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Modification and cross-linking parameters in hyaluronic acid hydrogels—Definitions and analytical methods

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ABSTRACT

Definitions and methods for the quantification of degree of modification and cross-linking in cross-linked hyaluronic acid (HA) hydrogels are outlined. A novel method is presented in which the HA hydrogel is degraded by the enzyme chondroitinase AC and the digest product analyzed by size exclusion chromatography combined with electrospray ionization mass spectrometry (SEC–ESI-MS). This method allows for the determination of effective cross-linker ratio (CrR) which together with the degree of modification (MoD), determined by, e.g. ^{1}H NMR spectroscopy, enables the calculation of the degree of substitution (DS) and degree of cross-linking (CrD).

The method, could be applicable to the major cross-linked HA hydrogels currently on the market, and is exemplified here by application to two HA hydrogels. The definitions and methods presented are important contributions in attempts to find relationships between MoD, DS and CrD to mechanical properties as well as to biocompatibility of HA hydrogels.

1. Introduction

Hyaluronic acid (HA, also known as hyaluronan) is a linear polysaccharide (Fig. 1) naturally occurring in mammalian tissues (Laurent & Fraser, 1992). It consists of a repeating disaccharide, →4)-β-D-Glc*p*A-(1→3)-β-D-Glc*p*NAc-(1→, and due to its properties HA has become important for many medical and esthetic applications such as eye surgery, viscosupplementation therapy of osteoarthritis, dermal filling, and tissue augmentation (Schanté, Zuber, Herlin, & Vandamme, 2011; Volpi, Schiller, Stern, & Šoltes, 2009). The molecular mass of HA used for these applications is usually high and a solution of such HA is highly viscous. Another important property of HA is its high ability to bind and retain water.

To improve the mechanical properties and prolong the duration of HA *in vivo*, a common method is to form a hydrogel by covalently cross-linking the HA polymer chains into a three-dimensional network (see e.g. Ågerup, Berg, & Åkermark, 2005; Edsman et al., 2011). HA hydrogels, formed by cross-linking, have become important for many medical and esthetic applications. The mechanical and physical properties of the hydrogels are dependent on the degrees of modification and cross-linking (La Gatta, Schiraldi, Papa, & De Rosa, 2011; Stocks et al., 2011). Although not yet shown, it has been suggested that too high a degree of modification and cross-linking could potentially affect the biocompatibility of the HA hydrogel (Tezel & Fredrickson, 2008). In the establishment of the relationships between degrees of modification and cross-linking to mechanical properties as well as to biocompatibility it is of great importance to have methods and definitions for the determination of the cross-linking parameters.

A frequently used method today for cross-linking HA into hydrogels for products currently on the market (Schanté et al., 2011) is the reaction with 1,4-butanediol diglycidyl ether, BDDE (Fig. 1) under alkaline conditions to yield a stable covalent ether linkage between HA and the cross-linker (Mälson & Lindqvist, 1986). In the reaction, nucleophilic groups of HA react with the epoxide groups of BDDE. In theory, six sites in every HA–disaccharide unit are available for the reaction with BDDE. The deprotonated hydroxyls are much stronger nucleophiles than both the anionic carboxylic group and the amide. Hence the hydroxyl groups are the most likely reaction sites, forming stable ether bonds with the cross-linker. Any esters formed with the carboxylate group of HA will probably be hydrolyzed under the alkaline conditions. The relative tendency

Abbreviations: MoD, degree of modification; DS, degree of substitution; CrD, degree of cross-linking by stoichiometric relationship; DC, degree of cross-linking by fraction cross-linked monomer units; CrR, effective cross-linker ratio.

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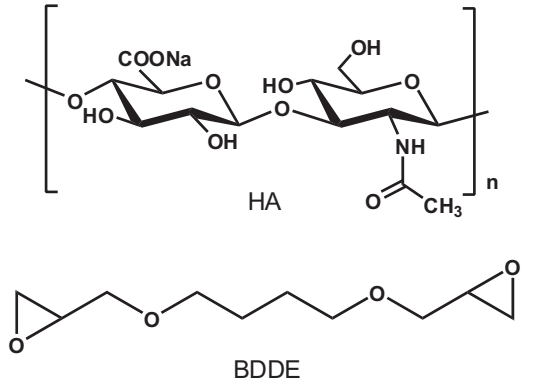
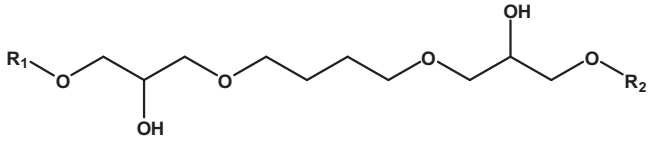


Fig. 1. Structures of the disaccharide repeating unit in HA and BDDE.

of binding to the four different hydroxyl groups has not been determined but may vary due to (1) steric differences between the primary and secondary alcohols, (2) conformational effects of the polymer, (3) differences in the acidity of the hydroxyl groups, and (4) differences in reaction conditions. During the cross-linking process the epoxide groups of BDDE react with nucleophiles forming derivatives of 1,4-butanediol di-(propan-2,3-diolyl)ether (BDPE) as shown in Fig. 2. Some portion of the added BDDE reacts only with water/hydroxide forming free BDPE (Fig. 2, **a**). Another portion of the BDDE reacts with water/hydroxide at one end and with HA at the other end forming mono-linked BDPE (Fig. 2, **b**). A third portion of the BDDE reacts with HA at both ends yielding disubstituted BDPE (Fig. 2, **c**), resulting in the cross-linkages found in the HA hydrogels.

