction also prevents the deprotonation of chitosan at neutral pH because its amino groups are shielded by the hydration shell formed by the GP-water interaction [18]. However, increasing temperature (~ 25-37 °C), promotes the definitive transfer of protons from amino groups to phosphate ions, losing the chitosan-GP interaction, leading to the formation of chitosan-chitosan interactions through hydrogen bonds due to the reduction in chitosan interchain electrostatic repulsion [32]. This proton transference is a direct consecuence of a thermosensitive drop in pKa of chitosan (~ -0.025 pK units/ °C), promoting neutralization, losing its cationic potential [33]. This chitosan-chisotan union, generates non reversible three-dimensional networks with a nonpolar character, which macroscopically translates into an increase in viscosity, known as the gel state [18].

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The significant increase in viscosity between 25 and 37 °C, as observed in Table 1, is advantageous because it allows the formulations to be injected in the sol state close to room temperature (~ <22 °C) and then substantially increase viscosity within the muscle tissue. If increased fluidity in IM injection is required, the CTH temperature could be reduced close to 4 °C. This aligns with what was previously described in the macroscopic appearance section, where, at room temperature (~22 °C), the formulations appeared viscous and exhibited the formation of small aggregates. This could indicate that the gelation process begins at temperatures <22 °C, with the peak occurring between 25-37 °C. The maximum values obtained by the formulations in this study are similar to those reported by [30], ranging from ~2000-5000 Mpa.s in CTH synthesized with different materials.

The determination of the time it takes for the CTH to reach the gel state at muscular temperature (~ 37 °C) is crucial for the development of a potential prolonged-release IDP supplement because the release of content is likely to be higher prior to the gel state due to the absence of a force opposing the diffusion of the compound at the injection site. As observed in Table 1, the sol-gel transition time of the CTH formulations increased with IDP content, being three times longer in CTH0.4 compared to the control. This phenomenon suggests that the reactions responsible for the increase in viscosity require more time to occur in the presence of IDP and aligns with the fact that the viscosity values were similar for all (Table 1). This could mean that the formation of a three-dimensional chitosan-chitosan network responsible for the gel state occurs independently of IDP content, delaying thermosensitivity but not preventing it. This delay in transition time could be explained by the presence of non-thermosensitive IDP and its interference in the chitosan-chitosan binding that leads to the gel state. Finally, these results are consistent with findings from other authors, where transition times of approximately 1-10 minutes were observed, when the chitosan molecular weight and concentration were higher [19]. In conclusion, the formulations are thermosensitive, and its sol-gel transition times are sensitive to IDP content, with values within normal and appropriate limits for its potencial IM injection.

### 3.2.6. Water-Gel Phase Separation

The effective entrapment of IDP within the CTH in the gel state is necessary to achieve sustained release. To demonstrate this entrapment, the formulations were centrifuged in the gel state to separate the hydrophobic gel and the water in the formulation, forcing the release of IDP with water since they are highly hydrophilic. This was done to determine whether the entrapment of IDP within the chitosan network is sufficient to prevent escape. In Figure 2, the formulations in the gel state before (Figure 2a) and after (Figure 2b) centrifugation are shown. All formulations were separated into two phases, except for CIDF, which keeps the IDP suspended in water because of its high hydrophilicity. In formulations with phase separation (CTHs), the upper phase corresponds to the hydrophilic portion of the hydrogel (white arrow, Figure 2b), consisting mainly of GP suspended in water. The lower phase (black arrow, Figure 2b) corresponds to the hydrophobic gel, which was completely separated from the water during centrifugation due to higher density. It is noted that in CTHs containing IDP, the lower phase (gel) retains the coloration of the initial formulation, indicating that the IDP (previously suspended) remain in the gel after centrifugation, unlike CIDF. Considering that a chemical interaction between IDP and chitosan is unlikely due to the respective hydrophilic/hydrophobic characteristics, it is hypothesized that this phenomenon is likely the result of the physical entrapment of IDP due to a not sufficient chitosan gel pore size to diffuse out during the water-gel separation. In summary, the action of the CTH seems to allows IDP retention in chitosan hydrophobic gel at 37 °C, conditioning the release of the IDPs to the structural degradation of the chitosan network.

