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Hyaluronic Acid Solutions—A Processing Method for Efficient Chemical Modification

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ABSTRACT: To ensure repeatability of these crosslinking procedures and thereby improve the degradation time of hyaluronic acid (HA)-based biomaterials *in vitro*, it is essential to have an understanding of the solution properties of HA. Control of HA dissolution is important because dissolution and solution degradation are concurrent processes during solution preparation. Complete dissolution is also important to maximize intermolecular crosslinking and reduce wasteful intramolecular reactions. Viscosity time profiles of HA solutions during dissolution have been obtained in order to optimize crosslinking efficiencies. Optimum dissolution occurs between 24 and 40 h when the HA is in a fully solvated disentangled state as indicated by the peak in the solvating curves which corresponds with Mark–Houwink α parameters of 0.76–0.85. The developed and characterized dissolution process was used to produce carbodiimide crosslinked HA films that were found to be reproducible and stable when immersed in distilled water. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 130: 145–152, 2013

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INTRODUCTION

Meyer and Palmer first described a procedure for isolating a novel glycosaminoglycan from the vitreous humour of bovine eyes[1] and they proposed the name hyaluronic acid (HA), which is sometimes referred to as Hyaluronan, as it exists *in vivo* as a polyanion. HA, a carbohydrate polymer, is present in all vertebrates and is a major constituent of the extra cellular matrix (ECM). Commercial HA is usually obtained from rooster comb or from bacterial sources such as *Streptococcus equi*[2] and *Streptococcus zooepidemicus*,[3] the latter eliminates the possibility of inter-species disease transfer.[4]

HA is a material of increasing importance to biomaterials science and is finding applications in diverse areas ranging from tissue culture scaffolds, which has been reviewed recently,[5] to cosmetic materials.[6] Its properties, both physical and biochemical, in solution or hydrogel form, are extremely attractive for various technologies concerned with body repair.[7,8] However, biomedical applications of HA have been hindered by its short residence time and lack of mechanical integrity in an aqueous environment and these drawbacks must be addressed in order to fully realize its potential. Therefore, various chemical procedures have been employed to protract the material's degradation and dissolution thereby improving its 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.[9–14]

Authors have reported the formation of HA-based hydrogels with limited stability.[13] This article, focuses on increasing the stability of the hydrogels by controlling the dissolution and ensuring complete dissolution of HA in distilled water. Complete dissolution is important in order to maximize intermolecular crosslinking and to reduce wasteful intra-molecular reactions that reduce gel stability. To achieve this, we measured and compared the hydrodynamic properties for different HA batches during a dissolution process. As there is likely to be a high level of inter-chain association rheological measurements were performed to give an indication of the level of molecular association involved. The results of this study can be used to produce consistent HA solutions, which can be further processed by drying to form films. These films can then be crosslinked by an immersion crosslinking procedure described by Collins et al.[13] for use in biomedical applications such as wound healing.

EXPERIMENTAL

Materials

The sodium salt of HA with an average molecular weight (Mw) of 2.0×10^6 was supplied by Clear Solutions (New York, USA).

Table I. Typical Dissolution Methods during Hydrogel Preparation Prior to Crosslinking Procedures

Temperature	Dissolving method	Comment
1 wt % aqueous solution of HA (2.0×10^6 Da)	**Method 1**	Solution did not appear uniform in viscosity. Incomplete dissolution
	HA sieved into double distilled water	
	Method 2	HA was fully dissolved
	HA sieved into double distilled water followed by agitation at 100 cycles/min in a water bath at 25°C for 6 h	Solution appeared uniform in viscosity
	Method 3	Resulting in a lower viscosity solution than method 2
	HA sieved into double distilled water	
	HA dissolved under high shear stirring	

This material is prepared in high yield from streptococcus bacteria by fermenting the bacteria under anaerobic conditions in CO_2-enriched growth medium.[15] 1.2×10^6, 0.85×10^6, 0.60×10^6 and 0.14×10^6 Mw samples were purchased from Bioiberica (Barcelona, Spain). This material has been extracted from rooster comb. All materials were supplied as dry powder.

