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# Study of the mechanism of formation of hyaluronan putty at pH 2.5: Part II—Theoretical analysis

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ABSTRACT

A new method was developed for quantitative determination of the viscoelastical characteristics of hyaluronic acid (HA) putty (formed at pH 2.5) using a stress relaxation approach. A concept of entanglement network numbers (ENN) was applied to provide a quantitative measure for the strength of HA putty. Based on the new approach, the relationship between ENN and HA concentration was established. The new approach was successfully applied to evaluate the effect of temperature, urea concentration and pH on putty strength as well as estimate the effects of glucose, β1-3-linked D-glucuronic acid and β1-4 linked D-*N*-acetylglucosamine, on the ENN of HA solutions quantitatively. The ENN established in the current study can be extended to other non-cross linked hydrogels, i.e. they are formed by hydrogen bonds, ionic interactions or other non-covalent bonds.

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## 1. Introduction

In a previous study, we used a stress relaxation rheological method (Wu, Ai, Chen, Kang, & Cui, 2013) confirmed earlier observations by Balazs and Gibbs (Balazs, 1966; Gibbs, Merrill, Smith, & Balazs, 1968) that hyaluronic acid (HA) formed a putty structure at pH 2.5. The results supported the notion that the intrinsic force for HA to form the well known putty structure at pH 2.5 was due to inter-chain hydrogen bonding of the two constituent monosaccharide units (Wu et al., 2013). We further elucidated the mechanism of the hydrogen bond formation: it was between the carboxyl group of glucuronic acid from one polymer chain and the acetomido group of *N*-acetyl glucosamine from the neighboring chain (Gibbs et al., 1968). Based on literature and previous studies, gel strength or viscoelasticity of HA putty at pH 2.5 can readily be described in a qualitative manner. However, it has been difficult to provide a quantitative measurement of the gel strength of any aqueous based hydrogel. It is generally believed that the observed HA putty at pH 2.5 was due to chain–chain association via hydrogen bonds at a specific condition and the consequential entanglements of the polymer chains which formed the three dimensional networks. The reduction of the carboxyl group dissociation at pH 2.5 favors the hydrogen bond formation which also caused the protonation of the–NH-group to give positive net charge to the molecule, which inter, could complex with the remaining negatively charged carboxyl group (Gatej, Popa, & Rinaudo, 2005). Both the quality and the quantity of the entanglement networks are important measures of the viscoelastical properties of the system (Ferry, 1979). In order to develop a quantitative method to characterize the HA putty system, existing literature was reviewed. Andrews and Tobolsky introduced two types of molecular networks in studying the quantity of the networks of vulcanized natural and synthetic rubbers (Andrews, Tobolsky, & Hanson, 1946), including cross-linked networks and physical networks formed by polymer chain entanglement; both networks were quantitatively treated by Berry, Scanlan, and Watson (1956), followed by a more detailed study by Flory (1960). The whole network was considered to have formed by the superposition of two networks of cross-linked strands and trapped entanglement strands (Ferry, 1979). The following expression links the density of elastically effective strands in a polymer network to the densities of random cross-links, main-chain scissions; and entanglements to the molecular weight distribution of the initial linear polymer (Langley, 1968):

$$\nu = \left(\frac{q\rho}{M_0}\right) w_g T_e^{1/2} + 2\,\varepsilon T_e \quad (1)$$

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where $q$ is the probability that a monomer unit participates in a cross link, $\rho$ the density, $M_0$ the monomer molecular weight, $w_g$ the gel weight fraction (=1 − $w_s$, where $w_s$ is the sol weight fraction),$T_e$ the probability that an entanglement is trapped and $\varepsilon$ the effective total concentration of entanglements.

According to the classical theory of rubber-like elasticity description, the equilibrium shear modulus ($G_e$) in infinitesimal deformations can be given by

$$G_e = g\left(\frac{\bar{\bar{r_E^2}}}{\bar{\bar{r_0^2}}}\right)\upsilon RT \tag{2}$$

where $\upsilon$ is moles of network strands per cubic centimeter and $g$ is a numerical factor not far from the unity, $\bar{\bar{r_E^2}}$ is the mean square end to end distance of a strands and $\bar{\bar{r_0^2}}$ is the mean square end to end distance of the strands of same length that were not constrained by cross-links.

