물리적으로 가교된 젤라틴 하이드로젤의 열안정성에 대한 염의 효과: DSC와 유변학 특성

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Effect of Salts on the Thermal Stability of Physically Crosslinked Gelatin Hydrogels: DSC and Rheology Study

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Abstract: The effects of preparation conditions, such as polymer concentration, annealing temperature, annealing time and pH, on the melting behavior of gelatin gels were investigated. An optimum condition of gel preparation for calorimetric test is obtained. In addition, the influence of salt including salt concentration and salt type on the melting behavior of gelatin gels was explored by DSC and rheometer. It was found that the melting temperature first increased and then decreased with an increase in salt concentration. This result indicates that electrostatic interactions are important for the formation and stability of gelatin gels. Moreover, the melting temperature of polymer gels with different salt addition conforms to the following orders: $Na^+ < Ca^{2+} < Cr^{3+}$. The different trend observed in respect of the storage modulus indicated that the triple helices dominated the gelatin gel strength, and the binding mechanism of Ca^{2+} may be different from that of Cr^{3+}.

Keywords: gelatin gels, thermal stability, polyelectrolyte, rheology.

Introduction

Gelatin is one of the most extensively applied biopolymers due to its availability, nontoxicity, and biocompatibility. Elastic gels can be readily obtained from gelatin aqueous solutions when the temperature is below roughly 30 °C. Much attention has been paid to these hydrogels in the past decades. The physically crosslinked gelatin hydrogels, which are thermo-reversible, have been widely used in food and pharmaceutical fields, due to the absence of the harmful chemicals.

It is known that gelatin gels are composed of an ensemble of physically interconnected triple helices,[1] which have the same conformation as collagen. Thus, gelatin molecules can partially revert to the ordered helix structures, which are held together by multiple hydrogen bonds.[2-5] Generally, these hydrogen bonds were suggested to be the main noncovalent source of structural stability in native proteins.[6] Recently, a lot of evidence has proved that hydrophobic interaction, rather than hydrogen bonds, plays a dominant role in the maintenance of the helical conformation in aqueous solution.[7] Moreover, as a polyelectrolyte, the electrostatic interaction should be considered in controlling of macromolecular conformation.[8] Accordingly, the chain conformations in the gelatin gels were the compromise results of these interactions including the hydrogen bonds, hydrophobic effects, and electrostatic forces, which can be regulated conveniently by experimental conditions. The gelatin hydrogels were found to be sensitive to different experimental preparation conditions such as polymer concentration,[9] the molecular weight of gelatin,[10] annealing time and temperature,[11] thermal history,[12] solvent quality,[13] pH,[14] and salt addition.[15] Among these conditions, the influences of salts on the structure of hydrogels are of great importance because salts are commonly present in many cases such as food industry and biological systems. In addition, the thermal stability of gelatin hydrogels, which is closely related to the gel structure, is one of the important factors determining the sig-

nificance of their applications. Thus it is necessary to explore the effects of salts on the thermal stability of gelatin hydrogels.

Generally, the melting temperature of a gelatin gel is accepted to evaluate the thermal stability of the triple helices present. The early observation of Bello *et al.*[16] on the melting temperature of gelatin gels indicated that the large differences in the effects of various ions on the melting temperature originated from the different interactions between the ions and the gelatin. This effect of salts was later generalized to a stabilization or destabilization influence on basic macromolecular conformational transitions.[17] Recently, Sarabia found that the melting temperature of gelatin gels was increased considerably with the addition of $MgSO_4$, $(NH_4)_2SO_4$, and NaH_2PO_4, whereas chloride salt acted to reduce it.[18] A similar phenomenon was observed by Sarbon *et al.*,[19] where $CaCl_2$ addition led to the decreasing of the melting temperature of the gelatin gels and $CaSO_4$ tended to increase it. Although the effect of different salts on the melting temperature of gelatin gels has been known for a long time, the possible mechanism of the interaction between gelatin molecules and saline ions is still unclear. The arguments on whether the ions are directly bound to the peptide backbone of gelatin or the ions affect gelatin folding indirectly by interacting with structurally bound water molecules, is not yet resolved.[20]

