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Effect of lyophilization on the physical characteristics of medium molecular mass hyaluronates

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Abstract

The effect of lyophilization on the physical characteristics of two different molecular mass hyaluronates (164 and 741 kDa) has been evaluated using dynamic oscillatory viscometry and size exclusion chromatography. The hyaluronates were prepared as either the free acid or the sodium salt. The rheological and SEC profiles of sodium hyaluronate were not affected by lyophilization. In contrast, the complex viscosity and related dynamic moduli were markedly decreased after lyophilization of the hyaluronates in the free acid form. Size exclusion chromatography indicated a reduction in molecular mass of these samples. These data indicate that lyophilization of hyaluronates as the free acid, as opposed to a sodium salt form, can have a detrimental effect on their physical characteristics.

Keywords: Hyaluronic acid; Lyophilization; Rheology

1. Introduction

Hyaluronic acid is a naturally occurring, high molecular mass glycosaminoglycan composed of a repeating disaccharide unit consisting of *N*-acetylglucosamine and D-glucuronic acid. It exists in solution as unbranched single chains stabilized by hydrogen bonds parallel to the chain axis which, together with water bridges, impart rigidity to the molecule (Scott, 1989). Hyaluronic acid can be prepared and isolated as either the free acid, or as a salt formed between appropriate counterions and the ionized carboxyl groups present in each repeating D-glucuronic acid unit. A variety of different hyaluronate salts and some ionic drug complexes have been reported (Saettone et al., 1991).

High molecular mass sodium hyaluronate has found wide clinical application in eye and joint surgery due to its unique rheological properties (Laurent and Fraser, 1992). Pharmaceutically, it has been employed as a prospective vehicle for topical ophthalmic drug delivery (Saettone et al., 1989, 1991), formulations of peptide growth factors (Prisell et al., 1992) and local anaesthetics (Johansson et al., 1985). Additionally, sodium hyaluronate has been utilized as a cosmetic ingre-

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dient because of its water-retaining capabilities (Balazs and Band, 1984). More recently, hyaluronates have been chemically modified through cross-linking and esterification (Kyyronen et al., 1992) to provide a potentially broad and novel spectrum of applications.

Lyophilization is a common method for isolating salts of acidic hydrophilic polymers formed after the addition of appropriate counterions to aqueous solutions of the free acid form of the hydrophilic polymer. Therefore, it was expected that lyophilization of the free acid form of hyaluronic acid would facilitate the preparation, isolation and subsequent evaluation of different hyaluronate salts. In the present study, the effect of lyophilization on the apparent molecular mass and rheological characteristics of hyaluronic acid was evaluated by freeze-drying the glycosaminoglycan in either the sodium salt or free acid form.

2. Materials and methods

2.1. Chemicals

Two molecular mass fractions of sodium hyaluronate (Na-HA) (average molecular masses: 164 kDa ($[\eta] = 3.4$ dl/g); and 741 kDa ($[\eta] = 10.8$ dl/g)) were generously supplied by Fidia S.p.A. (Abano Terme, Italy). The hyaluronic acid content of the Na-HA samples was $> 99\%$ (by weight) and the range in molecular mass was approx. $\pm 10\%$ of the average molecular mass value. All other chemicals were of analytical grade and water was obtained from a Milli-Q (Millipore, Milford, MA) water purification system.

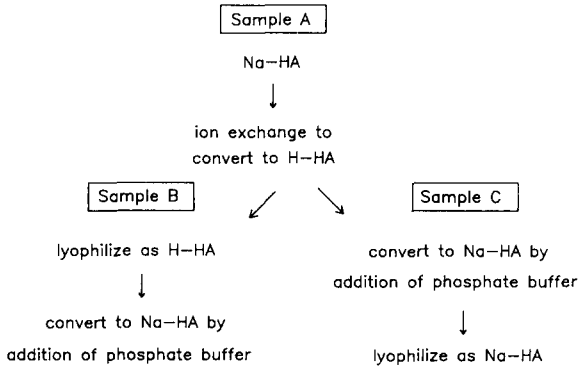
2.2. Preparation of hyaluronic acid (H-HA, free acid form)

Aqueous solutions (7–12 mg/ml) of the two different molecular mass fractions of Na-HA were passed through separate glass columns (8.5 cm × 2 cm), each containing 15 g of Dowex resin (16–40) prepared in the H^+ form. The pH of the eluent was approx. 2.5, and the solutions were filtered through Millex-PF 0.8 μm filter units (Millipore, Bedford, MA).

