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Study of the mechanism of formation of hyaluronan putty at pH 2.5: Part I. Experimental measurements

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ABSTRACT

This paper reports on the elucidation of the possible mechanism for forming the putty structure of hyaluronan (HA) at low pH (2.5) using rheological methods. Oscillation and stress relaxation tests on a series HA putty samples containing various concentrations of salt indicated that ionic forces were not the driving force for their formation, but hydrogen bonds were responsible for driving two or more HA molecular chains together to form a three dimensional network. Our results indicated/confirmed that the carboxyl group of the glucuronic acid and acetamido group of N-acetylglucosamine moieties on two neighboring HA chains are involved in the formation of the intermolecular hydrogen bonding, which lead to the formation of the putty at pH 2.5.

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1. Introduction

Sodium hyaluronate (Hyaluronan or HA), is a high molecular weight unbranched polysaccharide, which was initially discovered from the vitreous humor of cattle eyes by Karl Mayer (Meyer & John, 1934) in 1934. It occurs in the extracellular matrix of tissues in animals, especially in synovial fluid, cock's comb, umbilical cord, as well as in streptococci (Kendall, Heidelberger, & Dawson, 1937). HA is bioactive and has wide applications in the medical field and demonstrated applications for wound treatments (Chen & Abatangelo, 1999; Endre & Balazs, 2005; Necas, Bartosikova, Brauner, & Kolar, 2008; Solchaga et al., 2005; Soldati & Pasche, 1999).

HA is a disaccharide with β1-3-linked D-glucuronic acid and β1-4 linked D-N-acetylglucosamine repeating units polydispersed in molecular weight (Meyer, 1958) (Scheme 1). It has been shown to occupy a large hydrated volume with excluded volume effects, polyelectrolyte properties and viscoelastic properties (Hardingham, 2005). A special feature of HA, as reported by Balazs (1966) and Gibbs, Merrill, Smith, and Balazs (1968), has puzzled investigators: HA can form a viscoelasitic putty at pH 2.5, with an increased elastic behavior at a specific pH (Balazs, 1966). The "putty" texture disappears when the pH is higher or lower than a narrow pH range. This behavior was later confirmed by Hardingham (2005) and Gatej, Popa, and Rinaudo (2005). For example, Hardingham (2005) investigated the self-diffusion of the hyaluronan in NaCl solution at pH 2–4 by confocal fluorescence recovery after photo bleaching (FRAP) and observed a pronounced fall in self-diffusion and greater permeability of a tracer at pH 2.5 compared with a higher or lower pH values. This observation was consistent with the viscoelastic putty formation at this pH by Balazs (1966). Nevertheless, why HA forms a "putty" structure remains unclear. Balazs and Gibbs et al. noted that the HA molecule is considerably stiffer at pH 2.5 than at other pH conditions (Balazs, 1966; Gibbs et al., 1968). An X-ray diffraction study on films prepared from the umbilical cord HA putty revealed a left-handed antiparallel double helix which was slightly cross-linked to form the network. Conversion from solution to a putty was seen as a quantitative rather than a qualitative change of hydrogen bonds (Dea et al., 1973). NMR analysis of HA solutions identified a double helix at other pH values due to the formation of the donor-acceptor hydrogen bonds, particularly between intermolecular carboxyl group and acetamido groups (Heatey, 1999; Scott & Heatley, 2002). However, the mechanism of the formation of the putty is still unclear: which kind of force plays a dominating role in this process; and how it takes action. Previous literatures (Arthur Mashburn, Hoffman,

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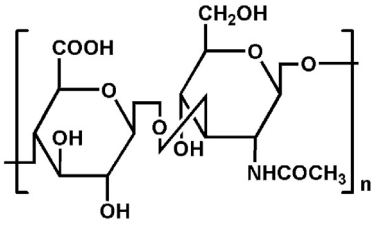

Scheme 1. The chemical structure of HA.