This paper deals with definitions and methods for the quantification of the different modifications occurring in cross-linked HA hydrogels. Different ways to describe the degree of modification or cross-linking of polymers exist in the literature and it is therefore important to clearly describe the basis behind the calculation of each value which is reported. One common way is to use the *stoichiometric relationship* between bound cross-linker and a defined monomer unit of the polymer (see e.g. Schanté, Zuber, Herlin, & Vandamme, 2012). Another way is based on the calculation of the *number of substituted monomer units* to the total number of monomer units in the polymer (see e.g. Tezel & Fredrickson, 2008). Furthermore, the monomer unit used in the calculations has to be defined and one approach is to set the *repeating* unit of the polymer as the monomer unit. This method is commonly used in the calculation of the degree of cross-linking in HA hydrogels and we also use this approach in this text. Another method would be to set the *monosaccharide* as the monomer unit and if so, this has to be clearly stated as it influences the outcome of the cross-linking



	R_1	R_2	Compound
a	H	H	Free BDPE
b	HA	H	Mono-linked BDPE
	H	HA	Mono-linked BDPE
c	HA	HA	Double-linked BDPE

Fig. 2. The different forms of BDPE (**a**–**c**) formed during the process of cross-linking HA with BDDE.

calculations. For cross-linkers that generate a certain amount of mono-linked residues, as is often the case with HA hydrogels, this factor has also to be taken into account. To address these issues, we propose four different defining terms (Table 1) that can be applied to HA hydrogels formed by reaction with BDDE:

(i) the degree of modification (MoD) which is the stoichiometric ratio between the sum of mono- and double-linked BDPE residues and HA disaccharide units;
(ii) the degree of substitution (DS) which is the proportion of the HA disaccharides that are substituted;
(iii) the degree of cross-linking described by the acronym CrD, which is the stoichiometric ratio between BDPE residues that are double-linked and HA disaccharide units;
(iv) the degree of cross-linking described by the acronym DC, which is the number of HA disaccharides actually involved in cross-linking in relation to the total number of HA disaccharides.

All four terms above deal with ratios in comparison to total amount of HA units. Two of the terms (MoD and CrD) provide the total amount of linked BDPE and double-linked BDPE, respectively, in comparison to total amount of HA. The other two (DS and DC) provide the amount substituted HA and cross-linked HA, respectively, in comparison to total amount of HA. If an additional modifier is used on an already cross-linked HA hydrogel we suggest that DS is used to describe the total degree of substitution.

The determination of DS, CrD and DC requires information on the *fraction* of cross-linker residues that are double-linked (forming cross-linkages in the polysaccharide network), compared to all linked cross-linkers. This is defined by the effective cross-linker ratio (CrR, Eq. (1)) which is reported as a value between 0 and 1. CrR is an important parameter that, besides its use in the calculation of DS, CrD and DC, shows the fraction of double-linked cross-linker residues. A value close to 1 for CrR will indicate that the procedure or the protocol employed in synthesis of the gel is an efficient one, whereas a value approaching 0 shows otherwise. For this reason the term can very well be seen as a measure of cross-linker efficiency.

$$\mathrm{CrR} = \frac{n_{\text{double-linked cross-linker}}}{n_{\text{mono-linked cross-linker}} + n_{\text{double-linked cross-linker}}} \quad (1)$$

Table 1 summarizes the definitions for the cross-linking parameters with examples for a hypothetical cross-linked HA segment. The examples demonstrate that MoD, DS, CrD and DC reveal different properties and hence the measured values for these are consequently different. CrD and DC are terms that both describe the degree of cross-linking but the two are defined in different ways resulting in different values for the same cross-linking pattern (20 and 40%, respectively, in the example). Hence this exemplifies the importance of clearly describing which of the two defining methods that has been used when reporting values for degree of cross-linking.

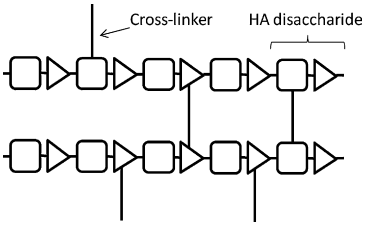
The relevancy is also different for the terms, with MoD and DS describing the total change in the polymer after modification, i.e. a gel with a low MoD or DS will resemble the intact polymer. CrD and DC are more important for the physical properties of the gel. For example, higher values of CrD (and of DC, since DC = 2 × CrD) for a certain gel would reveal that this gel is a stronger gel (i.e. more cross-linked) and would swell less than a weaker gel with a lower CrD.

To improve our knowledge of hydrogels, analytical methods for the determination of MoD and CrR are needed. However, the viscoelastic properties with an elastic modulus (G') dominating over the viscous modulus (G'') of the HA hydrogel make detailed structural analysis of the intact hydrogel difficult as there are no methods available which can directly analyze the complex network. Recently, a method for the analysis of the

Table 1
Definitions of MoD, CrD, DS and DC for modified HA hydrogels and values for these parameters obtained for a hypothetical structure.[a] Equations for the calculation of DS, CrD and DC from MoD and CrR are shown.

	Definition based on number of...	
	...cross-linker units/HA units	...substituted HA units/HA units
Parameter	Degree of modification (MoD)[b]	Degree of substitution (DS)[b]
Definition	$\frac{n_{\text{linked cross-linkers}}}{n_{\text{HA disaccharides}}}$	$\frac{n_{\text{sustituted HA discaccharides}}}{n_{\text{HA disaccharides}}}$
Example[a]	5/10 = 50%	7/10 = 70%
Determination	From NMR	DS = MoD + CrD = MoD × (1 + CrR)
Parameter	Degree of cross-linking (CrD)	Degree of cross-linking (DC)
Definition	$\frac{n_{\text{doubled linked cross-linkers}}}{n_{\text{HA disaccharides}}}$	$\frac{n_{\text{cross-linked HA disaccharides}}}{n_{\text{HA disaccharides}}}$
Example[a]	2/10 = 20%	4/10 = 40%
Determination	CrD = CrR × MoD	DC = 2 × CrD = 2 × CrR × MoD

[a] Schematic hypothetical structure of a HA hydrogel for exemplifying the different definitions: ▷ = GlcNAc, ☐ = GlcA, and | = BDPE:



[b] MoD and DS can also be used for modifications with monofunctional molecules. DS can in addition be applied if an additional modifier is used on an already cross-linked HA hydrogel to describe the total degree of substitution. DS is higher than MoD but will become equal when only monolinks are present, i.e. when CrR is 0.

