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Preparation and physical properties of hyaluronic acid-based cryogels

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ABSTRACT: Macroporous hydrogels based on hyaluronan (HA), a natural polysaccharide occurring in extracellular matrix, have attracted interest over many years owing to their numerous applications in the biomedical area. However, HA hydrogels produced so far suffer from low mechanical strength and slow rate of response against external stimuli, which limit their applications. Here, we prepared macroporous HA cryogels of high mechanical stability and fast responsivity from aqueous HA solutions at subzero temperatures using ethylene glycol diglycidyl ether as a crosslinking agent. HA cryogels are squeezable and no crack development was exhibited when compressed up to 80% strain. Depending on the synthesis parameters, the cryogels exhibit an elastic modulus between 0.2 and 2 kPa, and show fast swelling/deswelling behavior. The microstructure of the cryogels consists of large, interconnected pores on the order of 100 μm separated by thick pore walls, as observed by scanning electron microscopy and confocal scanning laser microscopy. © 2015 The Authors Journal of Applied Polymer Science Published by Wiley Periodicals, Inc. **2015**, *132*, 42194.

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INTRODUCTION

Hyaluronan, or hyaluronic acid (HA), is a naturally occurring linear polyelectrolyte that is found in biological "bodies/organs" such as the vitreous body of the eye, connective tissues, and the synovial fluid of articular joints.[1] HA is extensively used in the biomedical field, for example, in ophthalmic surgery,[2,3] arthritic treatment,[4,5] and dermal fillers,[6] as well as tissue engineering.[6,7] However, many applications show that hyaluronan is unable to give sufficient efficacy if used without chemical crosslinking owing to its incapability of forming physical gels over a wide pH range[8] and its fast degradation by hyaluronases that exist in the body. Hyaluronan is composed of repeating units of β-1,4-D-glucuronic acid and β-1,3-*N*-acetyl-D-glucosamine (Figure 1).

Depending on pH, HA carries a negative charge on every second monomer. The conformation of HA in solution is stabilized by intramolecular H bonds,[9] resulting in a relatively stiff polymer conformation.[10] The intramolecular H bonds are disrupted by the presence of NaOH[11] and/or by temperature,[12,13] resulting in a more flexible conformation with a markedly reduced radius of gyration at a pH of 13.[11] The rheological properties of hyaluronan, at intermediate pH values, are typical of polymer systems with no strong intermolecular interactions, i.e., the complex dynamic viscosity can be compared with the shear viscosity at comparable frequency and shear rates.[14] Furthermore, the samples are characterized as a solution at low frequency and as a weak gel at higher frequencies.[10] As pH is reduced toward 2.5, hyaluronan forms a gel owing to the decrease in carboxylate dissociation favoring intermolecular interaction; however, a further decrease in pH results in a gel-to-solution transition, probably related to protonation of acetamido groups causing an electrostatic repulsion.[10] Likewise, an increase of pH above 3 will convert the weak gel formed at pH 2.5 back to solution. As naturally occurring hyaluronan is a non-gelling polymer, besides this relatively limited pH range, it is either chemically modified or covalently crosslinked in order to form a less degradable gel for use in biomedical applications.[8]

The functional groups available for crosslinking are the hydroxyl and carboxyl groups. Hydroxyl groups may be crosslinked via an

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ether linkage and carboxyl groups via an ester linkage.[15] Successful crosslinking of hyaluronan cast into films has been achieved using, among others, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), glutaraldehyde (GTA), poly(ethylene glycol) diglycidil ether (EX),[15,16] and divinyl sulfonate (DVS)[15,17] as crosslinkers. Crosslinking of HA with poly(ethylene glycol) diglycidil ether (PEGDE) and ethylene glycol diglycidil ether (EGDE) for biomedical and cell growth purposes has been tested and cytocompatibility has been shown.[18–20] However, a relatively high concentration of PEGDE and EGDE was used[18–20] and there are concerns that a high degree of modification and crosslinking can lead to reduced biocompatibility of the HA,[21] which otherwise shows excellent biocompatibility.[6] There is thus an interest in reducing the amount of added crosslinkers. One route to obtain such gel is the use of butandiol diglycidil ether as a crosslinker instead of the longer PEGDE together with the use of organic base.[22] Requirement of gels targeted for tissue engineering and cell in-growth is the ability to form macropores (100–300 μm in size)[17,23] with a high interconnectivity to facilitate the 3D organization of cells and the facilitation of blood vessel in-growth.[24] Hyaluronic scaffolds with macropores have been obtained by lyophilization of preformed gels,[15,19,20], by pattering of gels,[18] or by crosslinking of foams obtained after freezing (100 min) and lyophilization.[23] It is of further advantage if the gels are of high toughness, i.e. reversible deformability, as this allows for the use of gauge needles when and if the scaffold is introduced in the body.

Cryogelation is another strategy that allows the formation of macroporous gels of high toughness and fast responsiveness.[25–27] During cryogelation, the crosslinking reactions occur at a temperature below the freezing point of the reaction solution, creating an apparently frozen system consisting of solvent crystals and unfrozen liquid microchannels.[26,27] The crystals formed as the solvent freezes give rise to a highly interconnected porous structure that after melting corresponds to the pores. As the main bulk of the solvent freezes, any solutes are concentrated into the microchannels.[28] While cryogelation has been used as a route to form stable physical gels of otherwise non-gelling polysaccharides,[29] the gels formed in such ways using HA alone[8,30,31] yield soft and formless weak gels[32] with poor stability in water (the gel disintegrated).

As far as we are aware, the preparation of chemically crosslinked hyaluronan cryogels has not been reported despite its potential to form gels suitable for biomedical applications, e.g. tissue engineering. Here, we outline how cryogels are prepared from frozen aqueous solutions of HA using ethylene glycol diglycidyl ether (EGDE) as a crosslinking agent. By tuning the synthesis conditions, we obtained responsive and mechanically stable HA cryogels of high toughness and with interconnected macropores.

EXPERIMENTAL

Materials

The sodium salt of hyaluronic acid (HA) from *Streptococcus equi* was purchased from Sigma-Aldrich, Sweden. Phosphate buffer saline (PBS), sodium hydroxide (NaOH), sodium chloride (NaCl), and rhodamine B were also obtained from Sigma-Aldrich, Sweden. Acetone was supplied by Fisher Scientific, Sweden and glycerol (87%) by BioChemica, Germany. Ethylene glycol diglycidyl ether (EGDE), with a molecular weight of 174.2 g/mol, was purchased from Polysciences Inc.. The PBS solution was made by dissolving one tablet of PBS in 200 mL Milli-Q water according to the supplier's instructions and contained 0.01*M* phosphate buffer, 0.0027*M* potassium chloride, and 0.137*M* sodium chloride. The pH of the PBS solution is 7.4 and simulates human body fluid.

