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A study on the nature of intermolecular links in the cryotropic weak gels of hyaluronan

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ABSTRACT

In this study, the influence of acidification and salting effect on the properties of hyaluronan (HA) aqueous solutions and cryotropic weak gels were investigated by dynamic rheometry, polarizing and optical microscopy, scanning electron microscopy (SEM), X-ray diffraction (XRD), differential scanning calorimetry (DSC) and FTIR spectroscopy. The forming mechanism of HA cryotropic weak gels was also discussed. Experimental results indicated that the HA weak gel showing a thermoreversible property was constructed by entangled bundle-like structures that could be melted at elevated temperature above 70 °C, and that the junction knots of three-dimensional polymeric network were not the ordinary microcrystalline zones that are generally of detectable crystallinity and thermal effect. The intermolecular hydrogen bonding induced from –COOH and $-NHCOCH_3$ in HA chains played a predominant role in respect to the network formation and stabilization of HA weak gel.

1. Introduction

Hyaluronan or hyaluronic acid (HA) is one of the most important and ubiquitous glycosaminoglycan with unbranched molecular chain composed of a repeating disaccharide unit of D-glucuronic acid (GlcUA) and *N*-acetyl-D-glucosamine (GlcNAc) (Lapčík, Lapčík, De Smedt, Demeester, & Chabreček, 1998). HA is a major component of extracellular matrix of vertebrates, where it is involved in maintaining osmotic balance and reducing friction in tissues such as vitreous humor, synovium and cartilage (Balazs, 2009; Fraser, Laurent, & Laurent, 1997). Its unique physiological and biomechanical properties attract not only the interests of scientists, but also interests of industries. Numerous products made from HA or including HA have been commercially available now in medical and cosmetic fields. However, studies on the application of HA, such as for osteoarthritis curing and postoperative adhesion prevention, show HA unable to give a sufficient efficacy due to its water solubility and degradation in vivo. In order to extend the half-life of HA in vivo, chemical modification and derivatization have been generally used, which result in various commercial products for medical use with the ability to slow its degradation in physiological condition (Himeda, Kaneko, Umeda, Miyata, & Miyoshi, 2005). The gelled HA has been proved not only to prevent degradation, but also to give a high molecular weight and controllable shape of HA molecules.

Although it is known that HA is a kind of non-gelling polysaccharide, cryotropic gels and viscoelastic putty gels of unmodified HA are found to be able to form under certain conditions. The viscoelastic putty gel can be obtained by adjusting the pH of the concentrated HA solution to 2.5 at physiological ionic strength (Balazs, 1966). But such a putty gel is unstable, and it will convert to the solution state again after adjusting the pH below 2.0 or above 3.0. Therefore, this considerable limitation of HA putty gel prevents its application. The cryotropic gel of HA, produced by once or repeating freeze-thawing, was firstly reported by Okamoto & Miyoshi (2002). This kind of gel overcame the drawbacks of putty gel due to its stronger stability under different pH or temperature conditions. It has been pointed out that HA cryotropic gel had the potentiality to be used as a substitute not only for native HA preparations but also for some biopolymers in a broad range of medical applications (Himeda et al., 2005). For instance, the studies of rat cecal abrasion model showed that HA cryotropic gel was effective in reducing postoperative adhesions (Himeda et al., 2003; Himeda, Umeda, Miyata, & Miyoshi, 2004; Himeda et al., 2005; Miyoshi & Okamoto, 2002).

It has been found that many polymers including both synthetic polymers and natural polymers such as polyvinyl alcohol (PVA) (Lozinsky, Domotenko, Zubov, & Simenel, 1996; Yokoyama, Masada, Shimamura, Ikawa, & Monobe, 1986), chitosan (Orrego & Valencia, 2009), xanthan (Giannouli & Morris, 2003), locust bean gum (Lozinsky, Damshkaln, Brown, & Norton, 2000c; Tanaka,

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Hatakeyama, & Hatakeyama, 1998), agarose (Lozinsky et al., 2008), amylopectin (Lozinsky, Damshkaln, Brown, & Norton, 2000a; Lozinsky, Damshkaln, Brown, & Norton, 2000b), amylose (Lozinsky et al., 2000b), cereal β-glucan (Lazaridou & Biliaderis, 2004; Lazaridou, Vaikousi, & Biliaderis, 2008; Vaikousi & Biliaderis, 2005) and carboxymethylated curdlan (Wu & Zhang, 2010) etc. are capable of forming cryotropic gels. These cryogels are generally interconnected macroporous and thermal-reversible with heterogeneous cryostructures (Lozinsky et al., 2000c). The formation and strength of these cryogels are affected by a series of factors, such as molecular weight, concentration, freezing and thawing temperature, thawing rate, freezing duration and the number of freeze-thawing cycle, etc. (Lozinsky et al., 2000c).

Among polymeric cryogels, the physicochemical bases of cryogelation of PVA solutions have long been investigated (Lozinsky & Plieva, 1998). Briefly, under freeze-thawing treatment, PVA chains are able to form ordered structures known as microcrystalline zone (Yokoyama et al., 1986), acting as junction knots of the network of the cryogel. The studies on the nature of intermolecular links in most of polysaccharide cryogels suggested that the major mechanism responsible for stabilization of junction zones was hydrogen bonding (Giannouli & Morris, 2003; Lazaridou & Biliaderis, 2004; Lazaridou et al., 2008; Lozinsky et al., 2000a, 2000b, 2000c, 2008; Orrego & Valencia, 2009; Vaikousi & Biliaderis, 2005; Wu & Zhang, 2010). However, the exact forming mechanism of HA cryogel has not been fully understood (Collins & Birkinshaw, 2008; Himeda et al., 2005; Okamoto & Miyoshi, 2002). The complexity of forming mechanism of HA cryogel might be mainly derived from its chemical structure, which includes not only massive –OH groups as in PVA and in galactomannan, but also $-COO^-$ and $-NHCH_3$ groups along with possible hydrophobic regions. Okamoto et al. tentatively suggested that the formation of HA cryogel was a cooperative phenomenon resulting from the interaction of hydrophobic and hydrogen bonds between HA molecules (Okamoto & Miyoshi, 2002). Collins and Birkinshaw (2008) presumed that either a crystallization process or at least a process analogous to crystallization occurred during the formation of HA cryogel. In the present work, in order to get a clearer understanding on this prospective material, the properties and forming mechanism of HA cryogel were further explored and discussed.

