Investigation of the Swelling Behavior of Crosslinked Hyaluronic Acid Films and Hydrogels Produced Using Homogeneous Reactions

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Received 23 June 2007; accepted 7 November 2007
DOI 10.1002/app.27631
Published online 11 April 2008 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: Hyaluronic acid (HA) has been crosslinked in solution with glutaraldehyde (GTA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), poly(ethylene glycol) diglycidylether (EX 810), and divinyl sulfone (DVS) to form hydrogels. Flory-Rehner calculations were used to determine molecular weight between crosslinks (M_c), the crosslink density (V_e), and mesh size (ε) of crosslinked hydrogels after 24-h swelling in distilled water. Generally, lower molecular weight films gave rise to decreased molecular weights between crosslinks as well as increased effective crosslink densities and decreased mesh size. The effects of pH and salt concentration were evaluated. Use of lower molecular weight HA gave rise to decreased molecular weights between crosslinks as well as increased effective crosslink densities and decreased mesh size. Water diffusion coefficients were measured for DVS and GTA hydrogels and were found to be 1.4×10^{-10} and 1.8×10^{-10} m^2 s^{-1}. Autocrosslinked and HA polyethyleneimine gels were also produced but had very limited stabilities compared with the covalently crosslinked materials. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 109: 923–931, 2008

Key words: biomaterials; hydrogels; crosslinking; swelling

INTRODUCTION

Hyaluronan (HA) is a member of a group of polysaccharides that have been termed ''connective tissue polysaccharides,'' ''mucopolysaccharides,'' or ''glycosaminoglycans.'' It is a linear, unbranched polymer composed of a repeating disaccharide that consists of *N*-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid (GlcA) linked by a β 1–4 glycosidic bond[1] and is an attractive building block for new biocompatible and biodegradable polymers with possible applications in drug delivery,[2] tissue engineering,[3–5] and visco supplementation.[6] Commercial HA is usually obtained from rooster comb and a procedure developed by Balazs et al. was the first industrially applied extraction method for the isolation and purification of pharmaceutical grade material.[7] Other isolation and purification methods have been described by Della Valle and Romeo.[8] The bacterial production of HA by *Streptococcus equi*[9] and *Streptococcus zooepidemicus*[10] enabled it to be produced in larger quantities than could be achieved with the extraction methods.

Biomedical applications of HA have been hindered by its short residence time and lack of mechanical integrity in an aqueous environment and to realize its potential for bioengineering applications crosslinking is required.[11–15] Crosslinking processes may be carried out using heterogeneous methods, where reactions are carried out on solid HA, cast in the form of films or membranes, or homogeneous methods using HA solutions. The former method has the advantage of allowing shaping of a product before crosslinking, whereas the latter method offers the advantage of better control of the chemistry with greater product homogeneity.

Crosslinking reactions have been accomplished under acidic, neutral, and alkaline conditions using carbodimides,[13,16,17] hydrazides,[17,18] aldehydes,[11] sulfides,[19] and polyfunctional epoxides.[20,21] Autocrosslinking[22,23] and photocrosslinking[24–26] have also been reported. Reactions for carbodimide, divinyl sulfone, epoxide, and glutaraldehyde are given in Scheme 1.

An earlier article[15] has dealt with heterogeneous reactions using cast films treated with some of the previously mentioned reagents and also a modification of the carbodimide reaction which introduces L-lysine methyl ester to form higher stability amide crosslinks.[13] The work reported here was concerned with homogeneous crosslinking using HA solution and utilized the same reagents with the parameters of interest being the effects of HA and reactant concentrations on swelling, and on water diffusion rates and crosslink density. Crosslinking of HA by solution

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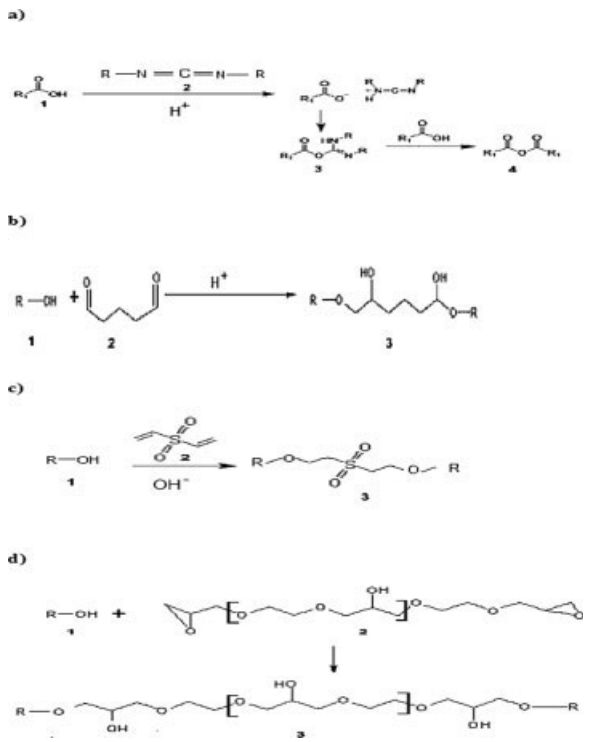

Scheme 1 **(a)** With water soluble carbodimides (WSC) (2) the crosslinking occurs through the initial formation of O-acylisourea (**3**) on the polysaccharide, through reaction with neighbouring carboxyl groups (**1**) an anhydride (**4**) is formed, and this anhydride then reacts with nearby hydroxyls to give both inter and intramolecular crosslinks, **(b)** The OH (1) group on the hyaluronic acid reacts under acidic conditions with glutaraldehyde (2) to give hemiacetal or ether crosslinks (3), **(c)** The OH (1) group on the hyaluronic acid reacts under alkaline conditions with divinyl sulfone (2) to give sulfonyl *bis*-ethyl crosslinks (3), **(d)** The OH (1) group on the hyaluronic acid reacts with the epoxy group of the poly(ethylene glycol) diglycidyl ether (2) to give ether crosslinks (3).

