1D NMR methods for determination of degree of cross-linking and BDDE substitution positions in HA hydrogels

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A R T I C L E   I N F O
Article history:
Received 29 August 2016
Received in revised form 25 October 2016
Accepted 10 November 2016
Available online xxx

Keywords:
Hyaluronic acid
Hydrogels
NMR spectroscopy
Cross-linking ratio
Substitution position

A B S T R A C T
Hyaluronic acid polymers cross-linked with BDDE are today among the most used hydrogels for biomedical applications. The physical properties of the hydrogels depend, among other parameters, on the degree of cross-linking of HA. Another parameter likely to affect the physical properties is the substitution position of the linker on the HA functional groups. A NMR-based method for the determination of these parameters in hyaluronic acid hydrogels is presented. The method is based on the degradation of HA cross-linked hydrogels by chondroitinase ABC followed by one-dimensional 1H and 13C NMR analysis. The necessary structural information to obtain both the degree of cross-linking and the substitution positions can be obtained from the same NMR sample and no chromatographic separation step is required prior to NMR analysis.

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I. Introduction

The use of cross-linked polysaccharide hydrogels for biomedical and biotechnological purposes has increased substantially in the last decades (Kogan, Soltés, Stern & Gemeiner, 2007; Viola et al., 2015). One of the most commonly used polysaccharides is hyaluronic acid (HA, also known as hyaluronan). HA is a natural linear polysaccharide with a repeating disaccharide unit consisting of α-glucuronic acid (GlcA) and N-acetyl-d-glucosamine (GlcNAc) linked by alternating 1 → 4 and 1 → 3 β-glycosidic bonds (Fig. 1) (Laurent & Fraser, 1992).

HA is naturally occurring in all vertebrates and in some bacteria. The primary structure is preserved through all species but the molecular weight (MW) of the polymer varies between different sources. Because of its high water holding capacity and viscoelasticity as well as its biocompatibility and biodegradability HA is used in a wide range of medical, pharmaceutical and biotechnological applications (Xu et al., 2012). Natural HA has a rapid turnover in vivo and therefore modifications such as cross-linking are utilized to obtain biomaterials with longer duration (Laurent, 1987; Segura et al., 2005). Some examples of methods for chemical cross-linking of HA are amidation, Ugi condensation, ether formation, ester formation etc (Schánté, Zuber, Herlin & Vandamme, 2011). The most common cross-linking method uses ether formation by reaction with 1,4-butanediol diglycidyl ether (BDDE) (Ågerup, Berg & Åkermark, 2005). HA hydrogels obtained by cross-linking using BDDE have proven to be biocompatible and non-toxic (De Bouille et al., 2013; Lan, Jou, Wu, Wu, Chen, 2015). Under alkaline conditions, epoxides of BDDE react with the HA hydroxyl groups to form derivatives of 1,4-dibutaneol di-(propan-2,3-diyl) ether (BDPE). Some of the BDPEs form true cross-links that bind to HA at both ends while others only bind at one end (mono-linked). There are many products based on BDDE cross-linked HA and even though they have the same starting material the physical properties of the resulting hydrogels vary considerably (Edsman, Nord, Öhrlund, Lärkner & Kenne, 2012). The variation of physical properties among the hydrogels may be attributed to variations in structural parameters such as i) MW and polydispersity of the HA starting material ii) the degree of cross-linking iii) the substitution position of the BDDE cross-linker on HA.

http://dx.doi.org/10.1016/j.carbpol.2016.11.029
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cross-linking (CrD) is defined as the stoichiometric ratio of cross-linked cross-linker molecules to the moles of HA repeating units (Kenne et al., 2013). CrD can be calculated from the experimentally obtained parameters degree of modification (MoD) and effective cross-linker ratio (CrR). MoD is defined as the stoichiometric ratio of all linked cross-linker molecules to the moles of HA repeating units (both cross- and mono-linked linkers are included in MoD), and CrR is defined as the ratio of moles of cross-linked linkers (forming cross-linkages in the polysaccharide network) to the total moles of linkers. CrD is then calculated as: 

$$\text{CrD} = \frac{\text{CrR} \times \text{MoD}}{\text{CrR}}$$

Kenne et al., 2013. The CrD of a HA hydrogel is reflected in its physicochemical properties such as swelling and viscoelasticity. Hence, the properties of a gel depend on the ability to control CrR and MoD, and methods that accurately determine these parameters are therefore important. Excessively cross-linked HA hydrogels greatly modify the chemical and physical properties and it has been suggested that this potentially could affect the biocompatibility of the HA hydrogel (Tetzl & Fredrickson, 2008).

