



## Macromolecular Nanotechnology

## Mechanically strong hyaluronic acid hydrogels with an interpenetrating network structure



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## ABSTRACT

Hyaluronic acid (HA) is a natural glycosaminoglycan of high molecular weight with important biological and physicochemical functions. Although hydrogels derived from HA are effective biomaterials for soft tissue regeneration, they are generally brittle, or require complicated synthetic procedures. A simple one-pot synthesis method of mechanically strong HA hydrogels would be attractive for many application areas. Here, we present preparation of HA hydrogels via free-radical copolymerization of methacrylated HA (GMHA) and N,N-dimethylacrylamide (DMA) in aqueous solutions. GMHA was synthesized by methacrylation of native HA at various levels of methacrylation between 4 and 25% corresponding to 115–721 pendant methacrylate groups per GMHA molecule. It was found that GMHA acts as a multifunctional cross-linker during its copolymerization with DMA leading to the formation of interpenetrated and interconnected polymer networks. The effective functionality of GMHA increases with its degree of methacrylation as well as with the DMA concentration. The viscoelastic and mechanical properties of HA hydrogels could be tuned by varying the degree of methacrylation of GMHA and DMA concentration. A significant improvement in the mechanical performance of the hydrogels was observed when DMA is replaced with methacrylic acid monomer. By adjusting the synthesis parameters, hydrogels with a Young's modulus of around 200 kPa could be prepared that sustain up to 20 MPa stresses at 96% compression.

## 1. Introduction

Hyaluronan or hyaluronic acid (HA) is a natural glycosaminoglycan of high molecular weight composed of disaccharide repeat units of  $\beta$ -1,4-D-glucuronic acid and  $\beta$ -1,3-N-acetyl-D-glucosamine (Scheme 1) [1]. In physiological solutions, HA has a highly extended random coil conformation due to the hydrogen bonding between disaccharide units, and polyanionic properties [2,3]. HA is the major component of the extracellular matrix of vertebrate tissues with important biological and physicochemical functions. Due to the polyelectrolyte nature, HA can absorb large amounts of water and hence acts as lubricant in native extracellular matrix and controls the viscoelasticity of connective tissues [3].

Although HA is an attractive biomaterial for regeneration of soft tissues [4–9], it has limited application areas due to its rapid degradation and poor biomechanical properties. To overcome this drawback, HA has been physically or chemically cross-linked to form HA hydrogels [3,10–12]. The hydrogels based on HA are however generally brittle, or easily dissolve in aqueous solutions. For instance, HA hydrogels prepared by photopolymerization of methacrylated HA in aqueous solutions fracture at around 35% compressive strain under < 60 kPa stresses [13]. Cross-linking of native HA in aqueous solutions using cross-linkers such as divinyl

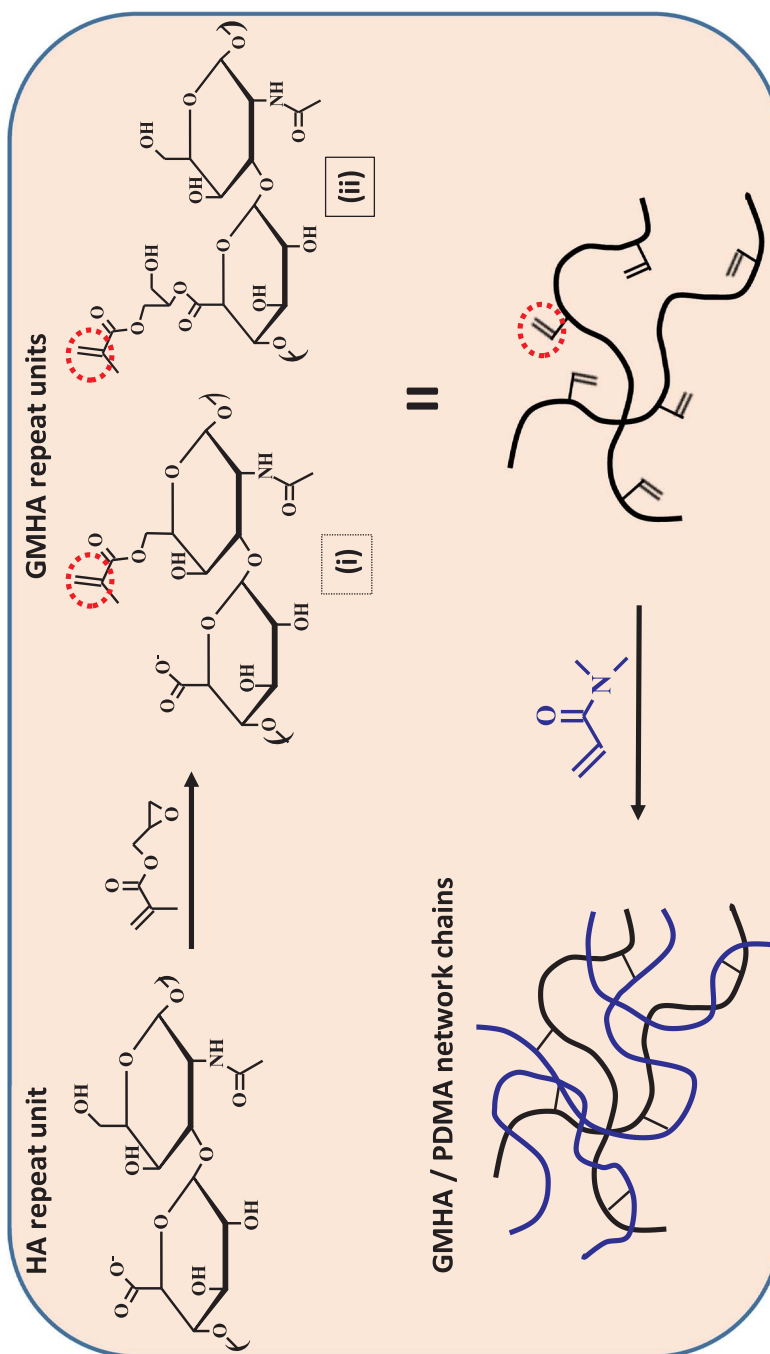
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**Scheme 1.** Formation of methacrylated HA (GMHA) by methacrylation of native hyaluronic acid (HA) using glycidyl methacrylate via transesterification (i) and ring opening (ii), and its copolymerization with DMA to form interconnected and interpenetrated GMHA/PDMA network hydrogels. Red circles indicate the methacrylate groups incorporated as pendant into GMHA molecules acting as potential cross-link points during the copolymerization with DMA.