& Hsu, 1974; Furth, Knierim, Buss, & Mayer, 2008; Guss et al., 1975; Pisarcik, Bakos, & Ceppan, 1995; Sheehan, Arundel, & Phelps, 1983; Sheehan, Gardner, & Atkins, 1977; Varga, Pietruszkiewicz, & Ryan, 1959; Virtanen & Paunio, 1980; Winter & Arnott, 1977; Winter, Smith, & Arnott, 1975; Zellermann, Bergmann, & Mayer, 2013) mainly focus on the rheological properties and conformation changes of HA solutions but no explanation of the mechanism of the formation of the putty was given. Although some studies were carried out on the effect of sodium chloride, guanidine hydrochloride and sucrose on the rheological properties of HA solutions, however, no specific effort was made to study those effects at the putty forming conditions (i.e. pH 2.5) (Fujii, Kobayashi, Okamoto, & Nishinari, 1995; Mi, Takaya, & Nishinari, 1996; Mo, Takaya, & Nishinari, 1995; Takaya & Nishinari, 1996). Based on above literature review, five small molecules, including sodium chloride, sucrose, urea, glucuronic acid and N-acetylglucosamine, were employed to elucidate possible mechanisms of hydrogen bonding between two or more HA chains at the putty forming condition, i.e. ~ pH 2.5.

2. Materials and methods

2.1. Materials

Hyaluronic acid with a weight average molecular weight of 3 million (protein content<0.5%) was purchased from Shandong Freda Biopharm Co.,Ltd. (Jinan, China), produced by streptococci fermentation. Glucuronic acid (purity 96%) and N-acetylglucosamine (purity 98%) were purchased from J&K Chemical Ltd (Shanghai, China). Sodium chloride (AR) was purchased from China National Medicines Co.,Ltd. (Shanghai, China). Purified water was prepared by Synergy Ultrapure Water Systems.

2.2. Sample preparation

In our experiments, 0.6% (w/v) HA in normal saline solution was prepared at various pH values to confirm the putty forming properties can be observed at pH 2.5 for the sample used in the current study as reported in the literature (Balazs, 1966; Gibbs et al., 1968; Hardingham, 2005). To study the influence of ionic strength on the formation of the putty, 0.6% HA solutions were prepared in purified water, 0.05 M NaCl solution, 0.10 M solution, normal saline (0.154 M), 0.25 M NaCl solution, and 0.5 M NaCl solution, respectively. Urea, known as a hydrogen bond breaking agent, was added to HA solutions prepared by normal saline at various concentrations at pH 2.5. Different concentrations of glucose, N-acetylglucosamine, and glucuronic acid were added to HA solutions separately to elucidate the possible site for hydrogen bonding formation. All of the prepared solutions were homogenously mixed over night, and adjusted to designed pH before rheological measurements.

2.3. Methods

ARES-G2 rheometer (TA instruments, DA, U.S.A), fitted with parallel plate geometry (diameter of 50 mm) was used for rheological measurements. To prevent drying of the samples, a special cover was used to seal the sample-holding region. After the sample was introduced on to the plate and the temperature reached the set value, the data were collected.

A strain sweep was performed to determine the linear viscoelastic region ranging from 0.01% strain to 1000% strain at set temperature at a low oscillation frequency (1 Hz), which was used to set the strain for the rest of the experiments.

A frequency sweep at a constant strain (2%) at set temperature was carried out to monitor the evolution of viscoelasticity, the storage modulus, G', and the loss modulus G'', and the tan δ in the frequency range of 0.1 Hz to 10 Hz.

Finally, the stress relaxation experiment was undertaken at a constant strain (20%) at set temperature to demonstrate the change of the networks of the putty structure: Sample was loaded onto the plate; gap size of the instrument was set at 1.1 mm. Extra-sample was trimmed carefully and the gap size was eventually set to 1 mm. When the normal force was close to zero, the stress relaxation test was started. Data were collected immediately and stopped when $G(t)$ value was lower than 0.01 Pa.

3. Results and discussion

3.1. Effect of pH on the viscoelastic properties of HA solutions

Fig. 1 shows the formation of HA "putty" and is reproducible: when pH is adjusted to 2.5 or near pH 2.5, the putty is formed.