2. Materials and methods

2.1. Materials

The HA sample used in this study was obtained from Shandong Freda Biopharm Co., Ltd. (China) in the form of sodium salt originated from bacterial fermentation. A detailed molecular characterization was carried out using both size exclusion chromatography–light scattering (SEC–LS) and horizontal agarose gel electrophoresis (AGE). For SEC measurement, the sample was characterized using the Viscotek TDA 305 instrument (Malvern Instruments, USA) equipped with two Viscotek A6000 M columns. Viscotek TDA 305 instrument consisted of two modules: (i) a SEC integrated system composed of a specific pump for size exclusion chromatography, an in-line solvent degasser and an autosampler; and (ii) a TDA module (triple detector array) consisting of an refractive index (RI) detector, a four-bridge viscosimeter (VIS), and an LS detector. The latter consisted of a right-angle light scattering (RALS) detector and an low-angle light scattering (LALS) detector that perform measurements of the scattered light at 7° with respect to the incident beam. The sample concentration was 0.5 mg/ml. The experimental conditions consisted of 0.15 M $NaNO_3$ as mobile phase, 30 °C of temperature, 0.7 ml/min of flow rate and 100 μl of injection volume. The refractive index increment dn/dc was set as 0.144 ml/g. Data was collected and analyzed by using OmniSEC software. The SEC results showed that the weight average molecular weight, the polydispersity index and the z-average radius of gyration of the HA sample were 1.29×10^6, 2.16 and 119.5 nm, respectively. In addition, the hydrodynamic radius (61.9 nm) and the intrinsic viscosity (1288.6 ml/g) were also obtained. AGE was performed using a Sub-Cell Model 96 horizontal gel electrophoresis system (Bio-Rad, UK) with a set of monodisperse hyaluronan standards, LoLadder (2.7×10^4 to 5×10^5), HiLadder (5×10^5 to 1.5×10^6) and MegaLadder (1.5×10^6 to 6.09×10^6) (Hyalose L.L.C, USA), following the procedures developed based on the work by Lee and Cowman (1994). The characterization results showed that the weight average molecular weight and the polydispersity index of this sample were 1.52×10^6 and 1.7, respectively, in agreement with those from SEC.

All the other chemicals used in the study were purchased from Sinopharm Chemical Reagent Co., Ltd. (China) and of analytical grade.

2.2. Preparation of HA cryogels

10 mg/ml HA solutions were prepared by weighing appropriate amounts of HA in vials and adding the solvent. The vials were immediately sealed and positioned on a roller mixer to tumble until complete solubilization of HA. The pH of the HA solutions was adjusted to 1.5 using 1 M HCl, then the acidified solutions were centrifuged at 10,000 rpm for 30 min to remove possible trapped air bubbles. 4.5 ml of centrifuged acidified solutions were transferred to 25 ml beakers. The beakers were tightly sealed with parafilms and subsequently stored at −20 °C for a required time. Subsequently, the frozen acidified solutions were allowed to thaw at 25 °C for one day, and then sponge-like cryotropic weak gels were obtained

The freeze-dried samples were obtained by introducing the above weak gels to a freeze-dryer for at least 24 h, and then were used in the following XRD, FTIR and SEM experiments.

2.3. Dynamic rheometry

The rheological measurements were carried out using a rotational rheometer AR G2 (TA Instruments, USA) with a parallel plate geometry (40 mm in diameter and 1 mm in gap). The temperature was regulated by a circulating water bath and peltier system. After removing the clear liquid phase and wiping off the gelled samples with a filter paper the obtained HA weak gels were ready for rheological measurements as HA solutions did. To avoid the evaporation of water from the sample during the measurement, a thin layer of low-viscosity silicone oil was placed on the periphery surface of the sample held between the plates. The measurements were performed within the linear viscoelastic region at 25 °C.

2.4. Polarizing and optical microscopy

The polarizing microscopic and optical microscopic observations were achieved by a Leica DMLP polarizing optical microscope (Leica Microsystems GmbH, Germany) with and without employing the polarizer, respectively. An automatic hot-stage (Linkam TH960, Linkam Scientific Instruments Ltd., UK) with a precision of ±0.1 °C was employed in the observation under elevated temperature. The samples that were sandwiched between pre-cleaned glasses were observed.

2.5. Scanning electron microscopy (SEM)

SEM was performed using an S-2150 microscope (Hitachi High-Technologies Corp., Japan) operated at 20 kV. The freeze-dried HA

weak gel was gold-coated using a sputter coater and then photographed.

2.6. X-ray diffraction (XRD)

XRD patterns were obtained using a *D*/max-2200/PC X-ray diffractometer (Rigaku Corporation, Japan) with Cu K_α ray. The voltage and current were 40 kV and 20 mA, respectively. The scan rate was 4°/min, and the 2θ scan range was from 5° to 50°.

2.7. Differential scanning calorimetry (DSC)

DSC measurements were carried out on a Micro DSC III (Setaram Instrumentation, Caluire, France). After removing the clear liquid phase and wiping off the gelled samples with a filter paper about 700 mg of HA weak gel was hermetically sealed into a standard Hastelloy cell and the same amount of distilled water was used as the reference. The temperature was raised and lowered in the range from 10 to 90 °C at a scan rate of 1 °C/min.

2.8. FTIR spectroscopy

FTIR spectra were recorded in a range from 4400 to 400 cm^{-1} on a Spectrum 100 FTIR spectrometer (Perkin Elmer, Inc., USA). The samples were fixed in KBr discs.

3. Results and discussion

Acidifying the HA aqueous solution using 1 M HCl not only protonates HA but also raises the concentration of NaCl in the solution. Therefore, before dealing with the HA cryotropic weak gel, to shed light on the influence of the addition of NaCl and acidification on the behavior of HA molecules, the rheological properties of HA solutions with and without NaCl, and with and without acidification were investigated, respectively (Fig. 1). Fig. 1(a) showed the comparison of frequency dependence of viscoelasticity for the 10 mg/ml HA solutions with and without 0.1 M NaCl. For the pure aqueous HA solution, it behaved like a typical viscoelastical fluid with the feature that at low angular frequencies, the loss modulus G'' was larger than the storage modulus G', and both of the moduli showed a pronounced frequency dependence whereas at high angular frequencies, G' was higher than G'' and both of the moduli only showed a limited frequency dependence. Once upon addition 0.1 M NaCl, both of the moduli were decreased, which is attributed to the screening effect of Na^+ on the electrostatic repulsion of ionized HA molecules and the HA molecules thus adopting a more compact conformation. Furthermore, the crossover frequency, at which G' and G'' are crossed to each other, shifted to a higher frequency, indicating that the viscosity property was more outstanding for HA solution in the presence of NaCl. These phenomena were in good agreement with the previous observations (Fujii, Kawata, Kobayashi, Okamoto, & Nishinari, 1996; Kobayashi, Okamoto, & Nishinari, 1994; Mo, Takaya, Nishinari, Kubota, & Okamoto, 1999). In addition, although NaCl is generally considered as a relatively weak salting-out salt that can increase the viscoelasticity of the solution of noncharged neutral polysaccharide of konjac glucomannan (Yin, Zhang, Huang, & Nishinari, 2008), its salting-out effect on polyanion of HA can be ignored in comparison with its screening effect of electrostatic repulsion in the present work. Both of the solutions with and without 0.1 M NaCl exhibited a slope very close to 2 in logG' versus logω plots and a slope very close to 1 in logG'' versus logω plots in the terminal region. This means that the addition of NaCl does not change the essential nature of the rheological property of the HA solution.

