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SEC determination of cross-link efficiency in hyaluronan fillers

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ABSTRACT

The cross-link efficiency in hyaluronan fillers was determined by means of triple detector (RI, LALS-RALS and a Differential Viscometer) GPC/SEC in aqueous buffer. The low water solubility of HA cross-linked with BDDE (HBC) was overcome with an alkaline hydrolysis step. The kinetics of linear HA hydrolysis was investigated at different NaOH concentrations (0.25–0.5–1 M), initial polymer Mws (200–700–1000–1200–1600 kDa) and polymer concentrations (0.1–0.3–0.5 mg/ml). As expected for first-order kinetics, the apparent hydrolysis constant (k_h) was independent of polymer concentration and initial Mws. The k_h was found to be linearly dependent on the NaOH concentration, suggesting a random polymer degradation. A similar behavior was observed for HBC polymers, synthesized with 5–7.5–10–14–18% mol/mol of BDDE for HA repeat unit. The degree of crosslinking was obtained using the Zimm–Stockmayer equation for random, tri-functional polydisperse polymers. The contraction factor (g) was determined after an accurate experimental measurement of the structure factor (ε). Comparative studies were performed by ^{1}H NMR spectroscopy and rheological measurements. Interestingly, the number of effective cross-links found is very low compared to the total BDDE linked; i.e. in a HBC sample only 0.04% of total BDDE linked (equal to 4.6% by ^{1}H NMR analysis) gives an effective cross-link.

1. Introduction

Hyaluronan (HA) is a natural heteropolysaccharide consisting of alternating residues of D-glucuronic acid and N-acetyl-D-glucosamine, discovered by Meyer and Palmer (1934). HA forms the capsule of some bacterial species and is also widely distributed throughout the human body: it plays an important role as the structural and mechanical support for tissues, as an active component in the cell physiology of skin, tendons, muscles and cartilages (Abatangelo, Martelli, & Vecchia, 1983; Goa & Benfield, 1994; Weigel, Fuller, & LeBoeuf, 1986) and it is one of the main non-protein constituents of synovial fluid, responsible for its viscoelastic properties (Lapčik, Lapčik, De Smedt, Demeester, & Chabreček, 1998). Even if the main fraction of HA is linear, the existence of natural cross-linked HA has already been studied in the human body, through its interaction with specific HA-binding proteins (hyaladherins). In a recent study, Day and de la Motte (2005) describe the protective role of naturally cross-linked HA in inflammatory processes (i.e. arthritis).

HA has been used in osteoarthritis for over 30 years to treat the associated pain (Adams et al., 1995) and in wound care to prepare dressings and to treat ulcers and skin lesions of various origins. HA is also used as a filler for wrinkles, furrows and small depressed areas of the face, as well as a volumizer for lips and cheeks, because it is immunologically inert, non-toxic, biodegradable, and bioresorbable. However its low residence lifetime, due to the action of the enzyme hyaluronidase and free radicals, often restricts its use in some medical applications that require a long-lasting effect. Currently the FDA has approved several HA filler agents for mid-to-deep dermal implantation (i.e. Restylane, Juvederm, Hylaform) for the correction of facial wrinkles and folds (Arron & Neuhaus, 2007; Falcone & Berg, 2009; Gold, 2007), where HA is subjected to chemical cross-linking processes which improve its viscoelastic properties and increase its half-life.

The possibility of obtaining cross-linked hyaluronan gels by chemical derivatization has been well known since 1964 (Laurent, Hellising, & Gelotte, 1964); in recent years, a wide variety of chemical modification and subsequent cross-linking have been proposed to achieve chemical and mechanical HA robustness (Serban, & Prestwich, 2008). The principal targets for HA chemical modification are the primary hydroxyl (N-acetylglucosamine residue) and the carboxyl (glucuronic acid residue) groups of the molecule.

Epoxides (i.e. 1,4-butanediol diglycidylether, 1,4-bisepoxybutane and polyethylene glycol diglycidyl ether) are the most important class of cross-linkers for HA due to their high reactivity and good biostability and mechanical properties. In acidic conditions (pH < 8), the epoxide reacts with carboxyl groups to form an ester linkage while an ether linkage is formed under alkaline conditions (pH > 10) (Kono et al., 2008; Zhao, Fraser, Alexander, Lockett,

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& White, 2002). Interestingly, the use of di-epoxide as a cross-linker for filler production demonstrated higher efficacy and higher subject satisfaction than the use of vinyl sulfone (Pouyani, Harbison, & Prestwich, 1994; Rao, Chi, & Goldman, 2005).