methods has been reported previously for divinyl sulfone[19] and carbodiimide[27] but not for glutaraldehyde and epoxides. Full reaction schemes for the various crosslinkers have been given in the previous article. Additionally, autocrosslinking and crosslinking using polyethylene imine were investigated. Autocrosslinking, induced by freezing and thawing, is postulated to occur through the interaction of hydrophobic and hydrogen bonds between hyaluronic acid molecules. Okamoto et al.,[28] maintain that during the freezing period at a low pH the electrostatic repulsive forces between the hyaluronic acid molecules are suppressed, so the molecules are packed closely together to facilitate the formation of a gel. Poly(ethylene imine) acts as a cation sponge and offers the possibility of crosslinking via salt bridges formed between protonated and positively charged amine groups of the poly(ethylene imine) and the negative carboxyl group on the hyaluronic acid.

MATERIALS AND METHODS

Materials

The sodium salt of HA with an average molecular weight of 2.06×10^6 was supplied by Clear Solutions, New York, NY as dry powder and was derived from bacteria. HA powders of average molecular weight 1.19×10^6, 8.5×10^5, and 1.4×10^5 were purchased from Bioiberica (Barcelona, Spain) and were obtained from rooster comb. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), glutaraldehyde (GTA), poly(ethylene glycol) diglycidyl ether, and divinyl sulfone (DVS) were purchased from Lancaster, UK. Solutions were prepared by sieving HA particles into double-distilled water to expose the maximum area for solvent interaction. This was followed by agitation, to minimize shear stress, in a shaking bath at 25°C for up to 102 h and was found to give reproducible solutions of uniform viscosity. Samples were fully dissolved after 24 h.

Crosslinking with divinyl sulfone

HA of molecular weight 2.06×10^6 Da was used to prepare solutions of 1, 2.5, and 5% concentration and DVS added to give a HA/DVS molar ratio of 5 : 1. The pH of the reaction medium was kept above 9 as this facilitates the crosslinking reaction. The crosslinking reaction was fast compared to the other crosslinkers used, and strong gels were formed quickly, particularly at higher HA concentrations. Generally, 1 h was enough for completion of the crosslinking reaction. The gels obtained were optically clear, with a smooth surface.

Crosslinking with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide

For crosslinking to proceed with EDC the aqueous system should be acidic, preferably having a pH between pH 4.0 and 5.0, adjusted with hydrochloric acid. At lower pH values EDC is unstable, and at higher values the reaction rate is diminished. To enhance the hydrolytic stability of crosslinked hydrogels through the introduction of a more hydrolysis resistant amide bond, L-leucine methyl ester hydrochloride was added to the crosslinking mixture. The mixture was held at room temperature for up to 5 h depending on the concentration and molecular weight of HA. Then, the crosslinking solution was cast into a Petri dish and allowed to react further and dry for up to 5 days. The thickness of the gel depended upon the amount of solution cast initially. Starting with HA of molecular weight 8.5×10^5 Da, a 4 wt % solution was used with HA/EDC mole ratios of 0.79 and 1.64 with the L-leucine methyl ester at a 1 : 1 mole ratio with the HA.

Crosslinking with ethylene glycol diglycidyl ether (EX-810)

Hyaluronic acid solutions were prepared as described earlier using HA of molecular weight of 2.06×10^6, solution concentrations of 4 wt %, and crosslinker 1 : 2 mole ratio. With epoxides, pH offers a possible method of control of crosslink density as the epoxy group hydrolytic stability is known to be a function of pH.[12] Reactions were carried out at pH 7.0 and 10.0. The crosslinking solution was poured into a Petri dish and allowed to react for up to 5 days. The resulting gels were washed to remove any unreacted crosslinker.

Crosslinking with glutaraldehyde

On the basis of initial survey experiments, a HA to crosslinker mole ratio of 1 : 2 was used and pH adjusted with 0.01*M* HCl to enhance the crosslinking reaction. HA molecular weight was 2.06×10^6, and solution concentration was 4 wt %. After 24-h reaction time, the gels were washed to remove any unreacted crosslinker. This crosslinker produced mechanically robust gels, and they were also easily moulded into any desired shape.

Autocrosslinking

Autocrosslinking was carried out by exposing the HA solution to freeze-thawing. Hyaluronic acid solutions were prepared using 2.06×10^6 Da polymer to give a 1 wt % aqueous solution. The pH of the solution was adjusted to 1.5 using 1*M* HCl and was then placed in a freezer at -20°C for 2.5 days and then thawed at 25°C.

Synthesis of hyaluronic acid/PEI complex

The cationic polymer polyethyleneimine (PEI) forms a complex with HA through an ionic bond between the carboxylic groups in hyaluronic acid and the amino or imino group in the polyethyleneimine. A 4 wt % solution of HA of molecular weight 2.06×10^6 was used with 1 : 2 mole ratio PEI.

Kinetics of gelation

The viscosity changes in the hyaluronic acid solution were monitored during gelation using a Haake Rotovisco 1. The solution was maintained at a constant temperature, and the viscosity was measured between two parallel plates using a low shear rate ($10\ s^{-1}$). A typical run shows an initial low viscosity followed by a rapid rise in viscosity and Figure 1 illustrates the type of behaviour observed. The time value obtained for the peak in the viscosity was taken as a measure of the gel point. After the gel point, there is normally a reduction in the indicated viscosity due to either the gel starting to break or gel slippage between the plates.

