Comparison of the Effectiveness of Four Different Crosslinking Agents with Hyaluronic Acid Hydrogel Films for Tissue-Culture Applications

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ABSTRACT: The effectiveness of four different reagents, glutaraldehyde (GTA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), poly(ethyelene glycol) diglycidyl ether (EX 810), and divinyl sulfone (DVS) as crosslinkers for cast hyaluronic acid (HA) films has been evaluated. Films were prepared by casting from solution and exposed to solutions of the crosslinkers in acetone–water solution. Swelling in water and in phosphate buffered saline (PBS) was then used to assess the effectiveness of the crosslinkers. GTA-crosslinked films were found to be of low stability compared with those treated with EDC, EX 810, and DVS. Results suggest that instability in GTA-crosslinked materials arises in part from residual acid catalyst. The effects of polymer molecular weight are not uniform. With GTA-crosslinked film produced from higher molecular weight HA swells more, and this is attributed to reduced diffusion of the crosslinker, but with EDC, the opposite effect is observed, implying some additional molecular weight dependent mechanism. Differential scanning calorimetry and dynamic mechanical thermal analysis results suggest that there are no significant structural difference between the gels for each crosslinker system and only the crosslink density and moisture content alters the transitions. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 104: 3183–3191, 2007

Key words: hydrogel; biological applications of polymers; biomaterials; mechanical properties

INTRODUCTION

Hyaluronan, also known as hyaluronic acid (HA), an abundant nonsulfated glycosaminoglycan (GAG) component of synovial fluid and extracellular matrices,[1,2] is an attractive building block for new biocompatible and biodegradable polymers with possible applications in drug delivery,[3] tissue engineering,[4–6] and visco supplementation.[7] As a polysaccharide of the extra cellular matrix (ECM), it plays a multitask role, having many structural, rheological, physiological, and biological functions in the body. It is a linear and anionic polymer consisting of two modified sugars, glucuronic acid and *N*-acetylglucosamine, with the molecular structure:-[D-glucuronic acid (1-*b*-3) *N*-acetyl-D-glucosamine (1-*b*-4)]$_n$.

Hyaluronan is synthesized by many types of cells in the body and extruded into the extracellular space where it interacts with the other constituents of the ECM to create the supportive and protective structure around the cells. It is present as a constituent in all body fluids and tissues and is found in higher concentrations in the vitreous humor of the eye and the synovial fluid in the joints. Commercial HA is usually obtained from rooster comb, although full details of its preparation are not always provided.

The biomedical application of HA is hindered by its short residence time and lack of mechanical integrity in an aqueous environment and these drawbacks must be addressed in order to realize its potential. In this article, we compare the effects of four different chemical crosslinkers to protract the material's degradation and dissolution and thereby improve mechanical stability. Crosslinking is the most common modification of hyaluronan to form a hydrogel and a number of mechanisms have been reported in the literature.[8–11] The functional groups, which are mainly responsible for crosslinking of HA molecules are the hydroxyl and carboxyl groups. Hydroxyl groups may be crosslinked via an ether linkage and carboxyl groups via an ester linkage. If desired, the HA may be chemically modified prior to crosslinking to form other chemically reactive groups. Thus, for example, HA may be treated with acid or base such that it will undergo at least partial deacetalization, resulting in the presence of free amino groups. It is said that amino groups may be crosslinked via an amide (—C(O)—NH—); imino (—N=CH—) or secondary amine (—NH—CH—) bond. An imino linkage can be converted into an amine linkage in

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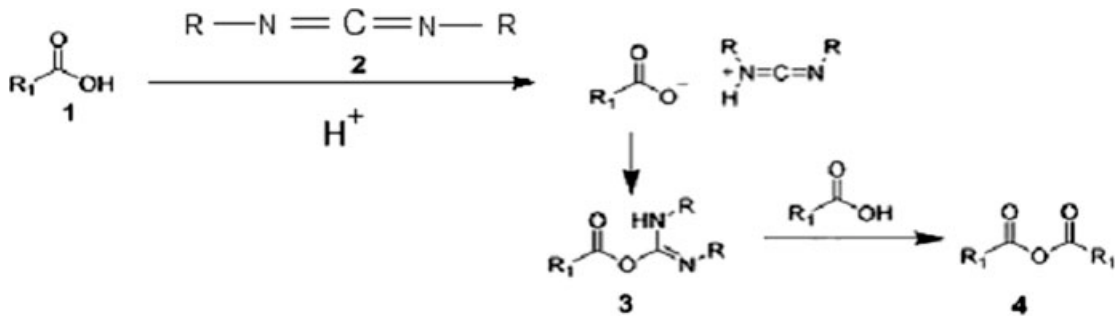


