Freeze-thaw-induced gelation of hyaluronan: Physical cryostructuration correlated with intermolecular associations and molecular conformation

Zhixiang Cai, Fei Zhang, Yue Wei, Hongbin Zhang*

Advanced Rheology Institute, Shanghai Key Lab of Polymer Dielectrics, Department of Polymer Science and Engineering, School of Chemistry and Chemical Technology, Shanghai Jiao Tong University, Shanghai 200240, People's Republic of China. *E-mail: hbzhang@sjtu.edu.cn; Tel: +86 21 54745005*

Table S1. Molecular parameters of different HA samples

Sample	$10^{-5}M_w$	$[\eta]$ (dL/g)	R_h (nm)	R_g (nm)
1	11.6	23.11	73.40	130.43
2	10.4	21.31	68.35	120.75
3	7.3	16.55	54.16	88.81
4	4.8	12.18	41.98	57.43
5	2.5	7.23	28.75	37.41

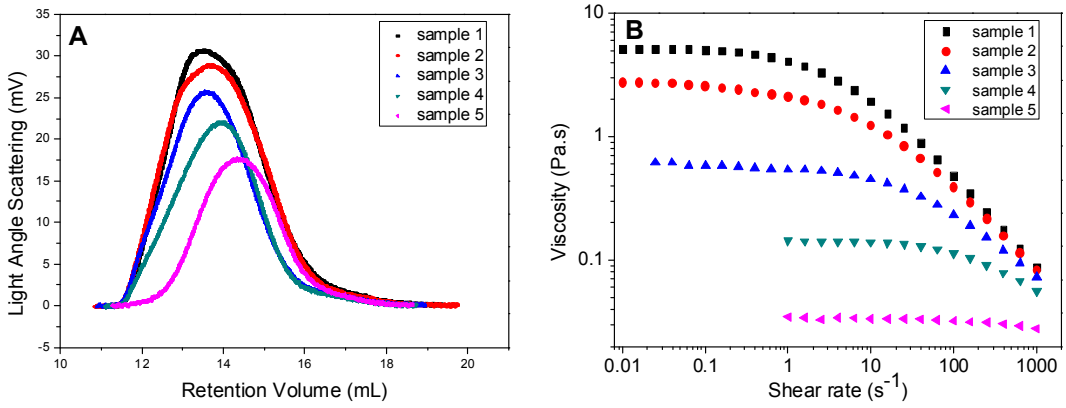

Fig. S1 (A) Intensity of laser scattering of HA with different molecular weights; (B) steady shear viscosity of 10 mg/mL

HA with different molecular weights.

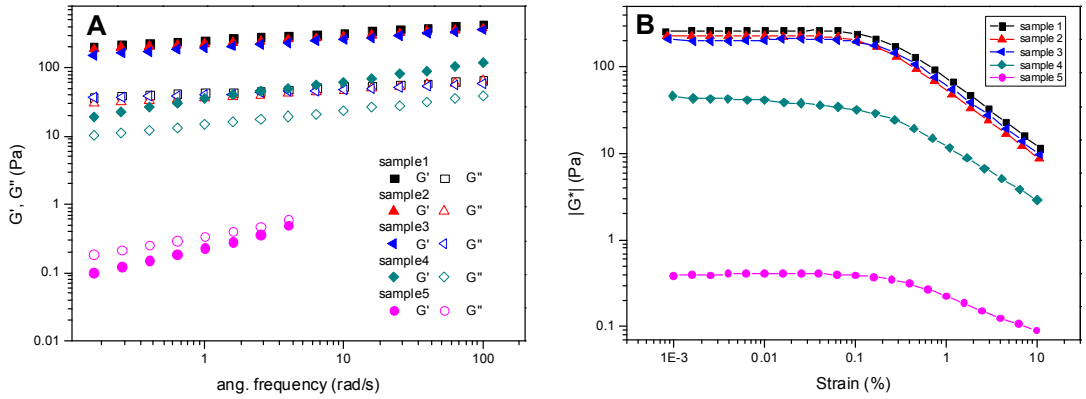

Fig. S2 Frequency dependence of G' and G'' (A), and the complex modulus G^* as a function of strain amplitude at a frequency of 1 rad. s^{-1} (B) for the cryotropic gels prepared by freezing for 6 days and with different molecular weights at 25 °C.