sulfone, glutaraldehyde, and ethylene glycol diglycidyl ether (EGDE) also results in brittle materials [14–21]. For instance, EGDE-cross-linked HA hydrogels rupture when compressed to 25–51% strain under 20–150 kPa stresses [21]. To improve the mechanical performance of HA hydrogels, cryogelation technique has been employed which bases on conducting the gelation reactions below the freezing point of aqueous HA solutions [22–24]. Another strategy is the preparation double-network HA hydrogels consisting of brittle and ductile network components [13,21,25,26]. Double-network HA hydrogels were prepared by swelling the brittle single-network HA hydrogel in a monomer solution and subsequent polymerization of the monomer to create an interconnected and interpenetrated network of HA and synthetic polymers [25]. Although double-network hydrogels based on HA exhibit improved mechanical strength as compared to the single-network ones [13,21,25], their synthetic procedure is lengthy and complicated. Thus, a simple one-pot synthesis of mechanically strong HA hydrogels would be attractive for many application areas.

Here, we describe preparation of mechanically strong single-network HA hydrogels via free-radical copolymerization of methacrylated HA and N,N-dimethylacrylamide (DMA) in aqueous solutions. The choice of the monomer DMA is due to the fact that poly(N,N-dimethylacrylamide) (PDMA) is a hydrophilic biocompatible polymer possessing strong associative properties and hydrogen-bonding interactions with HA [27–29]. The precursor of HA hydrogels, namely methacrylated HA (GMHA) at various levels of methacrylation between 4 and 25% was prepared by methacrylation of native HA using glycidyl methacrylate (Scheme 1) [30–32]. As will be seen below, GMHA acts as a multifunctional cross-linker during its copolymerization with DMA leading to the formation of interpenetrated and interconnected polymer network hydrogels. The effective functionality of GMHA increases with its degree of methacrylation as well as with the DMA concentration. The viscoelastic and mechanical properties of the hydrogels could be tuned by varying the degree of methacrylation of GMHA and DMA concentration. We also observed a significant improvement in the mechanical performance of the hydrogels when DMA is replaced with methacrylic acid (MAAc) monomer. By adjusting the synthesis parameters, hydrogels with a Young's modulus of around 200 kPa could be prepared that sustain up to 20 MPa stresses at 96% compression.

## 2. Materials and methods

### 2.1. Materials

Hyaluronic acid sodium salt (HA, Sigma-Aldrich, impurities:  $\leq 1$  protein) from *Streptococcus equi* has a viscosity averaged molecular weight of  $1.2 \times 10^6 \text{ g mol}^{-1}$  [22]. Glycidyl methacrylate (GM, Sigma Aldrich, 97%), N,N-dimethylacrylamide (DMA, Sigma-Aldrich, 99%), methacrylic acid (MAAc, Merck, 99%), triethylamine (TEA, Merck, 99%), tetrabutylammonium bromide (TBAB, Sigma-Aldrich,  $\geq 99\%$ ), 1-vinyl pyrrolidone (VP, Sigma-Aldrich), ammonium persulfate (APS, Sigma-Aldrich,  $\geq 99\%$ ), N,N,N',N'-tetramethylethylenediamine (TEMED, Sigma-Aldrich,  $\geq 99\%$ ), and acetone (Tekkim, 99.5%) were used as received. APS stock solution was prepared by dissolving 0.8 g of APS in 10 mL distilled water.

### 2.2. Methacrylation of HA

Methacrylated HA (GMHA) was synthesized by the reaction of HA with glycidyl methacrylate (GM), as described previously [13,30–32]. Typically, HA (0.5 g) was first dissolved under stirring overnight in 50 mL distilled water at room temperature. To prepare a reaction solution containing GM at a 6-fold molar excess to the disaccharide repeat unit of HA, TEA catalyst (1 mL), GM (1 mL), and TBAB (1 g) as phase transfer catalyst were mixed with the HA solution. The solution was then heated to 55 °C and stirred at this temperature for 1 h. After cooling to room temperature, the solution was precipitated twice in excess acetone and the precipitate was dissolved in water. The solution of GMHA in 10 mL water was then frozen at  $-25$  °C for 1 day and freeze-dried using Christ Alpha 2e4 LD-plus freeze-dryer at  $-40$  °C under 0.12 mbar vacuum for 1 day and at  $-60$  °C under 0.011 mbar for an additional 1 day.  $^1\text{H}$  NMR spectroscopy (500 MHz Agilent VNMRs spectrometer) was used to determine the level of methacrylation of

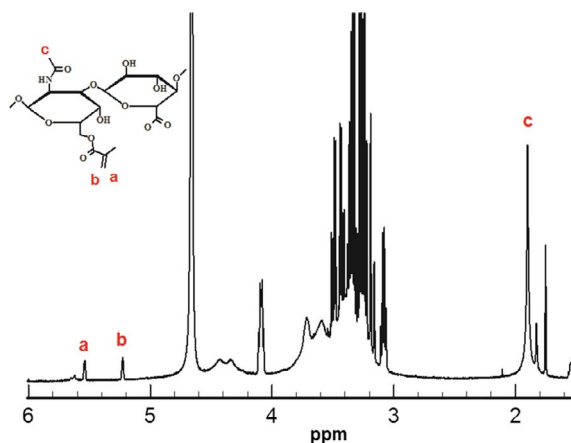


Fig. 1. Typical  $^1\text{H}$  NMR spectrum of methacrylated hyaluronic acid. GM/HA molar ratio = 24.

GMHA. Typical  $^1\text{H}$  NMR spectrum of GMHA is shown in Fig. 1. The two peaks at 5.2 and 5.5 ppm are due to the methacrylate groups (a, b), while the methyl group of HA appears at 1.9 ppm (c) [13,31]. The methacrylation degree (DM) was calculated from the integration of the methyl peak of HA and the methacrylate peaks. In accord with previous work [13], the level of methacrylation was found to be 4, 8, 14, and 25% for the GM/HA molar ratios of 6, 12, 24, and 49, respectively.