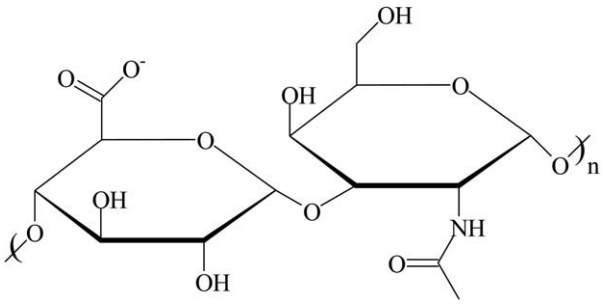

Figure 1. Disaccharide repeat unit of hyaluronic acid (HA).

Determination of Intrinsic Viscosity

An automated Ubbelohde viscometer (Schott-Geräte, Germany) with a capillary of 531 0a was used to determine the intrinsic viscosity of the hyaluronan used in the present study. The capillary was immersed into a water bath set at $T = 25°C$. The average of the flow-through time of the solvent (PBS) and dilute samples of hyaluronan was determined for the calculation of relative and specific viscosity, η_{rel} and η_{spec}, respectively. The flow-through time of each sample was repeated five times. The Hagenbach corrections were applied on the running times before calculating the relative viscosity (η_{rel}).

$$\eta_{rel} = \eta/\eta_0 = t/t_0 \quad (1)$$

where t equals corrected flow-through time and t_0 the corrected flow-through time of the solvent. The specific viscosity is given by

$$\eta_{spec} = (\eta - \eta_0)/\eta_0 = \eta_{rel} - 1 \quad (2)$$

The intrinsic viscosity, $[\eta]$ in dL g^{-1}, was determined by plotting η_{spec}/c and $\ln(\eta_{rel})/c$ against the concentration, (c in g dL^{-1}), and extrapolating to zero concentration.[33] The molecular weight of the hyaluronan was calculated using the Mark-Houwink-Sakurada equation

$$[\eta] = KM_w{}^a \quad (3)$$

where $K = 0.00034$ dL g^{-1} and $a = 0.79$.[34]

Preparation of HA Gels

HA solutions were prepared by weighing out HA using a Shimadzu AUW220D scale and adding it to deionized water (18.2 MΩ cm at 25°C). The dispersion was gently stirred at 4°C for at least 15 h, to ensure complete dissolution of the polymer. To obtain a final concentration of 1% or pH 13, NaOH was added to the cold HA solution; the EGDE was subsequently added. The dispersion was mixed for 5 min before being transferred into plastic syringes with an inner diameter of 6 mm and length of 78 mm. The syringes were immediately placed in a glycerol bath, precooled at a predetermined temperature (T_{prep}), and placed in a freezer (Labconco FreeZone Stoppering Tray dryer model 7948030) kept at T_{prep}. Experiments were

also carried out without precooling of the reaction solution in a glycerol bath, to determine the effect of the cooling rate on the cryogel properties. The syringes were kept at T_{prep} for 4 days. Thereafter, the gel was thawed by immersing the syringes in a water bath at room temperature. When thawed after 3 h, the cryogels were taken out of the syringes and placed in deionized water.

Swelling and Gel Fraction Measurements

Cylindrical gel samples were immersed in a large excess of deionized water at 20°C for at least 15 days by replacing water every other day to extract any soluble species. The mass, m, of each gel sample was monitored as a function of time by weighing the samples. The weight swelling ratio m_{rel} of gels with respect to their preparation state (m_0) was calculated as

$$m_{rel} = m/m_0 \quad (4)$$

Then, the equilibrium swollen gel samples $m_{rel:eq}$ (the mass of the gel after 15 days of swelling) were taken out of water and freeze dried for the determination of the gel fraction W_g, and the swelling degree Q. W_g, i.e. the conversion of soluble HA to the water-insoluble crosslinked HA, was calculated from the masses of dry extracted HA and from the HA in the feed. The solvent-holding capacity of the cryogels was further determined by the swelling degree Q, defined as

$$Q = (m_{eq} - m_{dry})/m_{dry} \quad (5)$$

where w_{eq} is the equilibrium weight of the gel immersed in water or PBS, and m_{dry} is the weight of the corresponding freeze-dried sample. The swelling of the freeze-dried samples in water and PBS was performed at 20°C and in triplicate. For the deswelling kinetics measurements, the gel samples were swollen in water at 20°C until equilibrium prior being transferred into acetone. The weight changes of the gels were measured gravimetrically after blotting the excess surface solvent at regular time intervals. For the measurement of the reswelling kinetics of the gels, the collapsed gel samples in acetone were transferred into deionized water or PBS solution at 20°C. The weight changes of the gels were also determined gravimetrically, as described above. The measurement of each gel sample was performed in triplicate.

Mechanical Tests

Uniaxial compression tests were performed using an Instron 5565A electromechanical system (USA) with a load cell of 2 kg at room temperature. The swollen cryogels were cut in cylinders of approximately 9 mm of height and 8 mm in diameter. The initial length, l_0, and diameter, D_0, of each cylinder was noted. The gel samples were aligned in the center of stainless steel compression plates. The samples were compressed at a displacement rate of 5% s^{-1} and the tests were performed in triplicate. The nominal stress, σ_{nom}, and strain, ε, were calculated from the force F required to compress the samples according to

$$\sigma_{nom} = F/A_0 = F/(\pi(D_0/2)^2) \quad (6)$$

and

$$\varepsilon = 1 - \lambda \quad (7)$$

where A_0 is the cross-sectional area of the undeformed gel specimen and λ is the deformation ratio (deformed length/initial length). The elastic modulus G of the gels was determined from the initial slope of linear dependence.[35,36]

$$\sigma_{nom} = G(\lambda - \lambda^{-2}) \quad (8)$$

Microstructural Determination

For the texture determination of freeze-dried cryogel samples, scanning electron microscopy (SEM) studies were carried out at various magnifications between 20 and 1000 times (Jeol JSM 6335F Field Emission SEM). Before the measurements, network samples were sputter-coated with gold for 3 min using an Edwards S150B sputter coater instrument. Confocal laser scanning microscopy (CLSM) measurements were also carried out, to investigate the morphology of swollen cryogel samples. The cryogels were stained with a 0.01% w/v solution of rhodamine B; the excess of stain was then removed from the gels and the pores by rinsing with deionized water. The analyses were performed at room temperature using a Leica confocal laser scanning microscope model TCS SP5 II or SP2 AOBS Germany. The light source was a HeNe laser with an emission maximum of 594 nm, and the signal emitted at a wavelength interval of 605 to 685 nm was recorded. The formats of the images were 512 × 512 or 1024 × 1024. These were recorded using a 20× water objective (NA of 0.50), and computer zooming was done at 1×, 2×, and 4×.