Several different methods have been proposed to assess MoD and/or CrD in HA hydrogels using techniques such as exclusion chromatography (SEC)-MS/TDA, LC-UV and IR (Barbucci et al., 2006; Kablik, Monheit, Yu, Chang & Gershkovich, 2009; Guarise, Pavan, Pirrone & Renier, 2012; Kenne et al., 2013). Kenne et al. developed methods to determine MoD using 1D 1H NMR, and CrR using SEC/LC–MS of enzymatically degraded HA gels. Chromatographic separations of degraded hydrogels followed by MS analysis can be time consuming. The response factors in the detection methods must also be considered and it is often assumed that all fragments independent of size give the same detector response. However, limited availability of standard oligomers for calibration implies that no suitable method exists for the determination of component ratios using UV, MS or LC techniques. In contrast, NMR spectroscopy is particularly useful because simple experiments such as single pulse experiments give reliable signal area integrals that are directly related to compound ratios. When signals are overlapping in the 1H NMR spectra, quantitative 13C NMR which has significantly higher signal dispersion can be used (Xia, Akim, & Argyropoulos, 2001; Caytan, Remaud, Tenailleau, & Akoka 2007; Zhang et al., 2015). The use of 13C NMR spectroscopy as a quantitative tool requires the elimination of the nuclear Overhauser effect by inverse-gated decoupling sequence and sufficiently long recycle delays (Freeman, Hill & Kaptein, 1972). Performing NMR analysis on intact HA hydrogels of high molecular weight is however difficult or even not feasible due to the high viscosity which leads to poor sensitivity and severe line broadening of resonances resulting in inaccurate area of signals from integration.

Degradation of HA by the lyase chondroitinase ABC (chABC) from Proteus vulgaris will cleave 1,4-linkages between GlcNAc and GlcA producing disaccharides with unsaturated uronic acids at the non-reducing end (Fig. 1) (Yamagata, Saito, Habuchi, & Suzuki, 1968). Cross-linked HA is however not completely degraded to disaccharides by chABC. In the final digest larger fragments with linkers attached to them are found besides the unsaturated disaccharide which is the main end product. For HA cross-linked with BDDE mono-linked end-products ($\Delta$HA$_2$A-B (2–B) and $\Delta$HA$_2$A-B (4–B)), cross-linked end products ($\Delta$HA$_2$A–B$\Delta$HA$_2$ (2–B), $\Delta$HA$_2$A–B$\Delta$HA$_2$ (4–B–2) and $\Delta$HA$_2$A–B$\Delta$HA$_2$ (4–B–4)) and also mono-cross-linked fragments such as $\Delta$HA$_2$A–B$\Delta$HA$_2$–B (4–B–B) have been observed after complete degradation (schematic representation in Fig. 2, the fragments being named according to the nomenclature used in Kenne et al., 2013). Using NMR and LC–MS analysis, we have recently shown that among mono-linked fragments, di- and tetrasaccharides (2–B and 4–B) are end products of complete enzymatic degradation by chABC (Wende et al., 2016).

Fig. 1. Repeating unit of hyaluronic acid polysaccharide (left) and unsaturated HA disaccharide obtained after incubation with chondroitinase ABC (chABC) (right).

Detailed structural analysis of the isolated mono-linked fragments 2–B, 4–B and 6–B demonstrated that BDDE substitution can occur at any of the four hydroxyl groups of the HA disaccharide repeating unit; C2, C3 of GlcA and C4, C6 of GlcNAc. Substitution occurred only on the terminal reducing and non-reducing end sugars and BDDE was not found to be linked on the internal sugars. The linker distribution over substitution positions was however found to be dependent on the size of the fragment. Substitution at position C4 of GlcNAc was not observed for 2–B but was dominant in larger fragments revealing that chABC is hindered from cleaving on the non-reducing end of a tetrasaccharide unit having BDDE substitution at C4 of GlcNAc at the reducing end. The overall favored substitution position in the investigated hydrogel was C4 of GlcNAc (73%) followed by C6 of GlcNAc (14%) and C2 of GlcA (10%). 1H and 13C NMR signals that could be used as chemical shift reporters of the position of substitution of BDDE on the sugars of HA were identified and used to determine the amount of substitution at different positions (Wende et al., 2016).