### 2.3. Hydrogel preparation

The hydrogels were prepared at 4 °C in aqueous solutions of DMA and GMHA using a redox initiator system consisting of 3.5 mM APS and 0.25 v/v% TEMED. The initial concentration of GMHA was set to 1 w/v% while both DMA concentration and the methacrylation degree of GMHA were varied between 5–50 w/v% and 4–25%, respectively. To illustrate the synthetic procedure, we give details for the preparation of hydrogels at 30 w/v% DMA concentration: GMHA (100 mg) was dissolved in 6.8 mL of distilled water overnight under continuous stirring. DMA (3.1 mL) and TEMED (25  $\mu\text{L}$ ) were then added and the aqueous solution was stirred for 30 min under bubbling nitrogen. After addition of APS stock solution (0.1 mL), a portion of the reaction solution was transferred between the plates of the rheometer for the rheological measurements. For the swelling and mechanical measurements, the remaining part of the solution was transferred into several plastic syringes of 4.6 mm internal diameter and the polymerization was conducted for 24 h at 4 °C.

### 2.4. Swelling and gel fraction measurements

After a reaction time of 24 h, hydrogel samples were immersed in a large excess of water at 25 °C for at least 4 days whereby the water was replaced every day to extract any soluble species. The swelling equilibrium was tested by weighing the gel specimens. The equilibrium swollen gel samples were taken out of water and dried at 80 °C under vacuum to constant mass. The equilibrium weight swelling ratios with respect to dry and as-prepared states,  $q_w$  and  $q_{w,o}$ , respectively, were calculated as

$$q_w = m/m_{\text{dry}} \quad (1a)$$

$$q_{w,o} = m/m_o \quad (1b)$$

where  $m$ ,  $m_o$ , and  $m_{\text{dry}}$  are the masses of the gel sample in equilibrium swollen, as-prepared and dry states, respectively. The gel fraction  $W_g$  was calculated from the masses of dry polymer network and from the comonomer feed.

### 2.5. Rheological experiments

The copolymerization reactions of GMHA and DMA were monitored at 4 °C within the rheometer (Gemini 150 Rheometer system, Bohlin Instruments) equipped with a cone-and-plate geometry (cone angle = 4°, diameter = 40 mm). The instrument was equipped with a Peltier device for temperature control. During the rheological measurements, a solvent trap was used and the outside of the upper plate was covered with a thin layer of low-viscosity silicone oil to prevent the evaporation of water. An angular frequency  $\omega$  of 6.3  $\text{rad s}^{-1}$  and a deformation amplitude  $\gamma_o$  of 0.01 were selected to ensure that the oscillatory deformation is within the linear regime. After a reaction time of 17 h, the elastic moduli  $G'$  of the reaction solutions approached limiting values. Then, frequency-sweep tests were carried out at 25 °C. The viscosity measurements on aqueous DMA and MAAc solutions containing 1 w/v% native HA or GMHA were conducted at 25 °C between shear rates  $10^{-2}$  and  $10^1 \text{ s}^{-1}$ . We have to mention that because the rheological tests were conducted between the metal plates while swelling and mechanical tests were carried out on gel samples prepared in plastic syringes, gelation dynamics in both cases may differ due to different environments.

### 2.6. Mechanical tests

Uniaxial compression and elongation measurements were conducted at 25 °C on a Zwick Roell Z0.5 TH test machine using a 500 N load cell. For the compression tests, the cylindrical gel samples in both as-prepared and swollen states were cut into cubic samples with dimensions 3x3x3 mm. Before the tests, an initial compressive contact of 0.01 N was applied to ensure a complete contact between the gel and the plates. The compression tests were performed at a constant cross-head speed of 0.3 and 1  $\text{mm min}^{-1}$  below and above 15% compression, respectively. The stress was presented by its nominal  $\sigma_{\text{nom}}$  and true values  $\sigma_{\text{true}}$  ( $= \lambda \sigma_{\text{nom}}$ ), which are the forces per cross-sectional area of the undeformed and deformed gel specimen, respectively, and  $\lambda$  is the deformation ratio (deformed length/initial length). The compressive strain  $\epsilon_c$  is defined as the change in the length relative to the initial length of the gel specimen, i.e.,  $\epsilon_c = 1 - \lambda$ . The compressive strength and strain of the hydrogels were calculated from the maxima in  $\sigma_{\text{true}}$  vs.  $\epsilon_c$  curves, as detailed before [33]. The uniaxial elongation tests were performed on cylindrical gel samples of 4.6 mm diameter in as-prepared state. The initial length of the gel samples between jaws and the cross-head speed were  $10 \pm 2 \text{ mm}$  and  $5 \text{ mm min}^{-1}$ , respectively. The tensile strain  $\epsilon$  is calculated as  $\epsilon = \lambda - 1$ . Young's modulus  $E$  of the hydrogels was calculated from the slope of stress-strain curves between 5 and 15% compression and elongation. For reproducibility, at least five samples were measured for each gel and the results were averaged.

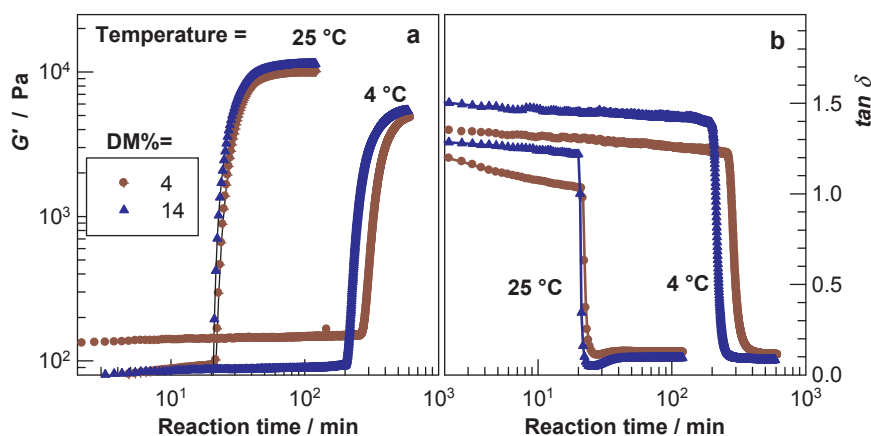


Fig. 2. Storage modulus  $G'$  (a) and the loss factor  $\tan \delta$  (b) during the copolymerization of GMHA and DMA shown as a function of the reaction time. The degree of methacrylation DM is 4 (circles) and 14% (triangles). DMA = 30 w/v%. GMHA = 1 w/v%. The reaction temperatures are shown in the Figure.