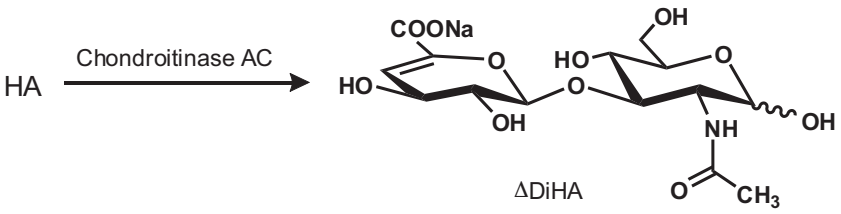
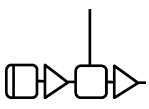
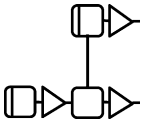
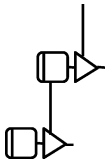
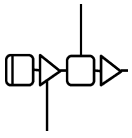


Fig. 3. ΔDiHA formed by the degradation of HA with chondroitinase AC.

cross-linking efficiency in BDDE cross-linked HA hydrogels, based on the chromatographic separation of polysaccharides formed by mild hydrolysis in alkaline conditions, was reported (Guarise, Pavan, Pirrone, & Renier, 2012). Enzymatic treatment with hyaluronidase of HA hydrogels cross-linked with BDDE (Kablik et al., 2009) and divinylsulfone (Chang et al., 2007) into oligosaccharides prior to chromatographic separation has also been described. A disadvantage with mild hydrolysis and hyaluronidase treatment of HA is that the degradation product consists of a complex mixture of larger oligosaccharides (>tetrasaccharides) making it difficult for a detailed analysis of the cross-linked residues. However, the enzyme chondroitinase AC (Hiyama & Okada, 1975) is a lyase which by an elimination reaction cleaves all the 1,4-linkages between GlcNAc and GlcA and generates a single product, namely the disaccharide with an unsaturated uronic acid at the non-reducing terminal (ΔDiHA; α-ΔUA-(1→3)-GlcNAc, ΔUA = 4-deoxy-L-*threo*-hex-4-enopyranosyluronic acid) (Fig. 3). When the HA hydrogel is formed by reaction with BDDE some disaccharide elements in the polymer are substituted with BDPE residues and the enzyme is faced to deal with a partially unrecognizable substrate. The normal degradation is altered in that cleavages close to the new ether linkages occur less frequently and consequently longer BDPE-substituted oligosaccharide fragments are generated. These oligosaccharides can be separated from the main disaccharide product, ΔDiHA, by different chromatographic techniques, e.g. size exclusion chromatography (SEC) and analyzed subsequently.

Table 2
Nomenclature for HA–BDPE fragments with examples. **X** represents the number of monosaccharides and **B** a BDPE residue. **—B** corresponds to mono-substitution and **—B—** to a cross-linkage.

HA–BDPE fragment Abbreviation	Mono-linked **X—B**	Cross-linked **X—B—X**	Mono- and cross-linked **X—B—X—B**	Two mono-linked **B—X—B**
Example	**4—B**	**4—B—2**	**2—B—2—B**	**B—4—B**
Structure				

▷ = GlcNAc, ☐ = GlcA, ☐ = ΔUA, | = BDPE.

To differentiate between the different forms of the HA–BDPE fragments a nomenclature, illustrated in Table 2, is introduced which describes the size of the oligosaccharide (**X**) and type of modification (—**B**). This nomenclature will be used throughout the text.

In this paper a new method is presented in which the HA hydrogel is degraded by chondroitinase AC and the digest product is analyzed by size exclusion chromatography combined with electrospray ionization mass spectrometry (SEC–ESI-MS). The method allows the determination of CrR which together with MoD (determined by ^{1}H NMR spectroscopy) enables the calculation of DS, CrD, and DC for HA hydrogels and this is exemplified by application to two HA hydrogels.

2. Experimental

2.1. Materials

Chondroitinase AC from *Arthrobacter aurescens* (Hiyama & Okada, 1975) (E.C. 4.2.2.5; Sigma–Aldrich) was used for the degradation of the sodium hyaluronate gels. Chromatographic buffer was made from analytical grade acetic acid (Merck, glacial, 100%) and ammonia solution (Merck, 25%). Gradient grade acetonitrile (LiChrosolv, Merck) was used in the make-up solution. Water was de-ionized and further purified by a Milli-Q system (Millipore). Sodium chloride solution (9 mg/mL) from Fresenius Kabi was used.

Two BDDE cross-linked HA hydrogels (Hydrogels 1 and 2), on which the analytical method was applied, were laboratory made test gels with a HA concentration of 20 mg/mL. The gels were made under alkaline conditions with a BDDE/HA ratio (w/w) of 0.01 and 0.18 (i.e. a BDDE/HA disaccharide molar ratio of 0.02 and 0.36) for Hydrogels 1 and 2, respectively. The weight average molecular weight (Mw) of the sodium hyaluronate (Na^+HA^-) used was approximately 1 MDa. The reaction mixture was kept overnight at room temperature, after which the pH was lowered to 7 by the addition of hydrochloric acid.