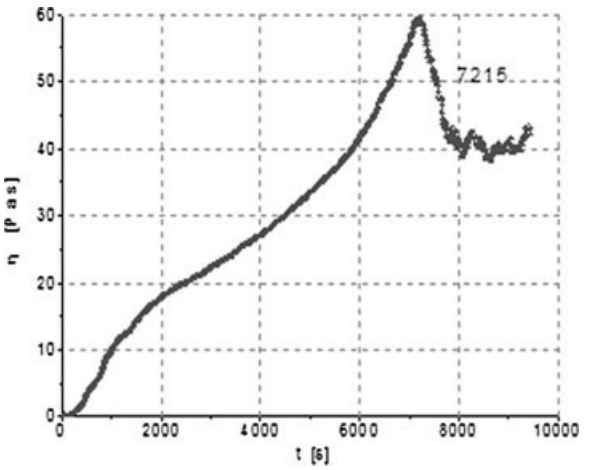

Figure 1 A typical viscosity curve for a crosslinking hyaluronic acid solution.

Swelling measurements

Swelling ratio (SR) was calculated via the eq. (1):

$$\text{Swelling ratio} = \frac{W_s}{W_d} \tag{1}$$

where W_s is the weight of the sample at equilibrium at each temperature and W_d is the weight of the dried sample and the equilibrium water content (EWC) defined by,

$$\text{EWC} = \frac{(\text{SR} - 1)}{\text{SR}} \tag{2}$$

Prepared gels were immersed in either distilled water or phosphate buffered saline solution for various periods of time at 25 and 37°C. The swollen gel was then carefully taken out from the solution, wiped with a filter paper for the removal of the free water on the surface, and then weighed. All measurements were made in triplicate. Most work reported here was carried out using distilled water, with a small number of experiments in buffered saline. Previous work[15] on heterogeneously crosslinked films has shown slightly slower gel degradation in buffered saline compared with water.

Diffusion rate measurement

Both DVS and GTA were found to be capable of producing gel samples of appropriate geometry for diffusion measurement. HA of molecular weight 2.06×10^6 Da was used to prepare solutions of 4 wt % concentration, and DVS and GTA were added to the

solution to give molar ratios of 1 : 2 with the pH adjusted as described previously.

The diffusion coefficient and the mode of transport through the samples were characterized for both gels by immersing the crosslinked materials in distilled water. Data analysis was performed by assuming that the water uptake can be described by a one-dimensional diffusion process; that is, radial diffusion can be neglected compared to axial, obeying Fick's first law of diffusion. This condition is satisfied if $d \ll H$; where d and H are correspondingly the sample diameter and thickness, and the reaction and gelation characteristics of the systems was such that this requirement were only fulfilled by the glutaraldehyde and divinyl sulfone gels.

When the appropriate conditions are met, data can be fitted to a linear correlation of the form,

$$B = \frac{4}{H}\sqrt{\frac{D}{\pi}} \tag{3}$$

where B is the slope of M_t/M_∞ (M_t = swelling after time t and M_∞ = equilibrium swelling) versus $\sqrt{t}$, H is the specimen thickness, and D is the diffusion coefficient. By plotting M_t/M_∞ versus $\sqrt{t}$ from the slope (B) of the initial linear portion of the curve, as shown in Figure 10, a solution for the diffusion coefficient, D is obtained via:

$$D = \frac{B^2H^2\pi}{16} \tag{4}$$

Crosslink density Flory-Rehner calculations

Thin films (M_w 1.19 $\times$ 10^6 and 1.40 $\times$ 10^5) were produced by casting 10-mL volumes of the reacting solutions, prepared as described previously, into a petri dish. The resulting crosslinked films were subsequently swollen in distilled water.

Crosslink density was assessed by measuring volumetric swelling and applying a simplified version of the Flory-Rehner equation.[28]

$${Q_v}^{5/3} \cong \frac{\bar{v}\overline{M}_c}{V_1}\left(\frac{1}{2} - \chi\right) \tag{5}$$

where Q_v is the volumetric swelling ratio, $\bar{v}$ is the specific volume of the dry polymer, M_c is the average molecular weight between crosslinks, V_1 is the molar volume of the solvent (18 cm^3/mol for water), and χ is the Flory polymer solvent interaction parameter.

Q_v was determined from the degree of mass swelling, Q_M:[29]

$$Q_v = 1 + \frac{\rho_p}{\rho_s}(Q_M - 1) \tag{6}$$

where ρ_p is the density of the dry polymer (1.229 g/cm^3) and ρ_s is the density of water. Q_M is the swelling ratio determined experimentally by comparing the mass of the material before and after immersion, is used to calculate Q_v. The value of χ for HA was estimated by Leach et al.[30] to be 0.473, based on several assumptions. It was assumed that χ for HA is comparable to dextran, since HA and dextran have similar ring conformations and are presumed to have similar molecular mobility. χ estimates for HA based on an analysis similar to those published by Gekko.[31] gave values within 2% of the value of χ for dextran. Differences between unmodified polysaccharides and crosslinked films were assumed to be negligible.