The combination of Eqs. (1) and (2) makes it possible for quantifying networks based on experiments. However, it was not an easy task to quantitatively determine the networks in aqueous polymer systems. To resolve this, Ferry developed the Flory theory and developed a new expression (Ferry, 1979):

$$\frac{E_s}{E_0} = \frac{(\lambda_0^2 - \lambda_0^{-1})}{(\lambda_0^2/\lambda_s^2) - (\lambda_s/\lambda_0)} \tag{3}$$

where $\lambda_0$ is the extension ratio during the introduction stage of chemical cross-links in the strained state; $\lambda_s$ is the extension ratio in the state of ease; $E_s$ is the equilibrium Young's modulus in small expressions at the state of ease; and $E_0$ is the equilibrium Young's modulus of the original entanglement network in a time scale where the entanglements behave as trapped. The cross-linked network ($\upsilon_x$) and entanglement network ($\upsilon_N$) have such relations with $\lambda_0$ and $\lambda_s$:

$$\lambda_s = \left[\frac{1 + \lambda_0\upsilon_x/\upsilon_N}{1 + \upsilon_x/\upsilon_N\lambda_0^2}\right]^{1/3} \tag{4}$$

Based on Eqs. (3) and (4), Ferry and co-workers determined the quantity of cross-linked and entanglement networks of 1,2-polybutadiene by the stress relaxation spectrum method (Greco, Taylor, Kramer, & Ferry, 1975; Kan & Ferry, 1978, 1979; Kramer & Ferry, 1975; Kramer, Carpenter, Ty, & Ferry, 1974; Nelb, Pedersen, Taylor, & Ferry, 1980). Since then, stress relaxation has been regarded as the most convenient method for evaluating the networks of rubber materials (Bjork & Stenberg, 1990).

However, to our best knowledge, there are no literature reports on quantifying the entanglement networks in hydrogels due to three possible reasons: (1) the objective of the original study was mainly for rubber-like materials, whose deformation can be easily measured (Ferry, 1979). In contrast, it was difficult to determinate the comparatively small deformation of hydrogels during stress relaxation tests; (2) the entanglement network in hydrogels differed from the idealized concept defined in the theory (Ferry, 1980); (3) the tested materials were not of a single polymer, instead, it contained only a few percent of natural polymers (mostly 0.1–5%) in large amounts of aqueous solvent (mostly 95–99.9%). The physical entanglement network "content" is not only related to the concentration and molecular weight of polymer, but also related to environmental factors such as ionic strength, temperature, pH in aqueous solution. Therefore, a new method was necessary for quantitative description of the entanglement networks in hydrogels or polymer solutions; this would provide a more accurate tool to evaluate network formation of biopolymeric materials. Based on the theory of Ferry (Greco et al., 1975; Kan & Ferry, 1978, 1979; Kramer & Ferry, 1975; Kramer et al., 1974; Nelb et al., 1980) we developed a new method using a stress relaxation approach to quantitatively study the entanglement networks of hyaluronic acid solution at pH 2.5 (which does not contain cross-linked networks). The new method can also be used to quantitatively evaluate the contribution of hydrogen bonds to the formation of entanglement networks which was identified in previous study (Wu et al., 2013), and examine various factors that could affect the viscoelastic properties of polymer solutions.

## 2. Materials and methods

### 2.1. Materials

Hyaluronic acid with average molecular weight of 3,000,000 (protein content <0.5%) was purchased from Shandong Freda Biopharm Co., Ltd. (produced by streptococci fermentation). Glucuronic acid (purity, 96%) and *N*-acetylglucosamine (purity, 98%) were purchased from J&K Chemical Ltd. Sodium chloride (AR) was purchased from China national medicines Co., Ltd. Superpurified water was prepared by Synergy Ultrapure Water Systems.