### 3. Results and discussion

#### 3.1. Formation of HA hydrogels

The precursor of the present hydrogels, namely methacrylated hyaluronic acid (GMHA) was synthesized at various levels of methacrylation between 4 and 25% using glycidyl methacrylate by a competing reaction mechanism between transesterification and ring opening (Scheme 1) [30–32]. Because the average molecular weight of hyaluronic acid (HA) used in the hydrogel preparation is  $1.2 \times 10^6 \text{ g mol}^{-1}$  and the molecular weight of the disaccharide repeat unit is  $416 \text{ g mol}^{-1}$ , 4–25% methacrylation indicate that 115–721 methacrylate groups were incorporated per molecule of GMHA as pendant vinyl groups. Thus, GMHA can be considered as a multifunctional macromolecular cross-linker able to form interpenetrated and interconnected polymer networks when copolymerized with vinyl monomers such as N,N-dimethylacrylamide (DMA) (Scheme 1).

Transparent hydrogels with tunable viscoelastic properties were prepared by copolymerization of GMHA and DMA in aqueous solutions using APS-TEMED redox initiator system. The amount of GMHA in the reaction solution was fixed at 1 w/v% while both the degree of methacrylation of GMHA and DMA concentration in the comonomer feed were varied. No gel formation could be detected by polymerization of GMHA alone, which we attribute to the low GMHA concentration making the intramolecular cross-linking reactions favorable. Because GMHA was insoluble in aqueous solutions containing more than 50 w/v% DMA, we conducted the copolymerization reactions below 50 w/v% DMA. We have to mention that the polymerization of aqueous 5–50% DMA solutions in the absence of GMHA resulted in semi-dilute PDMA solutions revealing that the self-cross-linking efficiency of DMA is insufficient for the onset of gelation [34]. The gelation reactions were initially carried out at both 4 and 25 °C. Typical gelation profiles of the reaction solutions obtained by rheometry using oscillatory deformation tests are shown in Fig. 2 where the storage modulus  $G'$  and loss factor  $\tan \delta$  ( $= G''/G'$ , where  $G''$  is the loss modulus) are plotted against the polymerization time. The initial reaction solutions contain 30 w/v% DMA and 1 w/v% GMHA with methacrylation degrees of 4 and 14%. It is seen that, although increasing the polymerization temperature from 4 to 25 °C significantly reduces the induction period of the reaction, the limiting values of both  $G'$  and  $\tan \delta$  are close together after 10 h. However, to eliminate the possibility of degradation of GMHA during gelation [35], all the hydrogels reported below were prepared at 4 °C.

Frequency-sweep results of the hydrogels after a reaction time of 17 h are shown in Fig. 3 where the storage  $G'$  and loss moduli  $G''$  are plotted against the angular frequency  $\omega$ . In Fig. 3a, the hydrogels were prepared at 5 w/v% DMA and at two different degrees of methacrylation (DM) while in Fig. 3b, DM was fixed at 14% while DMA concentration was varied between 5 and 30 w/v%. The general trend is that, at DMA contents below 20 w/v%, the hydrogels exhibit predominantly elastic or viscous nature depending on the frequency, i.e., on the time scale of the rheological tests. At low frequencies,  $G''$  attains very low values ( $\sim 10^1 \text{ Pa}$ ) and the loss factor  $\tan \delta$  approaches to 0.01 corresponding elastic, solid-like behavior. At high frequencies,  $G''$  approaches to  $G'$  and the gels exhibit a viscous character. This feature is opposite to what is observed in semi-dilute polymer solutions, but similar to hydrogel systems with strong hydrogen bonding interactions [21,36–38]. Thus, the intermolecular hydrogen bonds between GMHA and GMHA-PDMA molecules seem to act as physical cross-links at low frequencies and thus contribute to the gel elasticity. Because these bonds are broken at high frequencies, increasing amount of energy is dissipated with increasing frequency so that  $G''$  increases leading to the appearance of a strong-to-weak gel transition. Moreover, as indicated by the arrows in Fig. 3,  $G'$  increases with the methacrylation degree, or with the monomer concentration DMA%, while the loss modulus  $G''$  is only affected by DMA%.

To highlight the effect of the synthesis parameters on the viscoelastic properties of the hydrogels,  $G'$  and  $\tan \delta$  of all hydrogels measured at  $6.3 \text{ rad s}^{-1}$  are shown in Fig. 4a as a function of the DMA concentration. The arrows indicate direction of increasing methacrylation degree (DM) of GMHA. Increasing DMA% also increases the storage modulus  $G'$  while the loss factor remains almost unchanged revealing that the viscoelastic nature of the hydrogels is not much affected with increasing polymer concentration. However, when the methacrylation degree is increased at a fixed DMA%,  $G'$  increases while  $\tan \delta$  decreases indicating increasing

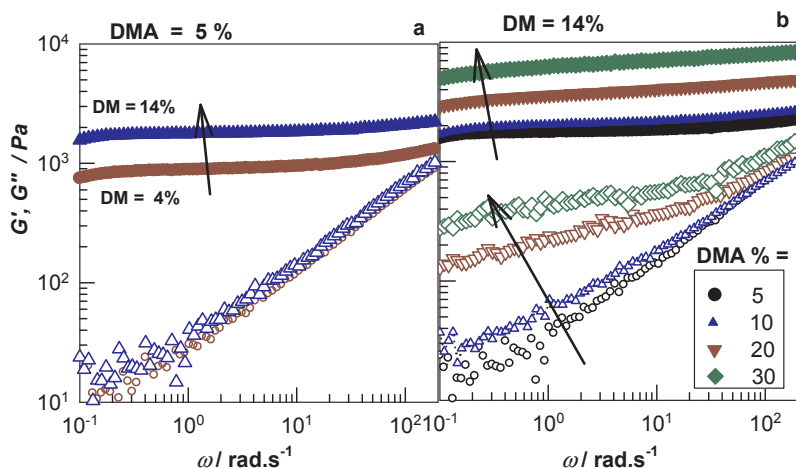


Fig. 3. Storage moduli  $G'$  (filled symbols) and loss moduli  $G''$  (open symbols) shown as a function of the angular frequency  $\omega$  measured after 17 h of reaction time. (a): DMA = 5 w/v%. DM = 4 (circles) and 14% (triangles). (b): DM = 14%. DMA concentrations are indicated. The arrows show the direction of increasing DM (a) and DMA% (b).