3.2. The influence of ionic strength

Due to addition of NaCl electrostatic shielding will increase, which usually causes the increase of the molecular stiffness and gel strength of HA solutions (Morris, Rees, & Welsh, 1980; Tigwell & Scott, 1978; Wik & Comper, 1982). However, the effect of Na^+ concentration on the chain stiffness is not significant as shown in Fig. 2. Thus, the gel strength of HA solutions increased with the salt concentration lower than 0.1 M and decreased with further addition of NaCl. (Fig. 2). The further addition of Na^+ ion (>0.1 M) may affect the formation of hydrogen bonds due to the present of excessive ions. The above observations are in accordance with previous data (Balazs, 1966; Gribbon, Heng, & Hardingham, 1999; Krause,

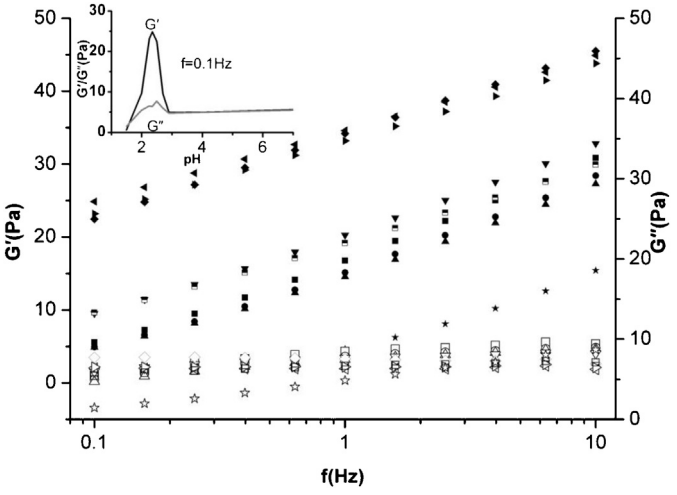

Fig. 1. Effect of pH on storage modulus G' and loss modulus G'' of HA solutions. HA concentration 0.6 g/100 mL in normal saline solution at 10 °C (■: pH 7.0 G';□: pH 7.0 G'';●: pH 4.0 G'; ○: pH 4.0 G''; ▲: pH 2.9 G';△: pH 2.9 G'';▼: pH 2.7 G';▽: pH 2.7 G'';◆: pH 2.5 G';◇: pH 2.5 G''; ◄: pH 2.35 G; ◁: pH 2.35 G'';►: pH 2.25 G'; ▷: pH 2.25 G';⬒:pH 2.0 G',⊟:pH 2.0 G''; ★:pH 1.5 G'; ☆pH 1.5 G''). Insert: changes of G' and G'' with pH at 0.1 Hz.

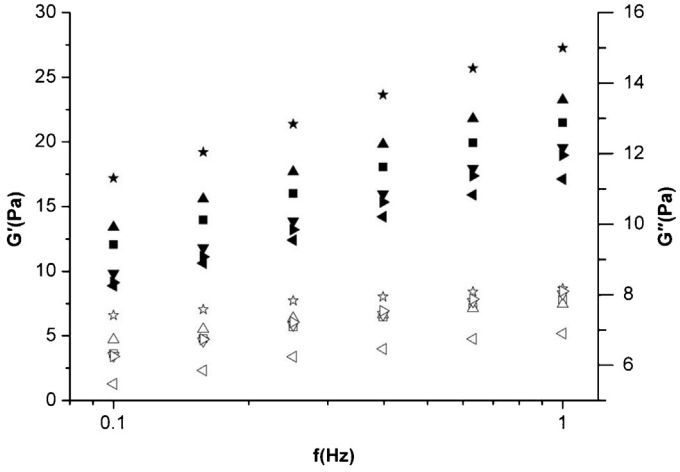

Fig. 2. Effect of NaCl on the storage modulus G' and loss modulus G'' of 0.6% HA in different saline solutions at pH2.5, 25 °C (■: ultrapure water G'; □: ultrapure water G''; ⋆: 0.05 M NaCl solution G';☆: 0.05 M NaCl solution G''; ▲: 0.1 M NaCl solution G';△:0.1 M NaCl solution G''; ▼:normal saline G';▽: normal saline G''; ◀:: 0.25 M NaCl solution G'; ◁:0.25 M NaCl solution G''; ▶: 0.5 M NaCl solution G'; ▷: 0.5 M NaCl solution G'').