A high HA cross-linking degree in the filler improves its viscoelastic properties and its long-lasting effect, but progressively denatures the HA to the extent of profoundly modifying its chemical, physical and biological properties. Excessively cross-linked HA matrices increase collagen deposition around the filler, which triggers inflammatory reactions with the formation of fibrotic capsules (Wang et al., 2007). The quality and safety of the filler depends on the ability to control the cross-linking degree; however, only a few analytical techniques are reported nowadays.

Rheological measurements (Falcone & Berg, 2009; Zhao et al., 2002) do not provide information on chemical structure/modification, solid state ^{13}C NMR spectroscopy (Barbucci et al., 2006; Pouyani et al., 1994) is an expensive and non-routine technique, with low sensitivity, while enzymatic degradation assay with hyaluronidase has been described as an indirect and comparative test (Kablik, Monheit, Yu, Chang, & Gershkovich, 2009; La Gatta, Schiraldi, Papa, & De Rosa, 2011; Tayal, Kelly, & Khan, 1999). GPC/SEC analysis has been extensively reported for several cross-linked polymers; (Clarke, 2008; Collins & Birkinshaw, 2008; Gaborieau & Castignolles, 2011; Gaborieau et al., 2008; Rodríguez-Hernandez, Angulo-Sanchez, & Perez-Chantaco, 2007; Šimkovic, Hricovini, Šoltes, Mendichi, & Cosentino, 2000; Wang, Kharchenko, Migler, & Zhu, 2004) however, it requires complete sample dissolution.

One of the cross-linked HA properties is low water solubility, which becomes critical with the increase in the branching degree; an alkaline hydrolysis step was set up in order to achieve water solubility of the cross-linked polymer. Actually, the mechanism of basic hydrolysis has been studied by Tokita and Okamoto (Stern, Kogan, Ledrzejas, & Soltes, 2007; Tokita & Okamoto, 1995); however the kinetics of this process for HA has not been reported.

In the first part of this work the kinetic of linear HA hydrolysis in alkaline conditions was investigated. In the second part, several HA polymers cross-linked with different amount of BDDE were analyzed with a new, fast and sensitive analytical method for the cross-linked HA characterization by means of triple detection GPC/SEC in aqueous buffer. The obtained data were compared with rheological and ^1H NMR measurements; the latter are performed for the first time in DMSO-d6.

2. Experimental

2.1. Materials

Hyaluronic Acid Sodium Salt linear polymers were provided by Fidia Farmaceutici s.p.a. with different Mw (200 kDa, 700 kDa, 1000 kDa, 1200 kDa and 1600 kDa); Mw were determined by means of Viscotek TDA Max analysis.

NaOH and HCl reagents were provided by Sigma–Aldrich; DMSO-d6 was provided by Merck.

2.2. Hyaluronan BDDE cross-linked (HBC) polymers synthesis

24 μl of 1,4-butanediol diglycidyl ether (BDDE) were dissolved in 7 ml of 0.25 M NaOH and added to 1 g of HA^-Na^+ (700 kDa) for HBC 5% synthesis. 35, 47, 66, 85 μl of BDDE were added to obtain HBC 7.5%, 10%, 14%, 18%, respectively. The reaction was carried out for 2 h at room temperature, and then by heating for 2 h at 45 °C. Finally, the solution was neutralized with 0.1 M HCl to a pH of approximately 7 and rehydrated in water reaching a final HA concentration of 22 mg/ml. The final product is a transparent gel.

2.3. Solution state ^1H NMR

Linear and cross-linked HA polymers underwent the same treatment prior to dissolution in DMSO-d6. 10 mg of HA were pre-swollen in 5 ml of H_2O. The obtained HA sample and about 500 mg of each HBC sample were washed and precipitated in 10 ml of ethanol. Solvent excess was removed under N_2 flow. The washing step is critical in order to remove excess BDDE (if present). Then, samples were re-swollen in acidic buffer and heated in a water bath to 70 °C for 1 h. Finally, all the polymers were lyophilized and dissolved in DMSO-d6. Solution state ^1H NMR spectroscopy was performed on a Bruker Advance spectrometer operating at 300 MHz. Spectra were acquired with 128 transients, relaxation time was set at 1 s; reprocessing was executed using XWinNMR software.