However, the effect of the addition of HCl on the rheological property of HA solution is very different. Fig. 1(b) showed the compared change in both of the storage and loss moduli for HA aqueous solutions with and without acidification. After the HA solution was acidified to pH 1.5, G'' became higher than G' in the whole tested range of frequency, still indicating a pronounced viscous fluid behavior. This phenomenon was also derived from the screening effect of hydrogen ions on the electrostatic repulsion of HA molecules during acidification. Notice, however, for the acidified HA solution, both of the moduli showed limited frequency dependence even especially at low angular frequencies, and the slope of logG' versus logω plots (0.67) was much smaller than 2 and the slope of logG'' versus logω plots (0.61) was much smaller than 1 in the terminal region. This fact of terminal effect strongly implied the occurrence of some association between HA molecules. Fig. 1(c) and (d) further showed that the addition of H^+ or Na^+ could screen the electrostatic repulsion of HA in the presence of NaCl or HCl and further thereby decreasing the moduli of the solution. On the other hand, while the slopes of logG' versus logω plots (0.67 for acidified HA solution, 1.03 for acidified HA solution in the presence of 0.1 M NaCl, ca. 2 for aqueous HA solutions with and without NaCl) in the terminal region are much less than 2, the degree of the deviation from 2 in the slope of logG' versus logω plots in the terminal region, to the large extent, reflects the degree of molecular associations between HA chains in the solutions. Such associations, likely hydrogen bonds as suggested for many polysaccharide hydrogels (Lazaridou & Biliaderis, 2004; Lazaridou et al., 2008; Lozinsky et al., 2000a, 2000b, 2000c, 2008; Vaikousi & Biliaderis, 2005; Wu & Zhang, 2010) in the light of its chemical structure, their enhancement as the HA concentration is increased by conversion of water to ice during freezing, and their sustainability on thawing, should be essential and responsible for stablilization of network structures in the hydrogels. An increase in hydrogen ions in solution would decrease ionization of carboxyl groupings of HA, thus contributing to hydrogen bonding between HA chains. Excessive Na^+ would occupy the sites of protonation of $-COO^-$ in HA, thereby hindering the formation of hydrogen bonding, resulting in the remarkable decrease in both of the G' and G'' in the terminal region as seen in Fig. 1(d).

Next, rheological properties of the HA weak gels were dealt with (Fig. 2). Here the term of weak gel is used since the loss tangent of the obtained gels is large compared with that of a true gel. The conspicuous changes from solution to freeze-thawing induced weak gel were then identified as shown in Fig. 2(a) by comparative rheological measurements. Fig. 2(a) showed the frequency dependence of both of the storage and loss moduli for acidified 10 mg/ml HA aqueous solution and the weak gels obtained by freezing such solutions for 3 days (gel-A3) or 8 days (gel-A8) and thawing. These mechanical spectra reflected the effect of freeze-thawing treatment on the formation of weak gels and indicated that the gel strength was increased with increasing the freezing time. Long freezing time seems to be propitious to force the alignment of HA chains to form associations, which then remain intact on thawing, as the junction zones of network in the gel.

As a comparison, the frequency dependence of G' and G'' for the acidified 10 mg/ml HA solution in the presence of 0.1 M NaCl and for the samples obtained by freezing such solutions for 3 days (solution-B3) and 8 days (gel-B8) and thawing, was shown in Fig. 2(b). It was found that in the presence of NaCl, gel from the acidified 10 mg/ml HA solution could not be formed by freeze-thawing treatment for 3 days (see solution-B3 with $G' < G''$ even at the highest frequency) and that the strength of gel-B8 formed in 8 days was significantly lower than that of gel-A8 (Fig. 2(a)), only about the same magnitude of that for gel-A3. The inhibitory action and weakening effect of NaCl with respect to the gel strength was also found in other hydrogel systems in which the hydrogen bonds played a dominant role in the stabilization of hydrogel network (Lozinsky et al., 1996, 2000c). The moduli-frequency profile for