Infrared Spectroscopy

Spectra of the as received powder samples were obtained using potassium bromide (Kbr) discs. All spectra were recorded on a Perkin Elmer spectrum 2000 FTIR spectrophotometer. The range 4000–400 cm^{-1} was scanned 10 times, averaged, and normalized using Perkin–Elmer's spectrum v1.30 software.

HA Solution Preparation and Analysis

Pre-sieved HA powder was dissolved in double-distilled water by gentle agitation in a 25 mL container to minimize shear stress, at 100 cycles/min in a water bath at 25°C. The resulting HA solutions were passed through a Viscotek size exclusion chromatograph (SEC) triple detection system at various dissolution intervals. All HA samples were tested at concentrations of 0.5 mg/mL at flow rates of 0.7 mL/min using PBS as the mobile phase. Two TSK gel columns (GMPWXL mixed bed column, 7.8 mm ID × 30 cm) maintained at 25°C were used for separation. The triple detection system Model 270 consists of a viscometer, light scattering detector (LALS and RALS), and a refractive index detector. Calibration was performed using polyethylene oxide (PEO) and dextran of known molecular weight. The dn/dc used for molecular weight determination was 0.147 and this was calculated from the slope of the refractive indices of a series of five dilutions of HA (0.2–0.8 mg/mL). The dn/dc was 0.147. The literature values for dn/dc ranged between 0.140 and 0.168.[16–20] Data acquisition and calculations were performed using Viscotek Omnisec software version 4.2.

Hydrodynamic radii were calculated as follows:

$$R_h = \left[\frac{3}{4\pi}\left(\frac{[\eta]M}{0.025}\right)\right]^{1/3} \quad (1)$$

where η is the intrinsic viscosity and M is the molecular weight.

For linear chain polymers, this can be related to the radius of gyration, using the Flory–Fox[21] and Ptitsyn–Eizner[22] equations, see equations as below:

$$R_g = \left(\frac{1}{6}\right)^{1/2}\left(\frac{[\eta]M}{\Phi}\right)^{1/3} \quad (2)$$

$$\Phi = 2.55 \times 10^{21}\left(1 - 2.63\varepsilon + 2.86\varepsilon^2\right) \quad (3)$$

$$\varepsilon = \frac{2a-1}{3} \quad (4)$$

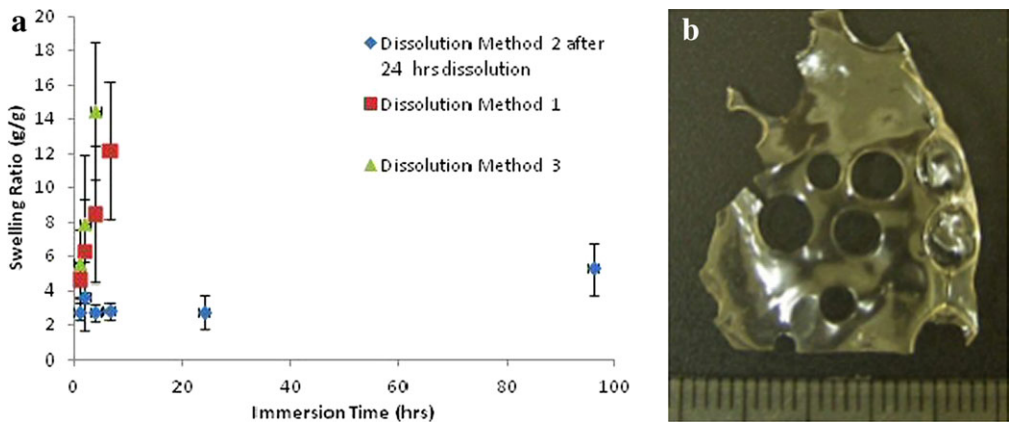

Figure 1. Stability of HA (M_w 2.25×10^6 Daltons) crosslinked films swollen in distilled water pH 6.2 at 25°C, monomer crosslinker mole ratio 1 : 4. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