2.4. Polymer characterization using size exclusion chromatography (SEC)

All the linear and cross-linked HA samples were analyzed by means of the Viscotek TDA Max 302 system, equipped with a triple detector (RI, LALS-RALS and a Differential Viscometer). 2 Viscogel GMPWxl columns were eluted at 40 °C with a buffer, composed of 0.1 M $NaNO_3$ and 3 mM NaN_3 buffered at pH 7 with $NaHCO_3$ 2% (w/v), at a flow rate of 0.6 ml/min; an injection loop of 350 μl was used. All samples were pre-filtered with 0.2 μm Nylon syringe filters. All the acquired chromatograms were processed with OmniSec 4.5 software using a refractive index increment (dn/dc) of 0.155.

2.5. Alkaline hydrolysis of polymers

75 mg of HA (700 kDa) dry samples were dissolved in 50 ml of 0.25–0.5–1.0 M NaOH respectively and heated to 45 °C. At fixed intervals (1.0–2.5–5.0–24 h) 2.0 ml of each sample were taken out, cooled down and neutralized to pH 6–7 with 2.0 ml of 0.25–0.5–1.0 M HCl respectively, and diluted to 10 ml with SEC elution buffer (final concentration 0.3 mg/ml). The same method was applied to the HA samples at different initial Mws. 125 mg of 200 kDa HA, 25–75–125 mg of 700 kDa HA, 75 mg of 1000 kDa HA, 50 mg of 1200 kDa HA and 50 mg of 1600 kDa HA were dissolved in 50 ml of 0.5 M NaOH respectively and heated to 45 °C. At fixed intervals (1.0–2.5–5.0–24 h) 2.0 ml of each sample were taken out, cooled down and neutralized to pH 6–7 with 2.0 ml of 0.5 M HCl, and diluted to 10 ml with SEC elution buffer.

Time zero samples for linear HA, at different MWs, were obtained by diluting the polymer to 0.3–0.5 mg/ml in elution buffer, after stirring for 24 h at r.t.

1.5 g of each HBC gel (at 22 mg/ml) were dissolved in 20 ml of 0.5 M NaOH and heated to 45 °C. At fixed intervals (1.0–2.5–5.0–6.0–7.0–24 h) 2.0 ml of each sample were taken out, cooled down and neutralized to pH 6–7 with equimolar amount of HCl, and diluted to 10 ml with SEC elution buffer (final concentration 0.3 mg/ml).

All the samples analyzed were processed with OmniSec 4.5 software and the data collected were processed using Origin 8SR4 and Microsoft Excel.

2.6. Rheological experiments

About 1 g of each HBC gel sample (22 mg/ml) was analyzed by means of a Thermo Haake Mars II Rheometer at 25 °C. The G' (elastic modulus) and G'' (viscous modulus) were measured (in Pa) from

0.07 to 90.0 rad/s at fixed strain value of 10% (an initial sweep strain with an oscillatory shear strain of increasing amplitude, γ, at the constant frequency of $\omega = 1$ Hz was applied to determine the region of linear response of the sample: at 10% the viscoelastic range is linear). All the samples analyzed were processed with Haake Rheowin Job Manager 4.0 software and data collected were processed using Origin 8SR4 and Microsoft Excel.

3. Results and discussion

3.1. Degree of crosslinking determined by ^{1}H NMR

An uncross-linked HA sample (700 kDa) and all HBC samples were investigated by means of NMR spectroscopy to measure the amount of BDDE cross-linked to the HA polymer.

The HBC polymers quickly absorb great amounts of water and dissolution in D_2O implies the formation of a gel which would require a solid state NMR (Barbucci et al., 2006; Pouyani et al., 1994) or a HR-MAS NMR technique. On the other hand, dissolution of these modified polymers has been previously described only in a DMSO-d6/D_2O mixture (Palumbo, Pitarresi, Mandracchia, Tripodo, & Giammona, 2006).

The pre-treatment of samples, already described in Section 2, enables dissolution in DMSO-d6.

^{1}H spectra show a slight difference at high fields: in the HBC 10% and 18% samples (Fig. 1a) a signal at 1.5 ppm coming from the $-(CH_2)_2$ moiety of the BDDE molecule can be noticed, while in the HA sample it is not present. Integration of the signal at 1.5 ppm with regard to the acetylglucosamine N—CH_3 signal at about 1.8 ppm gives the cross-link ratio (in mol/mol with regard to the HA repeat unit), which showed to be linear from 2.4% for HBC 5% to 8.65% for HBC 18%. As expected, not all the BDDE present during the reaction is able to react with the HA polymer.