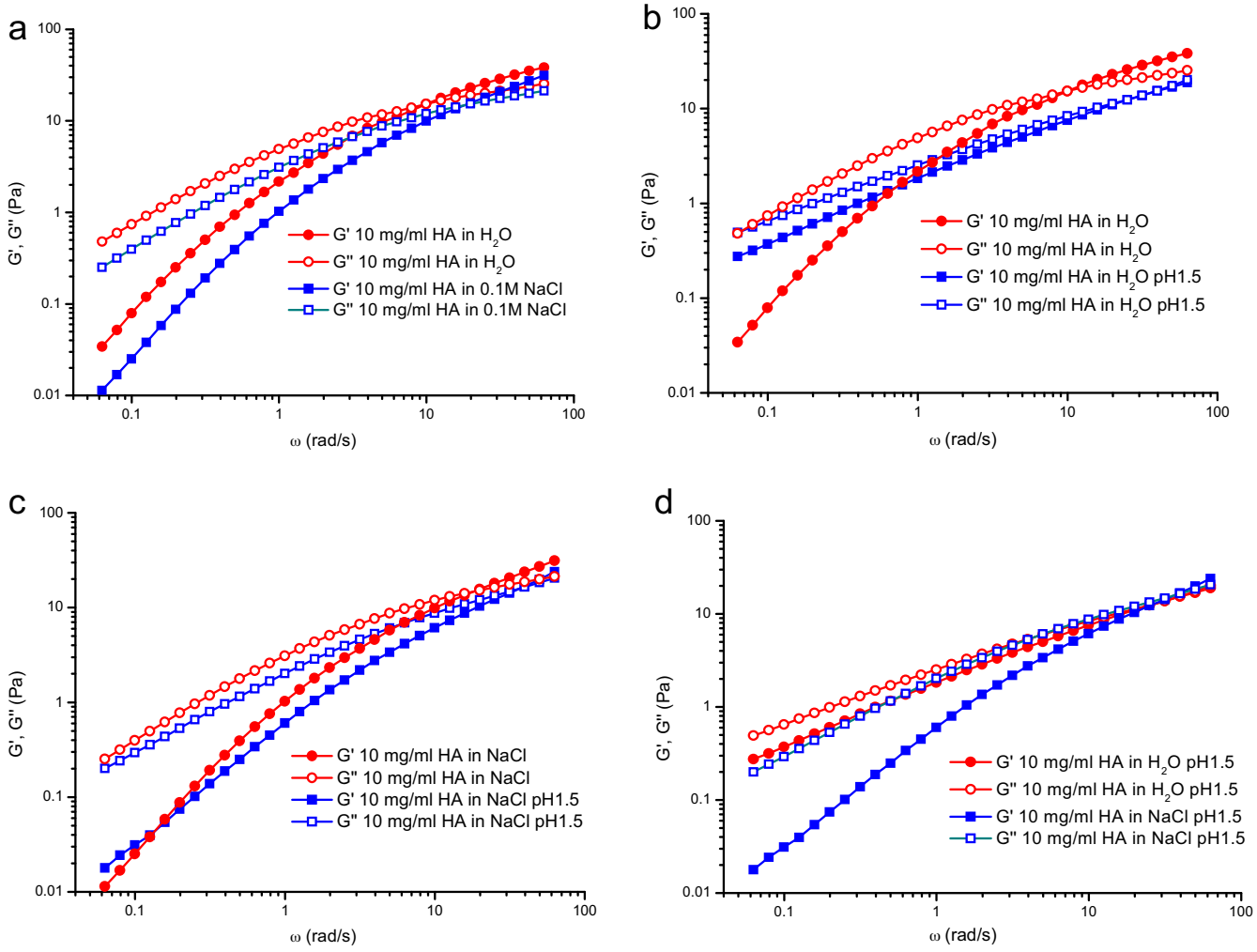


Fig. 1. The frequency dependence of G' and G'' for (a) 10 mg/ml HA aqueous solutions with and without 0.1 M NaCl, (b) 10 mg/ml HA aqueous solutions with and without acidification, (c) 10 mg/ml HA in 0.1 M NaCl solutions with and without acidification and (d) acidified 10 mg/ml HA solutions with and without 0.1 M NaCl.

solution-B3 with the feature of a viscous system strongly suggested that there existed less intermolecular association or entanglement in the solution, essentially indicating that the presence of 0.1 M NaCl remarkably interfered the formation of three dimensional network structures. As for the decrease in moduli for solution-B3 (lower than those for the initial solution), it is attributed to the decrease in molecular weight of HA by degradation that likely occurred mainly on thawing at low pH and 25 °C, during which many H^+ did not join in the protonation and association of HA.

It is reasonable to assume that the intermolecular links responsible for the formation of network in HA weak gel are multiple interchain hydrogen bonds. In order to prove this inference, we also tried to prepare HA weak gel by adding different chaotropic agents to the initial pure aqueous HA solution. Urea, a chaotropic agent, is known to interfere with the hydrogen bonds in aqueous media (Lozinsky et al., 2000c; Tanford, 1968) and Na_2SO_4, an anti-chaotropic agent (stronger salting-out salt than NaCl), was known to 'strengthen' hydrogen bonds and promote the structuring of water into clusters (Lozinsky et al., 2000c). In comparison with pure aqueous HA solution, Fig. 2(c) showed that when 0.1 M urea was added, no gel was formed. The sample after freeze-thawing still behaved like a viscous fluid with $G'' > G'$. Previous work has showed that urea can reduce the hydrodynamic domain size of the polymer and disrupt the hydrogen bonding between HA chains (Hardingham, 2004; Hirano & Kondoike, 1974). But upon addition of Na_2SO_4, cryotropic weak gel could form and its strength was enhanced in comparison with that from pure aqueous HA solution. These results strongly evidenced that the hydrogen bonding must play a dominant role in the formation and stabilization of HA weak gel network. By adding urea to HA solutions, however, Nishinari, Mo, Takahashi, Kubota, & Okamoto (2002) suggested that in HA solutions there are no intermolecular hydrogen bonds, and the disruption of the hydrogen bonds due to urea, even if it occurs, does not affect significantly the rheological behavior of HA solutions. This implied that intermolecular hydrogen bonds, mainly induced by OH groups, which lead to the formation of network in many gelling polysaccharides, do not seem to exist for HA in its solution state. Generally, for a 1% HA aqueous solution its pH is ca. 7.7. In our case, the solution is acidified (pH = 1.5), so the contribution of the involvement of –COOH groups is important for the intermolecular hydrogen bonding in the final gel. On the other hand, the lack of effective involvement of OH groups in the formation of intermolecular hydrogen bonds may explain why the freeze-thawing procedure of HA does not produce a strong gel but only a weak gel.

To investigate the likelihood of the scenario that the 3D structure of HA weak gels was stabilized mainly by multiple interchain hydrogen bonds in the junctions of the polymeric network (zones of microcrystallinity) as suggested for the well-studied cryogels of PVA (Lozinsky & Plieva, 1998; Yokoyama et al., 1986) and many other polysaccharide hydrogels (Lazaridou & Biliaderis, 2004;