Fourier Transform-Infrared (FT-IR) Spectrometer

Spectra of the freeze-dried cryogel samples were collected using a single bounce diamond attenuated total refractance (ATR) module on a Fourier transform infrared (FT-IR) spectrometer (Nicolet Nexus 6700) equipped with a liquid nitrogen–cooled mercury cadmium telluride (MCT) detector. The resolution of each spectrum was 4 cm^{-1}, and 64 interferograms were taken between 500 and 4000 cm^{-1}.

RESULTS AND DISCUSSION

Molecular Weight Determination of HA

Experimental measurements of solution viscosity of the HA at $T = 25$°C dissolved in PBS (yielding a pH of 7.4 and an ionic strength of 0.14M) were made within the range of $\eta_{rel} = 1.2$–2.0. Within this range, plots of η_{spec}/c and $\ln(\eta_{rel})/c$ against c (Huggins and Kraemer plot, respectively) should both be linear and extrapolate to a common intercept of $[\eta]$ as c approaches 0. The $[\eta]$ thus obtained was 21.2 dL g^{-1} (Figure 2).

This value of intrinsic viscosity gives a viscosity averaged molecular weight of the HA used in this study of 1.2 × 10^6 g mol^{-1}, agreeing with M_w values reported for bacterial HA falling within the range of 7.9 × 10^5 to 1.9 × 10^6 g mol^{-1}.[34,37]

Experimental Conditions for Gel formation

Different experimental conditions were tested in order to obtain hyaluronan (HA) gels of high mechanical stability and fast responsivity. As detailed in the experimental section, aqueous HA solutions in the presence of EGDE crosslinker were subjected to gelation in plastic syringes for four days. The varied experimental parameters were:

a. the crosslinker concentration C_{EGDE} between 0.25 and 3 (% w/v),

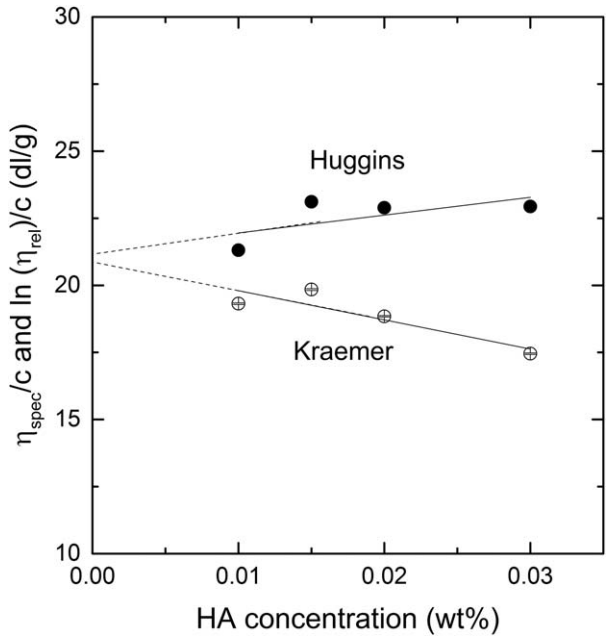

Figure 2. Determination of intrinsic viscosity of HA dissolved in PBS at 25°C from Huggins and Kraemer plots of, respectively, η_{spec}/c (filled symbols) and $\ln(\eta_{rel})/c$ (open symbols) against the concentration HA.

b. hyaluronan concentration C_{HA} between 2 and 9 (% w/v), and
c. gel preparation temperature T_{prep} between −24 and 20°C.

In addition, the effect of the pH of the reaction solution as well as the type of cooling (liquid or air cooling) were investigated. By the preliminary gelation experiments conducted at $C_{HA} = 7.3\%$ and $C_{EGDE} = 1.7\%$, three scenarios were observed depending on the temperature, pH, and type of cooling:

a. HA solution: The content of the syringe dissolved in deionized water; thus no gelation took place. Such a scenario was observed at low HA and EGDE content and at reaction pH <13.
b. HA hydrogel: Although gelation occurred, the gel formed was too weak and the swelling took place slowly with a large increase in the gel volume, i.e., the initial gel shape was not retained. Such gels are hereafter referred to as hydrogels and were obtained at reaction temperatures of above 0°C and at −24°C.
c. HA cryogel: Gelation also occurred within 4 days. The gel rapidly took up water with a small increase in its volume, i.e., the gels retained their initial shape. The gel appeared visually clear and did not dissolve while being kept in water over a period of 6 months. These gels are called cryogels and were obtained at reactions with pH >13 and at HA concentrations >3%, as well as reaction temperatures of −18 and −10°C.

The different experimental conditions used are summarized in Table I.

The first set of experiments shows that a pH as high as 13 is required for the onset of gelation in HA solutions. The reason for no gel formation at all at pH <13 could possibly be related to the intramolecular hydrogen bonds of native HA molecules in aqueous solutions. It is known that, at a pH of 12.5 or above, the hydroxyl groups on the HA backbone are ionized, resulting in a conformational change and a more flexible polymer as the intrinsic viscosity of hyaluronan is reduced about four times at pH 13.2 compared with pH 12 (at the same ionic strength and M_w of the polymer).[11,38] Thus, it appears that some flexibility of HA backbone is required for its functional groups to be available for crosslinking via the epoxy groups of EGDE crosslinker. The second set of experiments listed in Table I reveals that only in a certain range of subzero temperatures is cryogel formed, i.e., a gel showing a fast swelling rate at a reduced increase in gel volume. The gel formed at 20°C swells slowly in water with a large increase in its volume, as is typical for ionic hydrogels. This is expected as gelation proceeds in a homogeneous solution of HA and in the absence of solvent crystals acting as porogenes. A hydrogel is also formed when the crosslinking reaction takes place at very low temperatures, i.e., at −24°C; a possible explanation is that overly small solvent crystals are formed, which do not give rise to an interconnected gel structure (see Microstructural Determination section).

HA cryogels (at concentration and crosslinker used in this study) are formed at −10 and −18°C, while no gelation was observed at 0°C, at which the reaction solution was unfrozen.