Scheme 1 With water soluble carbodimides (WSC) (2) the crosslinking occurs through the initial formation of O-acylisourea (3) on the polysaccharide, through reaction with neighboring carboxyl groups (1) an anhydride (4) is formed, and this anhydride then reacts with nearby hydroxyls to give both inter- and intramolecular crosslinks.

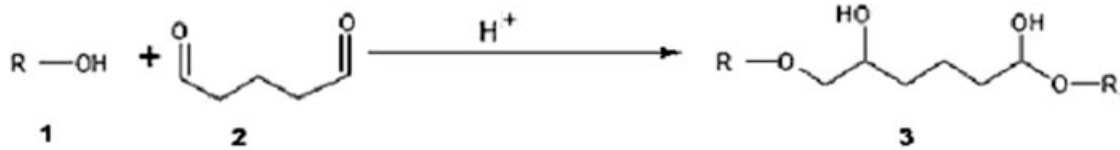
Reaction has been accomplished under acidic, neutral, and alkaline conditions using carbodimides,[10,12–14] hydrazides,[14,15] aldehydes,[8] sulfides,[16] and polyfunctional epoxides.[9,17–20] Autocrosslinking[21,22] and photocrosslinking[23–25] have also been reported. With carbodimides, the crosslinking occurs through the initial formation of anhydride on the polysaccharide, through reaction with neighboring carboxyl groups, and this anhydride then reacts with nearby hydroxyls to give both inter- and intramolecular crosslinks. It was postulated that crosslinking took place via ester groups. Scheme 1 gives some details of these reactions. A reported[10] modification of this reaction introduces L-lysine methyl ester, which offers the opportunity to form higher stability amide crosslinks. Dialdehydes are believed to crosslink through formation of acetal or hemiacetal groups on neighboring chains, with kinetic and spectroscopic evidence indicating a prevalence of the hemiacetal. Glutaraldehyde (GTA) is believed to form either a hemiacetal or an ether link with HA under acidic conditions[26] as shown in Scheme 2. With divinyl sulfone (DVS), the crosslinking occurs via the hydroxyl groups forming an ether bond as shown in Scheme 3. The epoxy group of EX 810 is known to react with —COOH and the —OH functional groups, therefore forming ester and ether bonds, respectively, and is shown in Scheme 4.

Reactions may be carried out using heterogeneous or homogeneous methods. Heterogeneous reactions are carried out on solid HA, cast in the form of films or membranes, in which case diffusion rates maybe at least as important as chemical kinetics, whereas homogeneous reactions are carried out 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.

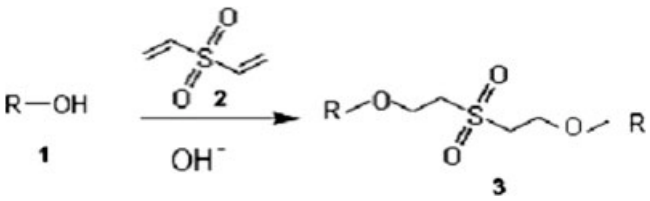
Experience has shown that the repeatability of reported experimental procedures and their outcomes is low, perhaps because of differences in the sources and preparation procedures of the HA and the HA solutions. Therefore, an important consideration in the work reported here was to compare, under a set of standard conditions, the performance of four important crosslinkers using well characterized HA from identified sources.