Furthermore, what the NMR technique gives is the total number of BDDE molecules linked to HA (total links), but it does not provide any information on how many of them are able to modify the rheological properties of the linear polymer (efficient cross-links). A study in the TDA-Viscotek system was designed and the low solubility of HBC polymer in the SEC aqueous buffer was studied after alkaline hydrolysis.

3.2. Alkaline hydrolysis of linear HA

The alkaline hydrolysis of Hyaluronic Acid linear polymers of different Mw was investigated at different NaOH and polymer concentrations (see Section 2.5).

The Mw drops quickly in the first 5 h of hydrolysis. Assuming that hydrolysis occurs randomly on β-(1 → 3) and β-(1 → 4) glycosidic bonds and considering that the NaOH is in great excess, the kinetic can be interpreted as a *n*th-order process (Tayal et al., 1999):

$$\frac{dl}{dt} = -kL^n \tag{1}$$

where L is the total number of hydrolysable linkages, k is the rate constant and n is the order of reaction.

Substituting:

$$L = N_0\left(\frac{M}{m} - 1\right) \tag{2}$$

where M is the average molecular weight of the chains, m is the monomer molecular weight and N_0 is the number of total molecules. By substituting (2) into Eq. (1), considering $(m/M) \ll 1$ (that means regarding only the first part of the depolymerization reaction) (Einbu, Naess, Elgsaeter, & Vårum, 2004) and integrating it as a first-order reaction, the following is obtained:

$$\frac{1}{\mathrm{Mw}} = \frac{1}{\mathrm{Mw}_0} + k_h t \tag{3}$$

where k_h is the apparent rate constant ($k_h = k/m$), Mw and Mw_0 are the weight-average molecular weights at time t and time 0, respectively.

$1/\mathrm{Mw} - 1/\mathrm{Mw}_0$ was thus plotted vs. the degradation time (until 24 h) at different initial concentrations (C_0) of HA polymer (700 kDa); k_h is the slope of the obtained linear plots. As expected for a first-order kinetic (Tayal et al., 1999), the rate constant is independent of C_0 (Fig. 2).

In the same way, the k_h proved to be independent of the initial Mw (200 kDa, 700 kDa, 1000 kDa, 1200 kDa and 1600 kDa) (Table in Fig. 2).

The efficiency of the alkaline hydrolysis depends on both the NaOH concentration and the temperature (Melander & Tømmeraas, 2010). The alkaline hydrolysis of the HA polymer (700 kDa) at 45 °C

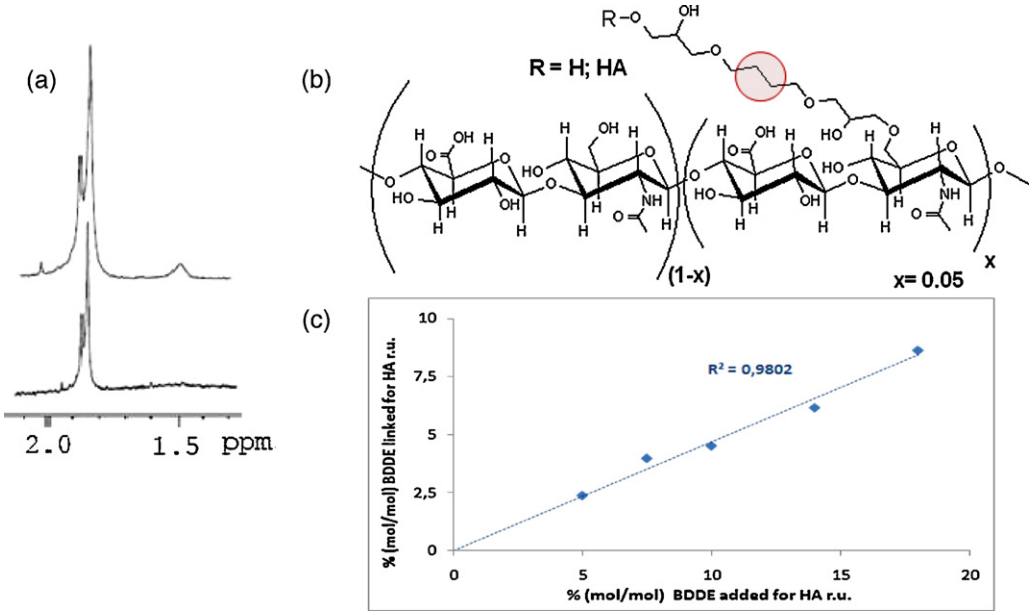
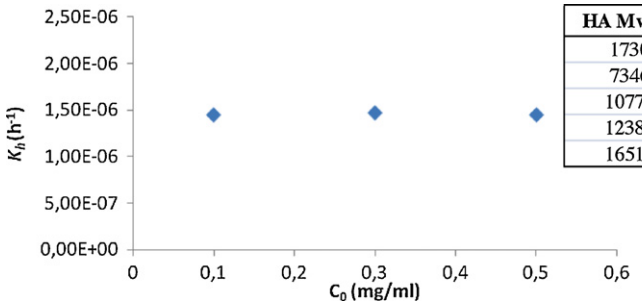