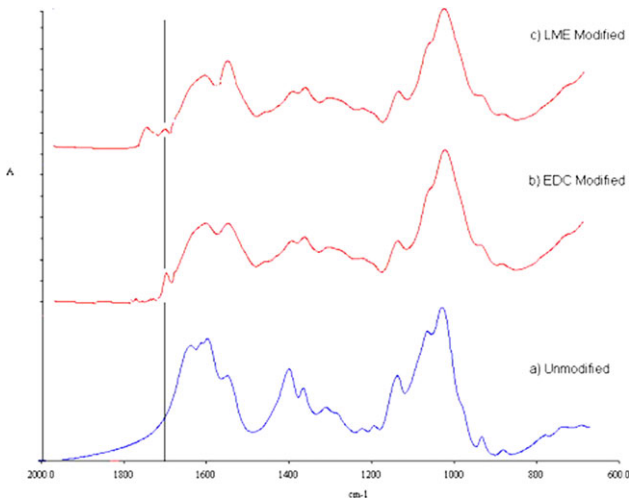

Figure 2. Uncrosslinked and crosslinked HA with crosslinking bands highlighted. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

a is the exponent of the Mark–Houwink equation:

$$[\eta] = KM^a \tag{5}$$

For dilute solutions, a Ubbelohde viscometer type 1C was used and a type 4 Ubbelohde was used to profile the dissolution of more concentrated HA solutions that are more representative of HA solutions used in crosslinking reactions to produce hydrogels. All measurements were carried out in a water bath held at a constant temperature using a water circulator at 25°C ± 0.1°C.

Rheology

Because HA crosslinking reactions are performed at different pH values depending on the crosslinker type used, rheological measurements were performed using a Haake–Rotovisco 1 on

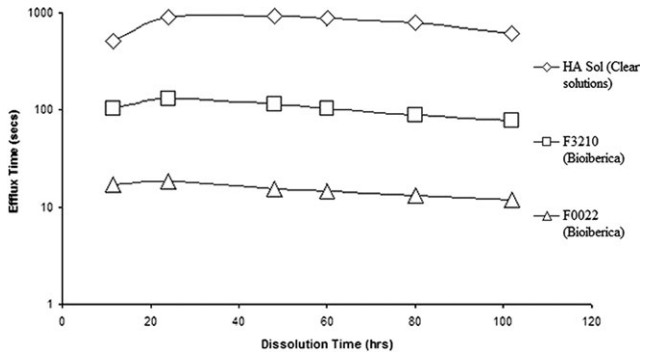

Figure 4. Dissolution of 1 wt % solutions of HA at 25°C reproduced with permission from Collins et al.[9]

1 wt % HA solutions at different pH levels. Shear stress and strain sweeps were measured between two flat plates with the distance between the plates of <1 mm.

Crosslinked HA films

The crosslinking procedure is described in detail elsewhere.[9,23–25] Briefly, films were prepared by casting a 1 wt % aqueous solution of HA onto a clean petri dish, followed by drying at 25°C under vacuum for 120 h. 10 × 10 × 0.2 mm samples of cast HA were placed in 10 mL of acetone–water solution (80 : 20 by volume) containing 0.01*M* HCl and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). L-leucine methyl ester hydrochloride (LME) was added to the crosslinking reaction to enhance the hydrolytic stability of crosslinked hydrogels, by the introduction of a more hydrolysis resistant amide bond.

Crosslinked films were immersed in distilled water and their stability was assessed by measuring their swelling ratio as a function of immersion time. The swelling ratios were calculated as follows:

$$SR = \frac{W_s}{W_d} \tag{6}$$

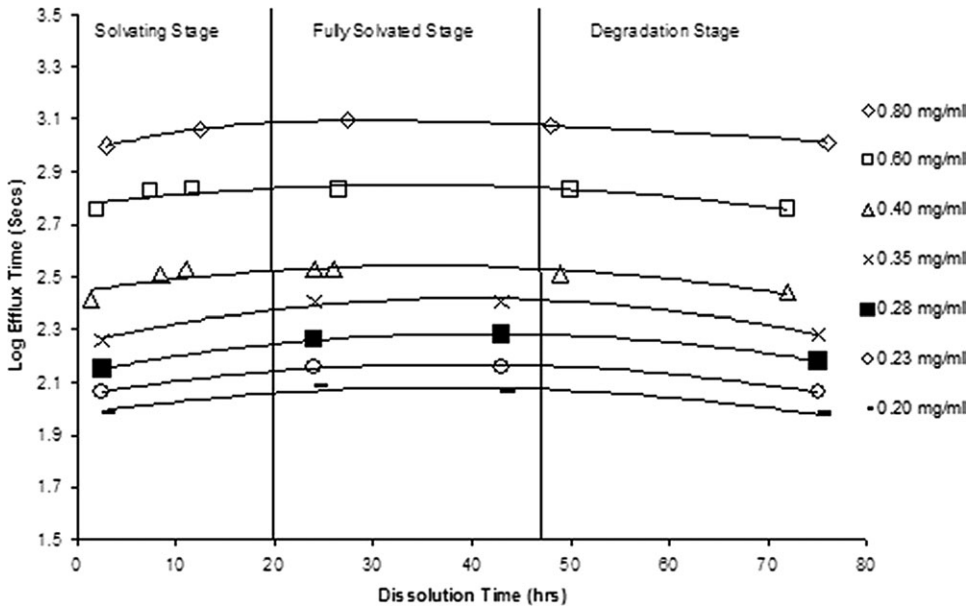

Figure 3. Dissolution profiling of dilute HA solutions of 2.0 × 10^6 Da.

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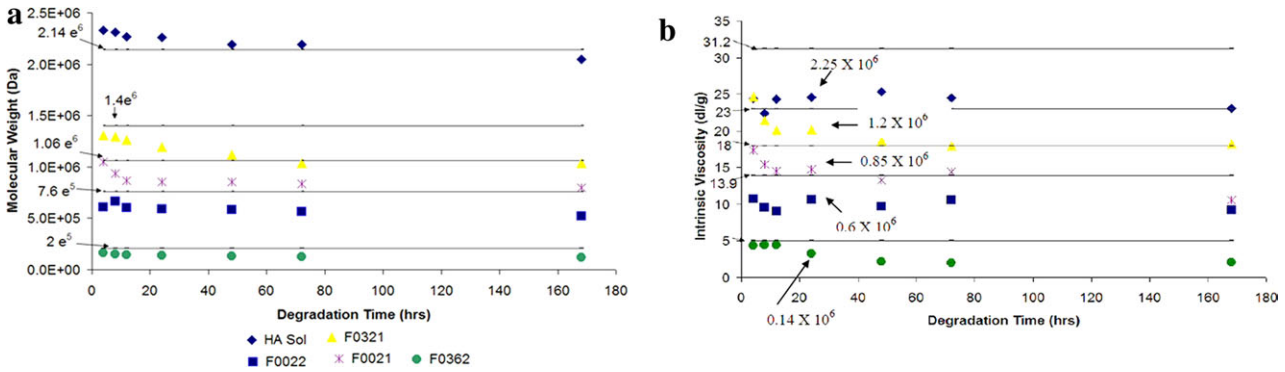

Figure 5. SEC analysis of HA solutions (a) Molecular weight degradation for up to 170 h dissolution. The straight lines represent the manufacturer's measurements for molecular weight (b) Intrinsic viscosity as a function of dissolution time. The straight lines represent the manufacturer's measurements for intrinsic viscosity. The molecular weights measured at 24 h dissolution are included in the diagram. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