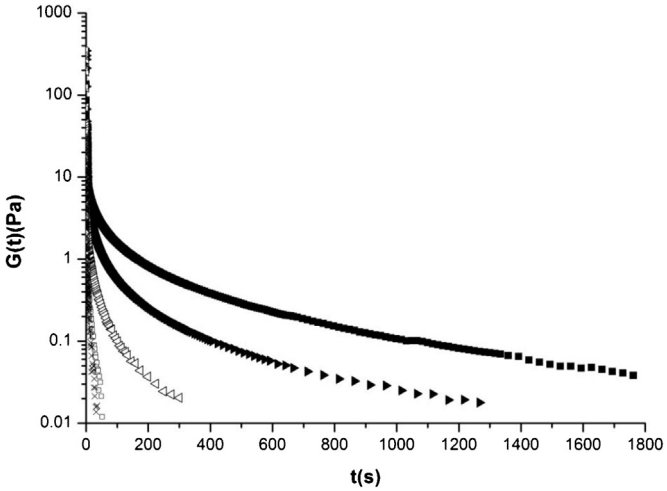

Fig. 4. Stress relaxation spectra of 0.6% HA normal saline solutions in different urea concentrations at pH2.5, 15 °C (■: 0% urea, urea:▶: 2.5% urea; ◁ 5.0%urea; □: 10%urea; ×20%urea).

Bellomo, & Colby, 2001; Varga et al., 1959). Ionic interaction is thus not the deciding force in forming the putty.

3.3. Breakdown of the hydrogen bonds between HA chains

Urea is known as a hydrogen bond breaking agent (Luo & Zhang, 2010; McGrane, Mainwaring, Cornell, & Rix, 2004; Sagle et al., 2009). Fig. 3 demonstrated the influence of urea on the network formation of HA solutions at pH 2.5. As the concentration of urea increased, the gel strength decreased. At a critical urea concentration the putty network was significantly weakened. Further increase of urea concentration to 10% and above completely destroyed the putty structure. This is more effectively demonstrated by the stress relaxation experiment in which the relaxation modulus $G(t)$ was measured against time, as described by Ferry (1980). The results of stress relaxation modulus $G(t)$ against time at various urea concentrations are shown in Fig. 4. With the increase of urea concentration, the relaxation modulus $G(t)$ decreased gradually indicating weakening of the putty networks. The decrease of

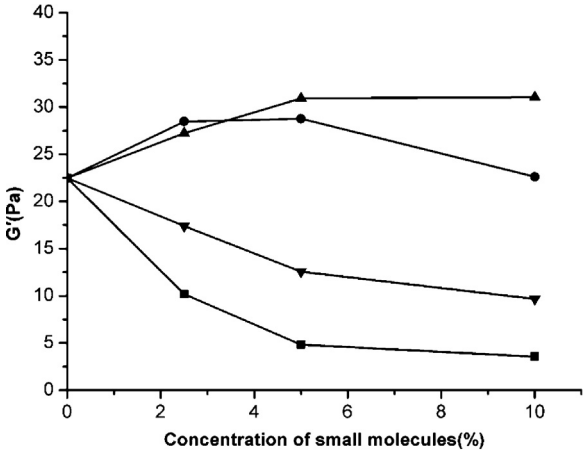

Fig. 3. Influence of added components on the storage modulus G' and loss modulus G'' of 0.6% HA normal saline solutions at pH 2.5, at 10 °C at 0.1 Hz (■: urea G'; ●:glucose G'; ▲: N-acetylglucosamine G';▼: glucuronic acid G').

$G(t)$ became more significant when urea concentration reached at 5%, indicating the complete breakdown of the putty structure. The relaxation modulus $G(t)$ fall to zero immediately when urea concentration was 10% or higher, indicating a viscous system at such conditions, as shown in Fig. 4.

It is well known that versatile substances with an active hydrogen-bond donor and/or acceptor, which have strong ability to break the existing hydrogen bonds and form new ones in aqueous solutions. These groups of compounds are often used as hydrogen bond breaking agents, such as urea, sulfo-urea and Guanidine hydrochloride (Luo & Zhang, 2010; McGrane et al., 2004; Sagle et al., 2009). Using the hydrogen bonding breaking agent urea showed that the structure of the putty is broken down when exceeding a certain urea concentration, illustrating that the main force for forming the putty structure is hydrogen bond. The effect of urea on HA putty structure can be explained below. The added urea would compete with the HA chain for forming hydrogen bonds, which consequently broke the inter-chain hydrogen bonds, thus, weakening or destroyed the putty structure. With a gradual increase of urea concentration, the gel strength of the putty decreases and the "putty" structure eventually disappears. The stress relaxation data showed in Fig. 4 as well as the frequency sweep results in Fig. 3 are further evidences for this conclusion.