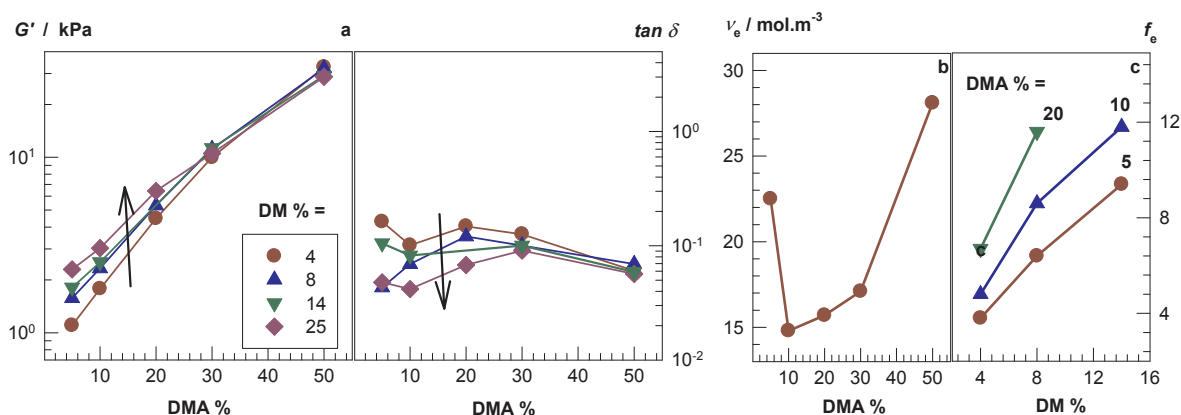


Fig. 4. (a): The storage modulus  $G'$  and loss factor  $\tan \delta$  measured at  $6.3 \text{ rad.s}^{-1}$  shown as a function of the monomer (DMA) concentration. The arrows indicate direction of increasing methacrylation degree (DM) of GMHA. Methacrylation degrees DM of GMHA are indicated. (b): Cross-link density  $\nu_e$  of the hydrogels calculated using eq 2 b plotted against DMA concentration. (c) Variation of the effective functionality  $f_e$  of GMHA with the level of methacrylation DM. The solid curves are guide to the eye.

elastic character of the hydrogels. Assuming that  $G'$  measured at  $6.3 \text{ rad.s}^{-1}$  corresponds to the equilibrium shear modulus  $G$ , one may calculate the effective cross-link density  $\nu_e$  of the hydrogels. According to the phantom network model,  $G$  at the state of gel preparation is related to  $\nu_e$  by [39,40]:

$$G = (1 - f_e) \nu_e R T \nu_2^0 \quad (2a)$$

where  $f_e$  is the average effective functionality of GMHA macromer, that is the number of elastically effective PDMA network chains per GMHA cross-link,  $\nu_2^0$  is the volume fraction of cross-linked polymer in the gel,  $R$  and  $T$  are in their usual meanings. At the highest degree of methacrylation of 25% corresponding to the existence of 720 pendant methacrylate groups per GMHA molecule, the functionality  $f_e$  is expected to be much larger than unity so that the first term at the right hand side of Eq. (2a) reduces to unity (affine limit), i.e.,

$$G = \nu_e R T \nu_2^0 \quad (2b)$$

Thus, using the modulus data of the hydrogels formed using GMHA with DM = 25% (Fig. 4a), the cross-link density  $\nu_e$  of the hydrogels can be calculated using Eq. (2b). Substituting these  $\nu_e$  values for each DMA concentration into Eq. (2a) allows estimation of the average functionality  $f_e$  as a function of the methacrylation degree. In Fig. 4b & c, the effective cross-link density  $\nu_e$  and the average functionality  $f_e$  are plotted against DMA% and DM%, respectively. For calculations, the volume fraction  $\nu_2^0$  of polymer in the as-prepared hydrogels was estimated using the equation  $\nu_2^0 = 10^{-2} (\text{DMA}\%)/d_2$ , where  $d_2$  is the density of PDMA ( $1.21 \text{ g mL}^{-1}$  [41]). Except the initial drop in  $\nu_e$  between 5 and 10% DMA,  $\nu_e$  continuously increases with increasing DMA% indicating formation of larger number of effective cross-links per dry polymer volume. Moreover, the effective functionality  $f_e$  of GMHA varies between 4 and 13, and increases both with the methacrylation degree of GMHA and DMA concentration. Thus, although GMHA macromonomer acts



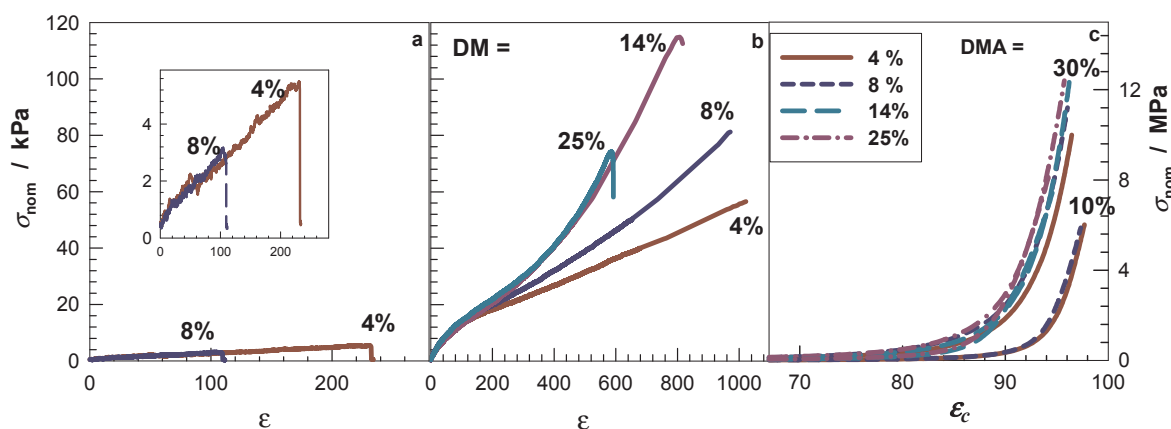


Fig. 5. (a, b): Tensile stress-strain curves of HA hydrogels formed at 10 (a) and 30 w/v% DMA (b) as the dependence of nominal stress  $\sigma_{nom}$  on the strain  $\epsilon$ . Methacrylation degree DM of GMHA is indicated (c): Compressive stress – strain curves of HA hydrogels as the dependence of  $\sigma_{nom}$  on the compressive strain  $\epsilon_c$ . DMA concentration and DM are indicated.

as a multifunctional cross-linker, its effective functionality is much smaller than the number of methacrylate groups incorporated as pendant into HA molecules. This is attributed to the cyclization reactions as well as reduced reactivity of pendant methacrylate groups that are generally observed in free-radical cross-linking copolymerization [42].