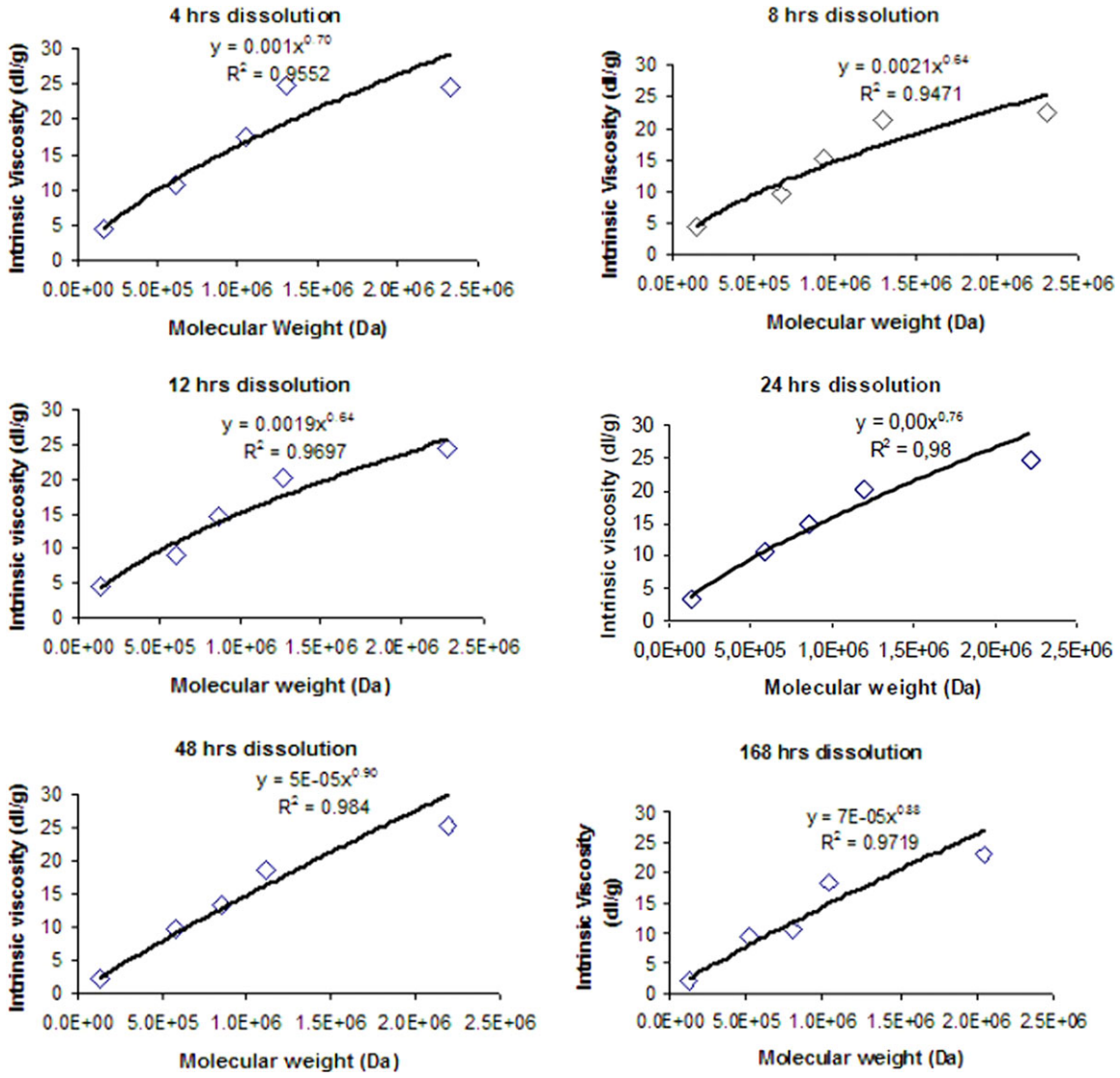

Figure 6. Mark–Houwink plots for HA in phosphate buffered saline (PBS) after various dissolution times. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