Table I. Experimental Conditions Tested for the Preparation of EGDE Crosslinked HA Cryogel Where Reaction pH was Varied, as well as Reaction Temperature and Freezing Rate (Air or Liquid Cooling)

Set #	T_{prep} (°C)	Solution pH	Type of cooling	Presence of a gel	Swelling rate	Swelling degree
1	−18	10	Liquid	No	-	-
	−18	12	Liquid	No	-	-
	−18	13	Liquid	Yes	High	Low
2	−24	13	Liquid	Yes	Low	High
	−18	13	Liquid	Yes	High	Low
	−10	13	Liquid	Yes	High	Low
	0	13	Liquid	No	-	-
	20	13	Liquid	Yes	Low	High
3	−18	13	Air	No	-	-
	−18	13	Liquid	Yes	High	Low

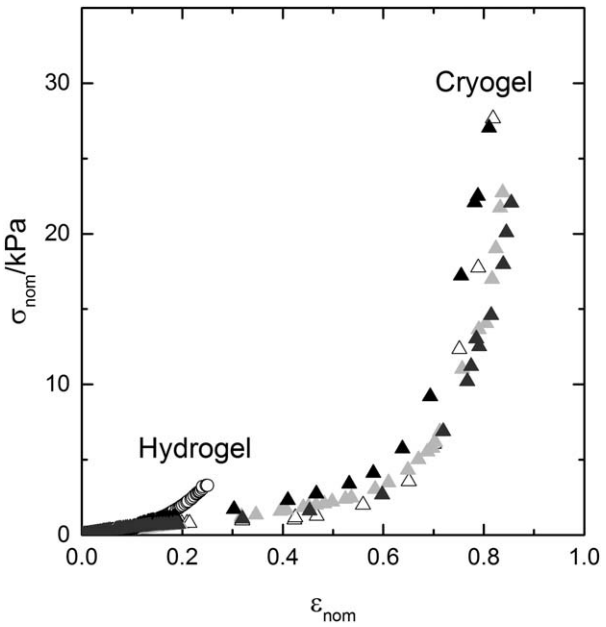

Figure 3. Stress–strain curves of HA cryogels (triangles) and HA hydrogel (circle) formed at 7.3% HA. $T_{prep} = -18°C$. C_{EGDE} 0.9% (open triangle), 1.7% (light gray), 2% (black), and 2.5% dark gray. The reference HA hydrogel was prepared at $T_{prep} = 20°C$ and $C_{EGDE} = 2.5\%$.

The reason for gelation occurring at temperatures below the freezing point of the reaction solution is the cryoconcentration of HA and EGDE in the unfrozen domains.[27] Thus, the actual concentrations of HA and EGDE in these domains are much larger than their average concentrations in the whole reaction system. As a consequence, the decrease in the rate of crosslinking reactions at low temperatures seems to be compensated for by the increased HA and EGDE concentrations in the reaction zones.[27] Another point worthy of mention is that rapid cooling of the reaction solution is required for the formation of cryogels (set 3 in Table I). For instance, freezing the reaction solution in glycerol at −18°C (liquid cooling) produces a cryogel, while freezing it in a freezer at the same temperature (air cooling) leads to no gelation. Note that the cryostat filled with a liquid at −18°C provides an eightfold more rapid initial cooling rate than in the freezer.[39] Thus, the cooling rate strongly influences the successful formation of HA cryogels. The reason for this finding needs to be investigated further, but one may speculate that excessive degradation of HA at pH = 13 is reduced under rapid freezing conditions so that gelation occurs.

For all cryogels and hydrogels reported here, the gel fraction W_g was found to be 0.89 ± 0.07, indicating that about 90% of the HA in the feed was incorporated into the gel network. The equilibrium swelling ratios $m_{rel;eq}$ of cryogels were 11 ± 1, independent of C_{HA} (in the range of 3–7%) of C_{EGDE} (above 0.5%), and at T_{prep} of −18°C and −10°C. The swelling was higher for the gel formed at −24°C ($m_{rel;eq} = 54 \pm 4$) and for the hydrogel formed at 20°C ($m_{rel;eq} = 140 \pm 40$).

Crosslinking of HA by the diepoxide crosslinker EGDE was analyzed by FT-IR. No significant differences in the spectra before

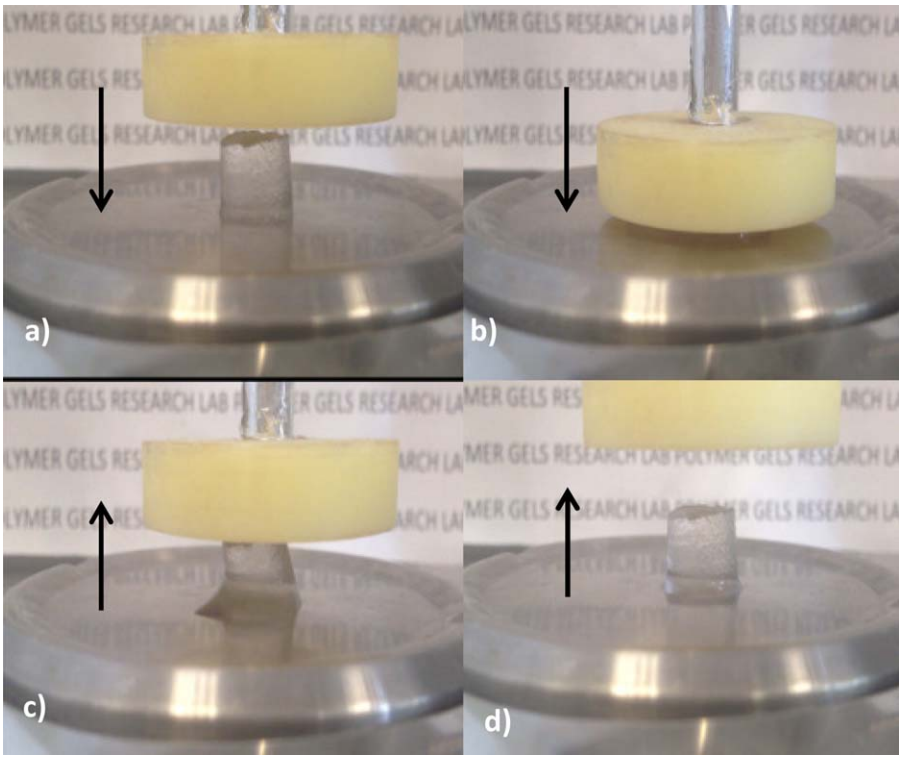

Figure 4. Photographs of a swollen HA cryogel sample formed at −18°C during the compression test (a, b). After the release of the load (c, d), the gel sample immediately recovers its original shape by absorbing the released water. $C_{HA} = 7.3\%$. $C_{EGDE} = 1.7\%$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