Several techniques have been employed to determine the melting temperature of gelatin gels: polarimetry,[17] rheology[18] and differential scanning calorimetry (DSC).[11] DSC appears to be the most convenient and widely used method, which has already been used to directly exploit the melting temperature of gelatin gels.[21-24] However, to the best of our knowledge, the influence of salt on the thermal stability of gelatin gels has not been systematically studied, and little information is available in relation to this respect.[19] The strategy for this study was the following. The effect of the preparation conditions of gelatin gels on its melting behavior is explored and an optimum condition of gel preparation for calorimetric test is obtained. Then the influence of salt additions was investigated under this preparation condition. The objectives of this study were to investigate whether and how the addition of salt at a wide range of concentration affects the thermal stability of the physical crosslinked gelatin hydrogels. A combination of DSC and rheology measurements of gel melting allows one to obtain physical insights into the structure of gelatin gels, which is closely related to the interaction between gelatin molecules and saline ions.

Experimental

Materials. Gelatin (type B, limed-hide hydrolysis) used was purchased from Sinopharm Chemical Reagent Co., Ltd. The average molecular weight is about 50000, and its isoelectric point (pI) was approximately pH = 5.0. The salts used in this work were purchased from Sinopharm Chemical Reagent Co., Ltd. All aqueous solutions were prepared using ultrapure water (18 MΩ·cm) from a Milli-Q system (Millipore). All of the chemicals were of analytical grade.

Preparation of Gels. The gelatin solutions with concentrations ranging from 5.0 to 40.0 wt% were prepared by dissolving gelatin powder in deionized water at 50 °C. In addition, 20.0% gelatin powder by weight was dissolved in different salt solutions of NaCl, $CaCl_2$ and $CrCl_3$ at different concentrations, respectively, to obtain the desirable solutions. The pH values of the gelatin solutions were tuned to be 2.0, 4.0, 6.0, 8.0 and 10.0, respectively, using hydrochloric acid and sodium hydroxide. The warm solutions were poured into a homemade Teflon mold and cooled to 20 °C and isothermal for 12 h to obtain the gels. The gel samples were stored in a refrigerator at 4 °C before performing further characterizations.

Differential Scanning Calorimetry (DSC). DSC measurements were performed using a DSC Q2000 (TA Instruments, USA) to investigate the melting behaviors of gelatin gels. The samples with mass about 10-15 mg were put into aluminum pans and hermetically sealed. All measurements were conducted under nitrogen flow of 50 mL/min. All the samples were first melted in the DSC cell at 50 °C for 5 min to erase previous thermal history. Solutions were then cooled at a rate of 20 °C/min to the desired temperature of annealing and held at this temperature for a certain period of time. The annealing temperatures were in the range 5-25 °C, and the annealing times varied from 15 to 200 min. After a given time the samples were reheated at a rate of 1 °C/min to 50 °C. The melting point was determined from the peak temperature of the DSC endotherm.[9] Temperature and heat flow calibrations were performed by using indium reference standard.
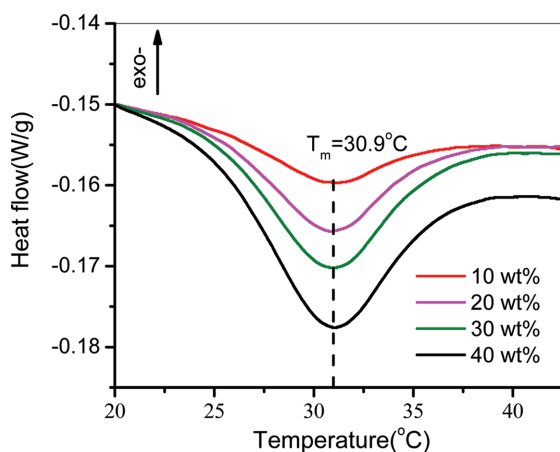
Rheometry. The small-strain oscillatory measurement was carried out by a rotational rheometer (DHR, TA Instruments, USA) with a Peltier temperature control. The parallel plate geometry(d = 40 mm) was used with a gap of 1 mm. Strain sweeps in the range of 0.1-100% at frequencies of 10 rad/s were carried out to determine the linear viscoelastic range of the sample solutions in both sol and gel states. Samples were first held at 50 °C for 10 min in a water bath to eliminate ther-

mal history effects. Then the hot solutions were poured directly onto the rheometer plates at 20 °C, and held at this temperature for 60 min. The gels were reheated from 20 to 50 °C at a scan rate of 1°C/min with a frequency of 1.0 rad/s and controlled strain 1% (within the linear viscoelastic range). The melting temperatures were taken as the point at which the phase angle peaked immediately after a sharp increase.

