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Research paper

Gel–sol–gel thermo-gelation behavior study of chitosan–inorganic phosphate solutions

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ABSTRACT

In present study, the feasibility of developing a novel thermo-sensitive hydrogel suitable for injectable formulations based on chitosan and inorganic phosphate was demonstrated. The physicochemical and rheological changes of chitosan/dibasic sodium phosphate solutions as in function with temperature were investigated in order to gain a better understanding of gelation process. According to the result of rheological study, there are two phase transition points as in function with temperature, corresponding to 30 °C and 43 °C. The system is gel state at ~4 °C. With the temperature increased to 30 °C, the gel–sol transition as well as the decrease in turbidity was observed. The sol–gel transition as well as the increase in turbidity was observed again as the temperature was above 43 °C. And the gel obtained at ~4 °C is reversible, but the gel obtained at ~43 °C is irreversible.

1. Introduction

Chitosan (CS), a semi-crystalline aminopolysaccharide obtained by deacetylation of chitin, has been widely used in the biomedical field owing to its nontoxicity, good biocompatibility, biodegradability, mucoadhesive, and permeation enhancing properties [1–3]. The hydrogel based on chitosan and its derivatives has been reported in the recent literature [4–7]. These hydrogels can be divided into two classes: chemical hydrogels, which forms permanently cross-linked gels, and physical hydrogels, which forms reversible cross-linked gels [8,9]. The cross-linking agent used in chemical hydrogels can interact with the encapsulated drug, especially bioactive molecule, which might lead to significant toxicity. For these reasons, physical hydrogels based on chitosan have gained particular interest [10,11]. Recently, the addition of a base such as inorganic phosphate solutions to chitosan solution could obviously increase the pH value of system and eventually form the gel [6]. The system remained in solution state at physiological pH (~7.2) and room temperature but transformed into a gel state as heated to 37 °C. It is one of the rare true chitosan physical hydrogels, obtained without any cross-linking agents as proposed very recently by Nair et al. [6].

Chitosan/inorganic phosphate system showed the gelation behavior at both 4 °C and 37 °C. It may involve several interactions such as screening of electrostatic repulsion, ionic cross-linking, hydrophobic effect, and hydrogen-bonding interactions. The dibasic sodium phosphate, a weak base (~9.2), can increase the pH value of chitosan solution to around neutrality (~7). It is negatively charged in solution and thus may screen the electrostatic repulsion between chitosan molecules. Several possible interactions have been proposed to explain the gelation behavior of the chitosan/β-glycerophosphate system, which might also be suitable for the explanation of gelation behavior of the chitosan/inorganic phosphate system [12,13]. In order to well understand the gelation behavior of chitosan/inorganic phosphate system, here we investigated the changes in ionization, turbidity and pH value as well as the rheological properties of system during the gel formation.

2. Experiments

2.1. Materials

Chitosan (deacetylation degree 86%, viscosity 200 cps) was supplied by Sigma–Aldrich (USA) and its molecular weight (Mw) with ~200 kDa was determined by a GPC (Agilent 110 HPLC, USA). Hexamethylenetetrammonium, dibasic sodium phosphate, acetic acid and hydrazine sulfate were purchased from KeLong Chemicals (Chengdu, China). All other chemicals used in this paper were of analytical grade. Ultrapure water from Milli-Q water system was used to prepare the aqueous solutions.

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2.2. Preparation of auto-gelling solution composed of chitosan and inorganic phosphate

Briefly, chitosan (1 g) was dissolved in 49 ml of 0.5% acetic acid solution at concentration of 20 mg/ml and filtered for further application. Auto-gelling solutions were prepared as follows: 2 ml chitosan solution was first placed in a glass vial and magnetically stirred in an ice bath. And then 0.2 ml of dibasic sodium phosphate (500 mg/ml) was added dropwise into chitosan solution. After the mixing, chitosan/dibasic sodium phosphate solutions were left for 4 h to degas without stirring at room temperature and stored in a refrigerator for the further investigation.