### 2.2. Sample preparation

As part of the development for the new ENN method, a series of concentrations of hyaluronic acid in normal saline solution at different pH values were prepared to establish the effects of concentration of polymer, pH value, and temperature on the entanglement networks. To validate the method, 0.6%HA in normal saline solution containing a series of concentrations of urea, glucose, *N*-acetalglucosamine and glucuronic acid with their pH adjusted to 2.5 was prepared overnight.

### 2.3. Methods

The rheological measurement of the HA samples was performed on a ARES-G2 rheometer (TA Instruments, U.S.A.) fitted with parallel plate geometry (diameter of 50 mm). To prevent drying of the samples during experiments, a special cover was used to seal the sample-holding region. After the sample was introduced onto the plate and the temperature reached the set value, data was collected. A strain sweep was used to study the linear viscoelastic region ranging from 0.01% strain to 1000% strain at oscillation frequency of 1 Hz to set the upper strain limit through out the experiments. The effect of HA concentration, temperature and pH value on the networks were determined by the stress relaxation experiments while the effects of the hydrogen-bond breaking agent (urea) and possible hydrogen-bond forming agents such as glucose, *N*-acetylglucosamine and glucuronic acid on formation of the networks of HA putty were quantitatively analyzed.

## 3. Results and discussion

### 3.1. The relation of network numbers and HA concentration

Graphs in Fig. 1 demonstrated the effect of concentration on the rheological behavior of hyaluronan in normal saline solutions at 10 °C at pH 2.5: (a) dynamic frequency sweep of four concentrations: 0.2%, 0.4%, 0.6% and 0.8% respectively; (b) stress relaxation spectra of the same samples as in (a). Both graphs demonstrated that with the increase of HA concentration, putty strength was increased; this is because increase of HA concentration will the possibility of entanglement between HA chains