The effective crosslink density, ν_e, was calculated as follows:[32]

$$\nu_e = \frac{\rho_p}{\overline{M}_c} \tag{7}$$

The swollen hydrogel mesh size, ξ, was determined with eq. (8):[33]

$$\xi = {Q_v}^{1/3}\sqrt{\bar{r}_0^2} \tag{8}$$

where $\sqrt{\bar{r}^2}$ is the root-mean square distance between crosslinks and depends on the molecular weight between crosslinks. For HA, the following root-mean-square end-to-end distance value was previously reported:[34]

$$\left(\frac{\bar{r}_0^2}{2n}\right)^{1/2} \cong 2.4 \text{ nm} \tag{9}$$

where n is the number of disaccharide repeat units for HA with a given molecular weight. For HA with the molecular weight (M_n) 2 $\times$ 10^6, n is 5305, and therefore,

$$\sqrt{\bar{r}_0^2} = 0.1748\sqrt{M_n}\ (\text{nm}) \tag{10}$$

A combination of eqs. (8) and (10) and a substitution of M_c for M_n gives

$$\xi = 0.1748\sqrt{\overline{M}_c}\ {Q_v}^{1/3}\ (\text{nm}) \tag{11}$$

RESULTS AND DISCUSSION

Swelling studies

Table I compares the gelation time for each of the chemical crosslinking systems where the time value is obtained from the peak of relative viscosity versus time curves. This method provides a clear ranking of relative reactivities with both DVS and EDC being

TABLE I
Gelation times for 10 wt % hyaluronic acid (HA : crosslinker 1 : 2) at a shear rate of 10 s^{-1}

Crosslinker	Gel point (s)
Divinyl sulfone	283
1-ethyl-3-(3-dimethylaminopropyl) carbodiimide	593
Glutaraldehyde	2300
EX 810	7215

much faster than the GTA and the epoxide systems. It is believed that with DVS, crosslinking occurs by sulfonyl-bis-ethyl linkages. With carbodimides, the crosslinking occurs through the initial formation of O-acylisourea on the HA, and then through reaction with neighbouring carboxyl groups an anhydride is formed, and this anhydride reacts with nearby hydroxyls to give both inter and intramolecular crosslinks. With GTA, it is thought that crosslinking occurs by formation of hemi acetal linkages and with the epoxide the OH group on the HA reacts with the epoxy group to give ether crosslinks. Although reaction conditions were optimized for each crosslinker, it is presumed that in all cases crosslinker efficiency is very low due to hydrolysis and dilution factors and this precludes a detailed kinetic analysis.

Considering in more detail the effectiveness of DVS, Figure 2 shows the effects of varying solution concentration on gel swelling with time in distilled water and indicates, through reduced swelling, that increasing solution concentration increases the crosslink density, even though the HA to crosslinker ratio is constant. The results also show that initially these gels exhibit a slight moisture uptake over the ultimate equilibrium value. This effect can be attributed to molecular relaxation. The water diffuses into the network before the chains of the network have enough time to relax; that is diffusion is faster than the relaxation, and the fractional uptake curve reaches a maximum, the overshoot value. When the chains do finally relax, the water is forced out of the network and the water uptake reaches its equilibrium value after $\sim$ 6 h. Gel integrity was retained throughout this process. A similar phenomenon was observed by Peppas et al., when examining the swelling dynamics of 2-hydroxyethyl methacrylate copolymerised with methyl methacrylate (MMA) and *N*-vinyl-2-pyrrolidone (NVP).[35] Here, the phenomenon becomes less pronounced as the hyaluronic acid content of the initial solution is increased, indicating that the water is absorbed more slowly allowing the chains time to relax.

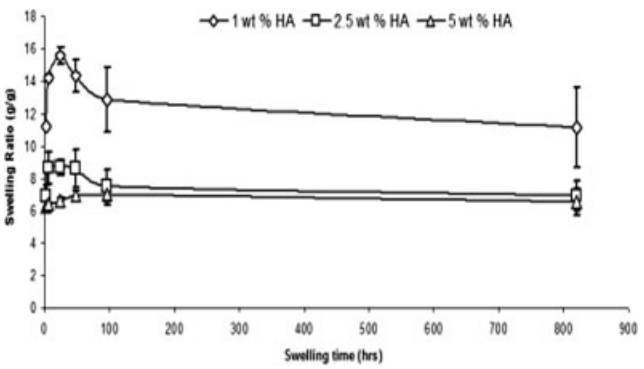

Figure 2 Swelling of DVS crosslinked HA (M_w 2.06 $\times$ 10^6) gels at 37°C in distilled water (HA : DVS mole ratio is 1 : 2).

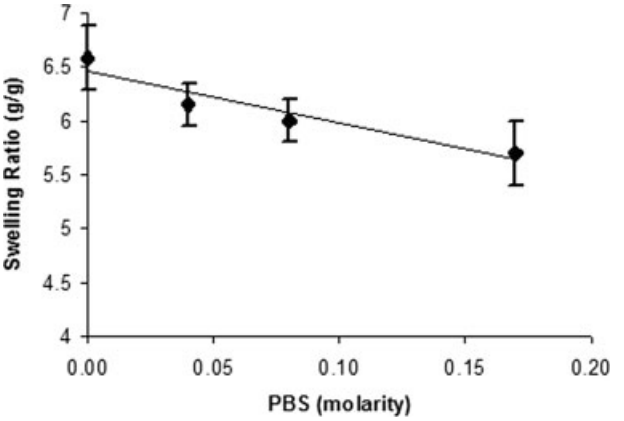

Figure 3 Swelling ratio of crosslinked DVS crosslinked HA (M_w 2.06 $\times$ 10^6) gels at different concentrations of PBS after 24-h swelling at 25°C (mole ratio 1 : 2), pH 7.

Figure 3 shows the 24-h swelling of DVS crosslinked gels immersed in different concentrations of PBS, and it can be seen that the swelling ratio of the gels decreases with increasing salt concentration. This means that a gel initially swollen in water will contract substantially when introduced into the body (because of the normal salt content of the body fluids and tissues), thus delivering its contents, perhaps an incorporated drug, into the body tissue.