In the current work, we present a novel and simple method for direct determination of degree of cross-linking by one-dimensional 13C NMR. Minimum sample preparation is needed and no chromatographic separation is required. Using the same sample, the relative distribution of the cross-linker over the four substitution positions on HA can also be obtained from the 1D 1H NMR spectra.

2. Experimental

2.1. Materials

Six laboratory made HA hydrogels cross-linked with BDDE were prepared. The gels (denoted hydrogel 1–6) had a HA concentration of 20 mg/ml and varying MoD and CrR. Chondroitinase ABC (chABC) from Proteus vulgaris (art. no. C2905) was obtained from Sigma Aldrich. D$_2$O (99.96% D) was purchased from Cambridge Isotope Laboratories, Inc. Water was de-ionized and further purified by a Milli-Q system (Millipore).

2.2. Sample preparation

Prior to enzymatic degradation 2–3 ml hydrogel was washed with 3 × 20 ml of aqueous NaCl (9 mg/ml) followed by 3 × 20 ml water. The liquid phase was removed in each washing step by filtration under vacuum using 0.22 µm Sterilon™ filter (Millipore, Darmstadt, Germany). The washed hydrogels were transferred to test tubes and gently mixed with 10 ml of 1 mM phosphate buffer (pH 7) and 300 µl chABC solution (4 units/ml). The samples were then incubated for 24 h at 37°C. The degraded samples were lyophilized, dissolved in 0.6 ml D$_2$O and transferred into 5 mm NMR
Fig. 2. Schematic representation of the degradation of a BDDE cross-linked HA hydrogel by chABC. The exemplified fragments are denoted 2-B, 2-B-2, 4-B-2 and 4-B.

2.3. NMR spectroscopy

The NMR spectra were recorded on a Bruker Avance III 600 MHz NMR spectrometer equipped with a 5 mm $^1$H/$^{13}$C/$^{15}$N/$^{31}$P cryo probehead with a z-gradient (Bruker BioSpin GmbH, Germany). The data was acquired and processed using the TopSpin 3.1 software (Bruker). The pulse sequences zg30, noesypppr1d and ledbpgr2s1d from the Bruker pulse sequence library were used for $^1$H NMR. To obtain quantitative $^1$H spectra a recycle delay (d1) of 30 s was used. A line broadening factor of 0.3 Hz was applied and base line correction was performed prior to integration of the $^1$H NMR signals. Quantitative 1D $^{13}$C NMR spectra were obtained with the inverse-gated decoupled experiment zgig30 from the Bruker pulse sequence library using a recycle delay of 15 s and between 512 and 8192 scans depending on the hydrogel sample. A spectral width of 36058 Hz (239 ppm) and an acquisition time of 1 s was used. Integration was carried out after applying a line-broadening factor of 1 Hz and automatic base line correction using the command c13cryn. Linear deconvolution was performed using the Bruker software. The start and end points for integration were kept constant for all spectra. The spin-lattice relaxations (T1) for $^{13}$C were determined by using the pseudo 2D experiment ttipg. The experiment uses inversion recovery to measure T1 and power gated decoupling to receive both NOE enhancement and decoupling. Nine different recovery delays (30, 15, 8, 4, 2, 1, 0.5, 0.1 and 0.05s) were used in the experiment, the number of scans was 1024 and the recycle delay (D1) 15 s. COSY, TOCSY, NOESY, HSQC, HSQC-TOCSY and HMBC were recorded with 4 K data points in t2 and 256 to 512 increments in t1, using a minimum of 16 scans per increment and a relaxation delay of 1.2 to 1.5 s. TOCSY spectra were recorded with mixing times of 120 ms and NOESY experiments were run with mixing times ($\tau_m$) of 500 ms.

2.4. Fractionation by LC–MS analysis

After completion of NMR analyses for MoD and CrR, the sample originating from the degradation of hydrogel 5 (~600 μl) was subjected to dialysis using a mini dialysis kit (1 kDa cut-off, GE Healthcare) in order to remove most of ΔHA2, which is formed as the major end-product of chABC degradation. After the dialysis, a second dose of 300 μl chABC solution (4 units/ml) was added and the mixture incubated for 24 h at 37 °C. No further dialysis was required before performing a micro–scale preparative purification of selected HA fragments using SEC.