2.3. Turbidity determination

The turbidity of chitosan/dibasic sodium phosphate solution in terms of temperature was determined by a modified method described by Wu et al. [12]. Briefly, the chitosan/dibasic sodium phosphate solution was added into a 96-well plate, and the absorbance at various temperatures was then measured at 680 nm with an ELISA microplate reader (Bio-Rad, USA). The turbidity was calculated from the standard absorbance–turbidity curve with formazin suspension as standard. Formazin suspension was self-prepared by reacting hexamethyenetetrammonium with hydrazine sulfate at 25 °C. And standards of formazin turbidity units (FTU) were prepared by appropriate dilution.

2.4. pH and conductivity measurements

All solutions were characterized in terms of pH and conductivity as in function with temperature. A water bath was selected to control temperature at a constant heating rate (1 °C/min). Temperature ranging from 0 °C to 50 °C and 0 °C to 37 °C was respectively selected to evaluate the changes of conductivity and pH of system. Since the conductance meter used was linearly temperature-compensated (referenced to 25 °C), we applied the following equation to recover the conductivity at a given temperature:

$$\kappa_{\mathrm{T}} = \kappa_{25}[1 + \alpha(T - 25)]$$

where κ_{25} the conductivity referenced to 25 °C, κ_{T} the conductivity measured at a given temperature T, and α the temperature coefficient of variation [14]. Therefore, the ionic strength of each component could be obtained by the calculation based on the pH value and conductivity measurements.

2.5. Rheological measurements

In this study, rheological measurement was performed with an AR-2000ex rheometer (TA Instruments, USA). Chitosan/dibasic sodium phosphate solutions were placed between parallel-plate of 40 mm diameter with a gap of 31 mm for investigation. During the gelation process, the evolution of the rheological properties was investigated as a function of temperature by oscillatory shear measurements. For time sweeping tests, storage moduli (G') and loss moduli (G'') were monitored as a function of time at a 1 Hz frequency under a constant temperature (37 °C). For temperature sweeping tests, the evolution of the G' and G'' was detected by increasing the temperature from 4 to 60 °C with a constant heating rate (1 °C/min). In order not to disturb the gel formation during these tests, small deformation (γ_o) of 0.01 and low frequency (1 Hz) were used.

3. Results and discussion

In recent years, thermo-sensitive hydrogels based on chitosan and its derivates have gained considerable attention for the pharmaceutical and biomedical application [6,12]. Chenite et al. first reported that chitosan solution, neutralized with β-glycerophosphate, could form a novel *in situ* gelling system suitable for subcutaneous administration [15]. This system was liquid at room temperature and turned into gel at body temperature (37 °C) suitable for *in situ* drug delivery. Here, we substituted β-glycerophosphate with dibasic sodium phosphate to develop a novel thermo-sensitive chitosan hydrogel. Compared with chitosan/β-glycerophosphate system, chitosan/dibasic sodium phosphate system showed two phase transition temperature as in function with temperature, corresponding to ∼4 °C and ∼37 °C, as shown in Fig. 1. The system is gel state at ∼4 °C. With the temperature increasing from 4 °C to 30 °C, the gel–sol transition of system was observed. Furthermore, the gel–sol transition behavior at this temperature range (4–30 °C) was reversible. As incubation at ∼37 °C for a period, the sol–gel transition was observed (Fig. 1). However, further increase in temperature to 50 °C or decrease in temperature to 4 °C, no changes in state of system (gel state) were observed. All these observed phenomenon suggest that gelation behavior of system at ∼4 °C was reversible but the gelation behavior of system at ∼37 °C was irreversible. This interesting phenomenon indicated that there are various driving forces for gel formation at these two phase transition temperatures.