Figure 4 shows the consequences of pH change on gel swelling. For the first cycle the gel was swollen to its equilibrium value in distilled water, with equilibrium being obtained in 6 h, and then placed in 0.1*M* HCl which caused shrinkage to a new equilibrium. Reimmersion in distilled water was then followed by reacidification and in the second and a subsequent cycle lower swelling equilibria were obtained. It has been postulated by Shah[36] that this may be a result of salt being leached out of the gel upon deswelling. Swelling is greater in distilled water because of ionisation of the carboxyl group (pK_a 2.9). The counter ion concentration is increased within the network on ionisation and the resulting osmotic pressure difference between solution within and outside the gel results in greater swelling. The pH cycling effect demonstrates the chemical robust-

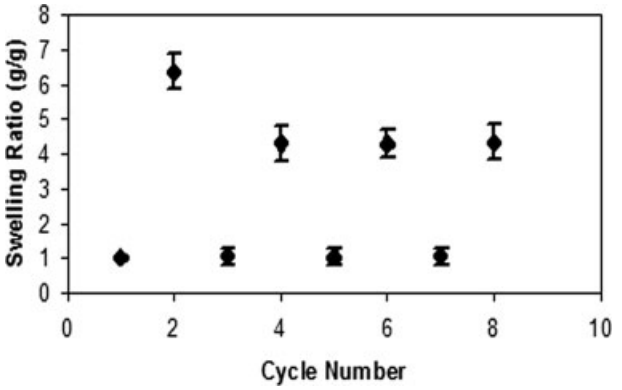

Figure 4 pH response and reversibility of the hyaluronic acid gels.

ness of these gels and the observed effect again may have some use in drug release.

The swelling behavior of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and L-leucine methyl ester crosslinked gels in distilled water is shown in Figure 5 and indicates that, as would be expected, more compact networks were obtained with the higher mole ratio of EDC. Onset of hydrogel degradation was also delayed by using the higher crosslinker mole ratio. Samples crosslinked with EDC alone (results not shown here) degraded much more quickly than the samples crosslinked with EDC and LME, proving the importance of amide bond formation. Figure 6 shows how HA molecular weight affects swelling and degradation properties of these gels in close to physiological conditions and indicates that gel stability is significantly reduced at 37°C compared with 25°C. The shape of the curves suggests that under physiological conditions two processes are occuring, possibly concurrently. When the crosslinked gel is placed in solution it firstly swells towards its equilibrium water content and then apparently contracts and this contraction is

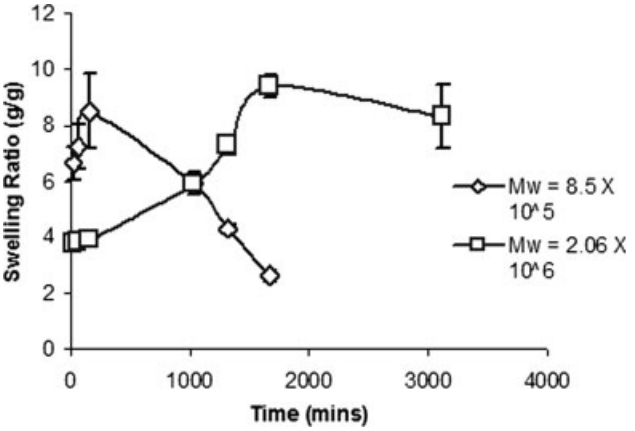

Figure 6 Effect of HA molecular weight on swelling and degradation at 37°C in PBS. HA : EDC : LME is 1: 1.5: 1 mole ratio.

taken to indicate breakdown of the structure and loss of degradation products to solution. Degradation of the gels was readily apparent on visual inspection as a loss of physical coherence and identity in the swelling medium. Increasing HA molecular weight retards both processes. The effects of crosslinker concentration on swelling in PBS at 37°C are shown in Figure 7 and indicates the range of gel densities that can be produced with this crosslinker system.

The swelling behavior of an EX-810 crosslinked gel is illustrated in Figure 8 and illustrates the role of pH as a controller of crosslink density. Contrary to the observations of Tomihata and Ikada,[12] working with heterogenous systems, it was found that these gels were less stable than those crosslinked with EDC. However, Tomihata reported that EX 810 crosslinked hyaluronic acid did not induce a significant tissue reaction suggesting that degradation products generated from HA and EX-810 are bioinert

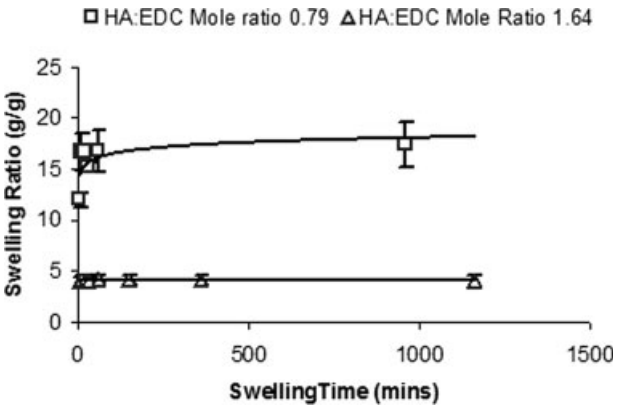

Figure 5 Effect of HA/EDC mole ratio on gel swelling properties at 25°C in distilled water. The molecular weight of HA was 8.5×10^5 Da.