SEC/ESI–MS in the negative ion mode was performed using a Superdex Peptide 10/300 GL column from GE Healthcare (Uppsala, Sweden) on an Agilent HP 1100 LC system coupled to an Amazon speed ETD ion trap mass spectrometer (Bruker Daltonik, GmbH). The mobile phase consisted of a 12 mM ammonium acetate buffer at pH 9.0 (adjusted with NH₄OH). The run was performed in an isocratic mode with a 400 μl/min flow and a run time of 42 min. The flow was split with a post-column splitter at a ratio of about 9:1. 10% of the sample entered the MS, here used as an ion monitor in real time to facilitate the collection of the appropriate HA fragment peaks as they eluted from the other splitter outlet. The ion trap was operated in a full scan mode from m/z 200–2000 with the following source parameters: spray capillary voltage 4000 V; end plate offset –500 V; nebulizer pressure 8 psi; dry gas 4.0 l/min; Dry temperature 350 °C. Around 500–700 μl fractions were collected.
following each injection volume of 50 μl. The collected fractions were lyophilized and subjected to NMR analysis.

3. Results and discussion

The HA hydrogels were washed with aqueous NaCl to remove free unreacted HA and BDDE prior to incubation with chABC. Complete degradation of the hydrogels is not necessary to determine the MoD and CrR values from the NMR spectra but better resolution and higher sensitivity are obtained. For correct analysis of linker distribution over the substitution positions, it is however important that the degradation is complete. Triplicate samples of six HA hydrogels with different degrees of modification and cross-linking were degraded and analyzed by NMR. The 1H and 13C resonances of the compounds resulting from the degradation were assigned from 2D TOCSY, HSQC and HSOQC–TOCSY spectra and using assignments from previous studies (Blundell et al., 2006). Signals from the unsubstituted disaccharide, ΔHA2, dominated the 1H NMR spectra (Fig. 3). Besides these major signals, signals from mono- and cross-linked HA oligosaccharides were also present.

3.1. MoD and CrR determined from 1D NMR spectra of the degraded gel

As previously shown by Kenne et al. MoD is easily determined from a 1H NMR spectrum by integrating one signal from BDDE, originating from the BDDE cross-linker, at δ 1.7 ppm and the signal from the N-acetyl group in HA at δ 2.1 ppm (Fig. 3). The ratio between the integrals for these two signals gives the MoD after correction for the number of protons responsible for each signal (Eq. (1)). The MoD obtained for the six investigated hydrogels (hydrogel 1–6) are reported in Table 1 and are between 3 and 30%.

MoD(%) = (I1H1.7) / (I1H2.1) · 100

(1)

While the MoD can be determined from 1D 1H NMR, the CrR was obtained from 1D 13C NMR spectra. Quantitative 1D 13C NMR spectra were obtained from inverse-gated decoupled NMR spectra (Freeman, Hill, & Kapteijn, 1972). The spin-lattice relaxation time for the carbons of interest was determined to be <2 s and therefore a recycle delay of 15 s was used. The area of the signals used for CrR determination was obtained by performing a deconvolution of the experimental Lorentzian. The CH2OH carbon farthest away from HA on mono-linked BDDE (C10, Figs. 3 and 4) has a carbon shift (62.7 ppm) which is well separated from other signals. When BDDE binds covalently to HA this signal is shifted downfield to ca 70 ppm. While the signal at 70 ppm overlap with other signals and cannot be easily integrated, the signal at 62.7 ppm can be integrated in the 1D 13C NMR spectra. The C5′ and C6′ at 25.2 ppm have the same chemical shift for both cross- and mono-linked fragments. Thus, integration of the CH3 signals at 25.2 ppm and the CH2OH signal at 62.7 ppm gives the fraction of mono-linked BDDE and CrR can be calculated (Eq. (2)). Since two carbons (C5′ and C6′) contribute to the signal at 25.2 ppm compared to one carbon (C10′) at 62.7 ppm, a 2:1 ratio between the two signals would indicate that all BDDE molecules are mono-linked while a 2:0 ratio indicate that all BDDE molecules are cross-linked.

CrR = 1−16C62.7 / [C6C25.2 / 2]

(2)

The MoD can also be calculated from the 13C NMR spectra by integrating the signal from BDDE at 25.2 ppm and the signals from the N-acetyl group of GlcNAc in HA at 21.9–22.6 ppm (Eq. (3), Fig. 4). The three signals observed between 21.9 and 22.6 ppm originate from the unsaturated disaccharide ΔHA2 (strongest signal) and from larger and BDDE substituted oligosaccharides. The degree of modification is obtained from the ratio between the integrals for these two signals (cross-linker/HA NAc) after correction for the number of carbons responsible for each signal.