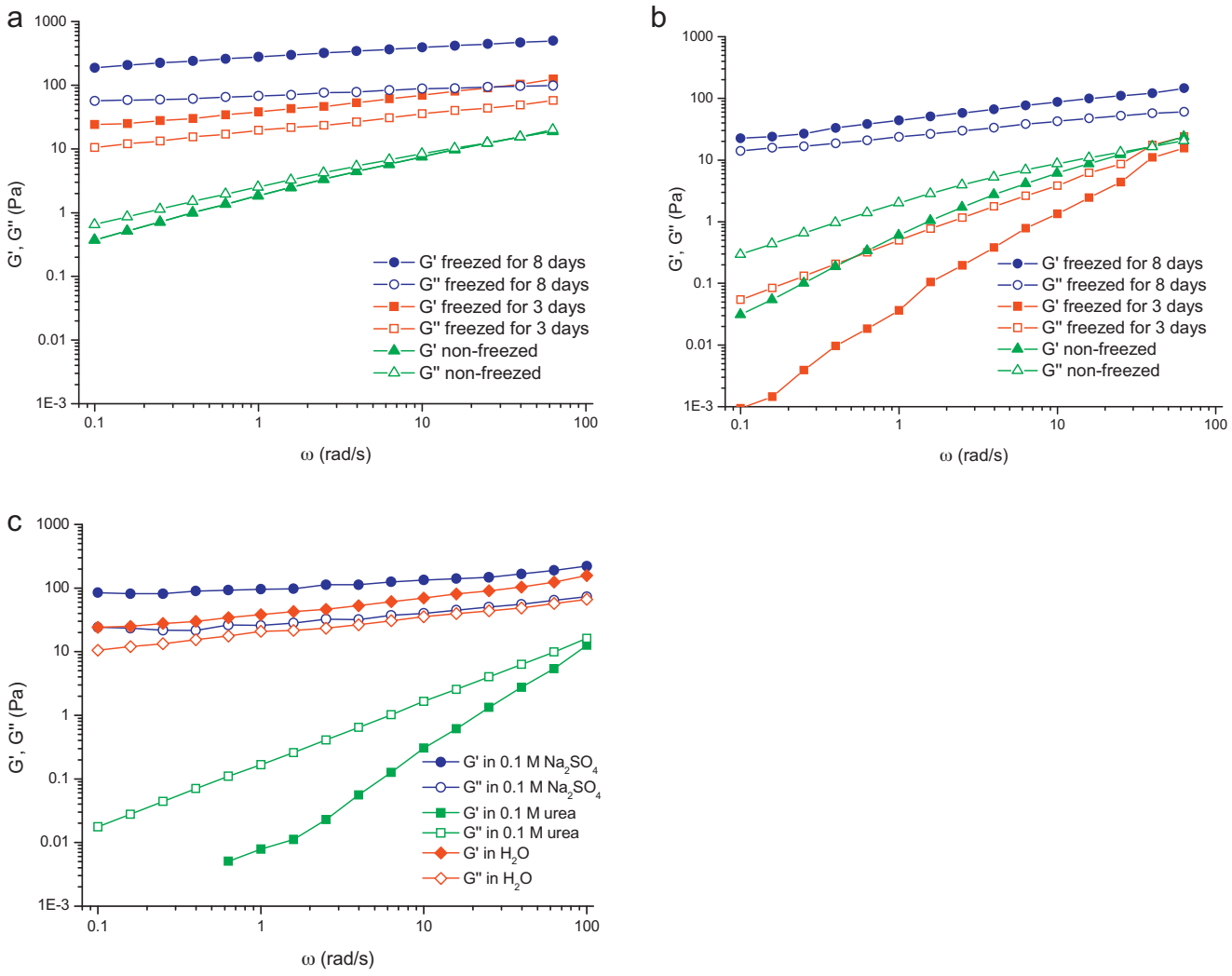
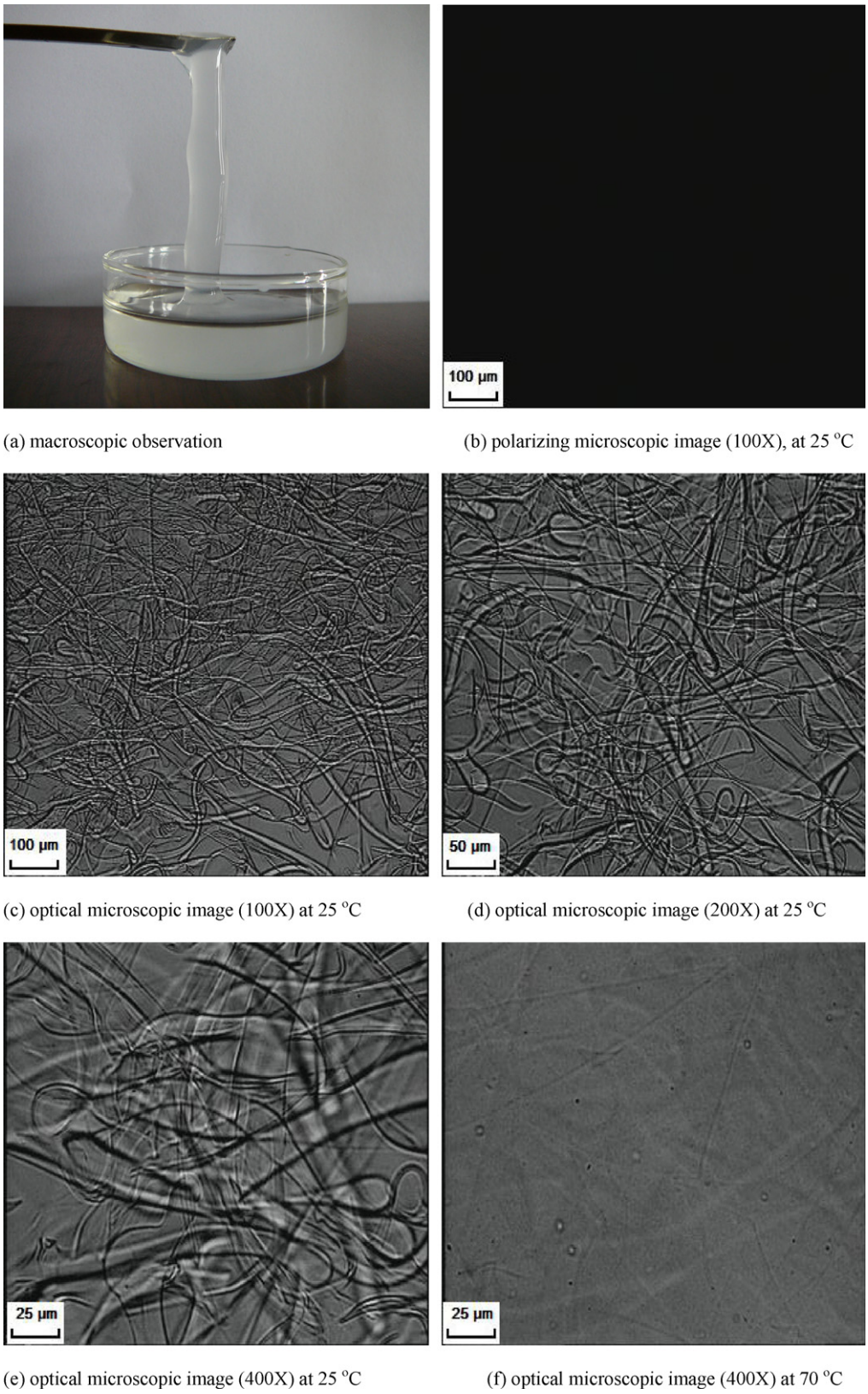


Fig. 2. The frequency dependence of G' and G'' for (a) the acidified 10 mg/ml HA aqueous solution and for the cryotropic weak gels obtained by freezing such solutions for 3 and 8 days and thawing, respectively; (b) the acidified 10 mg/ml HA solution in the presence of 0.1 M NaCl and for the samples obtained by freezing such solutions for 3 and 8 days and thawing, respectively; (c) the acidified 10 mg/ml HA in pure H_2O, in 0.1 M urea and in 0.1 M Na_2SO_4, respectively (the solutions were frozen for 3 days and then thawed).