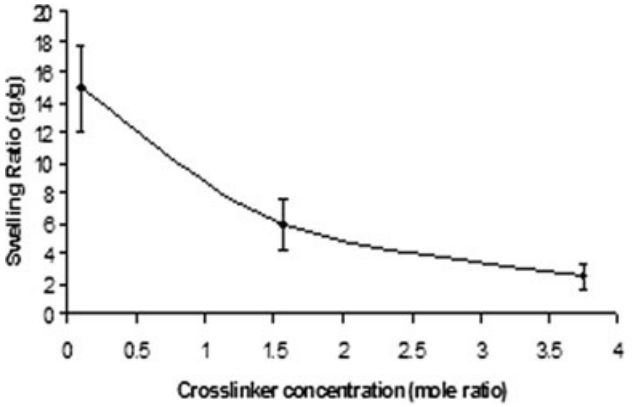

Figure 7 Crosslinker mole ratio effect on 24-h swelling of HA : EDC : LME gels, original HA molecular weight 2.06 $\times 10^6$ Da, at 37°C in PBS.

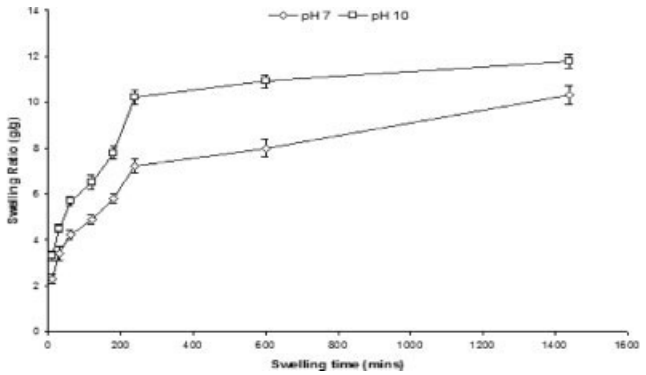

Figure 8 Effect of crosslinking pH on the swelling of EX-810 crosslinked gels Hyaluronic acid (M_w 2.06 × 10^6) and EX-810 were at a 1 : 2 mole ratio.

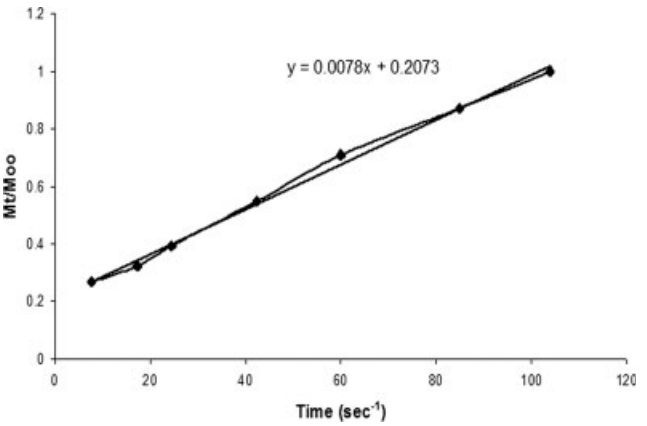

Figure 10 M_t/M_∞ versus $\sqrt{t}$ for glutaraldehyde crosslinked HA gels.

and that short term bioengineering application of these gels may be possible.

Figure 9 shows the short term swelling behaviour of GTA crosslinked gels. During swelling, the gels initially turned white in colour and then a colourless diffusion front moved inwards separating the highly swollen surface and less swollen core of the gel until equilibrium was reached after about 5 h in distilled water. Between 24 and 48 h, the gels began to swell more, and this is believed to be attributable to crosslink degradation.

Autocrosslinked gels

Autocrosslinked gels had stability times in the swollen state of not more than 2–3 h before dissolution. Swelling medium pH had a substantial effect on short term swelling, for example after 60 min at pH 2.0 the swelling ratio was 1.5, whereas at pH 9.0 it was 2.3. Tokita and Okamoto,[37] believed that hydrolytic degradation occurs in acid solution at the glucuronic acid residue while the degradation of the N-acetylglucosamine residue takes place in basic solution. Although the literature suggests[22] that autocrosslinking is a useful procedure it is questionable whether these are gels in the real sense and doubtful if they can have any value as bioengineering materials.

PEI–hyaluronic acid complex

The swelling of the PEI complex is shown in Figure 11. The gel remained stable for up to 2.5 h but subsequently degraded quickly to leave an insoluble white residue.

Diffusion through glutaraldehyde and divinyl sulfone crosslinked gels

Figure 10 shows a typical example of a diffusion rate plot and Table II gives the results obtained with GTA and DVS. Diffusion coefficients of 1.8 × 10^{-10} and 1.4 × 10^{-10} m^2 s^{-1} seem reasonable as Peppas calculated a diffusion coefficient of 4.78 × 10^{-10} m^2 s^{-1}

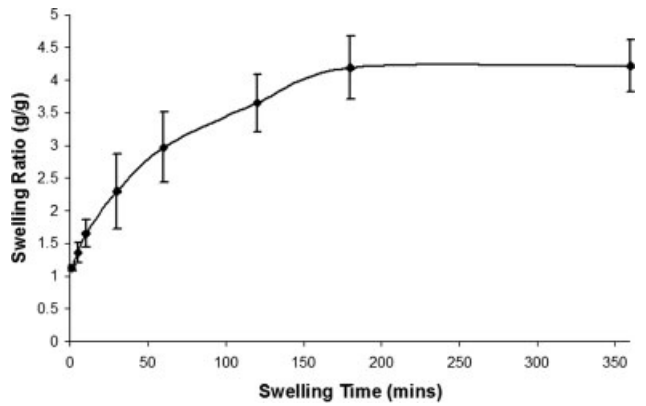

Figure 9 Glutaraldehyde crosslinked gels swollen at 25°C in distilled water. Hyaluronic acid (M_w 2.06 × 10^6) and glutaraldehyde were at a 1 : 2 mole ratio.