3.1. Turbidity of the hydrogel

The turbidity changes of system in the function of temperature were investigated, and the results were showed in Fig. 2. As depicted in Fig. 2, the turbidity of system was greatly affected by temperature. With the temperature increasing from 4 °C to 30 °C, the turbidity of system gradually decreased. However, further increase in temperature to 37 °C, the turbidity of system increased again. The turbidity changes of system as in function with temperature can by explained according to Chen et al. [16]. The reduced solubility and increased hydrogen bond of chitosan at low temperature tended to form physical junctions resulting in the increase in turbidity of system. With the temperature increasing from 4 °C to 30 °C, the weaker hydrogen bond between chitosan skeleton and water molecules could make chitosan molecules unfold freely, thus leading to the decrease in turbidity [13,17]. Raising the temperature to 37 °C, due to the increase in hydrophobic interaction between chitosan skeleton and possible ion bridge resulting in the gel formation (Fig. 1), the turbidity of system increased again [13,18].

Fig. 1. The gelation process of chitosan/dibasic sodium phosphate formulation as in function with temperature from 4 to 37 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

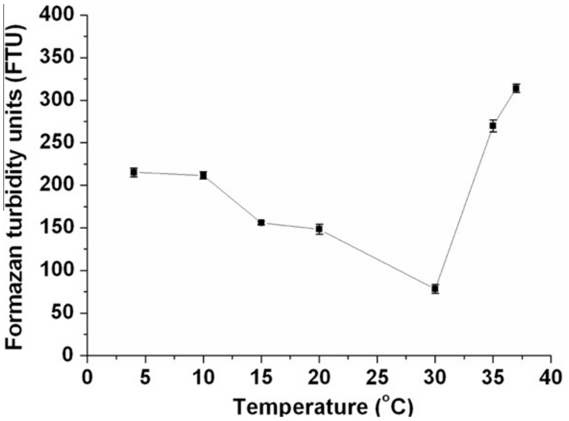

Fig. 2. Turbidity graph of chitosan/dibasic sodium phosphate system as in function of temperature from 4 °C to 37 °C.

3.2. Effect of temperature on the pH and conductivity

The pH and conductivity changes of solutions were monitored as in function with temperature. According to the Gibbs–Helmholtz equation (1), we could find that the equilibrium constant (K_a) was greatly influenced by the temperature.

$$\mathrm{p}K_\mathrm{T} = \log K_\mathrm{T} = -\frac{\Delta G}{2.303RT} \tag{1}$$

where ΔG represents the changes of Gibbs free energy of the equilibrium reaction; R is the gas constant (8.314 J mol^{-1} K^{-1}); T is the absolute temperature and K_T is the equilibrium constant (K_a) at give temperature. Thus, if the pK_a and ΔG values are known at some temperature, the pK_T can be estimated at temperature T (Table 1).

Table 1
pK_a and Gibbs free energy (ΔG) at 298.15 K.

Substance	pK_a	ΔG (kJ mol^{-1})
Acetic acid [13,23]	4.75	27.1
Chitosan [6]	6.5	37.1[a]
Dibasic sodium phosphate [27]	7.21	38.7
	12.66	307.6

[a] Estimated from Eq. (1).

For acetic acid, chitosan and dibasic sodium phosphate, the acid–base equilibrium reactions are respectively presented as follows:

$$\mathrm{CH_3COOH + H_2O \leftrightarrow H_3O^+ + CH_3COO^-} \tag{2}$$

$$\mathrm{CSNH_3^+ + H_2O \leftrightarrow H_3O^+ + CSNH_2} \tag{3}$$

$$\mathrm{HPO_4^{2-} + H_2O \leftrightarrow OH^- + H_2PO_4^-} \quad \text{and}$$
$$\mathrm{HPO_4^{2-} + H_2O \leftrightarrow H_3O^+ + PO_4^{3-}} \tag{4}$$

Therefore, the ionization of each chemical species can be estimated from the Henderson equation (2) as the follows:

$$K_\mathrm{T} = \frac{[\text{conjugate base}][\mathrm{H_3O^+}]}{[\text{acid}]} \quad \text{or}$$
$$\mathrm{pH_T} = \mathrm{p}K_\mathrm{T} + \log\frac{[\text{conjugate base}]}{[\text{acid}]} \tag{5}$$