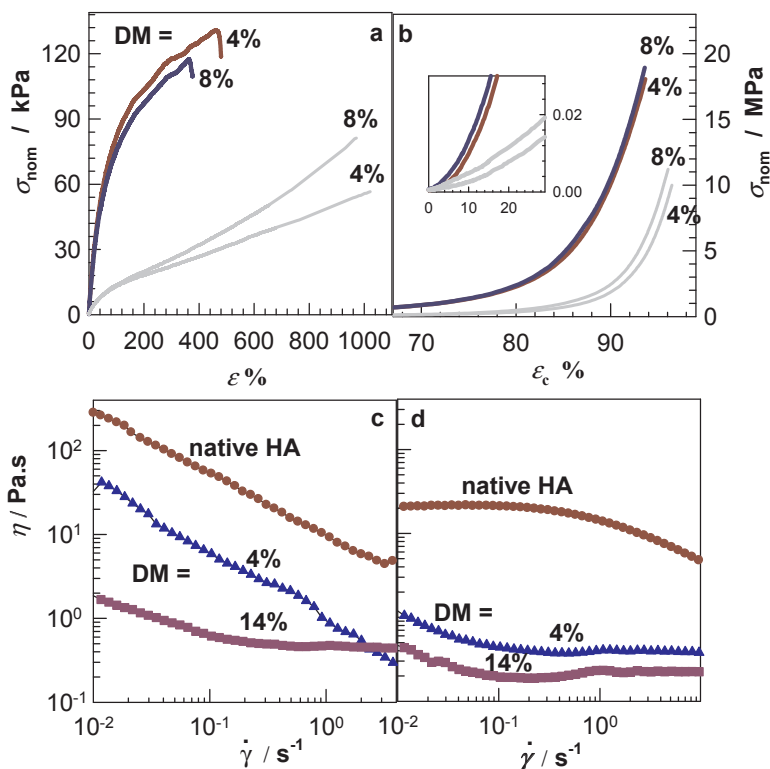
### 3.2. Mechanical properties of as-prepared HA hydrogels

HA hydrogels after a reaction time of 24 h were subjected to uniaxial compression and elongation tests. Fig. 5a & b show typical tensile stress-strain curves of the hydrogels formed at 10 and 30 w/v% DMA, respectively, where the nominal stress  $\sigma_{nom}$  is plotted against the tensile strain  $\epsilon$ . The methacrylation degree (DM) of GMHA used in the hydrogel preparation is indicated in the figures. The hydrogels formed at 10 w/v% DMA and at DM > 8% were brittle in tension and they already broke at the start of the mechanical tests. The maximum tensile strength  $\sigma_f$  observed at the lowest methacrylation degree of 4% was  $5 \pm 1$  kPa. Stronger hydrogels could be obtained at 30 w/v% DMA concentration (Fig. 5b); they all sustain above 600% elongation ratios and their tensile strength  $\sigma_f$  increases from  $55 \pm 3$  to  $111 \pm 13$  kPa with increasing DM from 4 to 14% while further increase in DM decreases  $\sigma_f$  of the hydrogels. Fig. 5c shows compressive stress  $\sigma_{nom}$  – strain  $\epsilon_c$  curves of the hydrogels formed at 10 and 30 w/v% DMA with varying DM of GMHA. The hydrogels formed at 10 and 30 w/v% DMA sustain  $7 \pm 1$  and  $11 \pm 1$  MPa compressive stresses, respectively, at  $96 \pm 1\%$  compressions.

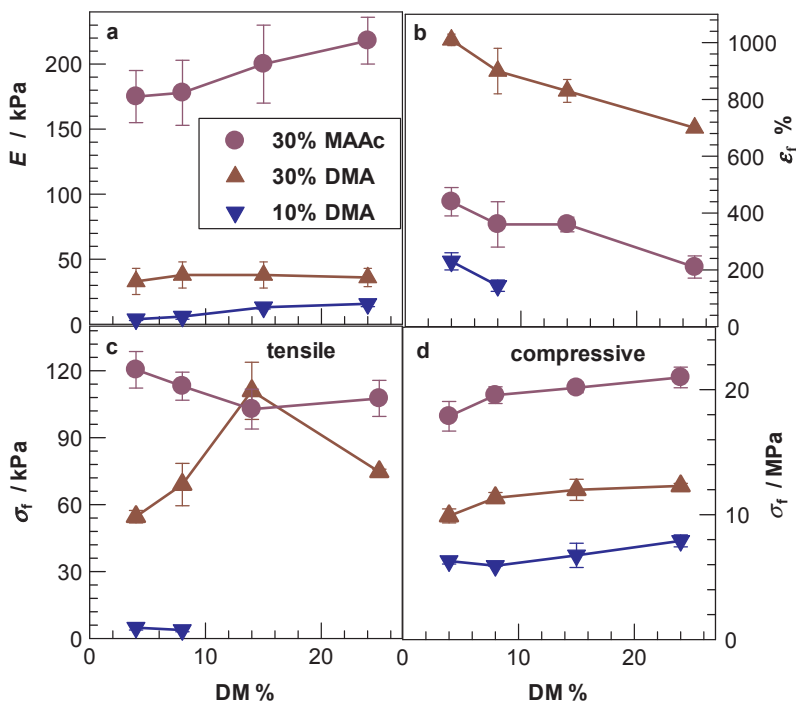
The results thus reveal that the simple one-pot free-radical copolymerization of GMHA and DMA provides formation of mechanically strong HA hydrogels by adjusting the degree of methacrylation of GMHA as well as the DMA concentration at gelation. Because of the existence of extensive hydrogen bonding interactions in HA solutions [2], and PDMA is a polymer with associative properties [27–29], the good mechanical performance of the hydrogels can be attributed to the existence of both hydrophobic and hydrogen bonding interactions acting as physical cross-links. These cross-links are reversibly broken under load and thus, resisting the crack propagation by dissipating energy and contributing to the mechanical properties [43,44]. Recently, Hu et al. demonstrated formation of tough physical hydrogels consisting of copolymer chains composed of DMA and methacrylic acid (MAAc) units [45]. We conducted gelation reactions by replacing half of the DMA with MAAc monomer but observed any further improvement in the mechanical properties of the final hydrogels. However, total replacement of DMA with MAAc resulted in stronger hydrogels. Tensile and compressive stress–strain curves of HA hydrogels formed using 30 w/v% MAAc and GMHA with methacrylation degrees of 4 and 8% are shown in Fig. 6a & b, respectively. For comparison, the data obtained using 30 w/v% DMA are also shown in the figures by the gray curves. The inset in Fig. 6b shows the portion of the curves below 30% compression. It is seen that the initial slope of the stress-strain curves corresponding to the Young's modulus significantly increases when DMA is replaced with MAAc monomer.