Fig. S2(A) shows the dynamic rheological properties of HA cryogels with different molecular sizes. The gels with $M_w \geq 730000$ display a mechanical spectrum of a comparatively strong gel, with its G' almost independent of frequency and much higher than G''. The gel with M_w of 480000 by the same process presented the characteristics of a weak gel, with both moduli showing obvious frequency dependence and slightly larger G' than G''. By contrast, the gel with M_w of 25000 at the same concentration displayed viscoelastic behaviour typical of polymer solutions, and no gelation was observed from it under the same freeze-thaw process. The complex modulus G^* also reflected these different behaviours of cryogels [Fig. S2(B)]. A higher M_w led to stronger gel, and HA with too low M_w was unable to form a gel under the present experimental conditions.

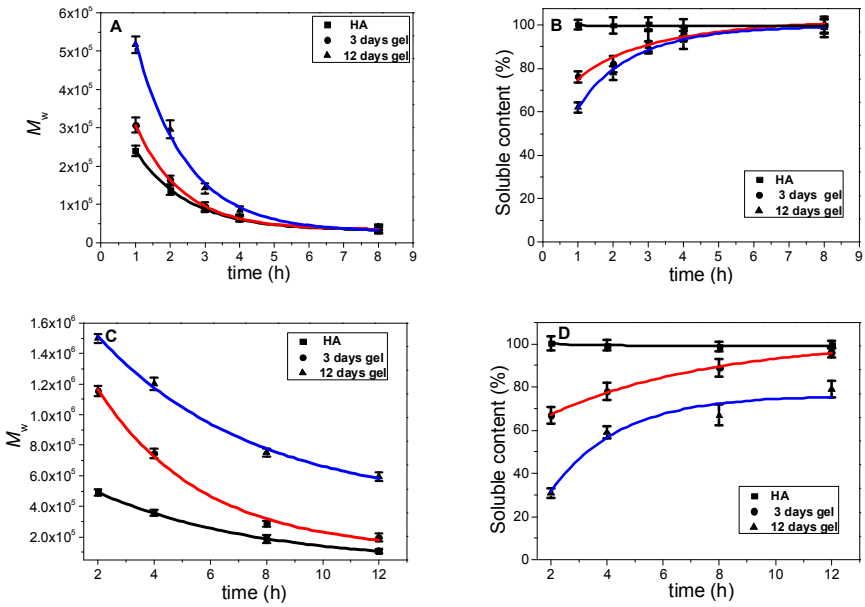

Fig. S3. The time evolution of acid degradation of native HA in solution and the neutral HA cryogels with different freezing days at different temperatures and pH 1: Change of Molecular weight and soluble content with time at 50 ^{o}C (A, B) and 37 ^{o}C (C, D), respectively.

Evaluation of acid decomposition in the obtained neutral HA cryogels was conducted based on a previously reported method,[1, 2] with minor adjustment. The HA cryogels obtained by freezing for different days were prepared at 15 mg/mL, added with 1 M HCl to reach a final pH of 1.0, and immediately placed in a thermomixer with stirring. Two temperatures (50 and 37 °C) were set for the degradation experiments. At different incubation times, solutions were withdrawn from the thermomixer, immediately neutralized with an alkali-buffer solution (1 M NaOH + 0.1 M PBS), and characterized.

The soluble content of HA was calculated as $[(m_t/m_0) \times 100\%]$, where m_0 is the initial weight of native HA or HA cryogel before degradation, and m_t is the weight of soluble HA in the solution at a defined period during degradation. The concentration of soluble HA and its corresponding M_w were determined with a Viscotek TDA 305 instrument

(Malvern Instruments, USA). Native HA was also tested as the control sample.