Results and Discussion

Melting Behavior of Gelatin Hydrogels. Effect of Polymer Concentration: The influence of gelatin concentration on the thermal stability of polymer gels was shown in Figure 1. The melting temperature of polymer gels was nearly invariable with gelatin concentration. This suggested that the concentration of the gel has no obvious effect on the stability of the network junctions. It can be found from Figure 1 that the 20 wt% sample has good DSC signal to noise ratio. Moreover, when the sample concentration was over 20 wt%, it was very viscous and difficult to handle it. Besides, the influence of polymer concentration on the thermal stability of gels was very insignificant. Thus the 20 wt% samples were considered to be representative in this work, and all the gelatin gels were prepared at this concentration in subsequent studies.

Effect of the Annealing Temperature: Figure 2 represented the effect of the annealing temperature on the melting behavior of gelatin gels. It was found that the melting temperature of gelatin gels increased evidently with the increasing of annealing temperature. This result, also reported by Gor-

nall,[9] Dranca[11] and Michon,[12] could be interpreted in terms of the increase in the length of triple helices increasing temperatures.[25] The maximum gelation rate of gelatin was found to lie between 14 and 19 °C.[26] The present study yielded the maximum temperatures of the gelation rate around 20 °C for 20 wt% solutions (data not shown). From Figure 2 it can be found that the enthalpy of melting of gels at 20 °C was large than that at 25 °C, although the melting temperature was high for the latter. Thus the annealing temperature was set to 20 °C for all the samples in subsequent experiments, which is believed to yield the larger amounts of gelatin gel at this temperature.

Effect of the Annealing Time: The effect of the annealing time on the melting behavior of gelatin gels was shown in Fig-
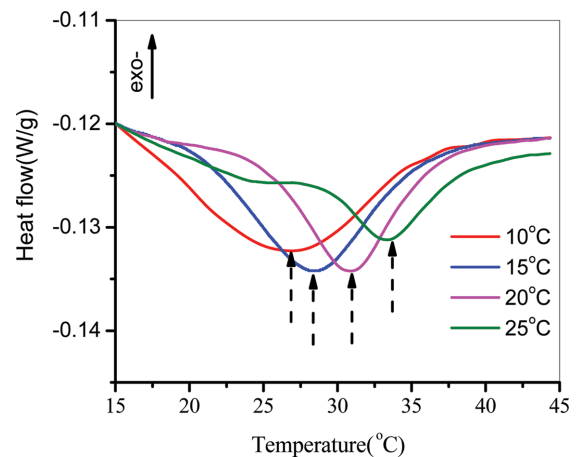
Figure 2. DSC thermograms for gelatin gels (20.0 wt%) formed on annealing for 1 h at different annealing temperature. The peak temperatures are indicated by the arrows. Heating rate is 1.0 °C/min.
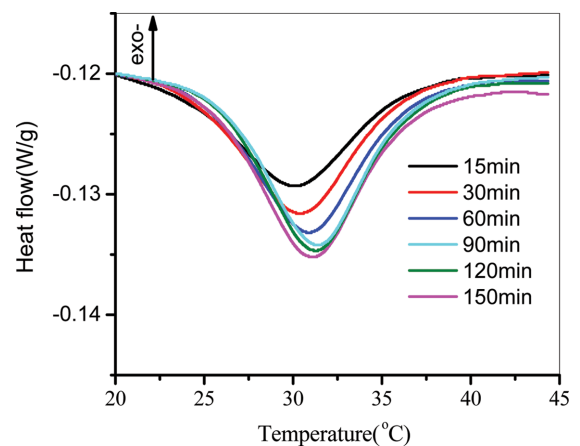

Figure 1. DSC thermograms for gelatin gels of different concentration formed on annealing for 1 h at 20 °C. The peak temperature is about 30.9 °C as indicated by the dash line. Heating rate is 1.0 °C/min.
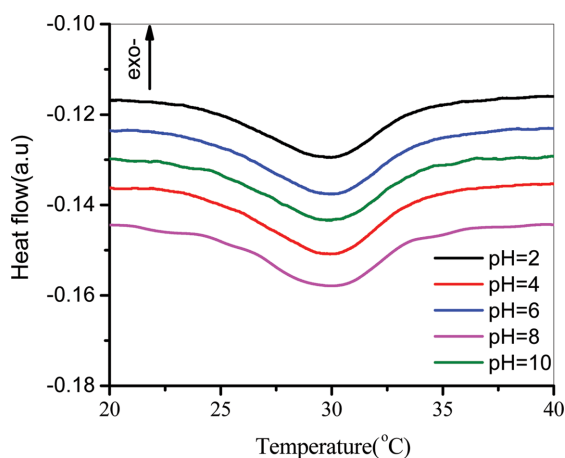