As depicted in Fig. 3, addition of dibasic sodium phosphate to chitosan solution could lead to the pH value of system increasing from 4.8 to 7.3 at 4 °C, due to the neutralization of dibasic sodium phosphate. Meanwhile, we also found that the pH value of system slightly decreased from 7.3 to 7.0 as the temperature increased from 4 °C to 37 °C. As well known to us, chitosan was almost deprotonated as the pH value was greater than 7, which indicated that the contribution of ionic cross-linking between $\mathrm{NH_3^+}$ and $\mathrm{HPO_4^{2-}}$ did not seem to be the main driving force for gelation no matter at ~4 °C or at ~37 °C [13]. Meanwhile, the conductivity of system was also detected in the function with temperature, as showed in Fig. 4. The conductivity of system showed the temperature dependency with a conductivity increase as in function with temperature. It is well known that ion species, ion concentration and ion migration rate as well as the charge number of ion have great effect on the conductivity of solutions [19–21]. On the other hand, the increase in ion migration rate at high temperature also could improve the conductivity of solution [22].

3.3. Measurement of ionic strength

According to Eq. (5), the ionization degree of each species as well as ion concentration in terms of temperature can be calculated. Hence, the ionic strength of solution can be estimated by Lewis equation as follows:

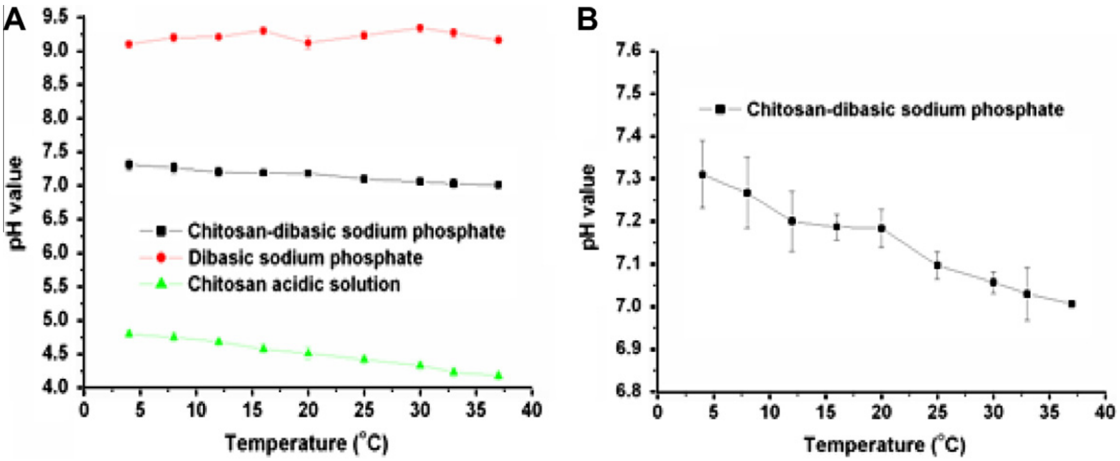

Fig. 3. (A) pH measurements of system and (B) chitosan/dibasic sodium phosphate solution as in function with temperature from 4 °C to 37 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