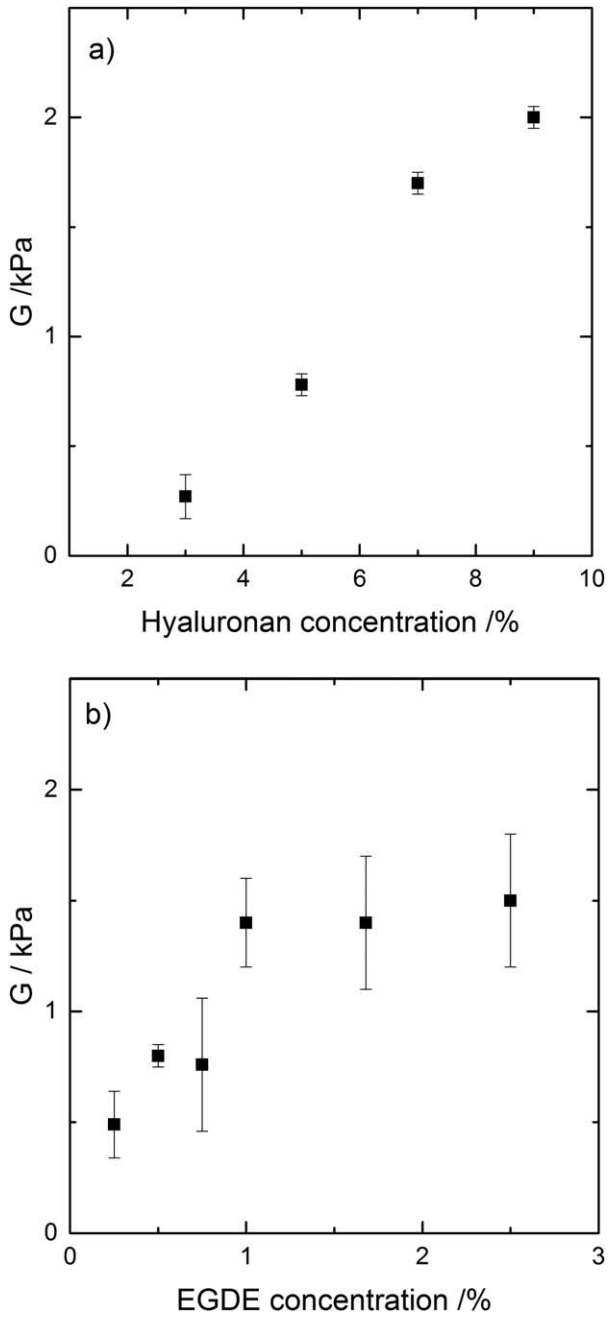

Figure 5. Elastic modulus G of swollen cryogels formed at −18°C plotted against the concentrations of (a) HA (C_{HA}) and (b) EGDE (C_{EGDE}) in the gelation solution. The crosslinker ratio $X = 0.28$ was fixed in the case of varying C_{HA}, and a C_{HA} of 7% was used in the case of varying C_{EGDE}.

and after the crosslinking reactions were apparent, indicating that the new chemical bond introduced by crosslinking with EGDE is similar to the bonds present in the native HA.[16] However, it is likely that the crosslinking occurs via an ether bond at the reaction pH used in this study.[16] It is worthwhile to note that no obvious peak at 1700 cm^{-1} was observed in FT-IR spectra of the cryogels, which would correspond to ester bond formation.

Mechanical Characterization of the Gels

Uniaxial compression tests performed on the cryogels show that they are highly resilient and can be compressed up to about 80% strain without any visual crack development. In comparison, HA hydrogels formed at 20°C were brittle and they fractured at a low deformation (30% strain). This behavior is shown in Figure 3, where the stress, σ_{nom}, is plotted against the strain, ε (% compression), for HA cryogels formed at −18°C and at various EGDE concentrations. The behavior of HA hydrogel formed at 20°C is added as a comparison.

The photographs in Figure 4 also demonstrate how the cryogels sustain a high compression. As the cryogel is squeezed under the piston or via manual hand compression, the gel releases its water so that it can be compressed up to large strains. After the release of the load, the gel sample immediately recovers its original shape by absorbing the released water. The toughness of the gel or the reversibility of the gel post deformation was further confirmed by stress–strain curves superimposed upon three

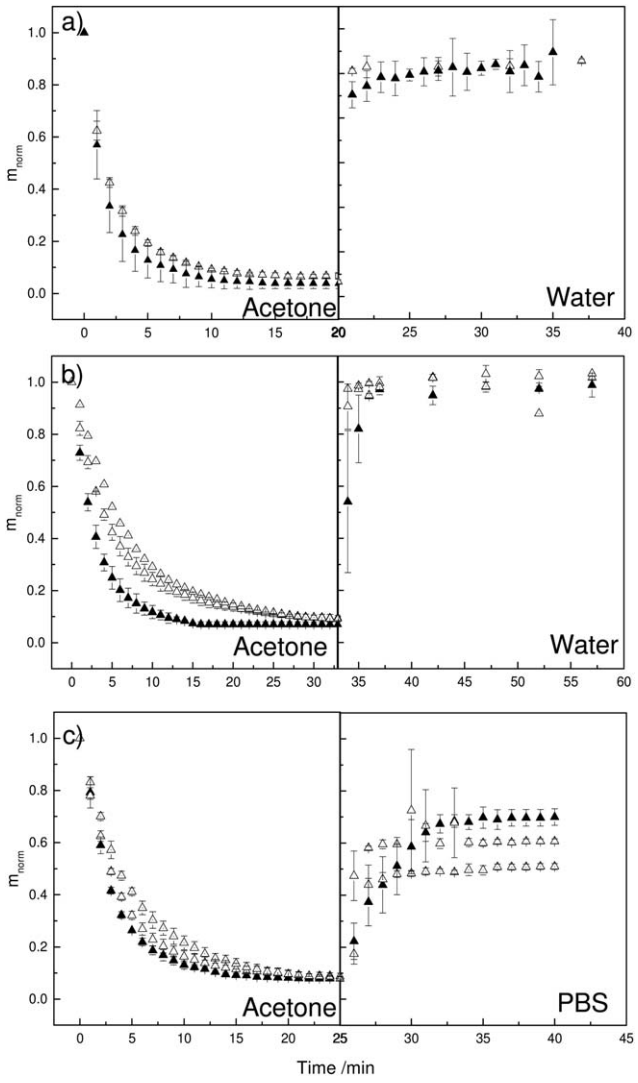

Figure 6. Deswelling–reswelling in (a) water of gels composed of $C_{HA} = 3\%$ (filled symbols) and 9% (open symbols) at a fixed X of 0.28. Deswelling–reswelling of gels composed of $C_{HA} = 7\%$ and $X = 0.12$ (filled symbols), 0.15 (open symbols), and 0.50 (open symbols with dot) in (b) water and (c) PBS. All deswelling and reswelling tests were performed at 20°C, and the gels were formed at −18°C.