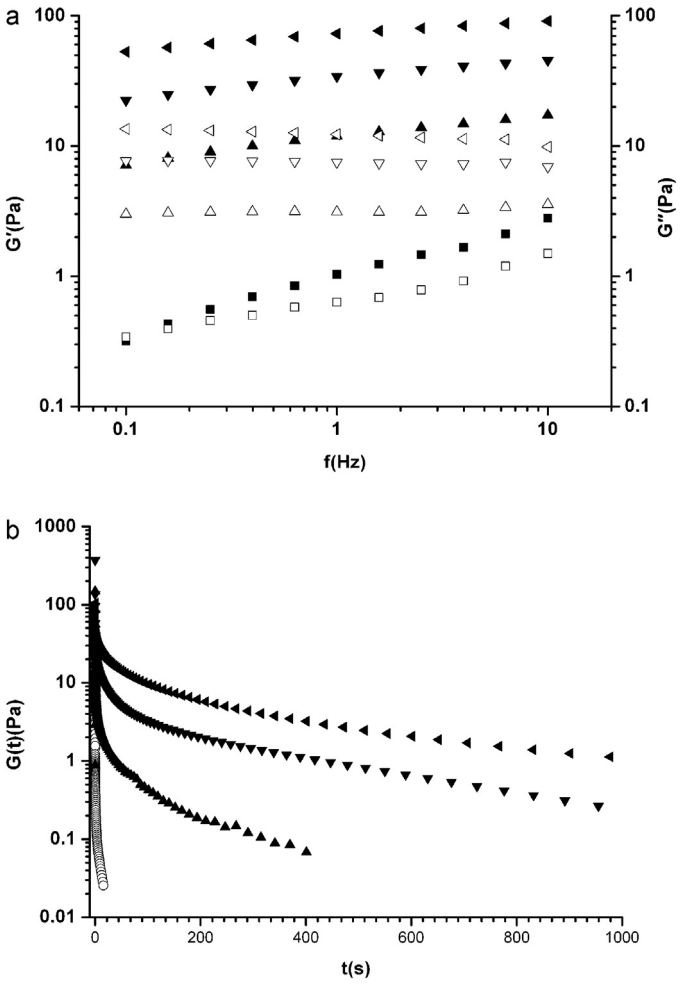


**Fig. 1.** (a) The effect of different hyaluronan concentration on $G'$ and $G''$ in normal saline solutions at 10 °C at pH 2.5 (■, $G'$ of 0.2% hyaluronan aqueous solution, □, $G''$ of 0.2% hyaluronan aqueous solution, ▲, $G'$ of 0.4% hyaluronan aqueous solution, △$G''$ of 0.4% hyaluronan aqueous solution, ▼, $G'$ of 0.6% hyaluronan aqueous solution, ▽$G''$ of 0.6% hyaluronan aqueous solution, ◀, $G'$ of 0.8% hyaluronan aqueous solution, ◁ $G''$ of 0.8% hyaluronan aqueous solution). (b) Strain relaxation spectra of different concentration hyaluronan in normal saline solution at 10 °C at pH 2.5. (◯, 0.2%, ▲, 0.4%, ▼, 0.6%, ◀, 0.8%).

increased greatly, hence, the increase of the strength of the putty structure (Hardingham, Garg, & Hales, 2004). On the other hand, the formation of entangled networks limits the free flow of water, hence, enhanced the gel strength as evidenced by prolonged the relaxation time. Although this data tell us the trends of the putty structure, it does not quantify the entanglement network.

In order to be able to quantitatively calculate the entanglement networks in a hydrogel system where the system does not contain cross-linked networks, we could hypothesize that the whole network can be considered as a series of parallel connections of Maxwell models. All the energy would be dispersed into each Maxwell model:

(5)$E = \Delta E \times n$where $E$ is the total energy contained in the network; $n$ is the numbers of entanglement networks; $\Delta E$ is the energy of each Maxwell model containing one entanglement network.

When the conditions of experiment are the same, the values of $E$ and $\Delta E$ will be constant. The potential energy would be transferred to kinetic energy at a value of $1/2mv^2$ (Bjork & Stenberg, 1990) after the release of stress. The force generated by the entanglement networks would impede the movement of the polymer chain until

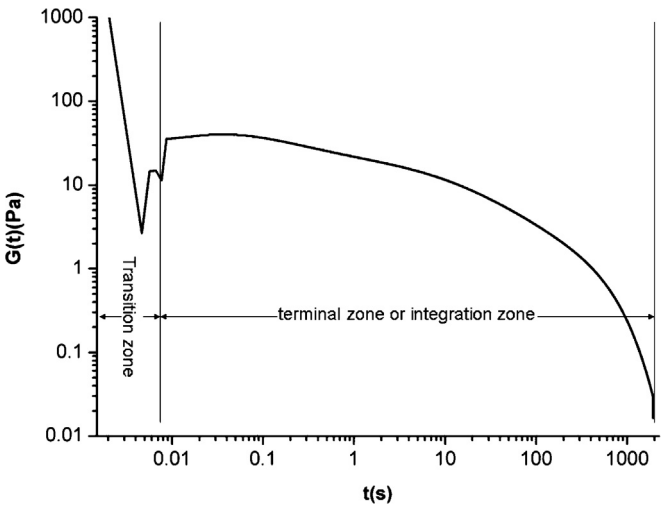


**Fig. 2.** The relaxation time spectra of 0.6 g/100 g HA normal saline solution at pH 2.5 at 10 °C. The entanglement networks $N$ = trapz ($t$,$G(t)$)= 1.6$E$+03.

it ceases. According to Newton's law of conservation of momentum, we have:

$$\int_{t_0}^{t_1} G(t) \times dt = n \times mv \tag{6}$$

We can further hypothesize that the mass of every Maxwell model is the same; therefore, the density of entanglement network is directly proportional to $\int_{t_0}^{t_1} G(t) \times dt$, assuming the polymer was not cross-linked. Based on the classical stress relaxation spectrum (Bjork & Stenberg, 1990; Ferry, 1980; Qihua Wu, 2002), the terminal zone of the curve reflects the structure of the entanglement network. As such, $t_0$ in Fig. 2 would be the start of the terminal zone (turning point from transition zone to terminal zone) of the stress relaxation spectrum. The relaxation time curve can be well fitted by the following model (Haider & Schugart, 2006):

$$G(t) = G_e + \int_{-\infty}^{+\infty} H(\ln \tau) e^{-t/\tau} d \ln \tau \tag{7}$$

If we define $N = n \times mv$, $N$ will reflect the relative content of the entanglement network if the conditions of experiment are the same. For example, if there were no cross-linked strands, $G_e$ would equal to zero (Ferry, 1980). Combining Eqs. (6) and (7), we could easily calculate the relative quantity of entanglement networks – it is defined as the entanglement networks number (ENN). Following is an explanation how to quantify the ENN using the stress relaxation rheological approach.