Infrared spectroscopy (Hitachi 270–30, Tokyo, Japan) was conducted using KBr discs and was used to confirm the presence of either H-HA or Na-HA. The free acid form displayed a strong band at approx. 1730 cm^{-1} due to the carbonyl stretching vibration, whereas the sodium salt form displayed a strong band at 1611–1638 cm^{-1} indicative of the carboxylate anion present in Na-HA (Sheehan et al., 1977). Due to the different IR frequencies of the unionized and ionized carboxyl groups present in H-HA and Na-HA (and because these frequencies were concentration-dependent), it was possible to develop a simple procedure for determining the ionization state of a hyaluronate sample. A standard curve was constructed using appropriately spiked samples (Na-HA/H-HA) and it was possible to quantitate the presence of less than 5% Na-HA in a sample of H-HA. Hyaluronic acid was designated as the free acid form (H-HA) when greater than 95% of the HA content was unionized.

2.3. Experimental design

The experimental approach and subsequent analyses conducted on either the free acid or sodium salt forms of hyaluronate is depicted in Scheme 1. Briefly, Na-HA was converted to the free acid by passage through an ion-exchange



Scheme 1. Experimental design employed for investigating the effect of lyophilization on the rheological and SEC characteristics of hyaluronate when prepared as either the free acid or sodium salt. Both the 164 and 741 kDa molecular mass samples were studied.

column, the sample divided, with approximately half being freeze-dried in the free acid form (sample B) and the remaining portion converted back to the sodium salt prior to freeze-drying (sample C). The samples (approx. 5 mg/ml) were subjected to lyophilization under a vacuum of 2 Torr for 12 h after initial freezing in a slurry of dry ice/acetone. Size exclusion chromatography and rheological measurements were then conducted on samples A–C.

2.4. Chromatography

Size exclusion chromatography (SEC) was conducted using a TSK G6000PW-XL column (Tosoh Co. Ltd, Japan) with an isocratic HPLC system (Beckman Instruments, San Roman, CA, Model 116 pump and 166 UV detector) fitted with a 20 μl injection loop (Rheodyne, Cotati, CA). The mobile phase consisted of 0.15 M phosphate buffer (pH 5.5), the flow rate was 0.5 ml/min and detection was by UV absorbance at 210 nm. The chromatography was linear over the concentration range 0.1–1.0 mg/ml ($r > 0.99$, $n = 6$) and the limit of detection was 20 μg/ml. For SEC analysis, samples of Na-HA and H-HA were prepared in 0.1 M phosphate buffer (pH 7.0) and deionized water, respectively.

2.5. Rheological measurements

The dynamic shear moduli of Na-HA, prepared as a 2.5% w/v solution in 0.1 M phosphate buffer (pH 7.0), were determined at 22 ± 0.1°C using a Rheometrics Fluids Spectrometer II (Rheometrics, NJ) equipped with a cone-and-plate apparatus (cone angle, 0.04 rad; diameter, 50 mm). Throughout the experiments, the torque sensitivity was varied by a decade by changing the mode of the force-rebalanced transducer.

During oscillating rheological measurements, the shear was applied sinusoidally at a maximum strain amplitude (γ_{max}) and angular frequency (ω) according to the standard relationship $\gamma(t) = \gamma_{max}\sin(\omega t)$. The amplitude of the shear stress (τ) and the phase difference (δ) between the stress and strain were monitored according to the relationship $\tau(t) = \tau_{max}\sin(\omega t + \delta)$. From these measurements, the complex viscosity (η^*) and its in-phase component (the elastic modulus, G') and out-of-phase component (the viscous modulus, G'') were obtained via the following standard relationships (Ferry, 1980) where, $\tau(t) = \gamma_{max}[G' \sin(\omega t) + G'' \cos(\omega t)]$ and $\eta^* = [((G')^2 + (G'')^2)^{0.5}]/\omega$. The Na-HA and H-HA samples were non-thixotropic as the rheological profiles were neither time nor shear-history dependent.