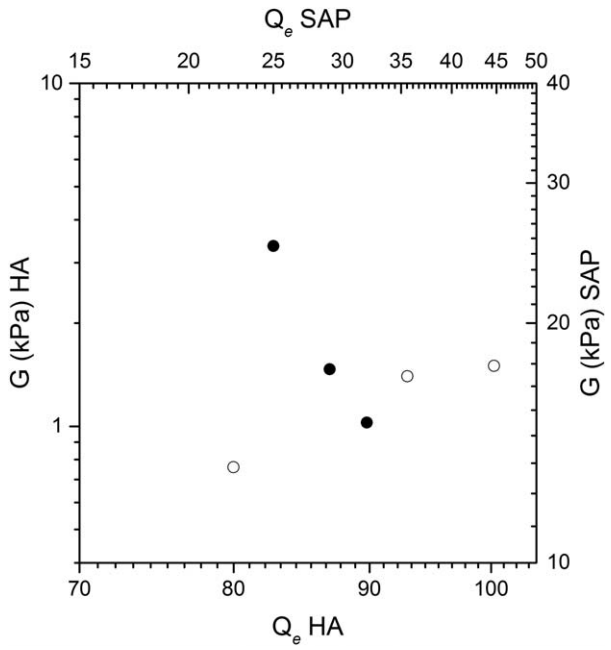

Figure 7. The moduli as a function of equilibrium swelling of a dried HA cryogel containing 7% HA (open symbols) and SAPs (filled symbols) with varying degree of crosslinking. The SAP data are used with permission of the publisher Soft Materials, Taylor and Francis Group.

consecutive deformation cycles up to a strain of 90%. The gels thus exhibit a reversible deformation, giving it the potential to be inserted in the body via a gauge needle or in separation processes.

The elastic modulus, G, of gels was determined from the initial slope of stress–strain curves, as detailed in the Material and Method section. At $C_{HA} = 7.3\%$ and $C_{EGDE} = 1.7\%$, cryogels formed at $T_{prep} = -10$ and $-18°C$ exhibited a similar modulus of 1.3 ± 0.5 kPa, while it decreased to 0.12 ± 0.05 kPa at $T_{prep} = -24°C$. Figure 5(a,b) show the modulus G of HA cryogels formed at $-18°C$ plotted against the concentrations of HA (C_{HA}) and EGDE (C_{EGDE}), respectively. The effect of HA concentration was investigated by fixing the crosslinker ratio, i.e. the molar ratio of epoxide to OH groups of HA at 0.28, to eliminate the crosslinker effect in the case of increasing HA concentration.

A part of the crosslinker used in this study will react with water/hydroxide-forming alcohols, while another part will form a bond only with one end to the HA and the other end will react with water. Another part will react with two ends to two different HA polymers, thus forming a crosslink. It is not trivial to determine the degree of actual crosslinks why only the theoretical maximal crosslinker ratio, X, is given in this study. Since the molecular weights of EGDE and the disaccharide repeat units of HA are 174.2 and 416 g mol^{-1}, respectively, and each repeat unit of HA contains four OH groups, X expressed as a molar ratio of epoxide and hydroxyl groups can be calculated as

$$X = \frac{[\text{epoxide}]}{[\text{hydroxyl}]} = 1.2\frac{C_{EGDE}}{C_{HA}} \quad (9)$$

where [epoxide] and [hydroxyl] correspond to the molar amount of epoxide and hydroxyl groups available. C_{EGDE} and C_{HA} correspond to the concentration (in weight) of EGDE and hyaluronan, respectively. No gel formation was observed at 1% HA, while the gels formed between 1% and 3% HA were too weak and unable to support their own weight. Therefore, mechanical tests cannot be conducted below 3% HA. In this range of HA concentration, even increasing the crosslinker ratio did not result in a stronger gel. However, increasing the HA concentration above 3% rapidly increases the modulus, and it becomes 2 ± 0.3 kPa at 9% HA [Figure 5(a)]. This increase in the modulus can be explained as a result of increasing the polymer concentration in the gel, as well as increasing the probability of intermolecular crosslinking reactions in concentrated polymer solutions.[27] As will be seen below, formation of thicker pore walls at high HA concentrations may also be responsible for the increased mechanical strength of HA cryogels. The influence of the crosslinker concentration (C_{EGDE}) on the modulus was investigated at a fixed HA content of 7%, while C_{EGDE} was varied between 0.25% and 3%, corresponding to a theoretical crosslinker ratio, X, of between 0.04 and 0.51 [Figure 5(b)]. This theoretical crosslinking ratio can be compared with those obtained for PEGDE crosslinked HA hydrogels, where theoretical crosslinking ratios ranging from 0.25 to 1.0[18] and BDGE crosslinked HA of 0.45–1.12[22] have been reported as necessary to yield gels strong enough to perform mechanical measurement. These studies showed that the mechanical properties increased with the theoretical crosslinking ratio of up to 1. Other studies have used four times[15] and 0.6 times[20] the molar ratio of crosslinker (EGDE) to HA. Figure 5(b) shows that under the conditions used in this study, the modulus G increases with the addition of EGDE up to a concentration of 1% EGDE, corresponding to a relatively low theoretical crosslinker ratio X of 0.17. The addition of more EGDE does not lead to a larger degree of crosslinking so that the modulus remains constant at around 1.5 kPa.

Gel Morphology and Its Impact on Swelling Capacities

HA hydrogels obtained under various synthesis conditions were subjected to deswelling and reswelling processes in acetone and in water (or in phosphate-buffered saline PBS), respectively. For this purpose, the equilibrium swollen cryogel samples in water were immersed in acetone and the weight changes of gel samples were determined as a function of the deswelling time. After reaching the equilibrium collapsed state in acetone, the collapsed gels were immersed in water or PBS, and the reswelling process was monitored by recording the weight increase over time. The results were interpreted in terms of the normalized swelling ratio $m_{norm} = m_{rel}/m_{rel;w}$, where $m_{rel;w}$ is the equilibrium swelling ratio of the gel sample in water. Thus, $m_{norm} = 1$ means a complete recovery of the mass of the collapsed gel upon reswelling in water. Typical results are shown in Figure 6, where m_{norm} for several cryogel samples is plotted against the time of deswelling or reswelling. Fast and complete swelling was observed for all collapsed cryogels, independent of HA concentration (3–9%) or the crosslinker ratio X (from 0.12 to 0.5 was tested). The initial swollen mass of the cryogels was obtained already within a couple of minutes. This fast swelling process indicates that all cryogels contain interconnected macropores, enabling fast inflow of water. However, as the gels are immersed