2.2. Determination of MoD

The degree of modification (MoD) of the HA hydrogels was determined with an NMR based method. In brief, HA hydrogels were washed first by the addition of sodium chloride solution and then filtration using a 0.22 μm Steritop filter (Millipore) under vacuum. Washed hydrogels (~0.1 mL in 0.6 mL D_2O) were degraded by chondroitinase AC overnight at 37 °C. Then ^{1}H NMR spectra were recorded on the enzyme digests on a Bruker 400 MHz instrument at 30 °C. MoD was determined by integrating the CH_2 signal at δ 1.6 ppm from the different BDPE residues and the signal at δ 2.0 ppm from the N-acetyl of the disaccharide units. The ratio between the integrals for these two signals (δ 1.6 ppm/δ 2.0 ppm) gave the MoD value after correction for number of protons responsible for each signal. The washing of the hydrogel is essential to remove free BDPE residues and any unreacted BDDE from the hydrogels which would otherwise contribute to the signal at δ 1.6 ppm giving an erroneous MoD determination.

2.3. Isolation and characterization of HA–BDPE fragments

Hydrogel 1 (14 mL containing 280 mg cross-linked HA) was treated with chondroitinase AC (11.6 U in 6 mL H_2O) in a sealed flask at 37 °C for 12 days with occasional agitation for a large-scale preparation. The reaction was followed by NMR spectroscopy. The solution was then diluted with water and freeze-dried. Portions of the product were fractionated on a Superdex 30 prep grade HiLoad 16/60 column (GE Healthcare) and an ÄKTA P-900 system (Amersham Pharmacia Biotech) using 0.1 M ammonium acetate as eluent (2 mL/min), UV detection at 210, 230 and 250 nm and a P-900 fraction collector (6 mL/tube). Fractions from several repeated runs were pooled and analyzed by SEC–ESI-MS and ^{1}H NMR spectroscopy.

The amount of oligosaccharides in each fraction was determined by ^{1}H NMR spectroscopy using 99.99% potassium hydrogen phthalate (Merck) as internal standard. ^{1}H NMR spectra were obtained as described in Section 2.2. The intensities of the N-acetyl signal and the signals from the standard were compared giving the amount of oligosaccharides.

2.4. SEC–MS analysis of degradation products

The different fractions were analyzed by SEC–ESI-MS in the negative ion mode using a Superdex Peptide 10/300 GL column from GE Healthcare on a LC10vp LC system from Shimadzu coupled to a Q-Trap 2000 LC/MS/MS system from AB Sciex. The mobile phase (400 μL/min) consisted of 12 mM ammonium acetate buffer (pH 9.0) filtered through a 0.2 μm Millipore filter prior to use. To improve the performance of the mass spectrometer a 100 μL/min make-up flow of acetonitrile and water in the ratio 4:1 (v/v) was added post-column and the total flow split so that ~350 μL/min entered the spectrometer. Single and multiple (two and three) charged negative ions were analyzed with the ion-spray voltage set to −4500 V and with a nebulizer temperature of 500 °C. The nebulizer and curtain gas pressures were set to 60 and 30 psi, respectively.

2.5. Analytical method for the determination of CrR and CrD

2.5.1. Enzymatic degradation of HA gel

A portion of 0.13 g hydrogel was allowed to swell for 30 min in 1.3 mL of Milli-Q purified water. Chondroitinase AC was added to a final activity of 0.3 U/mL. After gentle mixing the samples were incubated for 22 ± 2 h at 37 °C with gentle agitation. The digests were stored below 8 °C (<5 days) during the time between incubation and analysis. For the investigation of the enzyme degradation, a second portion of enzyme was added for some preparations, and the samples incubated again. The same amount of digests (50 μL) was injected and analyzed by SEC–ESI-MS in the SIM mode with the same MS settings as described in Section 2.4. Single and multiple (two and three) charged negative ions were analyzed. The ions analyzed were selected for all theoretical combinations of HA–BDPE fragments (<3500 Da) and declustering and entrance potentials were optimized for each ion.

2.5.2. Calculation of CrR and CrD

The peak areas in the reconstructed ion chromatogram (RIC) of the fragments were used to calculate the effective cross-linker ratio (CrR) by dividing the sum of the peak areas of the cross-linked HA–BDPE fragments (**X**—**B**—**X** and **X**—**B**—**X**—**B**, **c** in Fig. 2) with the total peak area of all detected HA–BDPE fragments (**X**—**B**, **B**—**X**—**B**, **X**—**B**—**X** and **X**—**B**—**X**—**B**, **b** and **c** in Fig. 2) according to Eq. (2). The cross-linking degree (CrD) was obtained by multiplying CrR with the degree of modification (MoD) obtained for the gel.

$$\mathrm{CrR} = \frac{\sum(\mathrm{area}_{\mathbf{X-B-X}} + \mathrm{area}_{\mathbf{X-B-X-B}})}{\sum(\mathrm{area}_{\mathbf{X-B-X}} + \mathrm{area}_{\mathbf{X-B}} + 2 \times \mathrm{area}_{\mathbf{X-B-X-B}} + 2 \times \mathrm{area}_{\mathbf{B-X-B}})} \quad (2)$$

3. Results and discussion

3.1. Isolation of HA–BDPE fragments

HA hydrogel formed by cross-linking with BDDE was degraded by chondroitinase AC in order to identify oligosaccharides substituted with BDPE. To allow the formation of as small HA–BDPE