Lazaridou et al., 2008; Lozinsky et al., 2000a, 2000b, 2000c, 2008; Vaikousi & Biliaderis, 2005; Wu & Zhang, 2010), further various experiments were undertaken involving polarizing and optical microscopy, SEM, XRD, DSC and FTIR. Fig. 3(a) showed that the appearance of HA weak gel formed by freeze-thawing was soft and formless. Furthermore, Fig. 3(b) showed that nothing could be found in polarizing microscopic image of the HA gel. The optical microscopy is a useful method and has been used to identify the microstructure features of polymer hydrogel in many studies (Lazaridou et al., 2008; Lozinsky et al., 2008; Podorozhko, Kurskaya, Kulakova, & Lozinsky, 2000). It could be seen in Fig. 3(c–e) that there were a lot of macroscopical bundle-like structures in the gel, which entangled with each other forming network. Upon heating the HA gel to 70 °C, the network structures melted and disappeared gradually (Fig. 3(f)). This phenomenon indicated that the obtained weak gel was thermal reversible as many other hydrogels (Giannouli & Morris, 2003; Lazaridou & Biliaderis, 2004; Lazaridou et al., 2008; Lozinsky et al., 1996, 2000a, 2000b, 2000c, 2008; Orrego & Valencia, 2009; Vaikousi & Biliaderis, 2005; Wu & Zhang, 2010; Yokoyama et al., 1986). In additions, SEM was also carried out to further verify the microstructure of HA gel. Fig. 4 was the SEM images of HA gel with different magnifications on the same position of the sample. It was seen that the freeze-dried gel was of entangled bundle-like structures with a polyporous feature (Fig. 4(a) and (b)), in agreement with the observation of our optical microscopic results (Fig. 3). According to the scale bars in SEM images, the diameters of the "bundles" were in the range from ca. 1 to 15 μm. It was known that the diameter of HA chain was of about 0.5 nm (Cowman & Matsuoka, 2005), therefore, these "bundles" should be the aggregates of HA molecular chains. To increase the magnification to zoom in on details of the "bundles" as seen in Fig. 4(c) and (d), some small granules on the surface of the bundles were observed, which are crystallized NaCl. The existence of NaCl crystals was also evidenced in the following XRD experiments.

XRD experiments were achieved for identifying whether the microcrystalline zones existed in HA weak gel. Fig. 5 showed that there were broad peaks at 2θ of ca. 10° and 23° for the native HA powders on account of their low crystallinity. This is in good agreement with the XRD pattern by Lee, Lee, Song, and Park (2003), low crystallinity showing weak peak intensity. And it is also in fair agreement with the work by Jin et al. (2008), in which their HA XRD peaks are sharper than ours, which can be attributed to the higher crystallinity of their sample. For freeze-dried HA gel (frozen for 3 days), the broad peaks were substantially the same with those of native HA powders. This result also suggested that the HA weak gel was of the same poor crystallinity with native HA powders, none of



(a) macroscopic observation

(b) polarizing microscopic image (100X), at 25 °C

(c) optical microscopic image (100X) at 25 °C

(d) optical microscopic image (200X) at 25 °C

(e) optical microscopic image (400X) at 25 °C

(f) optical microscopic image (400X) at 70 °C

Fig. 3. Typical images of gels of HA (pure water solvent, 3 days freezing). Macroscopic observation (a), polarizing microscopic image (b) and optical microscopic images with different magnification at different temperature (c–f).

new obvious crystalline structure being observed in the HA gel. This XRD result without obvious crystallinity in HA weak gel, though beyond imagination, has a good consistence with the above observations in polarizing light microscopy. It is known that the cryogels become stronger with increasing number of freeze-thawing cycles (Watase & Nishinari, 1985a, 1985b). For a PVA cryogel, the increase of crystallinity was also observed by XRD with increasing number of freeze-thawing cycles (Yokoyama et al., 1986). In order to

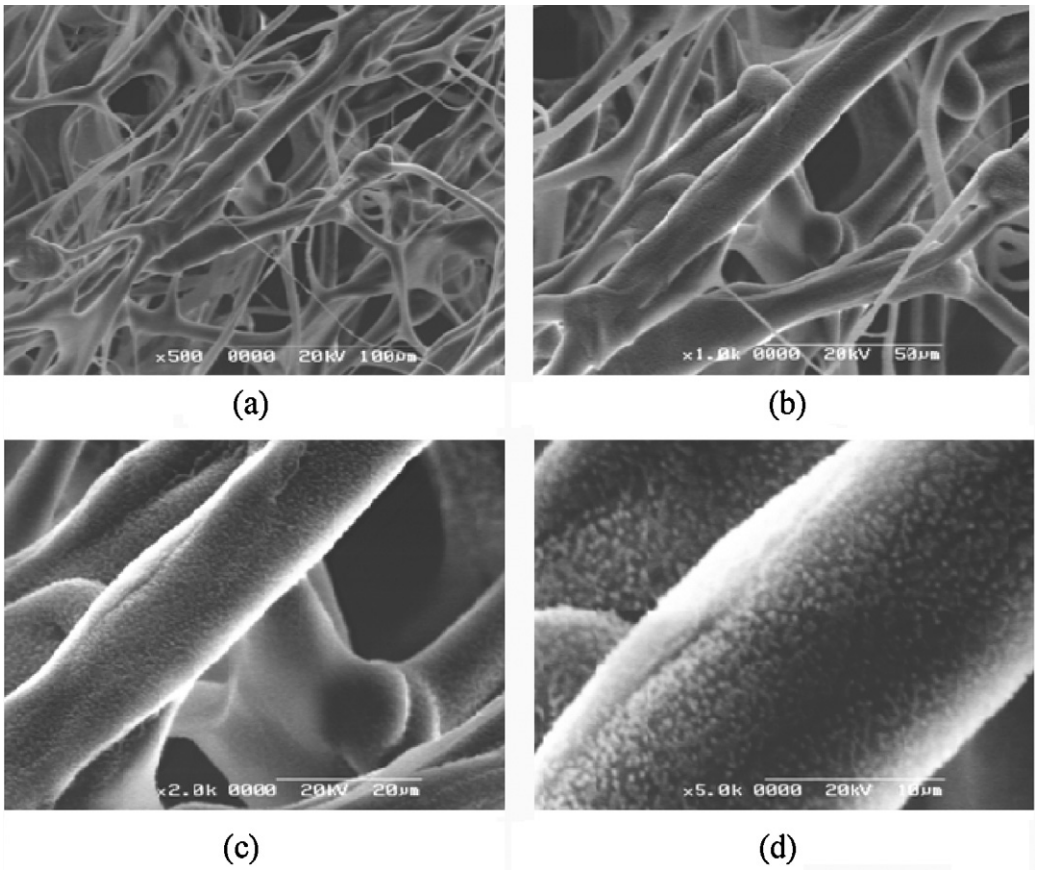


Fig. 4. SEM images of the freeze-dried HA weak gel with different magnification: (a) 500×, (b) 1000×, (c) 2000×, and (d) 5000×.

check whether the similar phenomenon can be observed for a HA weak gel, further XRD experiments were carried out for the HA gels prepared by repeating freeze-thawing cycles (one freeze-thawing cycle means freezing for 22 h and then thawing for 2 h). The XRD spectra of HA gels (5 cycles and 7 cycles) were also shown in Fig. 5. The result showed that the broad peaks were almost identical to that of the HA gel without repeated freeze-thawing (frozen for 3 days). This fact is different from that for the PVA cryogel. The difference may be related to that the PVA itself is a highly crystalline material (Yokoyama et al., 1986), but HA is not. In addition to this, the concentration of PVA solution for preparing a cryogel is generally in the range of 7–15% (Lozinsky & Plieva, 1998), below which it is difficult to obtain a PVA cryogel. However, to prepare such a highly concentrated HA solution is nearly impossible.