The first part of the study established a method for the controlled dissolution of HA and film casting, and then utilized the crosslinking reagents to give materials with increased stability with time. Control of dissolution procedure is considered to be important because dissolution and solution degradation may be concurrent processes. Complete dissolution is also important to maximize intermolecular crosslinking and reduce wasteful intramolecular reactions. Previously, crosslinking HA films by immersion in a crosslinking mixture has only been reported for GTA[8] and carbodiimide.[10]

The relationship between crosslink density and solvent swelling is described by the well-known Flory–Rhener equation, which demonstrates that increasing crosslinker effectiveness will be shown by a reduced volumetric swelling. For this work,



Scheme 2 The OH (1) group on the hyaluronic acid reacts under acidic conditions with glutaraldehyde (2) to give hemi-



Scheme 3 The OH (1) group on the hyaluronic acid reacts under alkaline conditions with divinyl sulfone (2) to give sulfonyl bisethyl crosslinks (3).

the swelling ratio (SR) was calculated via the equation:

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

where W_s is the weight of the sample at equilibrium at each temperature and W_d is the weight of the dried sample.

Huglin et al. defines the equilibrium water content according to the equation:

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

Therefore, the water content can be expressed as follows[27];

$$W_b(\%) = W_t - (W_f + W_{fb}) = W_t - (Q_{\text{endo}}/Q_f) \times 100$$

where W_b is the amount of bound water (%); W_f and W_{fb} are the amounts of free and freezing bound water, respectively, and W_t is the EWC (%).

The three types of water found in gels are defined as follows:

1. Bound water: this term refers to the water molecules that are bound to polymer molecules through hydrogen bounds. This kind of water shows no endothermic peak in the temperature range: −70 to 0°C.
2. Intermediate water or ''secondary bound water:'' other water molecules that interact with polymer molecules are referred to as intermediate water. This kind of freezing water has a melting point <0°C.
3. Free water: water molecules that do not take part in hydrogen bonding with polymer molecules are called free water because of their greater degree of mobility in comparison with other water molecules. Free water is freezing water showing a melting point at 0°C.[28]

An initial set of survey experiments compared the effects of the different crosslinkers and reaction environments, and the results obtained were then used in designing the experiments to determine the reaction conditions for further experiments.

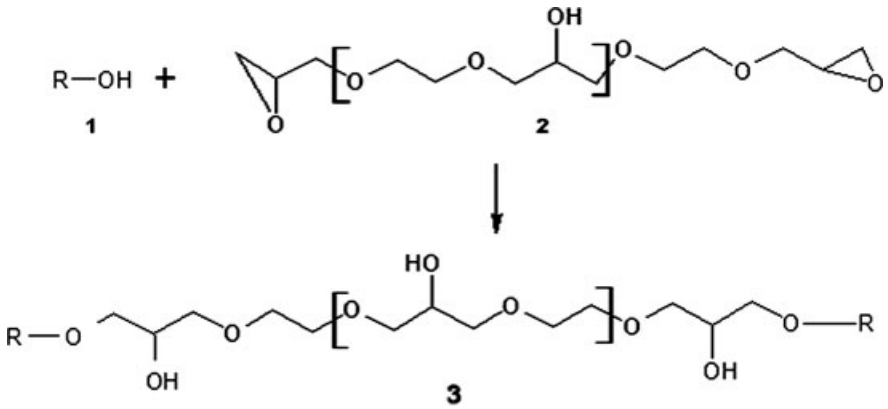
EXPERIMENTAL

Materials

The sodium salt of HA with an average molecular weight of 2.14×10^6 was supplied by Clear Solutions (New York, NY) as dry powder. This material is prepared in high yield from streptococcus bacteria by fermenting the bacteria under anaerobic conditions in CO_2 enriched growth medium.[29] HA powders of average molecular weight 1.2×10^6, 8.5×10^5, and 1.4×10^5 were purchased from Bioiberica (Barcelona, Spain). This material is obtained from rooster comb. HA has been mainly extracted from rooster combs for many years and many papers have been published on clinical application of HA from this source. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), GTA, poly(ethylene glycol) diglycidyl ether, and DVS were purchased from Lancaster (UK).