3. Results and discussion

The utility of hyaluronic acid as a viscosity-imparting formulation component has found wide application due to its unique biocompatibility profile. As part of a wider effort directed towards investigating formulation opportunities of medium molecular mass hyaluronates (up to 10^6 Da), the characteristics of two molecular mass

Table 1
Elution volumes (ml) for different molecular mass hyaluronate samples when lyophilized as either the free acid or sodium salt form (refer to Scheme 1 for details)

Hyaluronate samples		Elution volume (ml) of different molecular mass hyaluronates	
		164 kDa	741 kDa
Sample A	Na-HA	8.65	7.65
	after conversion to H-HA	8.71	7.74
Sample B	after lyophilization as H-HA	9.32	8.33
	after conversion to Na-HA	9.36	8.37
Sample C	after conversion to Na-HA	8.71	7.75
	after lyophilization as Na-HA	8.67	7.72

fractions of hyaluronic acid after lyophilization as the free acid or sodium salt were investigated.

3.1. Size exclusion chromatography

SEC analyses of the different molecular mass fractions of hyaluronic acid, when freeze dried as either the free acid or sodium salt, are presented in Table 1 and individual chromatograms are depicted in Fig. 1. It is notable that from a chromatographic standpoint, the elution volumes of either the H-HA or Na-HA form of a particular sample (A, B or C; Scheme 1) were indistinguishable from each other as conversion of the free acid to the anionic form occurred during chromatographic analysis.

After lyophilization of the free acid for either molecular mass hyaluronate, (sample B; Scheme 1) there was an increase in the elution volume indicative of a reduction in the apparent average molecular mass of the polymer. Since the addition of phosphate buffer to the freeze-dried H-HA sample prior to SEC analysis had no effect on the elution profile, it was apparent that an irreversible change in the polymer had occurred during lyophilization. In contrast, when the free acid form was reconverted to Na-HA prior to lyophilization (sample C; Scheme 1), the chromatographic characteristics were indistinguishable from the control (sample A) indicating that the process of forming the free acid had not affected the integrity of the polymer. Furthermore, the elution volume of hyaluronate was the same before and after lyophilization as the sodium salt (samples A and C; Scheme 1) indicating that the polymer integrity was unaffected by lyophilization.

3.2. Rheology

The rheological evaluation of the hyaluronates, when lyophilized as either the free acid or sodium salt, included a study of the complex viscosity (η^*), elastic modulus (G') and the viscous modulus (G''). Representative rheological responses of the different molecular mass hyaluronates are presented in Fig. 2 and 3. The elastic modulus is a measure of that part of the shear energy stored elastically in the polymer during application of the shear stress and it provides an indication of the rigidity of the polymer. The magnitude of G' is particularly sensitive to changes in the molecular mass of polymers when studied at low frequencies. The viscous modulus is a measure of the shear energy transformed into heat as a result of viscous flow and represents the energy that is irreversibly lost during deformation.

The 741 kDa molecular mass Na-HA exhibited typical viscoelastic properties across the frequency range studied (Fig. 2A). At lower frequencies, and where G'' predominates relative to G', all possible configurational rearrangements can occur within the period of cyclic deformation and consequently the material displays predomi-

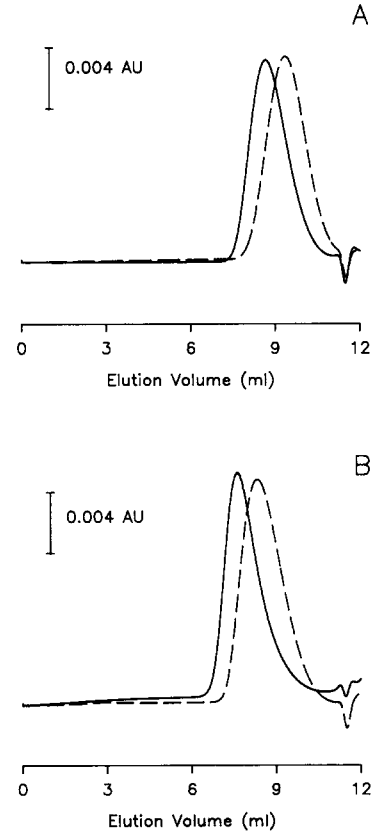


Fig. 1. Size exclusion chromatograms of the different molecular mass fractions of Na-HA prior to lyophilization (solid line) and after lyophilization as the free acid (dotted line). (A) 164 kDa fraction; (B) 741 kDa fraction.