In Fig. 7a–d, Young's modulus  $E$ , elongation at break  $\epsilon_f$ , compressive and tensile strengths  $\sigma_f$  of HA hydrogels are shown as a function of methacrylation degree DM of GMHA. The hydrogels formed using MAAc monomer exhibit a modulus  $E$  between 175 and 218 kPa that increases with increasing degree of methacrylation, as compared to 33–36 kPa obtained using DMA monomer at the same concentration. Thus, GMHA/poly(methacrylic acid) (PMAAc) hydrogels exhibit about 5-fold larger modulus as compared to GMHA/PDMA ones indicating the contribution of non-covalent cross-links to the effective cross-link density. Moreover, tensile strength increases from  $62 \pm 7$  to  $117 \pm 4$  kPa while compressive strength increases from 10 to 20 MPa when MAAc is used in the gel preparation instead of DMA.

Stronger extent of non-covalent interactions in hydrogels based on GMHA/PMAAc as compared to those based on GMHA/PDMA seems to be responsible for the improved mechanical properties of the resulting hydrogels. To compare the extent of non-covalent interactions, viscosity measurements at 25 °C were conducted on aqueous solutions of DMA and MAAc at a concentration of 30 w/v% containing 1 w/v% HA or GMHA. Fig. 6c & d present the viscosity versus shear rate curves for native HA and GMHA in MAAc (c) and DMA solutions (d). Note that pH's of the solutions are  $2.6 \pm 0.1$  and  $5.7 \pm 0.1$  for MAAc and DMA, respectively. The viscosity

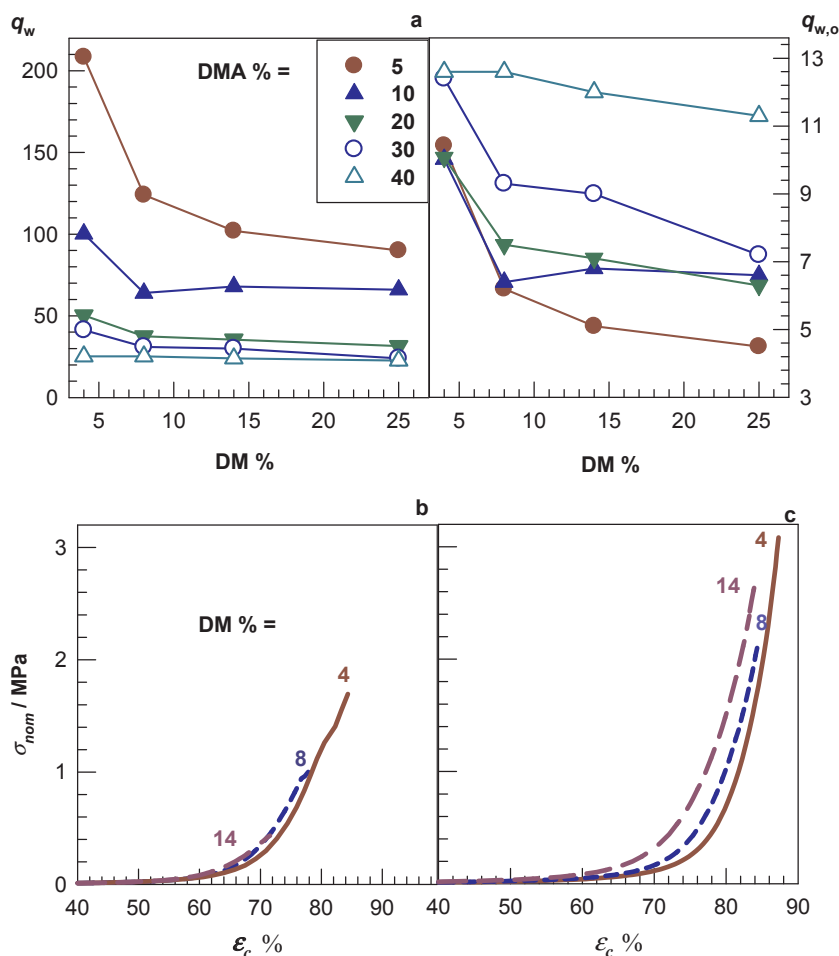


**Fig. 6.** (a, b): Tensile (a) and compressive stress-strain curves (b) of HA hydrogels formed using MAAc at a concentration of 30 w/v%. For comparison the data of the hydrogels formed using 30 w/v% DMA are also shown by gray curves. (c, d) Viscosities  $\eta$  of 1 w/v% native HA and GMHA at 2 different methacrylation degrees in aqueous solutions of 30 w/v% MAAc (c) and DMA (d) plotted against the shear rate  $\dot{\gamma}$ .



**Fig. 7.** Young's modulus  $E$  (a), elongation at break  $\epsilon_f$  (b), tensile and compressive strengths  $\sigma_f$  (c and d, respectively) of the hydrogels shown as a function of methacrylation degree DM of GMHA. The type and concentration of the monomer are indicated.