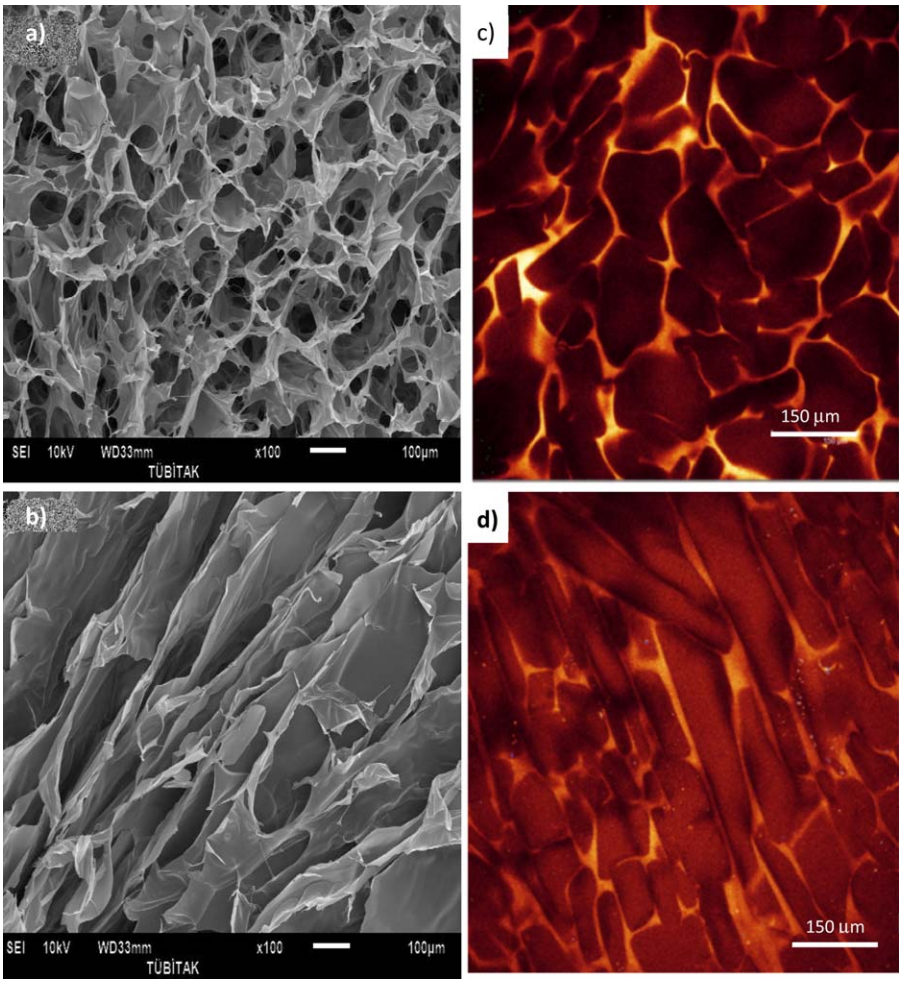

Figure 8. Scanning electron microscopy (SEM) of freeze-dried cryogels (a, b) and confocal laser scanning microscopy (CLSM) images (c, d) of a cryogel containing (a and c) 3% and (b and d) 7% hyaluronan. The samples have a HA:EGDE ratio of 4.3 and were prepared at $T = -18°C$. The parts stained yellow correspond to areas in which HA is present, and the darker areas are depleted of HA. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

in PBS solution instead of water [Figure 6(c)], the swelling was slower and the equilibrium value of m_{norm} remained below unity. This means that the cryogels swell less in PBS than in water. A lesser degree of swelling in salt solutions as compared with water is a typical behavior of polyelectrolyte gels owing to a decrease in the concentration difference of counterions inside and outside the hydrogel.[40] In the present case, the swelling pressure due to the counterions of the carboxyl groups of HA is reduced in PBS, leading to a 40 to 60% reduction in the gel volume. Furthermore, it was noticed that EGDE concentration used in the gel preparation, i.e., the crosslinker ratio X, has an impact on the equilibrium swelling degree of cryogels in PBS solution. The lower the crosslinker ratio X is, the larger the gel swelling in PBS is. For instance, when immersed in PBS, the mass of the collapsed cryogel formed at $X = 0.50$, 0.15, and 0.12 increases 50%, 60%, and 70%, respectively. This inverse relationship between the crosslinker ratio and the degree of swelling is also typical for polymer gels[41] and has also been reported for epoxide crosslinked HA hydrogels both in water and in salt solution.[22] Thus, HA cryogels exhibit characteristic features of polyelectrolyte gels in good and poor solvents, as well as in salt solutions.

The influence of solvent on the ability to reswell was further confirmed when studying the equilibrium swelling, Q_{eq}, of dry gels in water or PBS. A cryogel of 7% HA and a theoretical crosslinking ratio of 0.17 had a Q_{eq} of 90 ± 10 when immersed dry in water, whereas the value dropped to 30 ± 5 when immersed in PBS.

It is interesting to note that the relationship between the moduli and the swelling of the HA cryogels does not follow the common trend among hydrogels, e.g., polyacrylic acid (PAA). Polyacrylic acid is commonly used as a superabsorbent in hygiene products. Its ability to swell and retain liquid is strongly connected via the following equation,[41]

$$\pi = \pi_{mix} + \pi_{ion} + \pi_{elastic}$$

where π_{mix} is the osmotic pressure from the mixing of the polymer chains with the solvent, π_{ion} is the osmotic pressure derived from counterions within the gel, and $\pi_{elastic}$ is the opposing elastic pressure derived from the deformation of the polymer