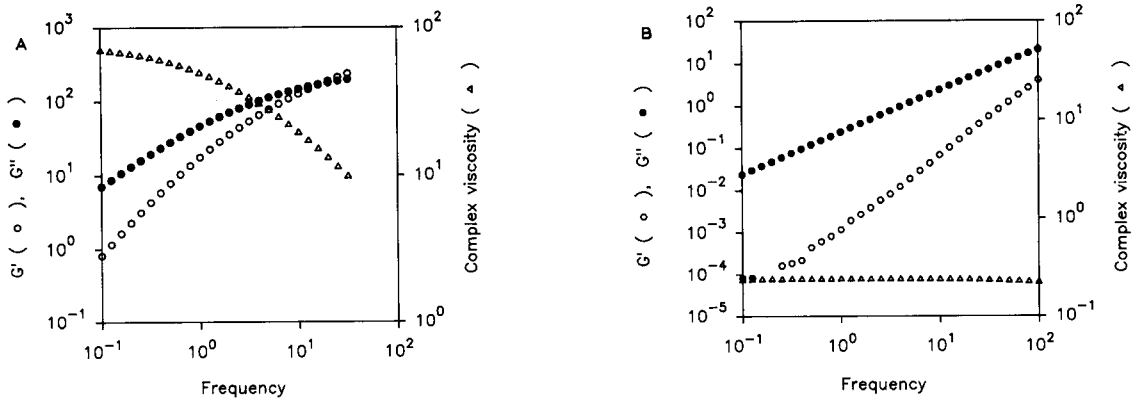


Fig. 2. (a) Frequency dependence (rad s^{-1}) of the elastic modulus (G', Pa), viscous modulus (G'', Pa) and complex viscosity (Pa s) of 741 kDa molecular mass Na-HA prior to lyophilization (sample A; Scheme 1). (b) Frequency dependence (rad s^{-1}) of the elastic modulus (G', Pa), viscous modulus (G'', Pa) and complex viscosity (Pa s) of Na-HA reconstituted after lyophilization of the 741 kDa fraction as the free acid (sample B; Scheme 1).

nately viscous characteristics. However, at higher frequencies there is insufficient time for internal rearrangements to relax within the period of cyclic deformation and this results in a predominantly elastic response. The microstructure providing this viscoelastic response was sensitive to shear as the complex viscosity decreased as the frequency of deformation was increased. Fig. 2B is the rheological profile of the same molecular mass hyaluronate which had been lyophilized as the free acid and then converted to the sodium salt for rheological analysis. The major features were a dramatic reduction in viscosity (from 49.2 to 0.2 Pa s measured at a frequency of 1 rad s^{-1}), conversion to an apparently Newtonian system and a decline in the elastic modulus relative to the viscous modulus. These changes are consistent with a reduction in the apparent molecular mass of the polymer which occurred during lyophilization as the free acid. In contrast, the rheological profile of sample C which had been lyophilized as the sodium salt was identical to the control sample (Fig. 2A) indicating no change in the viscoelastic properties of the polymer (data