Figure 3. DSC thermograms for gelatin gels (20.0 wt%) formed on annealing at 20 °C for periods of time from 15 to 150 min. Heating rate is 1.0 °C/min.

ure 3. The enthalpy of melting of gelatin gels was found to be increased markedly with increasing the annealing time, indicating the formation of more crosslinks with the annealing time. However, there was no notable improvement in thermal stability of polymer gels. The melting temperature of gelatin gels has only increased by a little over 1 °C when the annealing time increases from 15 to 150 min. A similar phenomenon has been observed for gels of 40 wt% gelatin.[11] In this work, the annealing time of 60 min was chosen so as to run all annealing experiments on DSC within one week.

Effect of pH Value: Figure 4 shows the influence of pH on the melting behavior of polymer gels. The role of pH on the thermal stability of gelatin gels was insignificant. Both the melting temperature and the enthalpy of melting of the gels remained to be invariable over the whole pH range, indicating a minor effect of pH variation on the gelation of gelatin. Our result was consistent with the early observation of Russell that the charge effects play a minor role in determining the thermal stability of isolated collagen molecules in solution.[27] The pH stability of gelatin gels over such wide range may contribute to its application in food.

Melting Behavior of Gelatin Hydrogels with Salt Addition. Effect of Salt Concentration: The effect of salt concentration on the melting behavior of gelatin gels is shown in Figure 5(a). There was a slight increase in the melting temperature of gelatin gels with salt addition when the concentration of salt was low (~10 mM), and it decreased with a further increase in salt concentration. However, this result is very different from the observations by Bello,[16] von Hippel,[17] Sarabia[18] and Haug,[28] which the melting temperature was

found to be decreased monotonically with the addition of salt. It should be noted that the concentration of salt used in these studies was much higher than 10 mM. In addition, a similar changing trend for melting enthalpy can also be found in Figure 5(a), and it indicated that the influences of salt addition were consistent with the nucleation and the growth of triple helices.

It is known that the gelatin molecules were prone to aggregate in aqueous solutions, especially at its isoelectric point. The gelatin chains were believed to be collapsed due to the inter/intrachain attractions. This electrostatic interaction can be shielded by salt addition, which opened up the protein chains more. Thus it increased the likelihood of a suitable formation of triple helices, which resulted in the increasing of the melting temperature of gelatin gels.[29] However, when the salt concentration was over 10 mM, the melting temperature of protein gels was found to be decreased with salt addition. Both hydro-
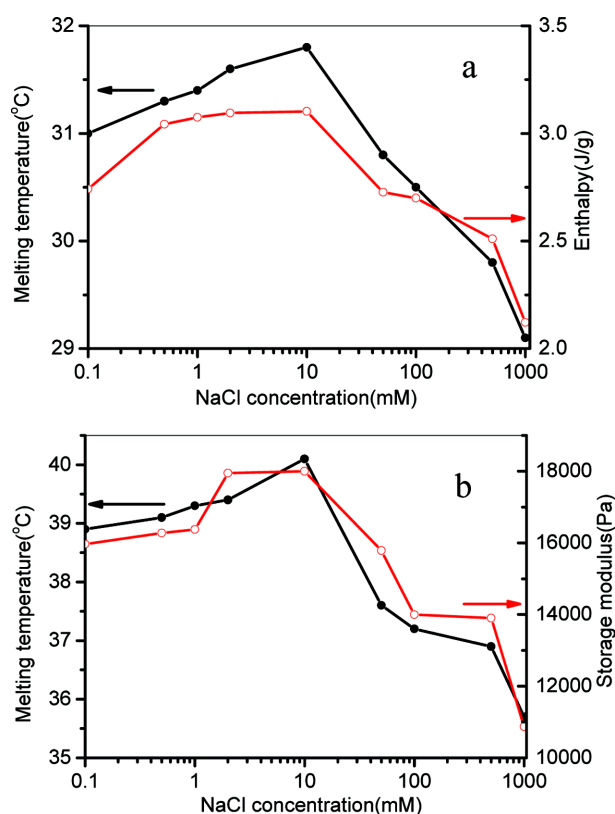
Figure 5. (a) Melting temperature (-●-) and melting enthalpy (-○-) of gelatin gels as a function of NaCl concentration. The melting temperature was determined from the peak temperature of the DSC endotherm; (b) Melting temperature (-●-) and storage modulus (-○-) of gelatin gels as a function of NaCl concentration. The melting temperatures were taken as the point at which the phase angle peaked immediately after a sharp increase.