In Fig. 5, the spectrum of freeze-dried HA gel showed that there were additional two sharp peaks at 2θ of ca. 32° and 46°, which compared well with the typical XRD peaks for NaCl. This result indicated that the Na^+ of HA and the Cl^- of added HCl in the

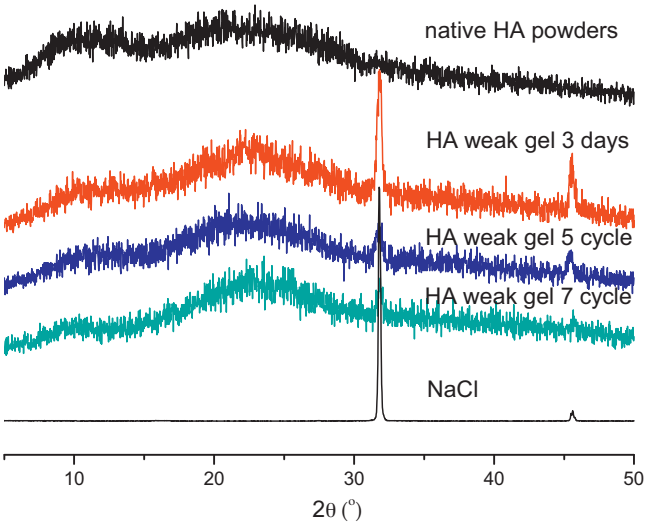


Fig. 5. X-ray diffraction spectra of freeze-dried HA gel (3 days, 5 cycles, and 7 cycles), native HA powders and the salt of NaCl.

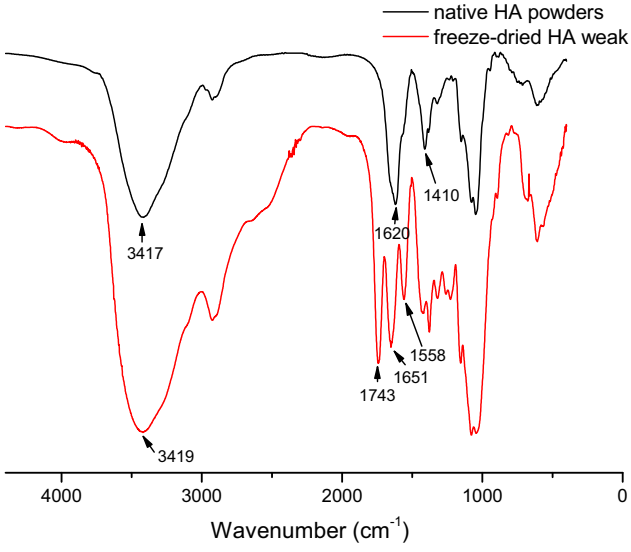


Fig. 6. FTIR spectra of native HA powders and freeze-dried HA gel.

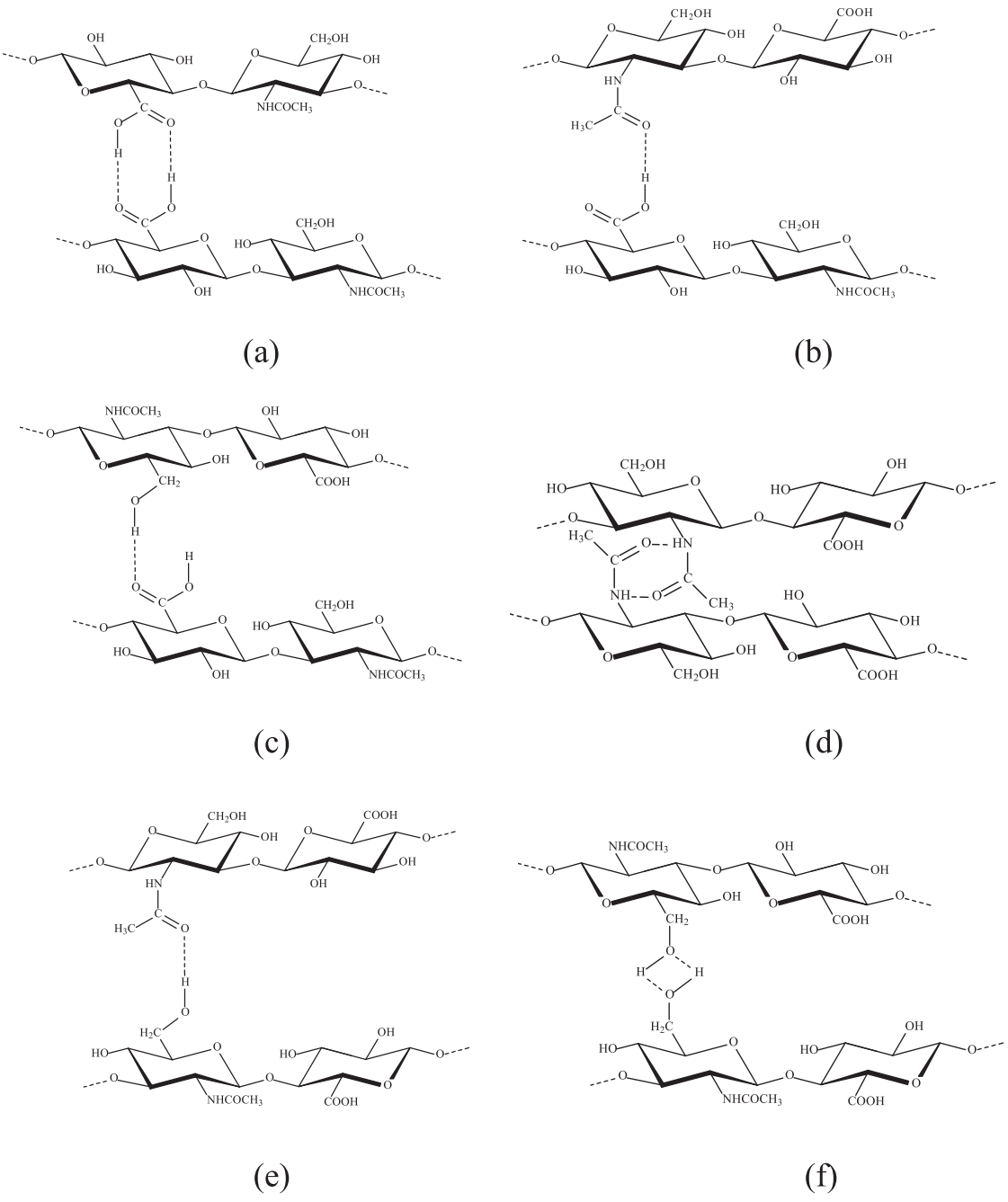


Fig. 7. The possible forms of hydrogen bonds between –COOH, –OH and –$NHCOCH_3$ groups in HA gel.

initial solution were crystallized out as NaCl crystal in the final HA gel sample, therefore, the remained H^+ of added HCl should bond with the anion of HA and participate in the formation of HA gel.