Fig. 1. (a) Overlapped ^{1}H NMR spectra in DMSO-d6 of linear HA (downside) and of cross-linked HBC 10% polymer (upside). (b) Structure of HA (cross-)linked with 10% of BDDE. (c) Plot of % BDDE linked for HA repeat unit (r.u.) by NMR as function of % BDDE added for HA r.u.



HA Mw_0 (Da)	k_h (h^{-1})
173092	1,51E-06
734622	1,45E-06
1077000	1,31E-06
1238000	1,37E-06
1651000	1,41E-06

Fig. 2. Plot of apparent hydrolysis constant (k_h) as function of initial HA concentration (C_0). The k_h as function of HA Mw_0 are reported in table in the upper left corner.

was investigated at different NaOH concentrations (0.25–0.5–1 M) and a linear relationship between the NaOH concentration and the k_h was observed (Fig. 3), as previously reported for a truly random degradation process in acidic conditions (Tømmeraas & Melander, 2008). This strengthens the hypothesis of a random polymer degradation over the entire Mw range, without any conformational effects.

3.3. Alkaline hydrolysis of HBC polymers

One of the HBC polymers properties is the great swelling capacity; though, sample dissolution in water is at least trivial. Mild alkaline treatment of HBC triggers hydrolysis of glycosidic bonds inside the HA main chain, but it is very likely not to break the cross-linking ether bond. For this reason basic hydrolysis of HBC has been studied as a way to reduce Mw of cross-linked HAs and increase their solubility.

All the samples (HA and different grade HBC) were treated and analyzed as yet described (see Section 2.5). The high cross-linked HBC samples (i.e. HBC 14 and 18%) analyzed after 0 and 2.5 h of alkaline hydrolysis did not show sufficient recovery, as complete dissolution was not achieved.

1/Mw values were plotted vs. hydrolysis time, as Mw_0 values were impossible to obtain. The slope of the linear equation obtained for each HBC hydrolysis provided a good approximation of the k_h, which is comparable to those shown (Fig. 2) for linear HAs (1.29×10^{-6} for HBC 5% and 1.08×10^{-6} for HBC 18%). These values are slightly lower than those of linear polymers, probably due to some conformational change brought on by the cross-linking.

Data reported in table (Fig. 4) were collected after 5 h of alkaline hydrolysis; all samples show recovery higher than 95% (by RI signal integration). In agreement with the literature (Gaborieau & Castignolles, 2011; Gaborieau et al., 2008), the Mw and the polydispersity (Mw/Mn) increase with the cross-link degree; a similar trend can be noted for the IV, the radius of gyration (Rg) and the hydrodynamic radius (Rh). Despite this, the values of the IV, Rg and Rh cannot be directly compared, because they depend on Mw.

As depicted in the graph below, the raw tri-detector signals (RI, DP and LS) for the cross-linked polymer (HBC 10%) are broader and show a higher polydispersity, compared to the linear polymer.

The calculation of the cross-link degree is based on the concept that in a polymer, at constant molecular weight, an increase in branching will result in a decrease in size and a subsequent decrease in the intrinsic viscosity (Wang et al., 2004). These topics are represented by the contraction factor (g), the calculation of which is based on how the radius of gyration (Rg) scales with Mw.

$$g = \frac{(\mathrm{Rg})^2\mathrm{branched}}{(\mathrm{Rg})^2\mathrm{linear}} \tag{4}$$

If any Rg information is absent (in many cases it is impossible to obtain a light scattering measure due to the low polymer Mw), g can also be determined from g', using the structure factor ε.

$$g' = \frac{[n]\mathrm{branched}}{[n]\mathrm{linear}} = g^{\varepsilon} \tag{5}$$

A correct cross-link determination requires comparing the IV and Rg for a cross-linked polymer with the same parameters obtained for a linear polymer at the same average Mw.