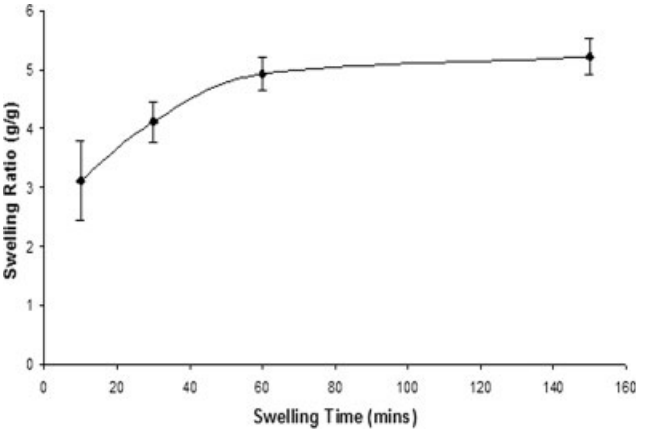

Figure 11 Hyaluronic acid (M_w 2.06 × 10^6) PEI complex insoluble for up 2.5 h at 25°C (n = 3). HA : PEI is 1 : 2 (w/v).

for polyHEMA in water with very light crosslinking at 34°C.[35] As expected there was little difference in the diffusion coefficient between the gels as they were both crosslinked using a 1 : 2 HA to crosslinker mole ratio.

Analysis of swelling using Flory-Rehner theory

Flory-Rehner calculations were used to determine molecular weight between crosslinks (M_c), the crosslink density (V_e) and mesh size (ε) of the crosslinked films in water after 24-h swelling. The calculations were based on the work carried out by Leach et al.[30] Q_M values were determined experimentally after 24-h swelling.

The general tendency of increased crosslink density with lower molecular weight was also evident in the Flory-Rehner calculations (Table III). Lower molecular weight films gave rise to decreased molecular weights between crosslinks as well as increased effective crosslink densities and decreased mesh size. Although, this did not hold true for all the samples, for example glutaraldehyde and EX-810 crosslinked films both had a higher than expected molecular weight between crosslinks (M_c) and crosslink density (V_e) and mesh size with a lower than expected effective crosslink density for the 1.2 million Da films. For glutaraldehyde it was attributed to crosslink hydrolysis, which was offset at the lower 0.1 million Da films by a higher crosslink density due to increased chain end free volume. This result arises in EX 810 films possibly due to molecular relaxation.

CONCLUSION

Chemical crosslinking greatly improves the *in vitro* stability of hyaluronic acid hydrogels. The gel point of each chemically crosslinked system was estimated at low shear rates and DVS was found to be fastest reacting crosslinker used in this study. The degree of swelling of DVS crosslinked gels can be controlled through swelling medium salt content and through pH and this may be of value for drug release purposes. As this behavior is primarily dependent upon the response of the polymer chain between crosslinks it is thought likely that these effects will apply to HA hydrogels produced using other crosslinker systems, but this hypothesis has not been tested. The carbodiimide L-leucine methyl ester hydrochloride crosslinker system yields gels of medium stability and to some degree stability is determined by the HA molecular weight.

Stable HA gels can be produced using GTA using solution methods and compared to those produced using DVS as crosslinker, they have a lower crosslink density and therefore a greater mesh size, but similar water diffusion rates. GTA solution crosslinked hydrogels may have merit as bioengineering materials and deserve more extensive study. Solution crosslinking using epoxides also yields gels of medium (up to 48 h) stability before decomposition and again the system merits further investigation. Poor stability with time suggests that auto crosslinked gels have little bioengineering merit although such systems would eliminate toxicity problems arising from residual crosslinker.

The crosslink density measurements show that all four covalent crosslinkers give broadly similar mesh sizes and within the limits of this study using a higher molecular weight polymer produces a more open gel, although the magnitude of the effect is not consistent from one crosslinker to another.

TABLE II
Diffusion Constant Values for Distilled Water Through GTA and DVS Crosslinked Hyaluronic Acid (M_w 2.06 × 10^6) Gels

	B^{2*}	H^{2**}	π	D (cm^2 s^{-1})***
GTA gel	0.00006	0.0016	3.14	$1.8 \times 10^{-6} \pm 0.2$
DVS gel	0.000036	0.0016	3.14	$1.4 \times 10^{-6} \pm 0.1$

HA : Crosslinker is 1 : 2 mole ratio.
* B = Slope of M_t/M_∞ versus $\sqrt{t}$.
** H = Thickness of sample (4 mm).
*** D = Diffusion coefficient.