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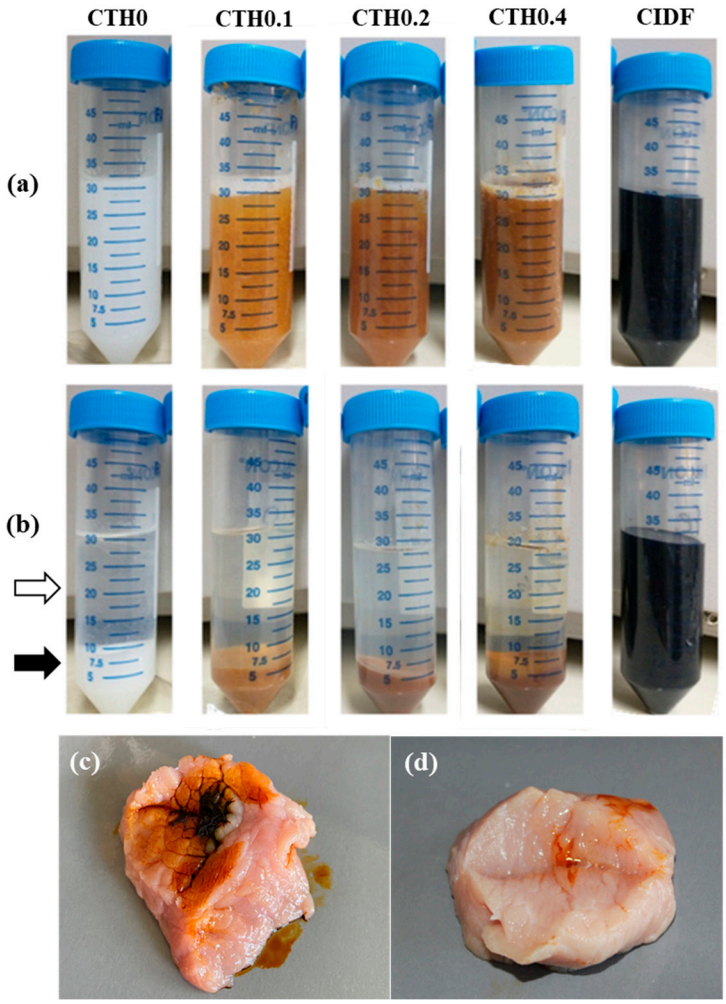


**Figure 2.** Macroscopic appearance of the chitosan thermosensitive hydrogel (CTH) formulations with increasing iron concentrations (0.1, 0.2 and 0.4 g of theoretical iron/g of chitosan) and the commercial iron dextran formulation (CIDF), showing the separation of the sol-gel phases at 37 °C before (a) and after (b) centrifugation. The white and black arrows indicate the upper (water) phase and the lower (gel) phase of CTH0, respectively.The appearance of CIDF (c) and CTH0.4 (d) injected into a piece of pork meat.

3.2.7. Fourier Transform Infrared Spectroscopy

The identification of IDP-chitosan interactions and the possible formation of new chemical bonds can help determine the nature of IDP entrapment and anticipate the characteristics of release at the injection site. In Figure 3, the spectra of CTH in the gel state and the materials used for synthesis (CIDF and chitosan as dry powders) are presented. First, in CTH formulations, an absorption band is observed in the region between 3700-3200 $cm^{-1}$, primarily reflecting O-H bonds from water molecules highly present in these formulations, and N-H interactions from the amino functional group of chitosan [34]. CIDF and CTH formulations show two absorption bands around 3000 $cm^{-1}$, corresponding to C-H bonds in aliphatic $CH_2$ and $CH_3$ groups present in the structures of chitosan and dextran [34].CTH formulations exhibits an absorption band near 1650 $cm^{-1}$, corresponding to vibrations of C=O bonds in the $NH_2$ amino group of chitosan monomeric units and O-H bonds from water molecules [34]. In the 1200-1550 $cm^{-1}$ range, CTH formulations, CIDF and chitosan, display absorption bands, mostly corresponding to characteristic vibrations of O-H, C-H, and C-O bonds inherent to chitosan and dextran structures [34,35]. Likewise, in CTH formulations, within the range of 800-1200 $cm^{-1}$, there are absorption bands corresponding to -O- and P-O-C bonds characteristic of chitosan and GP structures, respectively [34]. These data appear to reveal that the bonds present in CTH/IDP formulations exhibit absorption bands characteristic of CTH without IDP, and there is no

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evidence of the formation of new bonds or the disappearance of previously existing bonds and specific signals of iron-chitosan interactions, as described by Fahmy and Sarhan et al. [36]. In the CT/IDP formulations, there was no observed reduction in the intensity of the absorption band in the 3700-3200 $cm^{-1}$ region or the absorption band near 1650 $cm^{-1}$, indicating that the bonds of chitosan amino groups show no differences with the addition of iron and there was no evident appearance/variation of characteristic absorption bands of Fe-N or Fe-O interactions [36]. The formation of IDP-chitosan interactions through dextran is unlikely due to dextran's low reactivity, attributed to steric hindrance from its functional groups, and the loss of cationic potential of chitosan in the gel state. However, it is not possible to rule out the formation of hydrogen bonds between IDP and chitosan [29]. CTHs containing IDP exhibit bonds characteristic of CTH, and there is no evidence of chitosan-IDP interaction.