Fig. S3 shows acidic degradation at pH 1, different temperatures for native HA in solution, and two kinds of neutral HA cryogels obtained by freezing for 3 and 12 days. The molecular weights of all three samples decreased with prolonged time, whereas the soluble contents from the cryogels increased. In gels prepared by freezing for 12 days, the decrease in M_w and the increase in soluble content of the cryogels decreased compared with that of native HA in solution. Resistance to acidic degradation was also more obvious in gels frozen for 12 days.

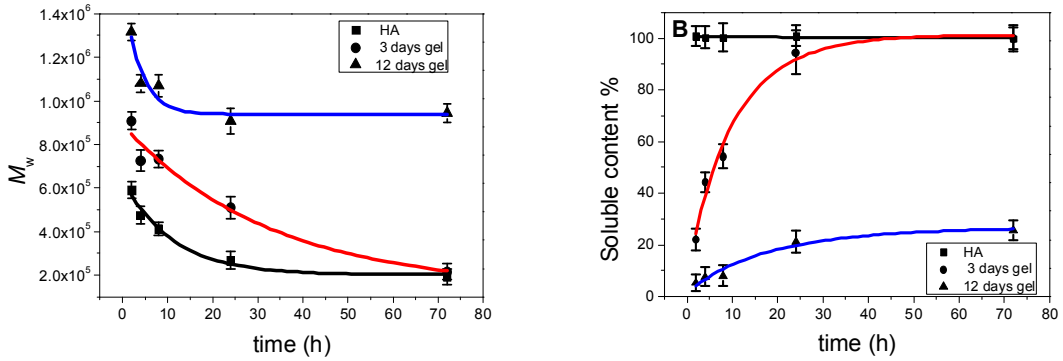

Fig. S4. The time evolution of enzymatic degradation of native HA in solution and the neutral HA cryogels prepared by different freezing time: Change of Molecular weight (A) and soluble content (B) with time

Enzymatic degradation was evaluated according to the method described by Okamoto and Miyoshi[1] and Gatta *et al*.[3] HA cryogels of 15 mg/mL in PBS (pH 7.0) were incubated in the presence of hyaluronidase in a thermomixer at 37 °C with stirring. Degradation tests were performed at a hyaluronidase concentration of 20 U/mL, which was calculated based on the specific activity indicated by the producer (300 U/mg). At different incubation times (20 min to 72 h), solutions were withdrawn from the thermomixer, boiled for 10 min to inactivate the enzyme, and characterized. The concentration of soluble HA and its corresponding M_w were also determined with a Viscotek TDA 305 instrument (Malvern Instruments, USA).

HA cryogels are biomaterials with promising *in vivo* applications; therefore, resistance to enzymatic degradation was

investigated. Fig. S4 shows the change in M_w of HA in solution and the soluble part from neutral cryogels with time in 100 U/mL hyaluronidase/PBS at 37 °C. The decrease in M_w in cryogels was significantly smaller than that of native HA throughout the entire experimental period, and the observed difference increased with prolonged freezing time [Fig. S4(A)]. These results indicated that the gelation of HA through freeze-thaw treatment dramatically improved the resistance of HA to enzymatic degradation. Soluble contents for HA neutral cryogel in the hyaluronidase solution showed the same trend [Fig. S4(B)].

References

1. Okamoto, A; Miyoshi, T. In *Hyaluronan 2000*; Kennedy, J. F., Phillips, G. O., Williams, P. A., Hascall, V. C. Eds.; Woodhead Publishing: Cambridge, UK, 2002; p285-292.

2 Martens, A.; Besseling, N. A. M.; Sudholter, E. J. R.; de Smet, L. C. P. M. Structure of hyaluronan during acid-catalyzed hydrolysis, a kinetic study of both chain scission and disaggregation. *Soft Matter* **2012**, 8, 1017-1024.

2. La Gatta, A.; de Rosa, M.; Marzaioli, I; Busico, T.; Schiraldi, C. A complete hyaluronan hydrodynamic characterization using a size exclusion chromatography-triple detector array system during in vitro enzymatic degradation. *Anal. Biochem.* **2010**, 404, 21-29.