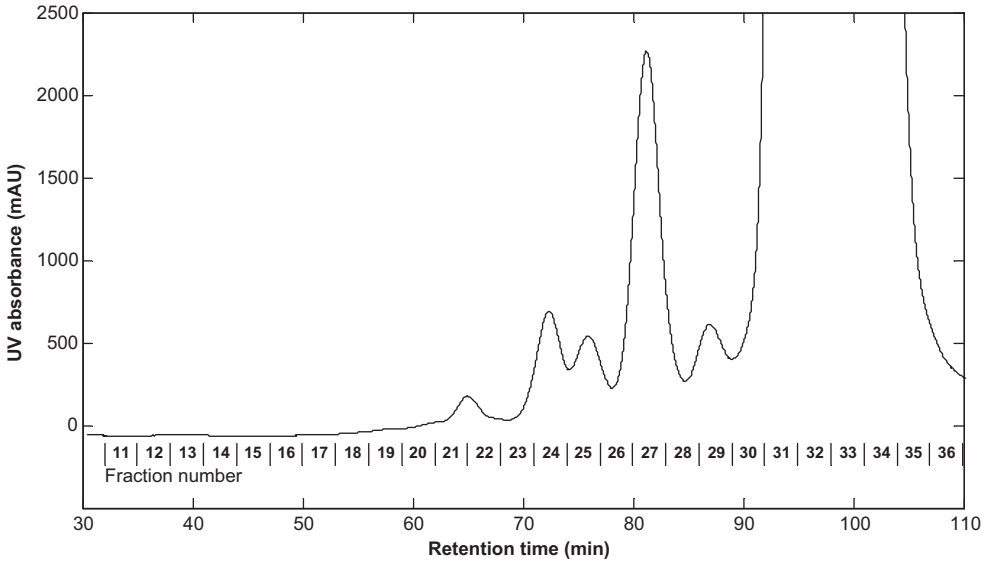


Fig. 4. UV-chromatogram of the SEC separation of the product obtained by treatment of HA Hydrogel 1 with chondroitinase AC.

fragments as possible Hydrogel 1, which has a low degree of modification, was chosen. The hydrogel was treated with chondroitinase AC until no further reaction occurred. The progress was monitored by loss of viscosity and measurement of the intensity ratio of the ^{1}H NMR signal from H-4 in the unsaturated new terminal (δ 5.8 ppm) and the N-acetyl signal (δ 2.0 ppm). When no further change of the ratio (δ 5.8:δ 2.0 intensity ratio ~0.9:3) occurred and all gel particles had disappeared, the digestion was considered as complete. The product mixture obtained by the enzymatic degradation was separated by size exclusion chromatography (SEC) and according to the UV-chromatogram several minor peaks corresponding to larger components were obtained in addition to a major peak in the disaccharide region (Fig. 4). Fractions 20–35, corresponding to the peaks, were collected for further characterization by ^{1}H NMR spectroscopy and MS.

3.2. Characterization of HA–BDPE fragments

Fractions 30–35 (Fig. 4) contained, according to ^{1}H NMR, the main product ΔDiHA, which is the expected product from treatment of HA with chondroitinase AC. The components in fractions 20–29 corresponded to BDPE-substituted oligosaccharide fragments indicated by the intensities of ^{1}H NMR signals from the unsaturated non-reducing terminal (δ 5.8) and from CH_2 of BDPE (δ 1.6). Analysis of fractions 20–29 by SEC–ESI-MS in the negative ion mode indicated oligosaccharides of different sizes (clusters with a mass difference of 379 Da, corresponding to the incremental mass residue of the disaccharide repeating unit in HA, Fig. 5) with higher mass for the faster eluting components. The ions formed from these components had masses corresponding to the oligosaccharide but with an additional 220, monosubstituted by a BDPE residue, or 202 Da, cross-linked with BDPE (Figs. 2 and 5). The masses indicated that the components in the fractions contained both mono- and disubstituted oligosaccharides as well as cross-linked oligosaccharides.

Analysis of SEC–ESI-MS data of the components in fractions 20–29 gave information on size and type of modification of the different fragments and their relative amounts in the fraction. The relative amounts were calculated from the abundances of ions formed (Table 3). Four different series of components were detected; monosubstituted, disubstituted, cross-linked and substituted with cross-linked oligosaccharides. Among the monosubstituted oligosaccharides the di- and tetrasaccharides (**2**—**B** and **4**—**B**) were the major components whereas only minor amounts of monosubstituted **6**—**B** and **8**—**B** were detected. The oligosaccharides with the higher masses were found to be cross-linked (**X**—**B**—**X**; **X** = 2–8) with the highest abundance of **4**—**B**—**4**. Disubstituted oligosaccharides, **B**—**X**—**B**, and disubstituted with cross-linked oligosaccharides, **X**—**B**—**X**—**B**, were also detected. The SEC–ESI-MS analysis of the fractions supported the conclusion from NMR that the disaccharide ΔDiHA was the main product but also that unsaturated oligosaccharides containing BDPE residues (consisting of mainly 1–8 disaccharides) were formed during the digestion of HA hydrogel with chondroitinase AC. The observed molar BDPE/HA disaccharide ratio obtained by NMR was found to resemble the calculated molar BDPE/HA disaccharide ratio from the assignments made by SEC–ESI-MS data for fractions 20–29 (Table 3).

In addition to the expected fragments, ions corresponding to the trisaccharide ΔUA-GlcNAc-GlcA (**3**) and its monosubstituted form (**3**—**B**) were detected. When HA is treated at alkaline conditions, it could be degraded by first a β-elimination reaction of the GlcA followed by a peeling reaction of the so formed reducing 3-substituted GlcNAc (Stern, Kogan, Jedrzejas, & Šoltés, 2007), leading to GlcA at the reducing end of the "peeled" poly- or oligosaccharide. Trisaccharides could then be formed by the enzyme degradation of these poly- or oligosaccharides. The amount of trisaccharides relative the amounts of the disaccharide ΔUA-GlcNAc and **4**—**B** could

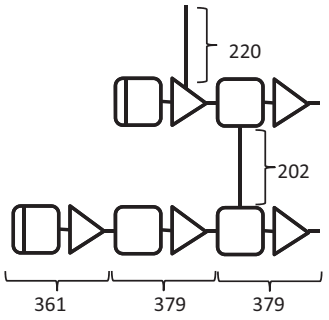


Fig. 5. Schematic representation of oligosaccharide BDPE fragments and the contributions of different parts to the masses: ▷ = GlcNAc, □ = GlcA, ◫ = ΔUA and | = BDPE.