Figure 4. DSC thermograms for gelatin gels (20.0 wt%) formed on annealing at 20 °C for 60 min at different pH values. Heating rate is 1.0 °C/min.

phobic interaction and hydrogen bond can be destroyed by NaCl addition, which prevented the stabilization of the gel junction sites.[30] Thus the thermal stability of gelatin gels decreased as the salt concentration was above 10 mM. On the other hand, Haug proposed that the reduction in melting temperature was probably resulted from the screening of electrostatic interaction preventing attractive ionic inter-chain bridging and gelation of gelatin.[28]

The raw phase angle of gelatin gels at various NaCl concentrations were explored in the melting process of gelatin gels. In this work the melting temperatures of gelatin gels were taken as the point at which the phase angle peaked immediately after a sharp increase. It was found that the melting temperature first increased with an increase in salt concentration up to 10 mM, and then decreased at higher salt concentration (Figure 5(b)). It is consistent with the DSC results.
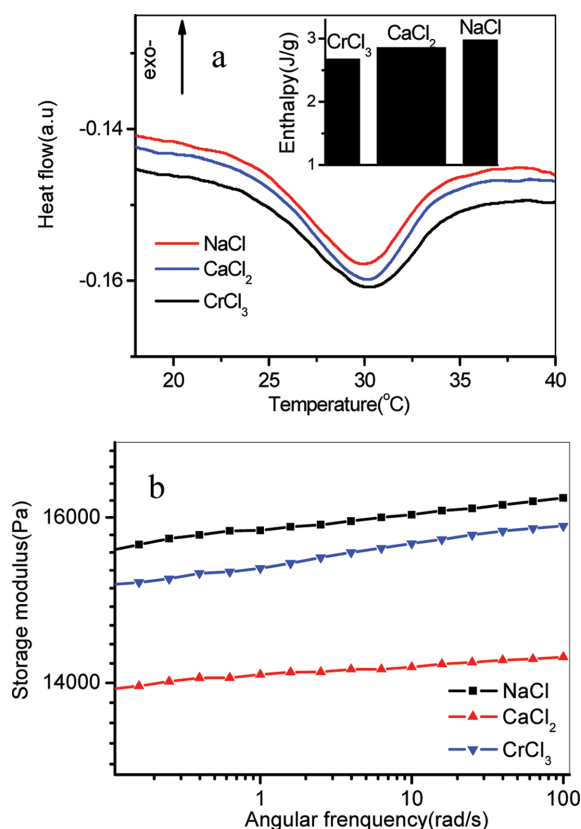
The changes of storage modulus (G') of gelatin gels at a frequency of 10 rad/s with salt concentration were also plotted in Figure 5(b). Similar to the melting temperature, the storage modulus of polymer gels first increased with increasing salt concentration up to 10 mM, and then decreased with further increasing of salt concentration. The slight increase in storage modulus in the presence of small amounts of salt may be resulted from a screening off of long range electrostatic interaction allowing the gelatin chains to be open and extended and promotion of formation of triple helices. When higher concentrations of salt were added, the storage modulus decreased probably due to the destruction of hydrophobic and hydrogen bonds and hence decreasing the ability for gelatin chains to come into contact and form junction zones. This result was in good agreement with earlier work[28] on the storage modulus for gelatin gels with NaCl addition, which was attributed to screening off of short range electrostatic interactions. Moreover, a monotonous decrease of the storage modulus with increasing NaCl concentration was frequently found in previous studies, where the concentration of salt addition was much higher than 10 mM.[15,18,31,32]