Fig. 2 represents a typical stress relaxation spectrum. Data obtained by the rheometer was exported to an excel file. The relaxation modulus $G(t)$ was plotted against relaxation time ($t$) using the software MATLAB 7.0. This software allows the integration of the area underneath the curve; the integrated area underneath the curve is defined as the ENN. A quantitative relationship between ENN and HA concentrations, temperature, urea concentration will be established, and the effect of D-glucose, D-glucuronic acid, D-*N*-acetylglucosamine on ENN of HA at pH 2.5 will be evaluated in the following sections.

Note:

1. The beginning time $t_0$ was selected as the start of terminal zone area (in a double logarithmic coordinate system).
2. The end time $t_1$ was selected at the point where $G(t)$ equals to 0.01 Pa; at this point, the process of relaxation was considered complete.

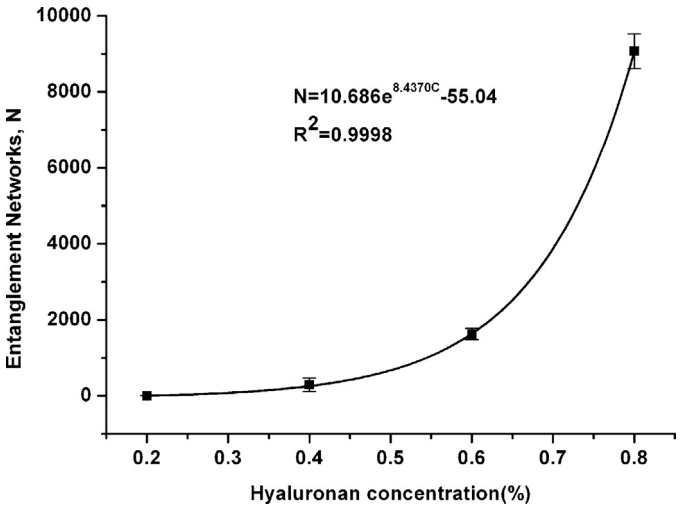


**Fig. 3.** The relation of relative network *N* and hyaluronan concentration in normal saline solutions at 10 °C at pH 2.5.

3. Since there are no cross-linked strands in HA chains, $G_e$ is assumed equal to zero. This assumption was proved by experiment data.

Fig. 3 represents a quantitative relationship between HA concentration and the ENN based on the new method:

$$N = 10.686e^{8.4370C} - 55.04 \quad (8)$$

where $N$ is the ENN, and $c$ is HA concentration (%). The $R^2$ of Eq. (8) was 0.9998, which indicates the curve fitted well with the experiment data and had good repeatability. The equation also tells us the concentration of HA had a major influence on the ENN, especially when the concentration of HA exceeded a certain value (about 0.4%), above which the ENN increased exponentially with the HA concentration. These results supported earlier observation by Balaz (Berry et al., 1956). In the following sub-sections, we will use the new method to quantify other relationships for HA solutions at pH 2.5, i.e. at the putty forming condition.

### 3.2. The relationship of the entanglement network numbers (N) and temperature

Fig. 4 illustrates the effect of temperature on the viscoelastical properties of HA solutions. Fig. 4a is the stress relaxation spectra of HA putty at various temperatures: with the increase of temperature, the gel strength decreased and relaxation time becomes shorter. If we apply the developed method to integrate the area underneath each curve to represent the ENN, and plot the ENN (log) against temperature (linear), as shown in Fig. 4b. A quantitative relationship between the ENN and temperature can be expressed by the following equation:

$$N = 3976.9e^{-0.09788t} \quad (R^2 = 0.9956) \quad (9)$$

This result suggested that the ENN will decrease with the increase of temperature in a quantitative manner; this result is also in agreement with the conclusion of Balazs and Gibbs (Balazs, 1966; Gibbs et al., 1968).