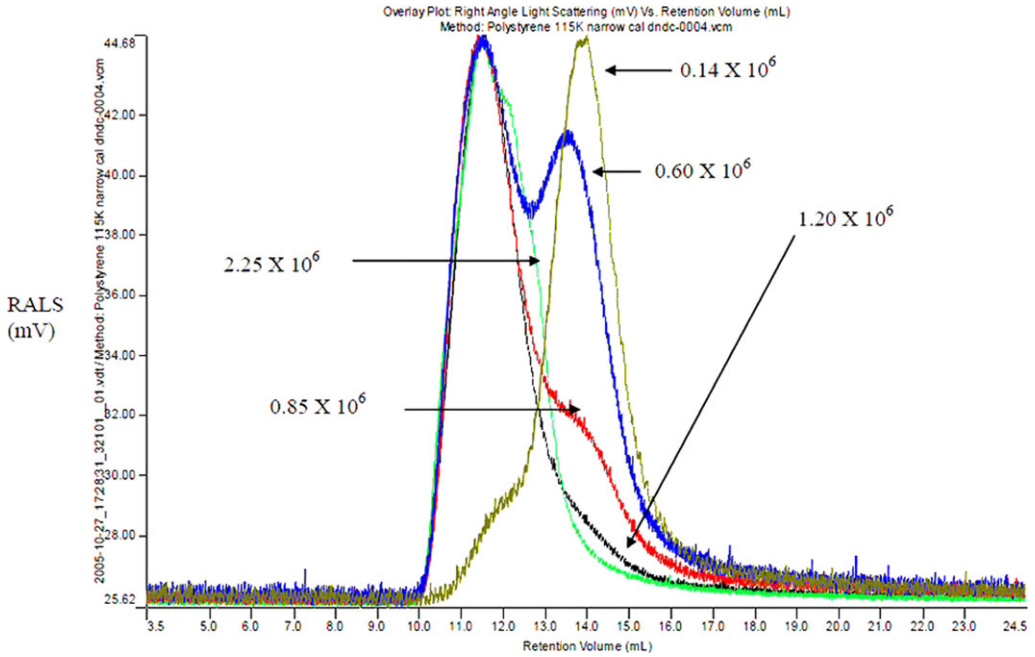

Figure 7. Comparison of the light scattering response for all samples after 24 h dissolution. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

where W_s = swollen weight at set intervals and W_d = dry weight.

RESULTS AND DISCUSSION

Table I outlines typical solution preparation methods that are used prior to crosslinking reactions to form HA hydrogels for biomedical applications. For each method the HA powder is sieved to increase the water contact surface area to allow faster dissolution times. Methods 1 and 3 were unsuitable for further analysis as the solutions displayed inconsistent viscosities because of incomplete dissolution in the case of Method 1 and chain scission as a result of high shearing in the case of Method 3 both of which resulted in wasteful intramolecular reactions, reduced crosslinking efficiency and subsequently hydrogels with poor stability in an aqueous environment as shown in Figure 1. Figure 1 clearly shows that Method 2, where the samples were gently agitated in a shaking water bath to minimize shear stress, promoted efficient crosslinking which produced stable hydrogels.

Figure 2 details FTIR analysis of uncrosslinked and crosslinked HA films produced using Method 2. The main absorption bands can be attributed to the following functional groups: carbonyl (1650 cm^{-1}), amide I (1610 cm^{-1}), amide II (1560 cm^{-1}), CH_2 bending (1413 cm^{-1}), amide III (1380–1210 cm^{-1}). The crosslinked films show an absorbance at 1700 cm^{-1}, which is attributed to an ester bond.[9,24] The presence of L-leucine methyl ester hydrochloride shows a reduction in absorption at 1700 cm^{-1} and an increase at 1740 cm^{-1} and 1560 cm^{-1} suggesting the formation of an amide bond.[9,24] The author has shown that crosslinked films produced in the presence of L-leucine methyl ester hydrochloride are more stable than EDC only crosslinked films.[13]

HA solutions resulting from Method 2 were further analyzed by viscometric analysis to optimize the HA solution preparation method. The viscosity time profiles of these solutions were obtained using a Ubbelohde 1C viscometer and are shown in Figure 3. The standard deviation was <0.5 s for each recorded flow time demonstrating reproducibility. The dissolution curves reflect two competing processes, initially the dissolution process is dominant as more and more polymer becomes fully solvated and solution viscosity increases accordingly. This solvating stage takes between 24 and 40 h approximately and a plateau viscosity is taken to indicate maximum salvation. The subsequent fall in viscosity arises from chain degradation becoming the dominant process, although it must be presumed that degradation commences at the same time as solvation. The onset of degradation is more apparent in solutions of lower concentration and with HA of lower molecular weight.

Representative 1% w/v solutions used in crosslinking reactions are profiled in Figure 3 (reprinted from a previous publication by the author).[9] The relative flow times for the HA solutions (Figure 3) are consistent with the molecular weights. All the viscosity–time curves have a similar shape with the samples becoming fully solvated and disentangled between 24 and 40 h. As the molecular weight is decreased, the HA reaches its fully solvated state more quickly hence the solvating stage does not appear for the lowest molecular weight. Up to 48 h degradation effects are insignificant for all solutions. After this, the solution viscosities are seen to fall and this is taken to indicate the dominance of polymer degradation through hydrolysis.[9]

Molecular properties were measured during the dissolution process for each HA sample via SEC. The effects of dissolution time on the intrinsic viscosity and molecular weight of the HA is shown in Figure 5. Results suggest that all the samples are