Table 3
Mono- and cross-linked BDPE fragments in fractions 20–29 observed by SEC–ESI-MS. Relative area-% is given for each fragment in the fractions (only amounts >0.5%).

HA–BDPE fragment type	Mono-linked						Cross-linked							Mono- and cross-linked				Two mono-linked		BDPE/HA ratio	
Fraction	3[a]	**2—B**	**3—B**	**4—B**	**6—B**	**8—B**	**2—B—2**	**4—B—2**	**4—B—4**	**6—B—4**	**6—B—6**	**8—B—6**	**8—B—8**	**4—B—2—B**	**4—B—4—B**	**6—B—4—B**	**B—4—B**	**B—8—B**	Calc.[b]	NMR[c]
20												12	88						0.1	0.1
21										8	58	22	8			3			0.2	0.2
22										14	70		1		1	5		8	0.3	0.2
23						47			18	14	3				6			11	0.3	0.2
24					1	17			80					1					0.3	0.2
25				24	36	1		16	21								2		0.3	0.2
26				48	25			22	3								2		0.4	0.3
27	1			98			1												0.5	0.4
28		7	2	81			10												0.5	0.4
29	2	91		5			2												0.9	0.4

[a] **3**=α-ΔUA-(1→3)-GlcNAc-(1→4)-GlcA.
[b] Calculated molar BDPE/HA disaccharide ratio from obtained area values for the fragments in each fraction.
[c] Observed molar BDPE/HA disaccharide ratio from NMR.

be related to the average length of the polysaccharide chains in the HA hydrogel. Another minor fragment detected, **4—B—(—B—)4**, consists of two double-linked BDPE residues in parallel bridging two tetrasaccharides (Table 4), showing that the distance between two cross-links can be within two neighboring disaccharide units or closer.

A total quantification of the different components in the fractions was done by ^{1}H NMR spectroscopy. An internal standard was added to each fraction and the intensities of the oligosaccharide signals relative the standard signals were determined. In this way the amounts of the components were determined. The response estimation by SEC–ESI-MS was complicated by incomplete purity data and low signals of the larger HA–BDDE fragments and the fragments with two BDDE residues. Since the major ions that contributed the most to the magnitude of CrR were found to have approximately the same signal response, all detected ions were assumed to have the same response factor in the CrR calculations.

3.3. Determination of CrR, analytical method

HA hydrogel was treated with chondroitinase AC and analyzed by SEC–ESI-MS using SIM. The ions which were monitored corresponded to the fragments found in the preparative experiment and some fragments which are theoretically possible (Table 4). The total amount of modified residues and cross-linked residues were calculated from the peak areas of the profiles obtained in the SIM analysis.

The size exclusion column used in the analysis for the determination of CrR did not resolve all the oligosaccharide fragments since some of the fragments have very similar molecular masses and hence some fractions inherently contained mixtures. However, this problem was overcome by use of SEC–ESI-MS in combination with the SIM technique where the individual areas under their specific m/z could still be obtained despite their overlapping retention times (see e.g. compounds **4—B—2—B** and **B—6—B** in Fig. 7 and Table 4). Selection of m/z values and optimization of signals were carried out during method development on the isolated fractions.

The CrR method showed a relative standard deviation of 5%, obtained from five separately prepared samples from the same HA hydrogel (Hydrogel 1) analyzed on five different occasions. The generation of small HA–BDPE fragments is to some extent dependent on the activity and amount of enzyme. This was observed when a further portion of enzyme was added. However, the relative amount of mono- and cross-linked fragments resulted in the same CrR value which shows that the exact enzyme amount or its activity is not critical.

3.4. Application of the method on two HA hydrogels

The two laboratory HA hydrogels, Hydrogels 1 and 2, were analyzed using the described method. In order to investigate the applicability of the method for the determination of CrR, hydrogels with different MoD were chosen.

3.4.1. Determination of MoD

A ^{1}H NMR spectrum of the enzymatic digest of Hydrogel 1 is shown in Fig. 6. The spectrum shows a signal at δ 2.0 ppm for the N-acetyl of the HA di- and oligosaccharides. The signal at δ 5.8 ppm is due to the unsaturation formed in GlcA by the enzymatic elimination reaction, as expected. The different BDPE residues are observed by the signal at δ 1.6 ppm. The inset in Fig. 6 shows the difference in BDPE signal intensity observed between an unwashed (a) and a washed (b) hydrogel. This difference is due to free BDPE (Fig. 2, **a**), but could also be due to unreacted BDDE, underlining the importance of washing the HA hydrogel before attempting enzymatic degradation in order to ensure that a true MoD value is obtained.