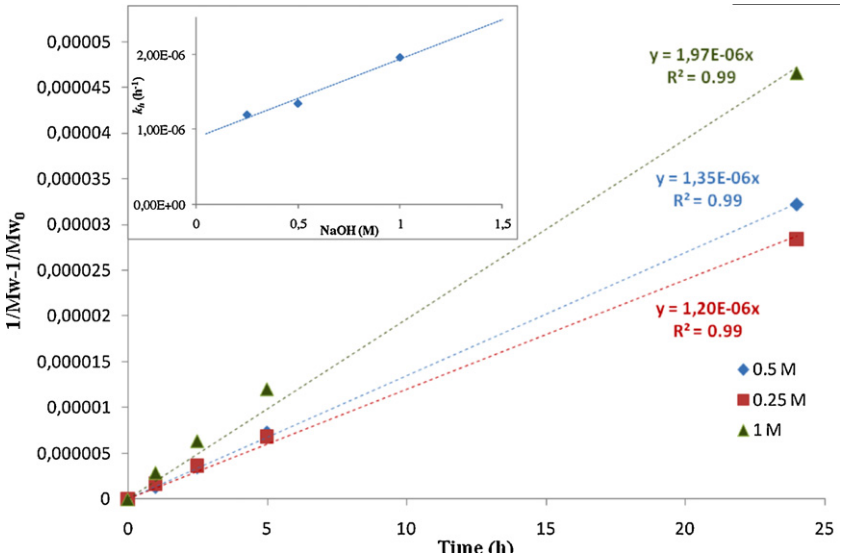


Fig. 3. Plot of $1/Mw - 1/Mw_0$ as a function of hydrolysis time and NaOH concentration at 45 °C. Upper box: plot of k_h as function of NaOH concentration.

Sample ID*	Mw (Da)	Mw/Mn	IV (dl/g)	Rg (nm)	Rh (nm)
HA	115239	1.62	3.46	20.25	17.78
HBC 5%	132631	2.06	3.10	23.99	17.63
HBC 7.5%	192854	2.59	3.42	36.23	20.12
HBC 10 %	252880	2.97	3.73	31.77	22.36
HBC 14%	535901	4.28	4.37	72.99	29.66
HBC 18%	693453	4.57	4.37	92.46	32.40

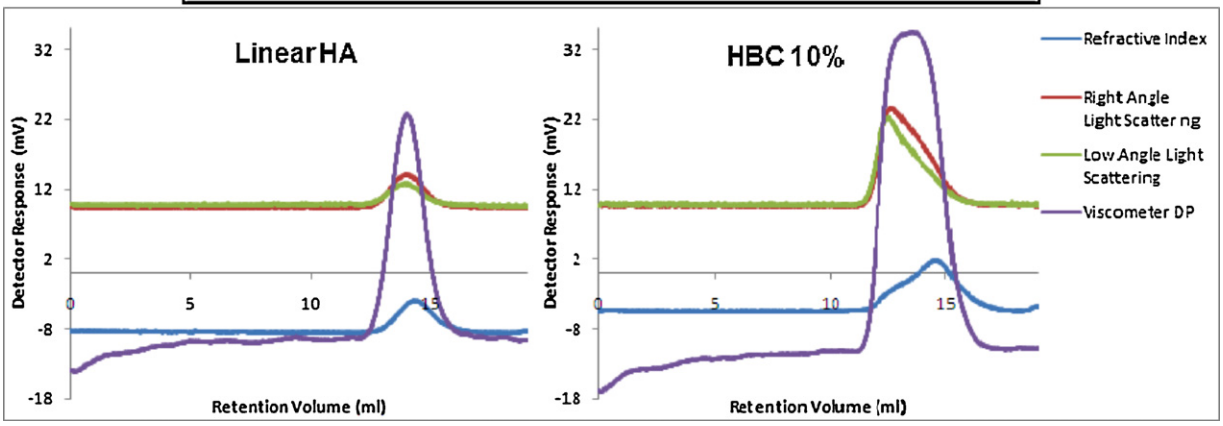


Fig. 4. Triple detector SEC chromatograms of linear HA (left) and cross-linked HA (right) after 5 h of hydrolysis. Data measured for HBC samples by SEC analysis after 5 h of alkaline hydrolysis are reported in table.