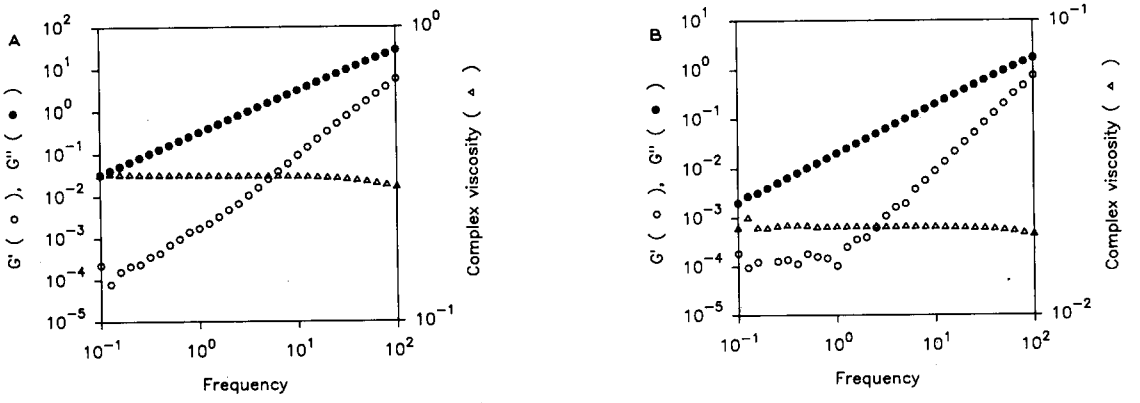


Fig. 3. (a) Frequency dependence (rad s^{-1}) of the elastic modulus (G', Pa), viscous modulus (G'', Pa) and complex viscosity (Pa s) of 164 kDa molecular mass Na-HA prior to lyophilization (sample A; Scheme 1). (b) Frequency dependence (rad s^{-1}) of the elastic modulus (G', Pa), viscous modulus (G'', Pa) and complex viscosity (Pa s) of Na-HA reconstituted after lyophilization of the 164 kDa fraction as the free acid (sample B; Scheme 1).

not shown). These observations are consistent with the size exclusion chromatography data presented in Table 1 for samples A and C.

As expected, the lower molecular mass Na-HA (164 kDa) did not demonstrate shear thinning or exhibit viscoelastic characteristics due to its lower molecular mass (Fig. 3A). However, after lyophilization as the free acid all rheological parameters were displaced to significantly lower values compared with the Na-HA control (Fig. 3B), and the profiles were broadly consistent with the data presented in Fig. 2. The rheological profile of sample C (lyophilized as the sodium salt) was identical to the control sample (Fig. 3A) indicating maintenance of the polymeric integrity (data not shown). Furthermore, these observations are consistent with the chromatographic characteristics of samples A and C (Table 1).

3.3. Basis for altered physical characteristics

When the major reductions and alterations in the rheological parameters G', G'' and η^* are viewed in the context of the size exclusion chromatography data presented in Table 1 and Fig. 1, it is probable that lyophilization of hyaluronate as the free acid led to irreversible changes in the molecular character of the polymer. As hyaluronates are linear polymers with no secondary or tertiary structure, conformational changes in the polymer would not be expected to account for the changes observed in these studies. Therefore, it is probable that cleavage of the polymer backbone occurred during lyophilization of the free acid (but not the sodium salt).

It is well known that hyaluronates are susceptible to degradation under a variety of conditions such as acid hydrolysis (Cleland, 1977; Longas and Myer, 1981), oxidative depolymerization reactions (Matsumura et al., 1966), and sonication (Kubo et al., 1993). The rate of acid catalyzed hydrolysis of hyaluronate in solution reported by Cleland (1977), which we have subsequently confirmed in 0.1 M HCl (unpublished data), is too slow to account for the extent of change in the apparent molecular masses observed in these studies. However, the rates of hydrolysis in solution cannot be directly applied to the rate of degradation in the amorphous matrix of the lyophilizate. During lyophilization, significant concentration effects would occur with respect to the hyaluronate, and it is possible that a combination of concentration effects and shifts in the effective pH of the amorphous reaction matrix could contribute to the apparent changes in molecular mass. Furthermore, this hypothesis concerning concentration and pH effects is consistent with the observed stability of Na-HA to lyophilization as the pH of those solutions was near neutral.

Oxidative depolymerization of hyaluronates has been reported to occur in artificial synovial fluid (Kvam et al., 1993), and it has been implicated in the apparent depolymerization of high molecular mass Na-HA (2×10^6 Da) during freeze-drying (Wedlock et al., 1983). The studies of Wedlock and co-workers employed a combination of electron paramagnetic resonance studies and the addition of specific free radical scavengers to implicate the likely role of oxidative depolymerization. The data from the current studies of the different ionic forms of the medium mass hyaluronates, where both oscillatory dynamic viscosity and the SEC profiles were examined, support and extend the preliminary high molecular mass Na-HA data of Wedlock et al. (1983). Our data indicate that the free acid form of medium molecular mass hyaluronate exhibited marked changes in molecular mass during lyophilization whereas the Na-HA appeared unchanged. Therefore, it appears that the putative depolymerization reaction is more facile when the medium molecular mass hyaluronates are present as the free acid as opposed to the sodium salt.

We have found some variability in the extent of change in the apparent molecular mass characteristics of the hyaluronates when lyophilized as the free acid and this may be related to variation in the pH of the eluent from the ion-exchange column used for preparation of the free acid. However, if the pH of the solution containing the free acid was increased to avoid these putative hydrolytic reactions during lyophilization, then some conversion to a salt form would occur

thereby contaminating the sample of the free acid.