### 3.3. The relationship between ENN and pH values

Changes of the ENN with pH of HA solutions are shown in Fig. 5. When pH values were changed from pH 7.0 to 3.0, there was essentially no change of the ENN However, when the pH values approached 2.5, the ENN increased sharply, reached a maximum at

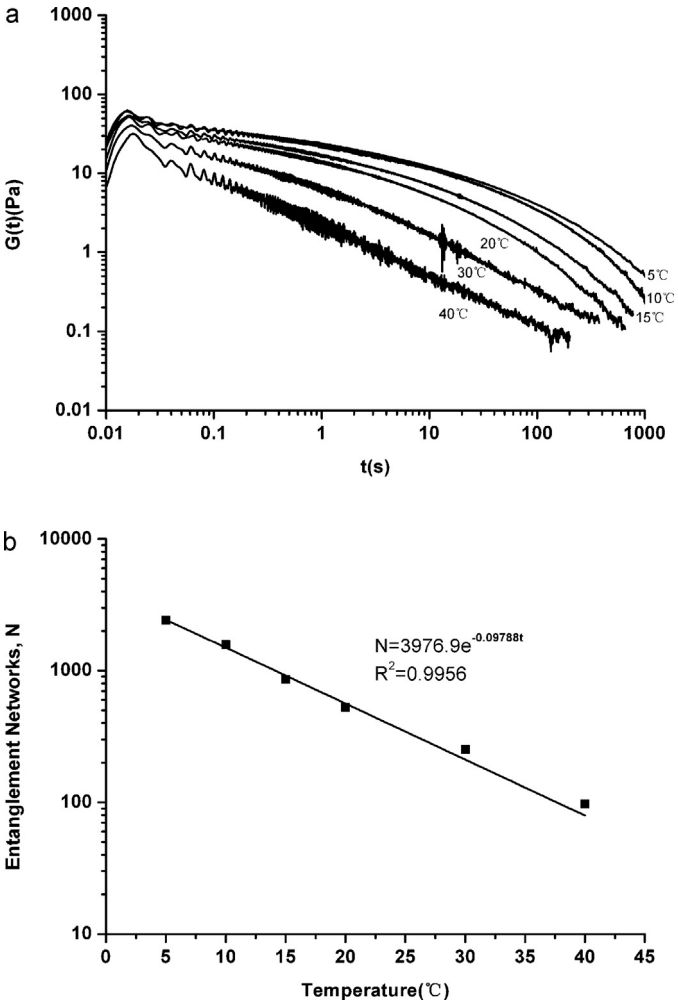


**Fig. 4.** (a) The relaxation time spectra of 0.6 g/100 g HA normal saline solution at pH 2.5 at different temperature. (b) The relation of temperature and the relative networks N of 0.6% hyaluronan in normal saline solutions at pH 2.5.

pH near 2.5. Further reduction of pH caused a dramatic decrease in ENN and reached baseline level when pH value was decreased to lower than 1.5. This observation appears to contradict the observation in regards to the self diffusion coefficient of HA with pH values (Hardingham et al., 2004). However, the increase of ENN translates