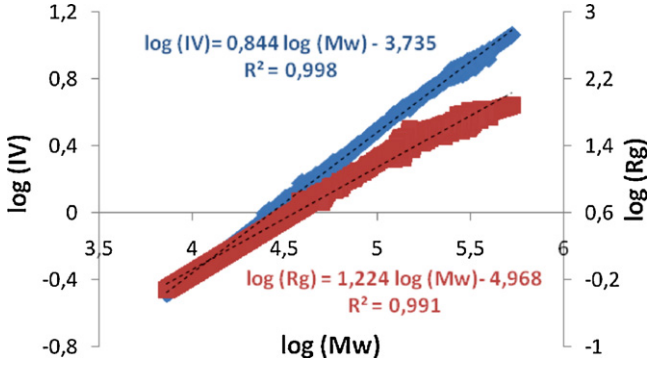


Fig. 5. Linear plot of log(IV) and log(Rg) as a function of log(Mw); data are referred to a linear HA (Mw_0 = 700 kDa) after 5 h of alkaline treatment.

Linear HA, after alkaline treatment (5 h at 45 °C in 0.5 M NaOH) shows linear correlations (Fig. 5) between log(Mw) and log(IV) and between log(Mw) and log(Rg). Using these equations, the theoretical Rg and IV values for a hypothetical linear polymer (bearing the same Mw obtained from the cross-linked polymer treated in the same way) have been extrapolated. In this case, for HBC 10% after 5 h of alkaline treatment (shown in table, Fig. 4), the simulated linear polymer (for Mw = 252,880 Da) shows an Rg of 44.37 nm and a IV of 6.67 dl/g. Applying Eqs. (4) and (5) the calculated ε value is 0.87.

The same procedure was applied to all the HBC, increasing the hydrolysis time as a function of the cross-link degree (from 1 to 7 h), obtaining polymers with a log(Mw) range comprised between 5 and 5.7. A mean ε value of 0.81 ± 0.1 was determined, considering that only g and g' values smaller than 1 were considered (Rodríguez-Hernandez et al., 2007).

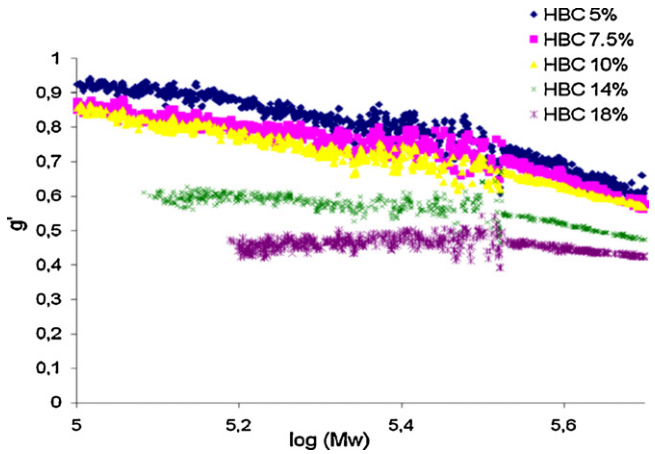


Fig. 6. Plot of g' as a function of log(Mw) for all the HBC samples.

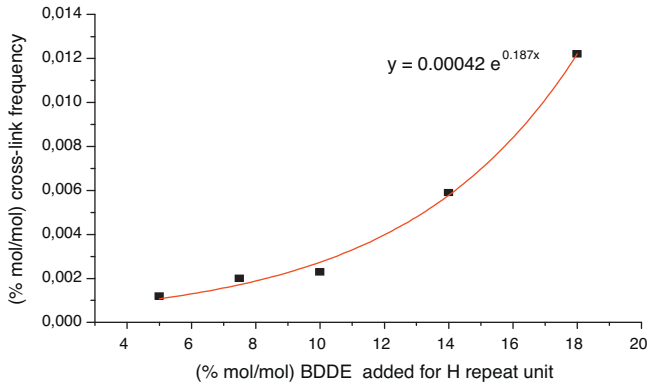


Fig. 7. Exponential plot of cross-link frequency (by SEC) as a function of BDDE added during synthesis.

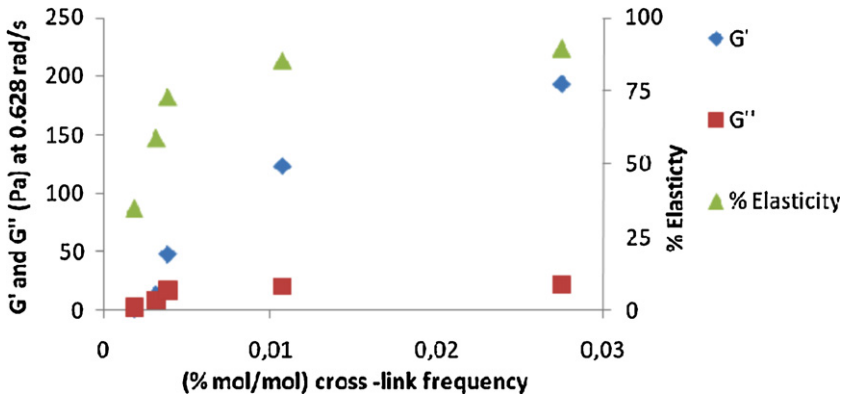


Fig. 8. G', G'' and % elasticity plotted as a function of cross-link frequency (by SEC).