In conclusion, lyophilization can have a detrimental effect on the molecular mass and rheological characteristics of medium molecular mass hyaluronic acid when freeze-dried as the free acid rather than the sodium salt. The mechanism(s) by which hyaluronic acid undergoes an irreversible apparent molecular mass reduction during lyophilization is unclear, although acid hydrolysis and oxidative depolymerization are likely major contributors to the process. Although our studies have been limited to sodium hyaluronate, it is possible that other salts of hyaluronic acid may also be stable during lyophilization as their in situ formation would necessitate a solution with a pH value approaching neutrality.

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References

Balazs, E. and Band, P., Hyaluronic acid: Its structure and uses. *Cosmet. Toilet.*, 99 (1984) 65–72.

Cleland, R.L., The persistence length of hyaluronic acid: An estimate from small-angle X-ray scattering and intrinsic viscosity. *Arch. Biochem. Biophys.*, 180 (1977) 57–68.

Ferry, J.D., *Viscoelastic Properties of Polymers*, Wiley, New York, 3rd Edn, 1980.

Johansson, A., Hassan, H. and Renck, H., Effects of adjuvants to local anaesthetics on their duration: IV. Effect of hyaluronic acid added to bupivacaine or prilocaine on the duration of nerve block in man. *Acta Anaesthesiol. Scand.*, 29 (1985) 736–738.

Kubo, K., Nakamura, T., Takagaki, K., Yoshida, Y. and Endo, M., Depolymerization of hyaluronan by sonication. *Glycoconjugate J.*, 10 (1993) 435–439.

Kvam, C., Granese, D., Flaibani, A., Pollesello, P. and Paoletti, S., Hyaluronan can be protected from free-radical depolymerisation by 2,6-diisopropylphenol, a novel radical scavenger. *Biochem. Biophys. Res. Commun.*, 193 (1993) 927–933.

Kyyronen, K., Hume, L., Benedetti, L., Urti, A., Topp, E. and Stella, V.J., Methylprednisolone esters of hyaluronic acid in ophthalmic drug delivery: In vitro and in vivo release studies. *Int. J. Pharm.*, 80 (1992) 161–169.

Laurent, T.C. and Fraser, J.R., Hyaluronan. *FASEB J.*, 6 (1992) 2397–2404.

Longas, M.O. and Meyer, K., Sequential hydrolysis of hyaluronate by β-glucuronidase and β-*N*-acetylhexosamidase. *Biochem. J.*, 197 (1981) 275–282.

Matsumura, G., Herp, A. and Pigman, W., Depolymerization of hyaluronic acid by autoxidants and radiations. *Rad. Res.*, 28 (1966) 735–752.

Prisell, P.T., Camber, O., Hiselius, J. and Norstedt, G., Evaluation of hyaluronan as a vehicle for peptide growth factors. *Int. J. Pharm.*, 85 (1992) 51–56.

Saettone, M.F., Chetoni, P., Torracca, M., Burgalassi, S. and Giannaccini, B., Evaluation of muco-adhesive properties and in vivo activity of ophthalmic vehicles based on hyaluronic acid. *Int. J. Pharm.*, 51 (1989) 203–212.

Saettone, M.F., Giannaccini, B., Chetoni, P., Torracca, M.T. and Monti, D., Evaluation of high and low molecular weight fractions of sodium hyaluronate and an ionic complex as adjuvants for topical ophthalmic vehicles containing pilocarpine. *Int. J. Pharm.*, 72 (1991) 131–139.

Scott, J.E., Secondary structures in hyaluronan solutions: Chemical and biological implications. *The Biology of Hyaluronan*, Wiley, Chichester, 1989, pp. 6–20.

Sheehan, J.K., Gardner, K.H. and Atkins, E.D., Hyaluronic acid: A double-helical structure in the presence of potassium at low pH and found also with the cations ammonium, rubidium and caesium. *J. Mol. Biol.*, 117 (1977) 113–135.

Wedlock, D.J., Phillips, G.O., Davies, A., Gormally, J. and Wyn-Jones, E., Depolymerization of sodium hyaluronate during freeze drying. *Int. J. Biol. Macromol.*, 5, (1983) 186–188.