3.4. Identification of the possible hydrogen bond contributors

Addition of small molecules with active hydrogen-bond donors and/or acceptors to the HA solutions could help to identify which group was involved in the hydrogen bonding of HA. Considering the similarity of structure and movability, glucose was selected as a competitive small molecule whose hydroxyl groups possess similar properties to those hydroxyl groups on the HA chain. With the addition of glucose, we could observe the role of the hydroxyl groups of HA chain in the formation of the putty structure. Similarly, N-acetylglucosamine and glucuronic acid were also added to HA solutions to examine the role of carboxyl and acylamide groups in the formation of hydrogen bonds. The effect of, glucose, N-acetylglucosamine and glucuronic acid on putty strcture at pH 2.5 are shown in Figs. 3–7. Fig. 3 shows the changes of storage modulus G' at a fixed frequency (0.1 Hz). The addition of glucose up to 5% did not weaken the putty structure; instead, the gel strength was slightly increased. In Fig. 3, the storage modulus G' initially increased with the addition of glucose, however,

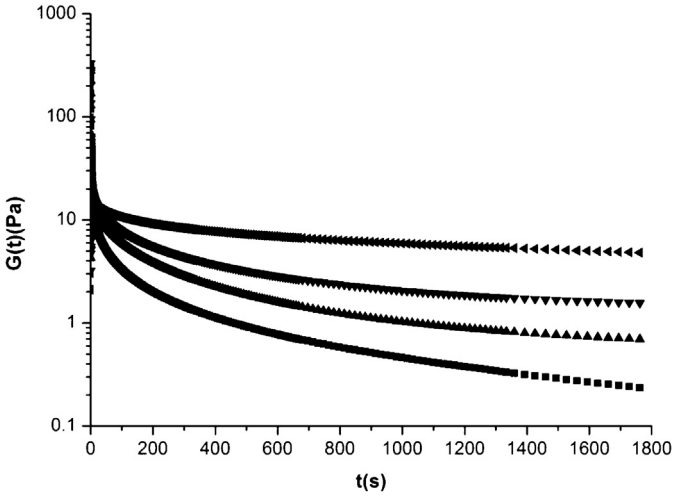

Fig. 5. The strain relaxation spectra of putty of HA containing different concentration glucose formed at pH 2.5 at 15 °C (■: no glucose; ▲: 2.4%glucose; ▼: 5.0%glucose; ◄: 10%glucose).

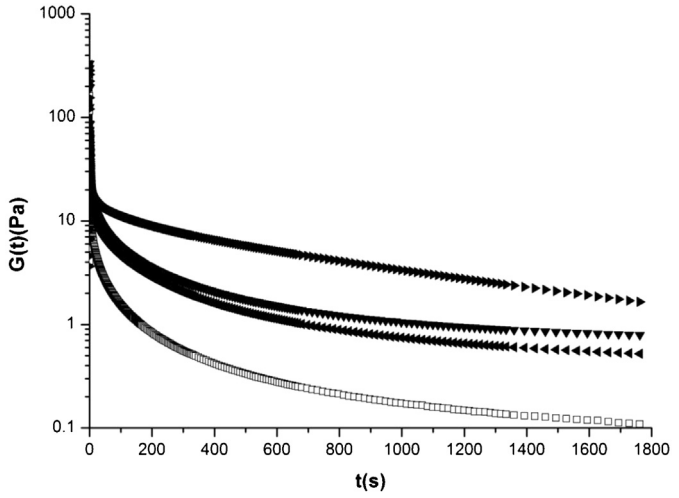

Fig. 6. Stress relaxation spectra of HA putty of containing N-acetylglucosamine(naga) at various concentrations at pH2.5, 15 °C (□: 0% naga; ◄: 2.5% naga; ▼: 5.0% naga; ►: 10% naga).