No thermal effect was detected on both heating and cooling processes for HA gels in the temperature range from 10 to 90 °C in the DSC measurements though the machine is of high sensitivity. The DSC curve of the HA gel was practically indistinguishable from the baseline (data not shown). Although the results from polarizing light microscopy, XRD and DSC showed some consistency, it is conceivable that the molecular associations, which are responsible for the stabilization of network, as the junction zones of the hydrogel, could include microcrystalline zones, intermolecular hydrogen bonding or even intermolecular hydrophobic interaction for a physically corsslinked gel. The formation of microcrystalline zones has been proved as the origin for the formation of PVA cryogel (Yokoyama et al., 1986). However, for the HA weak gel, crystalline structures in this work could not be found and traced by our polarizing light microscopy, XRD and DSC. Thus the present work might make clear that, there were no microcrystalline zones in the HA weak gel. Alternatively, the microcrystalline zones in HA gel might be too few, too small to be detected by the above techniques.

Since weak gels are formed, there must be some intermolecular links in the gels. In order to clarify the nature of these links further, the FTIR measurements were also carried out for native HA powders and freeze-dried HA gel as shown in Fig. 6. Our FTIR spectrum of native HA powders was the same with the previous report (Gilli, Kacurakova, Mathlouthi, Navarini, & Paoletti, 1994). The wide peak at 3417 cm^{-1} is corresponding to the stretching vibration of –OH

groups. No obvious shift of this peak was observed for freeze-dried HA gel (3419 cm^{-1}). The two peaks at 1620 and 1410 cm^{-1} were assigned to the asymmetrical C=O stretching and symmetrical C–O stretching of $–COO^-$, respectively. However, for freeze-dried HA gel, some obviously different features were observed. The peak at 1743 cm^{-1} was assigned to asymmetrical C=O stretching of –COOH, which indicated the existence of –COOH groups in the HA gel. In addition, the two bands at 1651 and 1558 cm^{-1} were assigned to amide I and amide II, respectively, which could not be observed for native HA powders. The fact that the amide I and amide II bands were clearly resolved in the protonated form of HA where the asymmetric stretching of carboxylate group greatly decreased (Gilli et al., 1994; Orr, 1954), further evidently confirmed the existence of –COOH groups in the HA gel.

The FTIR results indicated that the initial HA sample was transformed from the saline form to the acidified form in the HA gel. According to the chemical structure of HA, it was reasonable to infer that the intermolecular hydrogen bonding occurred among the numerous –COOH, $–NHCOCH_3$ and –OH groups on the backbone of HA, and it might be in several associated forms as shown in Fig. 7. Further, taking into account the FTIR results, no obvious band shift was found for the stretching vibration of –OH group in HA gel, the hydrogen bonding induced from –OH groups might be weak in comparison with those involving –COOH and $–NHCOCH_3$ groups. Based on the patterns of hydrogen bonding in Fig. 7, the location of hydrogen bonds between HA chains might be discrete due to steric hindrance, and the necessary regularity and the perfection to form microcrystalline zones might be scarce. In such a way, microcrystalline zones cannot be constructed effectively in the weak gels. Quite similarly, it is reported recently that carboxymethyl cellulose (CMC) gel can be prepared by immersing the CMC paste into an acid or an acid aqueous solution, or prepared by mixing CMC and acid solution (Takigami et al., 2007, 2008, 2009; Takigami, Nagasawa, Maehara, Takigami, & Tamada, 2010). According to them, the forming mechanism for the CMC gel also involves replacing the Na^+ in CMC with H^+ in the acid solution to promote hydrogen bonding. In addition, it can also be found that for both HA and CMC gels, the gel strength depends on the concentration of the polymer and the acid (Okamoto and Miyoshi, 2002; Takigami et al., 2007, 2008, 2009).

It is reported that HA from various sources is of different degree of purity. The main impurities, depending on the source and purification method, are bacterial endotoxines, chondroitin sulfates, proteins, nucleic acids, sodium chloride, and heavy metals (Lapčík et al., 1998). It is also demonstrated that HA isolated from different sources (human umbilical cord, bovine vitreous, bacterial and rooster comb) all contain some amount of protein, but human umbilical cord HA and bovine vitreous HA have by far more protein (Shiedlin et al., 2004). Although the amide and carboxyl groups of protein may provide another possibility of the intermolecular hydrogen bonding that may also contribute to the formation of the HA cryotropic weak gel, this contribution should be very small because of the very low amount of protein in bacterial HA used in the present work.

As for the effect of hydrophobic interaction on the gelation, it was known that the methylcellulose gel was formed mainly by the intermolecular hydrophobic interaction between the methyl groups in the molecules, and the gel strength was enhanced with elevating the temperature (Li, 2002). As for HA, the large hydrophobic regions on alternate sides of the single HA helices were also suggested (Scott, Heatley, & Hull, 1984; Scott, Cummings, Brass, & Chen, 1991) and it was also presumed that the formation of HA gel might be resulted from the intermolecular hydrophobic interaction (Collins and Birkinshaw, 2008; Okamoto and Miyoshi, 2002). However, according to our optical microscopic results and macroscopic observation along with the fact that the HA gel could be melted upon heating to high temperature of about 70 °C, the occurrence of hydrophobic interaction was not considered at present.

4. Conclusion

Based on the experimental results from dynamic rheometry, polarizing and optical microscopy, scanning electron microscopy (SEM), X-ray diffraction (XRD), differential scanning calorimetry (DSC) and FTIR spectroscopy, the obtained HA weak gels after the freeze-thawing cycling undoubtedly belong to the category of physically crosslinked gels whose three dimensional structure is stabilized mainly by multiple interchain hydrogen bonds in the junction zones of the polymeric network. Forced alignment of HA chains as the polymer concentration is increased by conversion of water to ice may provide a mechanism for the formation of side-by-side associations, which then remain intact on thawing, as the junction zones of the gel.

Protonation of the polyanion of HA is indispensable to the association of HA chains where hydrogen bonding between groups of –COOH and $–NHCOCH_3$ in HA chains plays a crucial role on such bridging. Although cryostructurates in HA gels (zones of microcrystallinity) resembling the much more well-studied gels on the basis of synthetic polymer, poly(vinyl alcohol) were not found, the feature of hydrogen bonding in HA gel were experimentally evident. Based on the different patterns of possible hydrogen bonding between HA chains, it may be reasonable to assume that the intermolecular links in the weak gels are discrete, lacking regularity and perfection to form microcrystalline zones, detectable microcrystalline zones thus unable to be constructed effectively.

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