The linear correlation applied is only true for polymers with Mw values high enough; in fact, for a low Mw, the cross-link degree became independent of the IV. This means that inside a specific range of Mw the cross-link degree can be calculated with good approximation.

The calculation of cross-link degree was obtained by applying, in a defined range of Mw, the following Zimm–Stockmayer equation (Zimm & Stockmayer, 1949), for random, tri-functional polydisperse polymer.

$$g = \frac{6}{n}\left[\frac{1}{2}\left(\frac{2+n}{n}\right)^{1/2}\ln\left(\frac{(2+n)^{1/2}+n^{1/2}}{(2+n)^{1/2}-n^{1/2}}\right)\right]-1 \quad (6)$$

where g is the contraction factor and n is the branching number for the polymer chain.

The selection of Mws range for calculating the branching degree was achieved by plotting the log(Mw) versus the g' ratio, considering only the range with a linear negative dependence. The graph obtained for every sample analyzed is zoomed below: g' decreases linearly with Mw in the limit range from 5.0 to 5.7 log(Mw) (equal to: 100–500 kDa), as shown in Fig. 6. In line with expectations, an increase in the BDDE amount used in the HBC synthesis leads to an increase in the viscosity and a consequent decrease in g'.

Fig. 7 shows an exponential plot ($R^2 = 0.995$) of the efficient crosslink measured by TDA Viscotek (after 5 h of alkaline hydrolysis) as a function of the amount of BDDE added in the HBC synthesis. The efficient cross-link or cross-linking frequency (λ) was determined by the expression $\lambda = nWF_R/M$ where M is the mass for each chromatographic slice, n is the branching degree and F_R is the repeat factor, defined as the mass of HA repeating unit (equal to 401.38 Da as sodium salt).

The cross-link reaction seems to be a cooperative process. In the 0–10% range, the cross-link generation linearly depends on the amount of BDDE added (as a random process). Over 10%, the frequency of efficient cross-link increases rapidly, probably due to a conformational change which brings the polymer chains closer.

According to expectations, the cross-link determination is independent from the alkaline treatment. The same polymer (i.e. HBC 10%) analyzed after 2.5 or 5 h of alkaline hydrolysis gives the same value of cross-linking frequency, meaning that the well controlled alkaline treatment does not modify the efficient cross-link measured.

Moreover, the branching contribute to contraction of the polymer was evaluated on a HA-derivative synthesized with Glycidol, as described for HBC 18%. No significant changes to IV, Rg, Rh has been observed compared to linear HA.

3.4. Rheological studies

It is reported that linear and cross-linked HA show different rheological behavior (Adams et al., 1995; Melander & Tømmeraas, 2010). All the samples of different HBC grade were analyzed, at the same concentration (22 mg/ml) by means of Thermo Haake Mars Rheometer. In Fig. 8 the % of cross-link frequency obtained by Viscotek analysis is plotted vs. G' (elastic modulus) and G'' (viscous modulus) at 0.628 rad/s. The % elasticity was calculated using the following formula: $(100 \times G')/(G' + G'')$. Increasing the cross-link frequency, the elastic modulus grows and becomes predominant over the viscous modulus. This would reflect in a longer residence lifetime of the injected filler (maintaining the same HA concentration).

4. Conclusion

We report for the first time an alternative approach to cross-link quantification, after alkaline hydrolysis. Alkaline degradation of linear HA was found to follow a pseudo-first order kinetics; similar behavior was observed for cross-linked polymers. HBC polymers were characterized by ^{1}H NMR spectroscopy, SEC and rheological measurements. Interestingly, the number of efficient cross-link found is very low compared to the total BDDE linked. In this case, HBC 10% shows an efficient cross-link frequency of 0.002% (by SEC analysis), while the total BDDE linked is 4.6% (by ^{1}H NMR analysis), implying that only 0.04% of total BDDE linked give an efficient cross-link. As expected the elastic modulus (G') increases with the efficient cross-link frequency; thus, only the BDDE efficiently cross-linked contribute to the elastic properties of the filler.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2011.12.004.

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