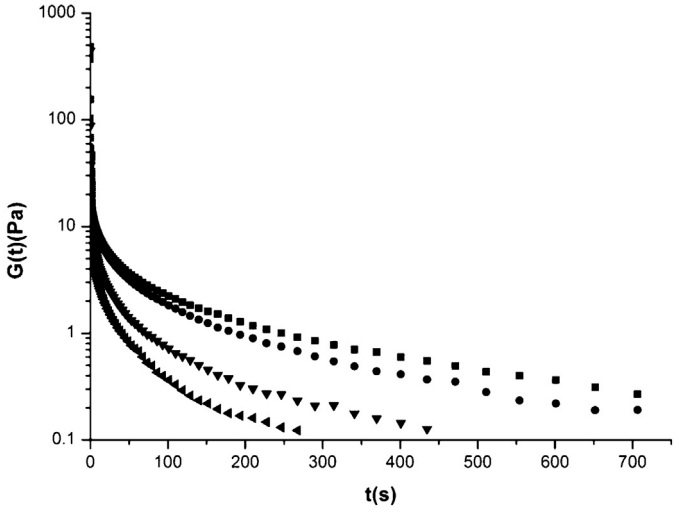

Fig. 7. Stress relaxation of HA putty containing glucuronic acid (GluA)at various concentrations at pH2.5, 10 °C (■: 0% GluA; ●: 2.5%GluA:5.0%GluA; ◄ 10%GluA).

the storage modulus decreased when glucose was added to 10%. This seems inconsistent with the data of stress relaxation (Fig. 5), which increased sharply. However, this could be explained by the transformation equation between oscillitation and the discrete relaxation time spectrum (Ferry, 1980):

$$G'(\omega) = \sum G_i \frac{\omega^2 \tau_i^2}{1 + \omega^2 \tau_i^2} \quad (1)$$

Because relaxation time τ_i is very small value, the stress relaxation modulus G_i would be a huge value when the $G'(\omega)$ is a constant value, which means that tiny difference in storage modulus of sample that instrument in oscillation mode is difficult to tell, huge difference would be detected by the instrument in stress relaxation mode. What's more, the relaxation time is related to the variety of solute and their concentration. So the experiment of stress relaxation is more effective for detection of the difference between samples. The two kind of experiments showed that the network density increased with the initial increase of glucose concentration. This is because the added glucose competed with HA for water, which in fact increased the effective concentration of HA, therefore, give rise to the gel strength. But the reason of the decrease of storage is still unknown when the concentration of glucose reached to 10%, the data of stress relaxation still showed the increase of network numbers in the putty. If the hydroxyl group was actively involved in the hydrogen bond formation in the putty, the addition of glucose (with numerous hydroxyl groups) would have competed with the hydroxyl group on the HA chain, therefore, weakened the inter-chain association (e.g. double helix).

When N-acetylglucosamine was added for competing with HA, the putty structure was not weakened by the addition of the acylamide groups (Figs 3 and 6); on the contrary, the putty structure was enhanced, as shown in Fig. 3. Stress relaxation data also demonstrated the similar trends, i.e. the putty structure was enhanced with the addition of N-acetylglucosamine, as shown in Fig. 6. This result seems indicating that the acylamide group did not actively take part in the hydrogen bonding formation in the HA putty, instead, it modified the solvent quality which resulted in the stiffen of HA chain. However, previous studies suggested that double helix were formed by the hydrogen bond between carboxylate groups and acetamido groups, both in HA putty and solutions (Dea et al., 1973; Gatej et al., 2005; Heatey, 1999; Scott, Cummings, Brass, & Chen, 1991). Considering the weak effect of pH on the acetamido groups and the importance of pH in forming the putty, we could speculate that acetamido group was passive in the process of forming the hydrogen bonds in the putty; however, once a hydrogen bond is formed, it will not be easy to break. This may explain why the added N-acetylglucosamine did not compete with that of HA chain in the compact double helical structure, possibly due to steric hindrance.