Preparation of HA-crosslinked films

Solutions were prepared by sieving HA particles into double-distilled water to expose the maximum



Scheme 4 The OH (1) group on the hyaluronic acid reacts with the epoxy group of the poly(ethylene glycol) diglycidyl

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. The viscosity time profiles of the solutions were then obtained using an Ubbelohde viscometer. Samples were fully dissolved after 24 h and the molecular properties of all the solutions were evaluated using size exclusion chromatography (SEC). Films were then prepared by casting a 1 wt % aqueous solution of each HA type onto a clean petri dish, followed by drying at 25°C under vacuum for 120 h. The volume of solution used determined the thickness of the resulting film and this was adjusted to give a thickness of ~ 0.2 mm.

For the initial survey experiments, $10 \times 10 \times 0.2$ mm^3 samples of cast HA ($M_W = 7.6 \times 10^5$ Da) film were weighed and placed in 10 mL of acetone–water solution (80:20 by volume) containing 0.01*M* HCl and varying mole ratios of 1-EDC, GTA, ethylene glycol diglycidyl ether (Denacol EX-810), and divinylsulphone (DVS). The acetone prevents the dissolution of the HA film into the reaction solution, and all the reaction vessels were sealed to prevent evaporation of the acetone. The acetone concentration of 80 vol % was selected after experimentation had shown that lower concentrations resulted in excessive water swelling of the cast HA film. Crosslinker molarity and mole ratio of crosslinker to HA were based on previously published data.[8] A high crosslinker concentration is used to drive the diffusion process. The crosslinking reaction was allowed to proceed at room temperature for 24 h unless otherwise specified. With EDC and GTA, crosslinking is favored by acidic conditions and 0.01*M* HCl was used as pH adjuster and catalyst,[8] while DVS requires alkaline conditions and in this case, 0.01*M* NaOH was used to adjust pH.

Characterization of films

On the basis of the initial observations, further series of crosslinked films were cast, using film thicknesses up to 0.2 mm, crosslinking times between 24 and 72 h, and crosslinking temperatures of 4 and 20°C. The crosslinker:HA mole ratio used was 2 : 1 to 4 : 1 and the medium was 80 : 20 acetone:water. These films were washed in distilled water for 1 h and then dried overnight in a vacuum oven at room temperature. They were then swollen in distilled water and in phosphate buffered saline (PBS) and the water content of the gel and the SR were measured.

DSC analysis was carried out on a TA Instruments DSC 10 differential scanning calorimeter. The thermal analysis profiles were of dried hydrogel samples and swollen samples. The temperature was increased from room temperature to 300°C at a rate of scanning calorimetry (DSC) (TA Instruments 10) was also employed to examine the state of water in the swollen hydrogels with different water contents. Samples sealed in aluminum pans were cooled to −20°C and then heated to 20°C at a heating rate of 5°C/min under 60 cc/min of nitrogen gas flow. Using peak areas, normalized for sample mass, the endotherm associated with water loss was obtained and compared with the theoretical value for water. The fraction of free water in the total water was then calculated using eq. (3) as the ratio of the endothermic peak area for water-swollen hydrogels to the melting endothermic heat of fusion (−334 J/g) for pure water. Bound water because of hydrogen bonding was expressed as the difference between the total water and the free water.

The dynamic mechanical thermal analysis (DMTA) of the materials in the form of films (0.7-mm thick) was carried out with a Polymer Laboratories DMTA MK-1 apparatus, operating in the parallel plate mode. The scans were performed on samples maintained under room conditions, at a frequency of 1 Hz, temperature range of −30 to 100°C, and a heating rate of 4°C min^{-1}.

RESULTS AND DISCUSSION

Preparation of HA films

The relative flow times for the HA solutions (Fig. 1) are consistent with the molecular weights indicated by the suppliers. All the viscosity-time curves have a similar shape with the samples becoming fully solvated between 24 and 40 h. After this time, the solution viscosities are seen to fall and this is taken to indicate the onset of polymer degradation through hydrolysis. As a result of these observations, all films used for crosslinking were cast after 24 h of dissolution and thus in the fully solvated and chain-disentangled state. The molecular properties of each