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Table II. Summary of Molecular Properties of HA During Dissolution

Dissolution time (hr)	Rh (nm)	Rg (nm)	M-H a
(a) 2.25 × 10^6 Da			
4	95.69	162.92	0.68
8	91.68	166.59	0.72
12	94.48	160.92	0.70
24	94.84	162.60	0.70
48	95.19	155.86	0.60
72	93.85	155.75	0.78
168	89.78	155.91	0.67
(b) 1.2 × 10^6 Da			
4	74.58	92.97	0.59
8	70.90	120.02	0.80
12	67.76	121.43	0.91
24	66.54	114.33	0.91
48	63.14	116.71	0.94
72	62.45	125.49	0.83
168	61.41	111.47	1.17
(d) 0.6 × 10^6 Da			
4	40.43	78.85	1.18
8	38.65	84.30	1.04
12	36.76	80.17	0.85
24	39.60	78.66	0.85
48	37.78	74.87	1.14
72	37.80	75.93	1.25
168	36.11	80.26	1.34
(c) 0.85 × 10^6 Da			
4	58.72	104.47	0.76
8	54.04	102.31	0.92
12	50.94	97.86	0.86
24	51.68	97.00	0.95
48	47.30	89.95	1.60
72	50.49	97.39	1.10
168	35.55	95.43	0.83
(e) 0.14 × 10^6 Da			
4	20.995	56.612	1.27
8	20.728	43.401	1.40
12	20.170	51.298	1.09
24	14.576	67.616	1.09
48	14.838	45.060	1.60
72	13.510	35.300	2.71
168	13.740	43.227	2.80

relatively stable over this time period with a slight reduction in molecular weight because of the onset of hydrolysis. It is worth noting that although the samples came from different suppliers and sources their dilute solution behaviors were similar.

In order to gather information on the overall behavior and conformation of HA molecules during dissolution, Mark–Houwink

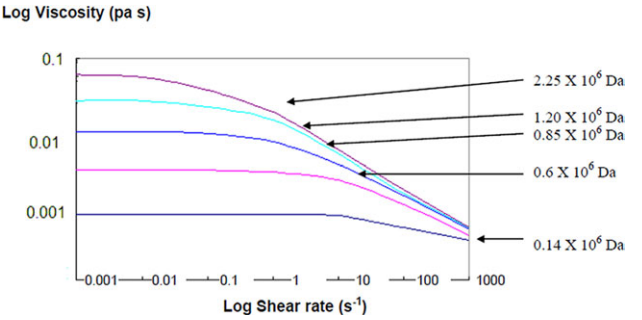

Figure 8. Shear dependence of the viscosity of hyaluronan solutions at constant concentration of 10 mg/mL and varying M_w at 25°C in distilled water. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

plots were constructed as shown in Figure 6. After 24 h, dissolution, see Figure 4 the α parameter is 0.76, indicating that the molecules have adopted a configuration.[26] As the dissolution proceeds the α parameter increases indicating HA chains become stiffer. This is because of the combined effect of (1) the polymer chains being in a fully solvated extended state and (2) the onset of degradation with lower molecular weight polymers appearing to be stiffer.

Figure 7 displays the light scattering response after 24 h dissolution and Table II shows the corresponding molecular information. Generally, the length of the polymer chain has a direct relationship with the radius of gyration, with shorter HA chains having higher α parameters indicating stiffer chains; however, as discussed in the previous paragraph degradation processes because of hydrolysis are also contributing factors. Taking the information from Figure 6 and Table II together, the Mark–Houwink parameters confirm the findings in Figure 3 obtained using the Ubbelodhe viscometer, and that is that generally the HA samples are fully solvated and disentangled between 24 and 40 h dissolution. Lower molecular weight HA reaches the fully solvated state quicker thereby displaying larger α values at earlier dissolution times. After 48 h, the α parameter increases further indicating that hydrolysis has begun.