The addition of glucuronic acid resulted in a decrease of both G' and $G(t)$ even at low concentrations (Figs. 3 and 7 respectively). This observation indicated that the carboxyl group of free glucuronic acid actively competed with the carboxyl group of the glucuronic acid residue on the HA chain as soon as it is added. When it's concentration reached a critical level, the putty structure collapsed. The observed results confirmed that glucuronic acid was actively involved in the formation of the hydrogen bonding in HA solutions, which led to the disruption of the network of the putty. This is becasue the movability of the glucuronic acid residues on HA chain in aqueous solution was severely limited by the chain conformation; in contrast, the coefficient of self-diffusion of the added free glucuronic acid molecules was far higher than that on the HA chain. As a result, it was easier for those small molecules to replace the residue of glucuronic acid on HA chain in competition for forming new hydrogen bonds. As a result, the

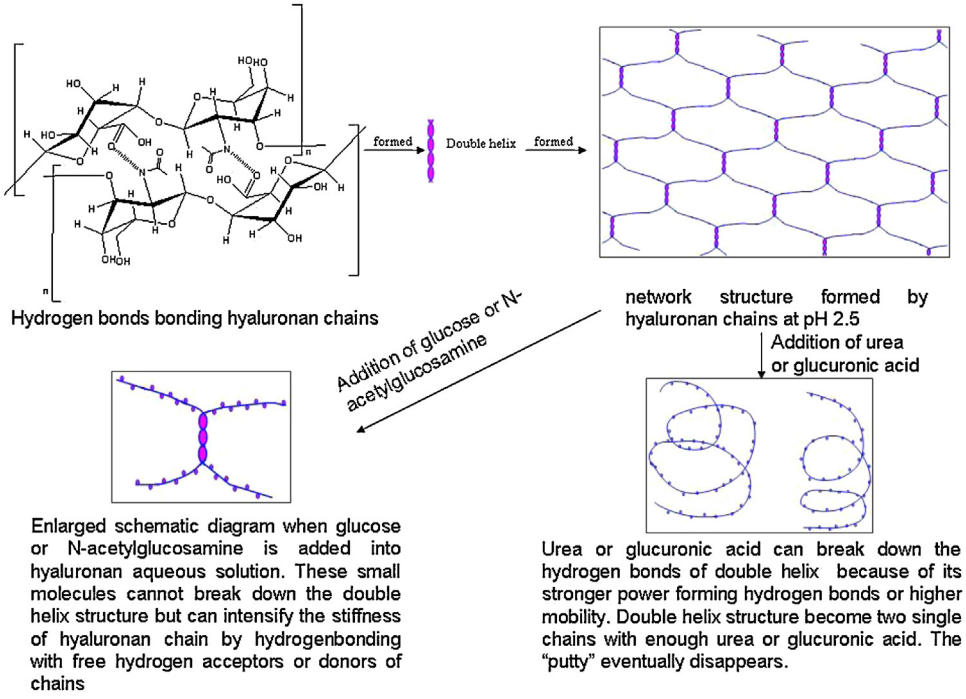

Fig. 8. Schematic diagram of the formation and disruption of HA putty networks.

excessive added free glucuronic acid can form new hydrogen bonds with the acetamido group on HA chain, which, acted like a wedge, dissociated the HA double helix, essentially led to the disappearance of the putty structure. Fig. 8 depicts the possible mechanisms for the formation of HA double helix structure and the effect of adding glucose, acetylglucosamine, and glucuronic acid on the stability of the putty structure at pH 2.5.

4. Summary and conclusions

Current study used a systematic approach to elucidate the possible mechanisms for a well-known but unresolved phenomenon: HA can form a putty at pH 2.5. The stepwise addition of NaCl, urea, glucose, N-acetylglucosamine and glucuronic acid in to the HA solutions at the putty forming condition (pH 2.5) helped to elucidated the individual roles of these four components. Based on the current study, following conclusions could be made:

(1) Ionic force was not the driving force of forming the putty, but it contributed to the strength of the formed putty; the extent of the effect varied with the concentration of ions.
(2) Hydrogen bonding was confirmed as the driving force for the formation of HA putty structure in solutions at pH 2.5.
(3) The competition of three components with HA chains demonstrates that the double helix structure formed by hydrogen bonds between carboxyl group of the glucuronic acid and acetamido group of the N-acetylglucosamine played an important role in forming the putty. The protonation of the carboxyl groups possibly promoted the formation of the double helix, which made the HA chains more stiffer, hence, formed three dimensional network, i.e. the putty structure. In the same principle, the excessive hydrogen ions, which are also hydrogen bond breaking agent, broke down the network structure of HA when pH was adjusted smaller than 2.5. Quantitative effect of pH, urea, N-acetylglucosamine and glucuronic acid on the putty structure will be reported in the subsequent study (Wu Shengfang, Chen, Kang, & Steve, 2013).

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