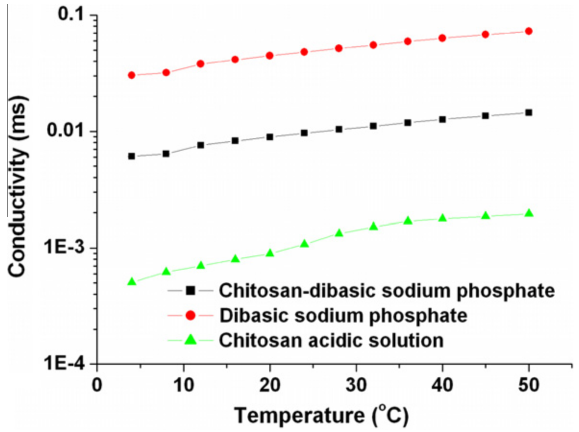

Fig. 4. Conductivity measurements of system as in function with temperature from 4 °C to 37 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$$I = \frac{1}{2}\sum m_B Z_B^2 \tag{6}$$

where m_B is the molar concentration of each ion and Z_B is the ion charge number. From Eq. (6), we could find that the ionic strength of solution showed the positive correlation with ion concentration as well as charge number of ion.

As depicted in Fig. 5, temperature did not greatly affect the ionic strength of chitosan acetic solution. For chitosan/dibasic sodium phosphate system, it showed the temperature dependence with an ionic strength increase as in function with temperature. The increase in ionic strength from 4 °C to 37 °C could efficiently screen the electrostatic repulsion forces between chitosan skeleton as well as increase the hydrophobic interaction between chitosan skeleton. Furthermore, temperature increase could probably modulate hydrogen bond distribution and favor polymer–polymer interactions over those of polymer–solvent via hydrophobic interaction [23]. Therefore, we speculated that the growing hydrophobic interaction might be the main driving force of chitosan gel formation at ~37 °C, and the physical conjunctions as well as the hydrogen bond between chitosan skeleton and water molecule are the main driving forces of chitosan gel formation at ~4 °C.

3.4. Thermal gelation rheology

Isothermal time dependence of elastic storage moduli (G') and loss moduli (G'') for chitosan/dibasic sodium phosphate system at 37 °C are presented in Fig. 6. Both G' and G'' are very low at the beginning of the gelation process ($t < t'$) with G' smaller than G'', corresponding to the sol state of system. Both G' and G'' increased as gelation proceeded: the growth rate of G' was much higher than that of G'' due to the hydrophobic interaction between chitosan skeleton. The differential on rates leads to a crossover of G' and G'', which could be defined as the gel point ($t = t'$), indicating the transition of chitosan/dibasic sodium phosphate system from the sol state to a gel state. From Fig. 6, as $t' > 15$ min a three-dimensional (3D) network formed successfully through physical cross-linking. Meanwhile, it also suggested that the gel formation is a progressive process. The rheological characterization of solutions as in function with temperature was also investigated, as shown in Fig. 7. As presented in Fig. 7, there are two phase transition points as in function with temperature, corresponding to 30 °C and 43 °C. The system was in gel state as the temperature was below 30 °C, as shown in Fig. 1. Both G' and G'' are very low with G' greater than G'' as temperature is below 30 °C, indicating the gel state of system. It is well known that increasing temperature could decrease hydrogen-bonding interactions between polymer and solvent [24,25]. At low temperature (<30 °C), water molecules are resumed to form enclosed structures that surround the polymer chains [26]. Meanwhile, chitosan possesses hydrogen bonding favoring groups such as —OH, —NH_2 and C=O, that could be associated by hydrogen bonding to form the physical junction [5,12].

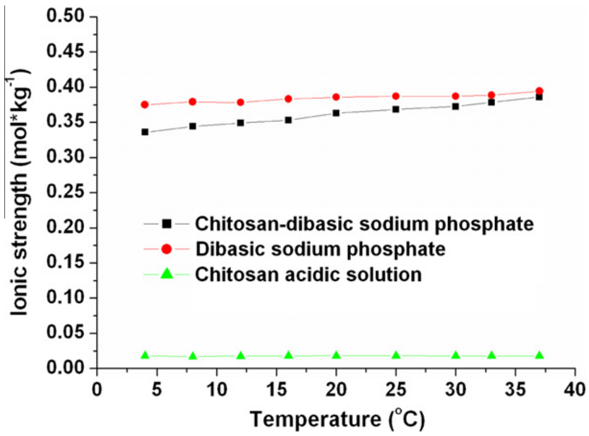

Fig. 5. The calculated ionic strength of system as in function with temperature from 4 °C to 37 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