Solutions of HA which are typically used during crosslinking reactions to produce biomaterials were chosen for rheological analysis as shown in Figure 8. At low shear rates, each sample approaches a Newtonian plateau where viscosity is independent of shear rate. The zero shear viscosity values all occur below 0.1 at these low shear rates the HA molecules remain in an entangled state. Once a critical shear rate is reached the HA

Table III. Steady Shear Rate Results of HA Samples

Molecular weight (×10^6)	η_o (Pa s)	n
2.25	0.030	0.45
1.2	0.016	0.53
0.85	0.010	0.61
0.60	0.005	0.58
0.14	0.002	0.55

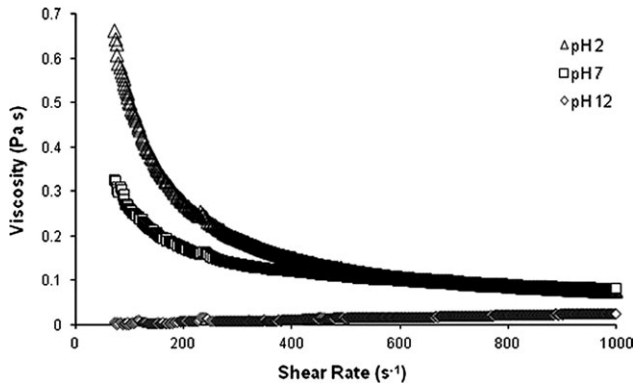

Figure 9. Viscosity dependency on shear rate at 25°C sample concentration 10 mg/mL in varying pH.

begins to disentangle; as shown by a viscosity reduction in the shear thinning region.

Table III displays the zero shear viscosity and power law index, *n*, compared to molecular weight. The power law index, *n* is a measure of the flow behavior and its relationship with shear stress and shear rate is defined in equation below:

$$\tau = k\dot{\gamma}^n \tag{7}$$

The highest molecular weight sample exhibits the lowest power law index, this is probably because of it having a larger degree of entanglement, which contributes to an increased viscoelastic effect. The lower "*n*" value for the low molecular weight samples is indicative of shear thinning behavior because of the break-up of HA chains resulting in less entanglement.

After dissolution, HA crosslinking reactions are conducted in varying pH levels to suit the chemistry of the crosslinker being used, therefore viscosity profiles of HA as a function of shear strain at different pH values is shown in Figure 9. At pH 2 the HA solutions were more elastic and exhibited a paste-like nature on gentle shaking or stirring, while at pH 12 and at pH 7 the solutions showed entirely different dynamic rheological behavior. The paste-like behavior may be attributed to a pronounced stiffening of the HA chains because of a critical balance between the repulsive forces (provided by the ionized carboxyl groups) and the attractive interactions (electrostatic or hydrogen bonds mediated) operating between the molecular chain elements.[27] At alkaline pH, hydrogen bonds are destroyed and this results in a large loss of the chain stiffness and the formation of a more compact, flexible random coil.[28]

Figure 1 displays swelling results in distilled water for crosslinked HA films using EDC as a crosslinker. The developed dissolution method produced films that had higher stability and better reproducibility and therefore greater potential as biomaterials. The increased film stability in aqueous solution is directly related to increased crosslinking efficiency. The improved performance is attributed to complete dissolution of the HA which maximizes intermolecular crosslinking and reduces wasteful intramolecular reactions associated with the other methods. Figure 1b displays the films prior to swelling tests.

CONCLUSION

A simple reproducible dissolution method of HA for subsequent chemical crosslinking reactions has been developed and characterized. It consists of 1% w/v HA agitation in double distilled water at 25°C for a specific time interval which is dependent on molecular weight. Viscosity time profiles of HA solutions during various stages of dissolution have been obtained in order to optimize and reproduce crosslinking reactions. Concurrent processes of HA dissolution and degradation have been mapped in terms of hydrodynamic parameters, and it was found that using the established dissolution procedure a minimum dissolution time of 24 h is required to achieve a fully solvated extended state which is optimum for crosslinking reactions. After 48 h, dissolution hydrolysis becomes significant for all solutions with lower molecular weights being particularly susceptible. Rheological methods have been utilized to assess the effects of molecular weight, shear rate, and pH on molecular chain association. The developed dissolution method was used to produce HA films, which, once crosslinked, were stable when swollen in water for up to 4 days. The improved crosslinking efficiency is attributed to complete dissolution of the HA which maximizes intermolecular crosslinking overcoming wasteful intramolecular reactions which result in instability and lack of reproducibility of HA-based biomaterials.

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