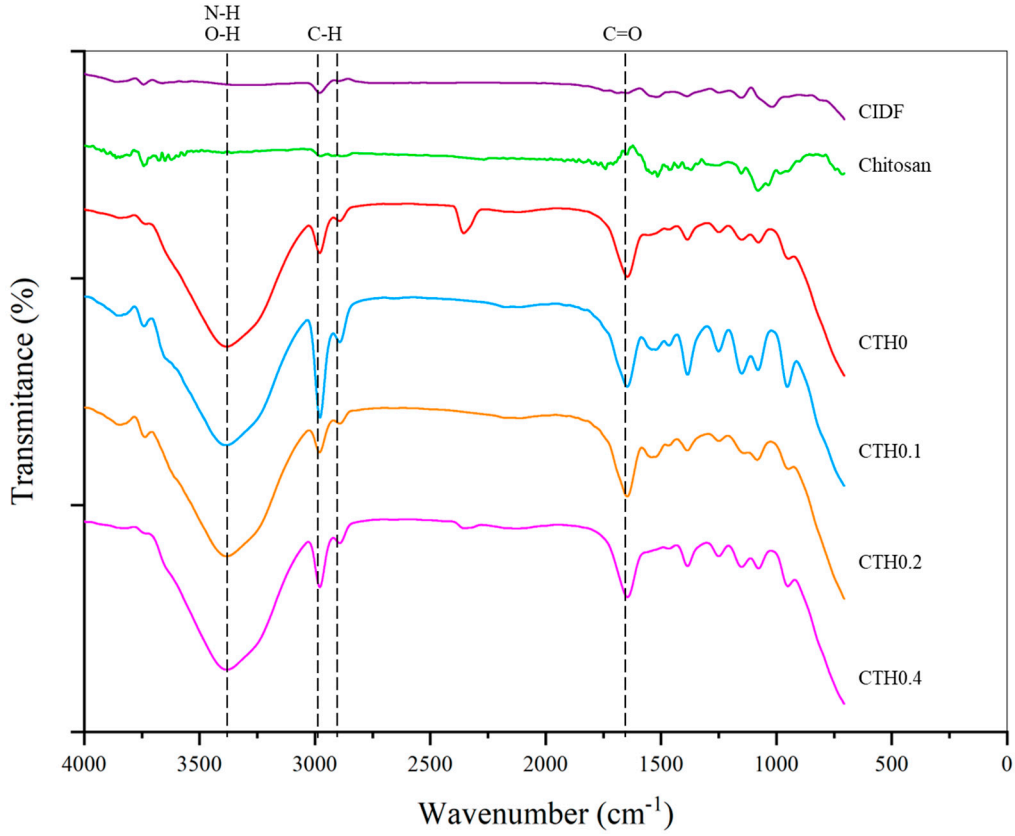


**Figure 3.** Spectrograms of the chitosan thermosensitive hydrogel (CTH) formulations with increasing iron concentrations (0.1, 0.2 and 0.4 g of theoretical iron/g of chitosan), the commercial iron dextran formulation (CIDF) and precursors. Dotted lines mark the main absorption bands. Data obtained through infrared spectroscopy.

3.2.8. Injectability in Pork Meat

The injectability of the formulations was confirmed through an ex vivo test. It was possible to inject the formulation with the higher iron content (CTH0.4) and the gold standard treatment (CIDF) into pork meat incubated at 37 °C, as shown in Figure 2, which shows distribution after injection. CIDF (Figure 2c) is widely distributed in the muscle tissue, while CTH0.4 (Figure 2d) is mostly localized at the injection site. The extensive distribution of CIDF in muscle tissue can be explained by its high hydrophilicity and low viscosity, which facilitates rapid diffusion through muscle fibers from the injection site. In contrast, CTH0.4 is localized mostly at the injection site, probably as a consecuence of the increase in temperature when it comes into contact with the muscle tissue (preheated to 37 °C), triggering the transition to the gel state. The high viscosity in the gel state at 37 °C (Table 1) reduces the diffusion of IDP through the muscle tissue. Furthermore, contrasting these results with the water-gel phase separation, it is hypothesized that after injection of the CTH, the

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chitosan polymer network formed after gelling, expels water molecules through difussion due to its hydrophobic nature, concentrating the gel portion charged with IDP at the injection site and confining it in a potential beneficial manner for prolonged release.