TABLE III
Crosslink Density Data for Hyaluronic Acid (M_w 2.06 × 10^6) Films After 24-h Swelling in Distilled Water

M_w	Q_m	Q_v	M_c (g/mol)	V_e (mol/cm^3)	ε (nm)
GTA crosslinked					
1.19 e^6	5.03	5.95	2.6×10^4	4.6×10^{-5}	51.52
1.40 e^5	3.16	3.65	7.1×10^3	1.7×10^{-5}	22.69
DVS crosslinked					
1.19 e^6	1.30	1.4	1.3×10^3	8.9×10^{-4}	07.21
1.40 e^5	1.70	1.86	2.3×10^3	5.3×10^{-5}	10.32
EDC crosslinked					
1.19 e^6	3.62	4.2	9.0×10^3	1.4×10^{-4}	26.87
1.40 e^5	2.72	3.11	5.4×10^3	2.3×10^{-5}	18.82
EX-810 crosslinked					
1.19 e^6	6.95	8.3	2.7×10^4	4.4×10^{-5}	59.20
1.40 e^5	3.71	4.33	9.4×10^3	1.3×10^{-5}	27.66

References

1. Rapport, M. M. M.; Weismann, B.; Linker, A.; Meyer, K. Nature 1951, 169, 996.
2. Palumbo, F. S.; Pitarresi, G.; Mandracchia, D.; Tripodo, G.; Giammona, G. Carbohydr Polym 2006, 66, 379.
3. Ibrahim, S.; Joddar, B.; Craps, M.; Ramamurthi, A. Biomaterials 2007, 28, 825.
4. Ji, Y.; Ghosh, K.; Shu, X. Z.; Li, B.; Sokolov, J. C.; Prestwich, G. D.; Clark, R. A. F.; Rafailovich, M. H. Biomaterials 2006, 27, 3782.
5. Takagi, A.; Yamashita, N.; Yoshioka, T.; Takaishi, Y.; Nakanishi, K.; Takemura, S.; Maeda, A.; Saito, K.; Takakura, Y.; Hashida, M. J Controlled Release 2006, 115, 134.

6. Fernandez Lopez, J. C.; Ruano-Ravina, A. Osteoarthritis Cartilage 2006, 14, 1306.
7. Balazs, E. A.; Leshchiner, A.; Leshchiner, A.; Band, P. (to Biomatrix, Inc.). U.S. Pat. 4,713,448 (1987).
8. Della Valle, F.; Romeo, A. U.S. Pat. 5,202,431 (1987).
9. Krahulec, J.; Krahulcová, J. Appl Microbiol Biotechnol 2005, 71, 415.
10. Akasaka, H.; Seto, S.; Yanagi, M.; Fukushima, S.; Mitsui, T. J Soc Cosmet Chem 1988, 22, 35.
11. Tomihata, K.; Ikada, Y. J Polym Sci Part A: Polym Chem 1997, 35, 3553.
12. Tomihata, K.; Ikada, Y. Biomaterials 1997, 18, 189.
13. Tomihata, K.; Ikada, Y. J Biomed Mater Res 1997, 37, 243.
14. Prestwich, G. The science of hyaluronan today, 2001. Available at http://www.glycoforum.gr.jp/index.html.
15. Collins, M.; Birkinshaw, C. J Appl Polym Sci 2007, 104, 3183.
16. Prestwich, G. D.; Marecak, D. M.; Marecek, J. F.; Vercruysse, K. P.; Ziebell, M. R. J Controlled Release 1998, 53, 93.
17. Hamilton, R.; Fox, E. M.; Acharya, R. A.; Walts, A. (to Genzyme Corporation). U.S. Pat. 4,937,270 (1990).
18. Luo, Y.; Kirker, K. R.; Prestwich, G. D. J Controlled Release 2000, 69, 469.
19. Balazs, E.; Leshchiner, A. (to Biomatrix). U.S. Pat. 4,582,865 (1986).
20. Zhao, X. B.; Fraser, J. E.; Alexander, C.; Lockett, C.; White, B. J. J Mater Sci: Mater Med 2002, 13, 11.
21. Simkovic, I.; Hricovini, M.; Soltes, L.; Mendichi, R.; Cosentino, C. Carbohydr Polym 2000, 41, 9.
22. Mensitieri, M.; Ambrosio, L.; Nicolais, L. J Mater Sci: Mater Med 1996, 7, 695.
23. Sikkink, C. J. J. M.; de Man, B.; Bleichrodt, R. P.; van Goor, H. J Surg Res 2006, 136, 255.
24. Matsuda, T.; Moghaddam, M. J.; Sakurai, K. (to Seikagaku Kogyo Kabushiki Kaisha). U.S. Pat. 5,462,976 (1995).
25. Hubbell, J. A.; Pathak, C. P.; Sawhney, A. S.; Desai, N. P.; Hill-West, J. L. (to Board of Regents, The University of Texas System). U.S. Pat. 5,567,435 (1996).
26. Yui, N.; Okano, T.; Sakurai, Y. J Controlled Release 1993, 26, 141.
27. Burns, J. W.; Cox, S.; Walts, A. (to Genzyme Corporation). U.S. Pat. 5,017,229 (1991).
28. Okamoto, A.; Miyoshi, T. In Hyaluronan; Kennedy, J., Phillips, G., Williams, P., Eds.; Woodhead: Cambridge, 2002; p 21.
29. Marsano, E.; Gagliardi, S.; Ghioni, F.; Bianchi, E. Polymer 2000, 41, 7691.
30. Leach, J.; Bivens, K. A.; Patrick, C. W., Jr.; Schmidt, C. Biotechnol Bioeng 2003, 82, 578.
31. Gekko, K. Am Chem Soc Symp Series 1981, 150, 415.
32. Huglin, M.; Rehab, M.; Zakaria, M. Macromolecules 1986, 19, 2986.
33. Lowman, A.; Peppas, N. In Encyclopedia of Controlled Drug Delivery; Mathiowitz, E., Ed.; Wiley: New York, 1999; p 397.
34. Cleland, R. L.; Wang, J. L. Biopolymers 1970, 9, 799.
35. Peppas, N. Hydrogels in Medicine and Pharmacy; CRC: Boca Raton, 1987.
36. Shah, C.; Barnett, S. J Appl Polym Sci 1992, 45, 293.
37. Tokita, Y.; Okamoto, A. Polym Degrad Stab 1995, 48, 269.