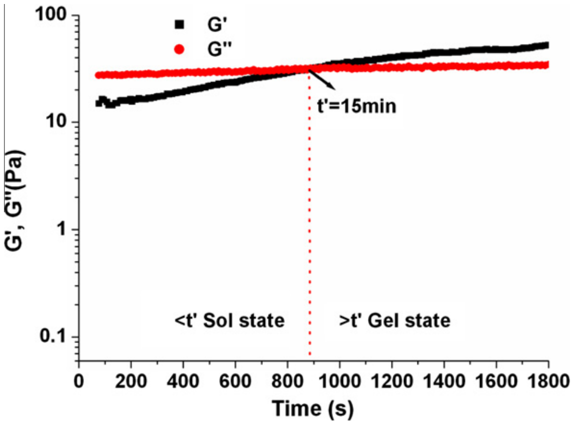

Fig. 6. Time evolution of elastic storage moduli (G') and loss moduli (G'') for chitosan/dibasic sodium phosphate system at 37 °C. The time (t') where G' and G'' crossover is defined as gelation point. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

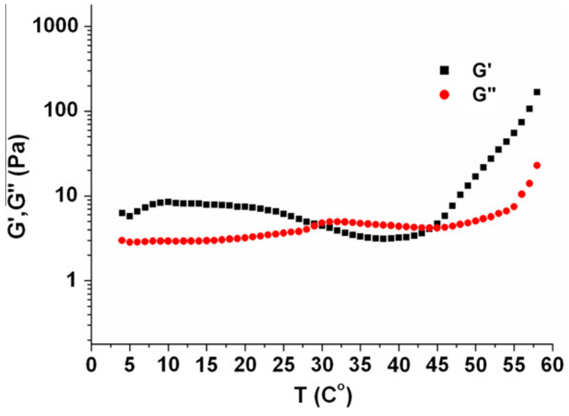

Fig. 7. Evolution of elastic storage moduli (G') and loss moduli (G'') for chitosan/dibasic sodium phosphate system as in function with temperature from 4 °C to 60 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Gel–sol transition of system was observed with G'' greater than G' as the temperature increases from 30 °C to 43 °C. With the temperature increase to 43 °C, the vibration and rotation energy of water increased obviously resulting in the escape of water molecules surrounding the chitosan chain [24,25]. Therefore, the hydrophobic polymer segments begin to associate with each other to form the 3D gel structure at the temperature above 43 °C. The transition temperature of system obtained by rheometer (Fig. 7) is different with that obtained by naked eye observation (Fig. 1), which might be attributed to the fact that the gel–sol–gel transition behavior is a progressive process as demonstrating by Fig. 6. Thus, the phase transition temperature as in function with temperature determined by rheometer could be pushed to the higher temperature ($\sim$43 °C) rather than body temperature ($\sim$37 °C). Based on the these results, it seems that the hydrogen bond between chitosan skeleton and water molecule as well as the physical conjunctions might be the main driving force to the gel formation at low temperature (<30 °C) but the hydrophobic interaction might contribute to the gel formation at high temperature (>43 °C).

4. Conclusion

In present study, the physicochemical and rheological properties of a novel thermo-sensitive hydrogels based on chitosan and inorganic phosphate were studied as in the function with temperature. There are two phase transition points as in function with temperature, corresponding to 30 °C and 43 °C. And the gel formation of system at low temperature (<30 °C) was reversible, but the gel formation of system at high temperature (>43 °C) was irreversible. Based on the studies on the changes of pH value, conductivity and ionic strength of system as in function with temperature, it seems that the hydrogen bond between chitosan skeleton and water molecule as well as the physical conjunctions might be the main driving force to the gel formation at low temperature (<30 °C), but the hydrophobic interaction might contribute to the gel formation at high temperature (>43 °C).

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