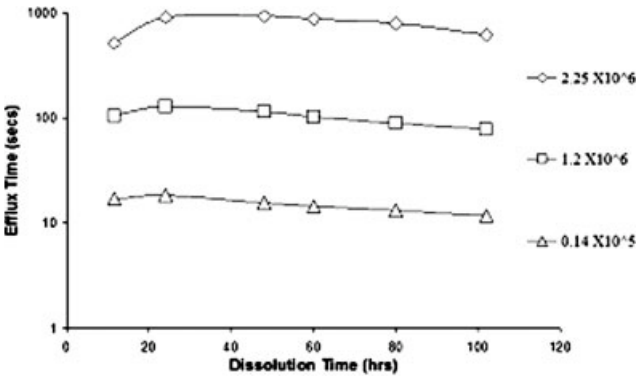


Figure 1 Dissolution time profile of 1 wt % hyaluronic

TABLE I
Molecular Properties of Hyaluronic Acid After 24 h Dissolution, Measurements were in PBS

Sample	M_w/M_n	Intrinsic viscosity (dL/g)	Radius of hydration (nm)	Radius of gyration (nm)	Mark–Houwink (α)	Mark–Houwink (log K)
2.25×10^6	1.08	24.61	94.84	162.61	0.61	−2.49
1.20×10^6	2.14	20.13	66.54	114.33	0.91	−4.23
0.85×10^6	3.27	14.80	51.68	97.01	0.95	−4.44
0.14×10^6	1.38	3.26	18.31	26.94	1.09	−9.884

sample is summarized in Table I and the values given were calculated using the Viscotek Omnisec software, version 4.2.

Table II summarizes the results of the survey experiments and details the swelling ratios of the films after they had been submerged in the solution of crosslinker in 80% acetone and 20% water for 24 h. The results show that the molarity of the crosslinker solution has only small effect indicating that film surface area is the critical issue. With both DVS and GTA, the need for acidification to optimize crosslinking is apparent.

Swelling studies

In Figures 2 and 3, the swelling in water and PBS of films, crosslinked under optimum conditions, is compared and in both media all three crosslinkers give an initial volume swelling of between 2.0 and 2.5, indicating similar crosslink densities. Considering the behavior in distilled water, both the DVS and EDC-crosslinked materials shrink with time and this is taken to indicate continuing crosslinking action, suggesting that the crosslinker diffusion into the film is faster than the crosslinker reaction with the constituent polymer.

GTA-crosslinked materials are clearly very sensitive to the nature of the swelling medium and it is thought that the lower swelling and greater stability in buffer arises from neutralization of residual acid in the film. With these materials, progressive swelling occurs in water and this is thought to arise from network scission. On the basis of Tomihata and Ikada's proposal that the crosslinks in this case are hemiacetals,[8] as shown in Scheme 2, it is reasonable to presume that these are undergoing hydrolyitic scission with time, catalyzed by residual acidity, and then the gel SR increases as the crosslink density falls. Taken overall, these results suggest that the crosslinking reagents remain reactive in the gel and that swelling with buffer has a stabilizing effect through neutralization.

TABLE II
Crosslinking Conditions for HA Films (∼ 0.1-mm Thick) $M_w = 8.5 \times 10^5$ Da

Crosslinker	Mole Ratio crosslinker/polymer	[crosslinker] M	pH	Equilibrium swelling ratio after 24 h in 80% acetone/20% water mixtures
DVS	02:01	0.26	12	1.53
DVS	04:01	0.57	12	1.37
GTA	02:01	0.19	2	1.28
GTA	02:01	0.19	2	1.28
GTA	02:01	0.21	2	1.27
GTA	02:01	0.22	2	1.26
GTA	02:01	0.22	2	1.26
GTA	04:01	0.40	2	1.20
EDC	01:01	0.14	2	1.67
EDC	02:01	0.22	2	1.47
EDC	02:01	0.30	2	1.33
EDC	03:01	0.26	2	1.57
EDC	04:01	0.46	2	1.28
EDC	04:01	0.46	2	1.28
EDC	04:01	0.58	2	1.31
EX 810	04:01	0.28	2	1.17
EX 810	04:01	0.32	2	1.25
EX 810	04:01	0.34	2	1.21
EX 810	04:01	0.36	2	1.21
EX 810	02:01	0.17	7	1.33

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