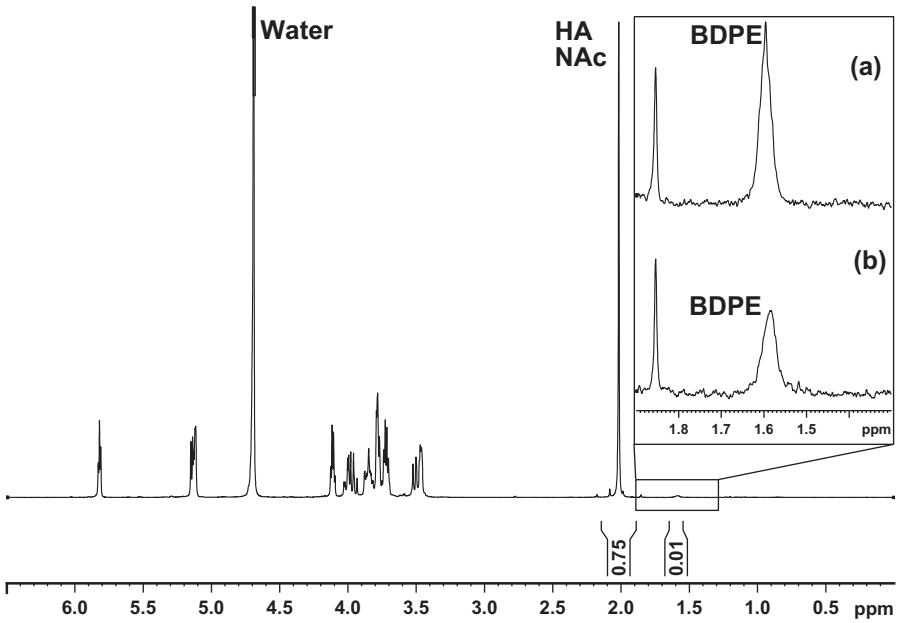


Fig. 6. ^{1}H NMR spectrum of enzymatic digest from Hydrogel 1. The inset shows the BDPE signal for the unwashed hydrogel (a) and the corresponding signal for the washed hydrogel (b). The signal at δ 1.85 ppm is the upfield ^{13}C satellite of the N-acetyl signal and was used to normalize the two spectra.

Table 4
Detected HA and HA–BDPE oligosaccharide fragments in elution order with fragment specific MS settings. HA and HA–BDPE fragment nomenclature according to Table 2.

HA/HA–BDPE fragment				Observed ion			
Name	Retention time (min)	Mw (Da)	Mono-isotopic mass (Da)	*m*/*z*	Charge	DP[a]	EP[b]
8—B—8	23.6	3236.8	3235.0	1077.3	−3	−40	−10
8—B—6	24.4	2857.5	2855.9	1427.0	−2	−40	−10
6—B—6	25.2	2478.1	2476.8	1237.4	−2	−50	−10
6—B—4—B	25.7	2319.1	2317.8	1157.9	−2	−50	−10
6—B—4	26.4	2098.8	2097.7	1047.8	−2	−50	−10
B—8—B	26.8	1957.8	1956.7	977.4	−2	−100	−10
4—B—4—B	27.2	1939.8	1938.7	968.3	−2	−80	−10
4—B—(—B—)4	27.4	1921.8	1920.7	959.3	−2	−60	−7
8—B	27.6	1737.5	1736.6	867.3	−2	−40	−10
4—B—4	28.1	1719.5	1718.6	858.3	−2	−60	−7
B—6—B	28.4	1578.5	1577.6	787.8	−2	−90	−10
4—B—2—B	28.8	1560.5	1559.6	778.8	−2	−80	−10
6—B	29.5	1358.2	1357.5	677.7	−2	−45	−10
4—B—2	29.9	1340.2	1339.5	668.7	−2	−60	−7
B—4—B	30.6	1199.2	1198.5	1197.5	−1	−90	−10
2—B—2—B	31.5	1181.1	1180.5	589.2	−2	−80	−10
4—B	32.3	978.9	978.4	977.4	−1	−100	−10
2—B—2	32.7	960.9	960.3	959.3	−1	−60	−7
3—B	32.8	775.7	775.3	774.3	−1	−60	−10
3	35.5	555.4	555.1	554.1	−1	−60	−10
2—B	35.8	599.6	599.2	598.2	−1	−100	−10
2[c]	39.1	379.3	379.1	378.1	−1	−30	−10

[a] DP = declustering potential.
[b] EP = entrance potential.
[c] **2** = ΔDiHA.

The MoD values found for Hydrogels 1 and 2 were 1 and 14%, respectively (Table 5), which can be compared to the BDDE/HA disaccharide molar ratios (2 and 36%, respectively) used for the formation of the hydrogels. The difference between the MoD value and the BDDE/HA disaccharide molar ratio used for the formation of a HA hydrogel gel represents free BDPE and/or unreacted BDDE as discussed above.

3.4.2. Determination of CrR

Some of the obtained SEC–ESI-MS reconstructed SIM profiles of the selected HA–BDPE fragment ions found in the analysis of the enzymatic digests are shown in Fig. 7. The same pattern of

Table 5
MoD and CrR, as determined by the analytical methods, together with the parameters DS, CrD and DC, which were derived from MoD and CrR for the two HA hydrogels investigated.

	Analytically determined parameters		Derived parameters		
	MoD (%)	CrR	DS (%)	CrD (%)	DC (%)
Hydrogel 1	1	0.20	1.2	0.2	0.4
Hydrogel 2	14	0.12	15.7	1.7	3.4