Figure 4 proposes the interaction dynamics between the different components of the formulation (chitosan, GP, IDP and water) in sol and gel states. In the sol state (Figure 4a), the dominant forces are the interactions of water-GP and water-IDP hydrogen bonds that keep these components suspended, in addition to the electrostatic GP-chitosan bond that prevents the precipitation of the polymer as it is anchored to water through the GP [18]. When transitioning to the gel state (Figure 4b), the increase in temperature generates a decrease in the cationic potential of chitosan, resulting in the loss of the chitosan-GP interaction and the generation of chitosan-chitosan hydrogen bonds, which give origin to the high-viscosity hydrophobic network [18]. Based on the results obtained and discussed in the present study, it is proposed that IDPs are confined in the chitosan network which is a physical barrier that stands in the way of diffusion. Thus, the release of IDPs may be dependent on the degradation time of chitosan, prolonging the release of IDPs in situ.

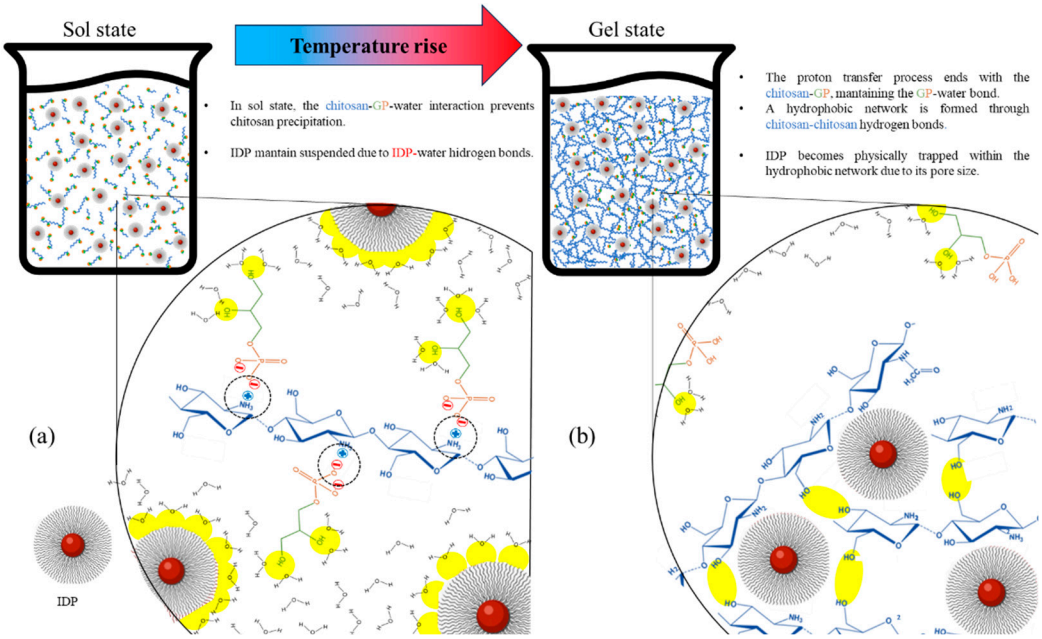


**Figure 4.** 2D schematic representation of interactions between chitosan, glycerophosphate (GP), water, and the iron dextran particles (IDP) in a chitosan thermosensitive hydrogel in sol state (a) and gel state (b). Dotted circles represent chitosan-GP ionic bonds, and yellow areas represent interactions involving GP-water, chitosan-chitosan, and water-IDP hydrogen bonding.

## 4. Conclusions

It was possible to obtain CTHs loaded with IDP, resulting in formulations with neutral pH, that are thermosensitive and suitable for IM injection in the sol state at ~4 °C, and that significantly increase in viscosity between 25 and 37 °C until reaching the gel state. It was concluded that the loaded IDP is effectively trapped in the chitosan polymer network formed in the gel state. Furthermore, no chitosan-IDP or GP-IDP chemical interactions of any kind were evident. Therefore, it is hypothesized that the physical confinement of IDP is highly beneficial for prolonged release. These results provide useful knowledge for the study of CTH as an iron supplementation strategy and serve as a basis for the development of new micronutrient supplementation strategies through prolonged release for pigs, humans, and other mammals.

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