**Fig. 8.** (a): Equilibrium weight swelling ratios of HA hydrogels with respect to their dry  $q_w$  and as-prepared states  $q_{w,o}$  shown as a function of the methacrylation degree of GMHA. DMA concentrations are indicated. (b, c): Compressive stress-strain curves of swollen HA hydrogels formed at 10 (b) and 30 w/v% DMA (b). Methacrylation degree of GMHA is indicated.

decreases with increasing methacrylation degree of GMHA due to the increasing hydrophobicity of HA upon incorporation of methacrylate groups [13]. Moreover, both native HA and GMHA exhibit higher viscosities at low shear rates and marked shear-thinning in the presence of MAAc as compared to DMA. This indicates increasing associativity of GMHA chains in MAAc environment and thus support the experimental findings.

### 3.3. Swelling behavior and mechanical properties of HA hydrogels in equilibrium swollen state

Gel fraction  $W_g$  of the hydrogels formed using DMA monomer was above 0.95 after a reaction time of 24 h and they contained 94–99% water in their equilibrium swollen states in water. Hydrogels formed using MAAc exhibited very large swelling ratios due to the osmotic pressure of counterions of carboxylate groups. They also became too weak to perform any meaningful gel fraction, swelling and mechanical tests. In Fig. 8a, the equilibrium weight swelling ratios of GMHA/PDMA hydrogels with respect to their dry ( $q_w$ ) and as-prepared states ( $q_{w,o}$ ) are plotted as a function of the methacrylation degree DM of GMHA. As expected,  $q_w$  decreases with increasing methacrylation degree as well as with increasing DMA concentration due to the simultaneous increase of the cross-link density  $\nu_c$  and cross-link functionality  $f_c$  (Fig. 4). However, when considering the swelling ratios with respect to the preparation state of the hydrogels, the higher DMA concentration, the higher is the degree of swelling  $q_{w,o}$ . This is due to the fact that the translational entropy of DMA monomer upon its polymerization decreases so that more water enters into the as-prepared hydrogel with increasing DMA% to assume its new thermodynamic equilibrium [33].

Fig. 8b & c show compressive stress-strain curves of swollen HA hydrogels formed at 10 and 30 w/v% DMA, respectively. In accord with the mechanical behavior of as-prepared hydrogels, those formed at 30 w/v% DMA exhibit better mechanical properties as compared to 10 w/v% DMA hydrogels. In Fig. 9, Young's modulus  $E$ , compressive strength  $\sigma_f$ , and strain at break  $\epsilon_f$  of swollen HA hydrogels formed at 10 and 30 w/v% DMA are shown as a function of methacrylation degree DM of GMHA. For comparison, the data obtained from as-prepared hydrogels are also shown by gray symbols. It is seen that the ultimate properties of the hydrogels, e.g., the

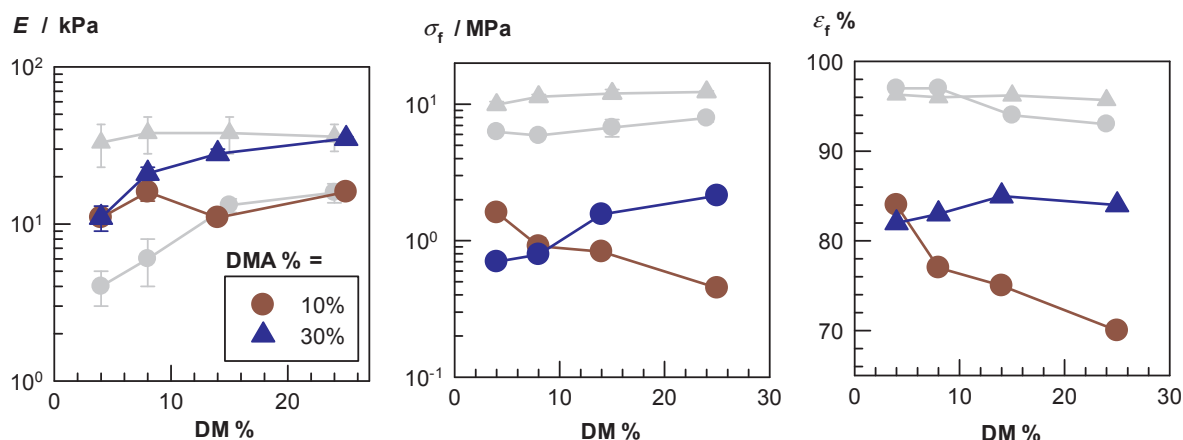


Fig. 9. Young's modulus  $E$ , compressive strength  $\sigma_f$ , and strain at break  $\epsilon_f$  of swollen HA hydrogels formed at 10 (circles) and 30 w/v% DMA (triangles) shown as a function of methacrylation degree DM of GMHA. Gray symbols represent data of the hydrogels in their preparation states.

fracture stress and fracture strain decrease after swelling of HA hydrogels. However, the modulus  $E$  does not change much and remains at around 10–30 kPa although swelling results in a 7- to 9-fold dilution of the hydrogels (Fig. 7a). This suggests increasing effective cross-link density of the hydrogels upon their swelling which is attributed to increasing extent of hydrogen bonding and hydrophobic interactions between GMHA and PDMA chains acting as additional cross-links [27–29].

#### 4. Conclusions

We presented a simple one-pot procedure for the preparation of mechanically strong HA hydrogels with tunable viscoelastic and mechanical properties. The precursor of the hydrogels, namely methacrylated hyaluronic acid (GMHA) was synthesized by methacrylation of native HA using glycidyl methacrylate at various levels of methacrylation between 4 and 25%, corresponding to 115–721 pendant methacrylate groups per GMHA molecule. The hydrogels were prepared via free-radical copolymerization of GMHA and N,N-dimethylacrylamide (DMA) in aqueous solutions. It was found that GMHA acts as a multifunctional cross-linker during its copolymerization with DMA leading to the formation of interpenetrated and interconnected polymer networks. The average functionality of GMHA for intermolecular cross-linking reactions increases with its methacrylation degree as well as with the DMA concentration. Both the methacrylation degree of GMHA and DMA concentration strongly affect the properties of HA hydrogels both in their as-prepared and equilibrium swollen states. We also observed a significant improvement in the mechanical performance of the hydrogels when DMA is replaced with methacrylic acid monomer. By adjusting the synthesis parameters, hydrogels with a Young's modulus of around 200 kPa could be prepared that sustain up to 20 MPa stresses at 96% compression.

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