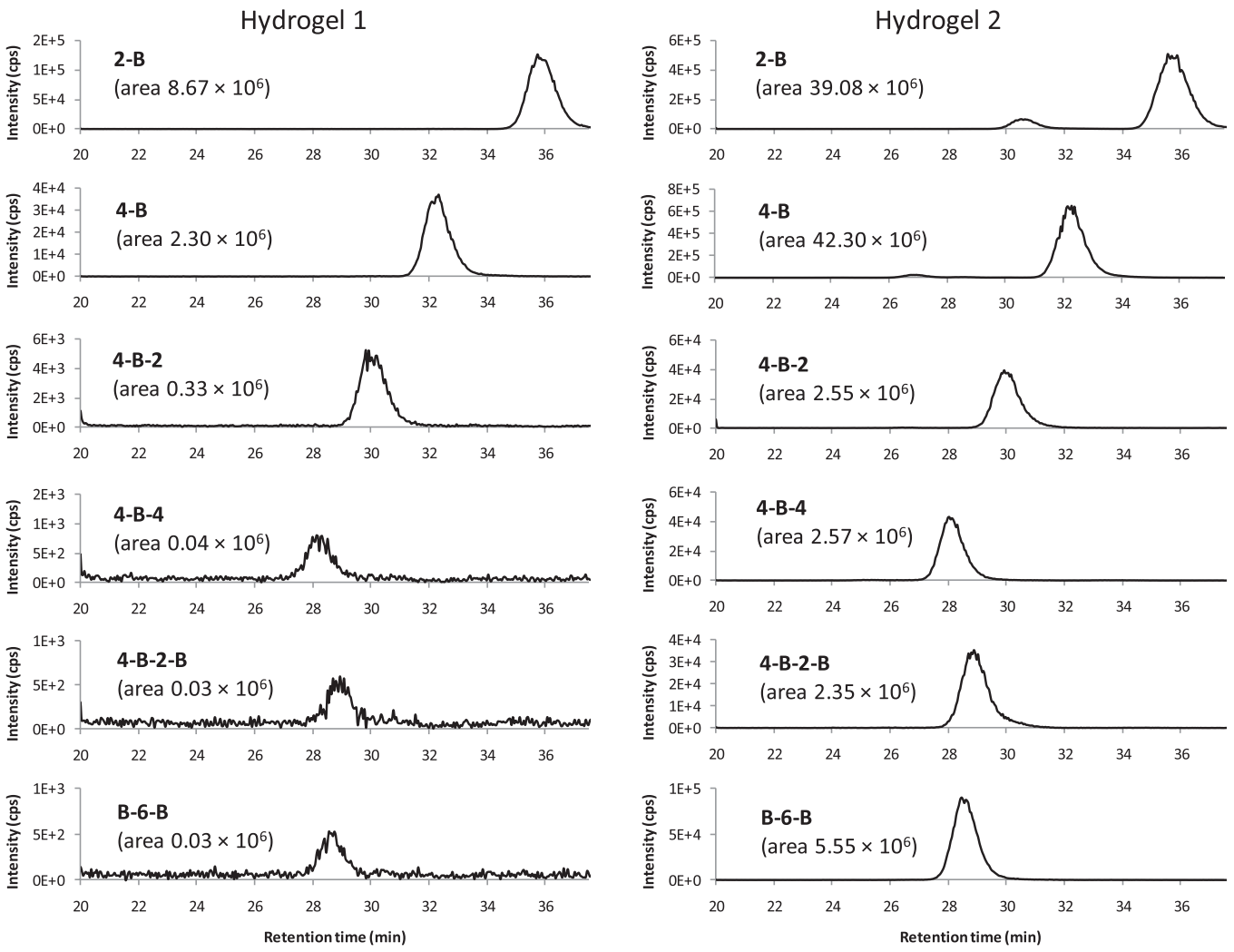


Fig. 7. Some of the SEC–ESI-MS reconstructed SIM profiles of selected HA–BDPE fragment ions found in analysis of the enzymatic digests of Hydrogels 1 and 2.

fragment ions was observed for both samples showing that the enzyme degradation worked for both hydrogels. Obviously, the intensities of all HA–BDPE fragments ions from Hydrogel 2 are higher compared to those of Hydrogel 1, reflecting the higher MoD of the former hydrogel.

The analysis of Hydrogels 1 and 2 resulted in CrR values of 0.2 and 0.1, respectively (Table 5). This means that two out of ten BDPE residues are double-linked in Hydrogel 1 whereas in Hydrogel 2 only one out of ten BDPE residues is double-linked. However, the CrD values (Table 5), calculated from the MoD values, show that the absolute number of cross-linkages is higher in Hydrogel 2 than in Hydrogel 1, reflecting the higher degree of modification of the former hydrogel.

4. Conclusions

Detailed structural analysis of intact HA hydrogels is a challenging task due to the high molecular mass and the viscoelastic properties of the hydrogel. Degradation of the hydrogel with mild hydrolysis (Guarise et al., 2012) or an enzyme (Kablik et al., 2009) partly overcomes the problem. The enzyme chondroitinase AC generates disaccharides from the unmodified parts of HA and various oligosaccharides from the modified parts where BDPE residues can slow down the enzymatic degradation. These low-molecular products can be separated by SEC and studied by NMR and MS. As the degradation of the hydrogel is done under very mild conditions the modifications remain intact in the oligosaccharide fragments and analysis of these gives information on the hydrogel. We have also found that the enzyme chondroitinase ABC generates the same fragments and in similar proportions, as obtained with chondroitinase AC, after the degradation of HA hydrogels.

By the methods described in this study the degree of modification (MoD) is first determined by NMR analysis of the product obtained after treatment of the hydrogel with chondroitinase AC. The MoD value includes both mono-linked BDPE residues and those involved in actual cross-linkages. The proportion of BDPE-fragments involved in cross-linkages (CrR) is then determined by SEC–ESI-MS. Knowing MoD and CrR, the values for DS, CrD and DC can be calculated. The methods for the determination of MoD and CrR have recently been applied to several commercially available HA hydrogels for dermal filling (Edsman, Nord, Öhrlund, Lärkner, Helander Kenne, 2012) and information on the cross-linking parameters for each hydrogel was obtained.

The definitions and methodology for analysis described here for BDDE cross-linked HA could also be used and adopted for the numerous other modified and cross-linked variants of HA reported (Schanté et al., 2011), and possibly also on other modified polymers. The results for the examples analyzed in this study show the importance to clearly describe how the different parameters for the quantification of modifications of polymers were calculated when reporting values for the respective parameter.

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