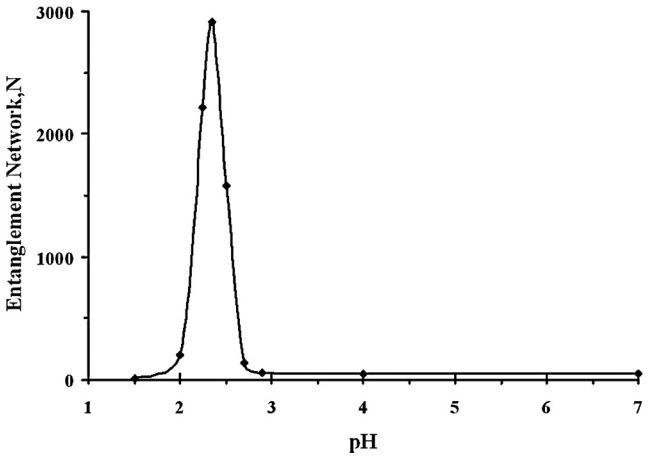


**Fig. 5.** The relation of pH and the relative networks *N* of 0.6% hyaluronan in normal saline solutions at pH 10 °C.

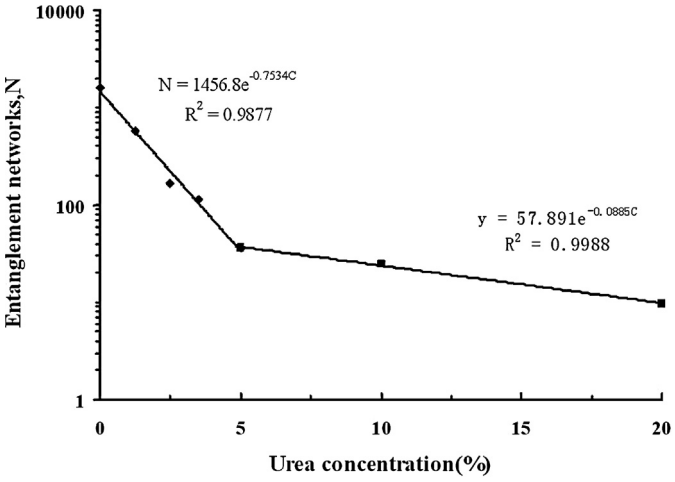


**Fig. 6.** The relation of urea concentration and the relative networks *N* of 0.6% hyaluronan in normal saline solutions at 10 °C at pH 2.5.

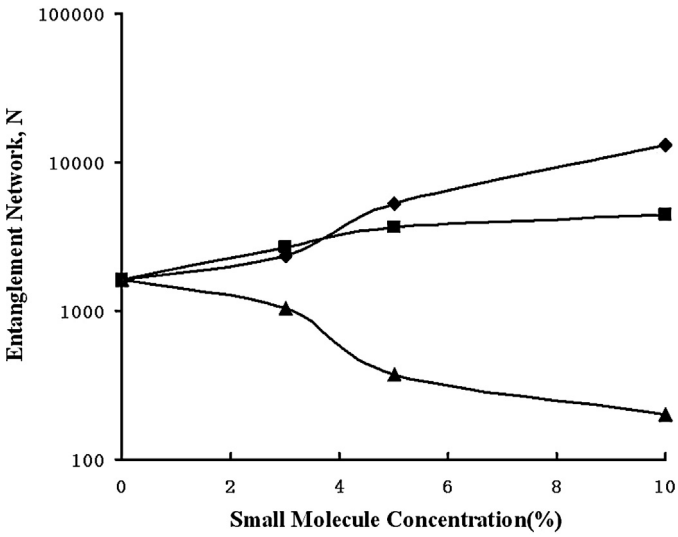


**Fig. 7.** The relation of competitive small molecules concentration and the relative networks *N* of 0.6% hyaluronan in normal saline solutions at 10 °C at pH 2.5 (♦, glucose, ■, *N*-acetyl-glucosamine, ▲, glucuronic acid).

to the limited mobility of HA chains. Therefore, both of these observations demonstrate the same phenomenon: putty was formed in the narrow pH region. When pH value was greater than 3, most carboxyl groups were ionized, thus, only a few hydrogen bonds could be formed. Through the process of protonation of carboxyl groups (reduced pH), more entanglement networks were able to form due to the formation of a large quantity of hydrogen bonds at the isoelectric point; this observation confirmed the explanation of an earlier study that at pH 2.5 the dissociation of the carboxyl group was greatly reduced which favors the hydrogen bond formation (Gatej et al., 2005). However, $H^+$ is also a hydrogen-bond-breaking agent (which led to the equilibrium of the hydrogen bond formation and subsequent breakdown). As a result, ENN was significantly reduced at pH 2.0 at which point carboxyl groups were completely protonated.