Generally, an increase in gel strength of a gelatin gel is accompanied by an increase in melting temperature.[33] In this work, the changes in the storage modulus of gelatin gels under varying salt concentrations seem to have the same salt dependence as melting temperature. It is known that the competition among hydrogen bond, hydrophobic and electrostatic interaction dominates the gelatin solution properties. The salt addition will undoubtedly shield the electrostatic interaction and bring the competition to a new balance. When a small amount of salt was added in solution, the shielding of long range electrostatic interaction will help to open up the gelatin chains more and promote the formation of junction zones. As a result both the storage modulus and melting temperature increased with NaCl addition. However, when the concentration of salt addition was high, the salt ions could break hydrogen bond and hydrophobic interaction, which hampered the growth of triple helices, and thus result in a decrease of both the storage modulus and melting temperature. At 1.0 M NaCl the melting temperature and the storage modulus of gelatin gels decreased to 29.1 °C and 10873 Pa, respectively (30.9 °C and 15792 Pa for salt free sample, respectively. The data of melting temperature is coming from DSC). This result indicates that electrostatic interactions may be essential for the formation and stability of gelatin gels.

Effect of Salt Type. The influence of salt type on the thermal stability of gelatin gels was shown in Figure 6(a).

It shows that the melting temperature for gels bound to various cations followed $Cr^{3+}>Ca^{2+}>Na^+$. When the multivalent cations were introduced into gelatin solutions, two effects should be considered. One was the strong screening effect, due to its more charges. This effect contributes to the formation and stability of triple helices. On the other hand, the multivalent ions such as Ca^{2+} and Cr^{3+} can bind to carboxyl groups on the gelatin chains, which act as crosslinks. This complexation of multivalent ions to protein hampers the movement of gelatin chains, and thus prevents the formation of triple helices. As the concentration of salt addition was low (2.0 mM), and the shielding effect may dominate, which lead to the melting temperature of gels increases with the increasing of valence of salt ions. However, an opposite trend was observed for the melting enthalpy of gels, which is proportional to the helix concentration. The melting enthalpy for gels bound to various cations followed $Na^+>Ca^{2+}>Cr^{3+}$ (inset of Figure 6(a)).The decrease of crosslinks (junction zones) may be due to the binding of multivalent ions to gelatin. It should be noted that the binding mechanism of Ca^{2+} may be different from that of Cr^{3+}. It was found that the gelatin solution at 1.0 M $CaCl_2$ was unable to form gel at 20 °C. However, the same protein solution at 50 °C was prone to from gel at 3.0 mM $CrCl_3$. The crosslink effect of Ca^{2+} probably originated from electrostatic interactions with a character of ionic crosslinking. This crosslinking was assumed to be occurred majorly within a chain. For $CrCl_3$, a strong interchain crosslinking effect coming from multimeric chromium (III) set in, which resulted in the formation of strong gels at high temperature. The result of the vis-

Figure 6. (a) DSC thermograms for gelatin gels at various salts with the same concentration of 2.0 mM. Heating rate is 1.0 $^{\circ}$C/min; (b) Change of storage modulus as functions of frequency for gelatin gels at various salts with the same concentration of 2.0 mM.

coelasticity of gelatin gels with multivalent ions validated above speculation. From Figure 6(b) it was generally observed that the storage modulus of polymer gels with different salt addition conformed to the following orders: $Ca^{2+} < Cr^{3+} < Na^+$. Although there were secondary crosslinks in gelatin gels with Cr^{3+}, the storage modulus of gelatin gels were lower than that with Na^+. This result indicated that the triple helices dominated the gelatin gel strength at low salt concentration.

Conclusions

The preparation conditions, including polymer concentration, annealing time and pH have been found to have insignificant effect on the melting temperature of gelatin gels. However, an increase in annealing temperature will result in an increase in thermal stability of protein gels. As a sequence, an optimum condition of gel preparation for calorimetric test is obtained.

The effect of salt (NaCl) addition was twofold. When the

concentration of salt was low (~10 mM), the shielding of long range electrostatic interactions led to a slight increase in the melting temperature of gelatin gels. However, hydrogen bond and hydrophobic interaction may be destructed at higher salt concentration, which caused a decrease of thermal stability of gelatin gels. The rheological data is consistent with the DSC results.

The thermal stability for gels bound to various cations followed $Cr^{3+} > Ca^{2+} > Na^+$. The slight increase in melting temperature suggested that the shielding effect may dominate. Compared with Na^+, it may be the crosslinking effect of Cr^{3+} which leads to a reduction in the storage modulus of polymer gels. This result suggests that the triple helices dominate the gelatin gel strength at low salt concentration.

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