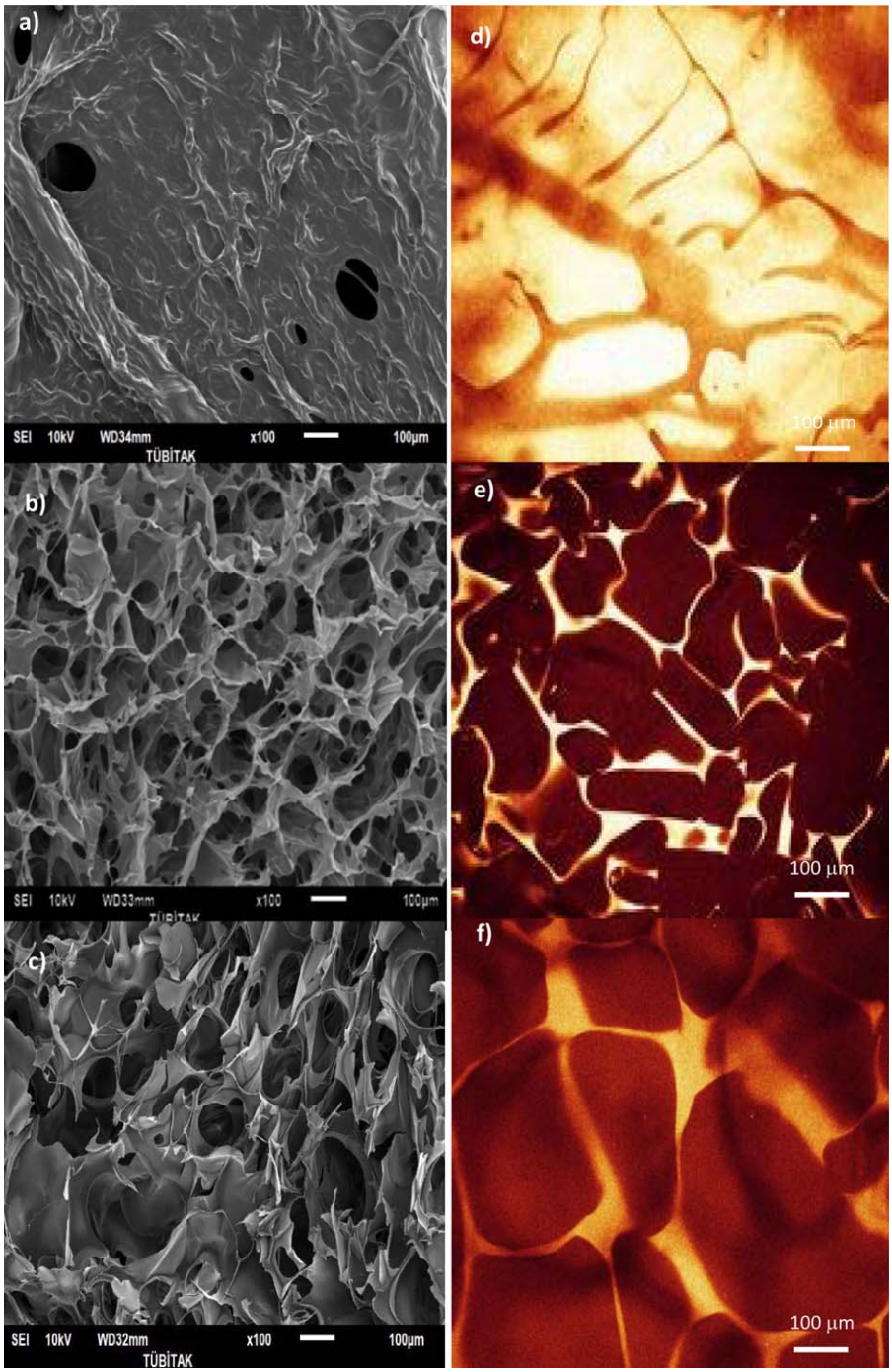

Figure 9. Scanning electron microscopy (SEM) of freeze-dried cryogels (a–c) and confocal laser scanning microscopy (CLSM) images (d–f) of cryogels containing 3% hyaluronan and with a HA:EGDE ratio of 4.3. The gels are prepared at a temperature of (a, d) −24°C, (b & e) −18°C, and (c & f) −10°C. The parts stained yellow correspond to areas in which HA is present, and the darker areas are depleted of HA. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

network during swelling. At a high degree of crosslinking, which results in high moduli, the opposing pressure from deformation increases, which in turn reduce the ability of swelling of, e.g. a PAA gel, as shown in Figure 7.

Such behavior is true also for chemically crosslinked hydrogels of HA.[22] However, in the case of HA cryogels, the increase in swelling of a dried to wet cryogel is not necessarily related to the moduli of the swollen gel. The partial decoupling of swelling and gel moduli as observed for the cryogels can possibly be explained by the majority of the water being located in large pores rather than in the pore wall consisting of crosslinked HA.

Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) were used to examine the morphology of dried and swollen cryogel samples, respectively. Figure 8(a,b)

show SEM images of cryogel samples formed at −18°C and at 3% and 7% HA concentrations, respectively. The images reveal a heterogeneous morphology consisting of large pores in the range of 100 μm and a high degree of interconnectivity, in accordance with the rapid swelling results. Furthermore, HA concentration in the feed solution appears to strongly affect both the size and the shape of the pores. A low concentration of HA produces relatively rounded pores with a pore size of about 100 μm, whereas an increase in HA concentration gives needle-shaped pores with a length of about 400 μm and a width close to 100 μm. CLSM was also used in order to visualize the pore structure of the cryogels while minimizing the risk of artifacts resulting from the required drying and sample preparation for SEM. Visualizing the structures with CSLM allows observations of the pores without any sample preparation other than soaking the gel in water solution with rhodamin B for staining of the HA polymer. Figure 8(c,d) show CSLM images of two cryogel samples formed at 3% and 7% HA, respectively, confirming the observations made using the SEM images. The size and the shape of the pores in cryogel formed at 3% HA are more rounded and appear to be more uniform in size, at around 100 μm, throughout the sample. In contrast, the cryogel formed at 7% HA has large needle shaped pores of about 200 μm in size alternating with smaller pores of less than 100 μm. Moreover, both the SEM and the CLSM images reveal that the pore wall in the cryogel formed at 7% HA is thicker than that formed at 3% HA. Generally, at a high polymer concentration the initial solution becomes more concentrated, and thus there is less solvent that can freeze, resulting in smaller pores with thicker pore walls.[42–44]

Figure 9 shows SEM and CLSM images of cryogels formed at various temperatures, T_{prep}. It is seen that the temperature at which the crosslinking reaction takes place plays an additional role in determining the microstructure of the cryogels. Decreasing T_{prep} from −10 to −24°C leads to a decrease in the average pore size owing to the formation of smaller spherical pores of 20 to 40 μm in diameter, in addition to the large pores of about 100 μm in size. Decreasing pore size with decreasing T_{prep} is consistent with the fact that a larger number of solvent crystals are formed as the temperature is decreased, i.e. as the freezing rate is increased. Previous reports also show similar behavior for various cryogelation systems.[27] Moreover, since solvent in large voids is preferentially frozen relative to that in small capillaries, decreasing T_{prep} leads to the freezing of solvent located in small voids and thus produces smaller pores in the final material.[27] In the case of $T_{prep} = -24$°C, a less interconnected structure is also illustrated.

CONCLUSIONS

Hydrophilic, biocompatible polymer scaffolds are important for tissue engineering, as these materials mimic the gel-like mechanical and chemical properties of the extracellular matrix (ECM). Preparation of macroporous gels based on hyaluronan (HA), a polymer naturally present in the ECM, could thus be an ideal material for tissue engineering purposes. However, HA gels for application purposes need to be mechanically stable and insoluble in water, and should exhibit tunable porosity. In the present study, we prepared macroporous cryogels based on HA in frozen aqueous solutions using EGDE as a crosslinking agent. The effects of different preparation conditions including HA and EGDE concentrations, as well as the gel preparation temperature, on the gel properties were investigated. The cryogels prepared at −10°C and −18°C are tough (strain resilient) and can be compressed up to about 80% strain without any crack development. HA cryogels exhibit completely reversible deswelling–reswelling cycles in acetone and water, respectively. They also behave as typical polyelectrolyte gels, showing reduced reswelling in salt solutions and an inverse relationship between the crosslink density and the degree of swelling. The cryogel microstructure, as revealed by SEM and CSLM, consists of large interconnected macropores (pores having at least one dimension >100 μm). The pores are separated by thick pore walls, which provide structural support to the material. The pore size decreases as the gelation temperature is decreased from −10 to −24°C. Furthermore, the pores in HA cryogels change in shape from near-spherical to ellipsoidal with increasing HA concentration in the feed.

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