### 3.4. Effect of urea concentration on entanglement network numbers (N)

Urea was used to confirm the hydrogen bonds in HA putty (Wu et al., 2013) based on the knowledge that urea is a classical hydrogen-bond breaking agent. Fig. 6 gives the quantitative relationships between urea concentration and ENN. Two distinct regions in the graph can be observed: the first region is below 5%. In this region, the ENN decreased sharply with the increase of urea concentration. The second region is above 5% in which the curve flattened indicating that further addition of urea decreased the ENN due to continued reduction of hydrogen bonds. The two regions can be quantitatively described by the following two equations:

$$N = 1456.8.3e^{-0.7534C} \quad (R = 0.9877, \quad C = 5\%) \tag{10}$$

$$N = 57.891e^{-0.0885C} \quad (R = 0.9988, \quad C = 5\%), \tag{11}$$

These graphs and equations showed that addition of urea effectively breaks down the hydrogen bonds. The turning point at 5% urea could be explained as such that when urea concentration reached 5%, most of the hydrogen bonds contributing to the three dimensional network structures of the putty were effectively broken down. Further increase of urea concentration continued to break down the remaining hydrogen bonds, which led to the continued decrease of ENN (Fig. 6)

### 3.5. Effect of small molecules on entanglement network numbers (N)

*N*-Acetylglucosamine, glucuronic acid and glucose were added into HA in normal saline solution at pH 2.5 to evaluate the effect of small component molecules on hydrogen bonding. As shown in Fig. 7, a small quantity of glucose did not change the network strength; however, large quantities of glucose prominently affected the networks by increasing the ENN. The influence of *N*-acetylglucosamine was similar to that of glucose, but to a lesser extent at higher concentrations. These results illustrated that the hydroxyl groups on the sugar ring have limited contribution to the formation of the hydrogen bonds necessary for forming entanglement networks or double helix. The increase in gel strength could be explained by the following: added glucose competed for the availability of water in the aqueous system, which would simply increase the effective concentration of HA in the mixed system (Wu et al., 2013). Earlier studies indicate that the amido groups of N-acetyl- glucosamine were actively involved in the double helix formation by interacting with the carboxyl groups on the HA chain and therefore playing an important role in the formation of networks (Balazs, 1966; Wu et al., 2013). The curve of ENN upon addition of glucuronic acid clearly demonstrates that glucuronic acid was involved in hydrogen bond formation. Initially, the carboxyl groups of glucuronic acid and amido groups of N-acetylglucosamine of HA chains formed stable hydrogen bonds which is the driving force for the formation of the double helix or the ENN of the putty. The added free glucuronic acid competed with the glucuronic acid on the HA chains. As a result the free glucuronic acid gradually replaced the glucuronic acid on the HA chains in the double helix or chain-chain entanglements, which led to the gradual breakdown of the entanglement networks (Wu et al., 2013). The reduction of the ENN was more significant when the added glucuronic acid was at a concentration of 5% and above, as shown in Fig. 7.

## 4. Discussion and conclusions

The above experimental results showed that the relationships between ENN and its influencing factors can be quantitatively described by the new method. The ENN is not an absolute value

but a relative measure which is affected by experimental conditions. The concept of entanglement network number described in this paper is different from the grid numbers of fishing net. According to the new method, higher ENN also can be translated to greater restriction in mobility of the molecules. We understood that both the intensity of the junction points and grid numbers in the entanglement networks contributed to the total ENN. As far as HA solution is concerned, the ENN reflects the quantity of hydrogen bonds that formed double helices of HA chains which limits the glide of joints and provide strength to the network structure.

In summary, we developed a new method to quantify the entanglement networks in polymer solutions: $N = \int_{t_0}^{t_1} G(t) \times dt$. This new method is based on stress relaxation measurements which can be carried out with a modern rheometer. Based on this method, a series of quantitative relationships were established: the relationships between entanglement network numbers ($N$) and HA concentration, temperature, pH values, addition of urea, glucose, glucuronic acid and *N*-acetyl-glucosamine. The new method can be applied to other hydrogel systems that do not contain cross-linked networks.

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