MoD(%) = (C6C25.2 / 2) / [C6C21.9−22.6 · 100

(3)

The MoD and CrR obtained from integration in the 13C NMR spectra are listed in Table 1 for the hydrogels 1 – 6. The MoD are very similar to the values obtained from the 1H NMR spectra. While there is no advantage of determining the MoD from 13C NMR instead of 1H NMR, the excellent agreement between the two methods indicates that the procedure can be used with confidence for CrR calculation. The cross-linking ratio was also evaluated by the SEC/LLC–MS method (Kenne et al., 2013) and Table 1 shows that similar results are obtained using the two different techniques.

For all samples, with the exception of hydrogel 4, NMR spectra with sufficient signal-to-noise (S/N) ratios could be obtained with 512 scans giving an experimental time of two hours. For hydrogel 4, a larger number of scans was necessary due to the low MoD value.

3.2. Substitution position of BDDE in cross-linked fragments

We have recently identified chemical shift reporters of position of attachment of BDDE on the sugars of mono-linked oligosaccharide fragments (Wende et al., 2016). To be able to determine the overall distribution of BDDE in HA from 1H NMR spectra of degraded gels, it is necessary to also determine the position of sub-

Table 1

<table>
<thead>
<tr>
<th>Hydrogel</th>
<th>MoD (%)</th>
<th>CrR</th>
<th>CrD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1H</td>
<td>13C</td>
<td>1H</td>
<td>13C</td>
</tr>
<tr>
<td>1</td>
<td>8.7 ± 0.2</td>
<td>7.8 ± 0.3</td>
<td>0.2 ± 0.02</td>
</tr>
<tr>
<td>2</td>
<td>7.4 ± 0.0</td>
<td>6.8 ± 0.3</td>
<td>0.2 ± 0.05</td>
</tr>
<tr>
<td>3</td>
<td>5.5 ± 0.1</td>
<td>5.1 ± 0.4</td>
<td>0.2 ± 0.03</td>
</tr>
<tr>
<td>4</td>
<td>3.4 ± 0.2</td>
<td>2.6 ± 0.1</td>
<td>0.3 ± 0.07</td>
</tr>
<tr>
<td>5</td>
<td>5.0 ± 0.0</td>
<td>5.0 ± 0.5</td>
<td>0.5 ± 0.03</td>
</tr>
<tr>
<td>6</td>
<td>32.0 ± 0.2</td>
<td>31.4 ± 0.3</td>
<td>0.2 ± 0.02</td>
</tr>
</tbody>
</table>

Values are presented as means from three samples with their respective standard deviations. MS analysis according to Kenne et al. gave CrR 0.1 for hydrogels 1–3 and 6, 0.2 for hydrogel 4 and 0.5 for hydrogel 5.
Substitution in true cross-linked oligosaccharide fragments. For this, a highly cross-linked HA hydrogel (CrR 0.5 and MoD 5%, hydrogel 5, Table 1) was degraded by chABC. Dialysis with 1 kDa cut-off devices permitted the removal of most of ΔHA2 which, due to its very high content, otherwise overlapped with the other peaks in the chromatogram rendering the isolation of pure fragments difficult. Smaller mono- and cross-linked fragments such as 2-B and 2-B-2 were also almost completely removed by dialysis. The SEC/LC–MS extracted ion chromatogram of the mixture of compounds obtained after dialysis showed that most of the larger HA fragments could be detected as their doubly charged ions (see Supplementary material). Two fractions corresponding to 4-B-4 and 4-B-2 were collected using LC–MS (Supplementary material) and further analyzed by NMR.

The 1H NMR spectra of 4-B-4 was nearly identical to that of 4-B (Fig. 5) and analysis of the data showed that BDDE substitution occurs almost exclusively at C4 of GlcNAc at the two reducing ends. As expected, the H10/C10′ cross-peaks at 3.58–3.66 ppm present in the HSQC spectrum of 4-B had vanished in 4-B-4 (Fig. 5). Substitution at GlcNAc-OH6 was not observed for 4-B-4 as it was in 4-B. NMR analysis of 4-B-2 revealed substitution at all four hydroxyl groups. The disaccharide part of 4-B-2 show substitution at ΔGlcA-OH2, GlcNAc-OH6 and ΔGlcA-OH3 which is similar to what was observed for 2-B. The tetrasaccharide part of 4-B-2 shows predominant substitution at GlcNAc-OH4 as observed in 4-B (Fig. 5).

3.3. Substitution position of BDDE in hydrogels

Having established the position of substitution of BDDE in mono- and cross-linked HA oligosaccharides and identified chemical shift reporters that can be used to determine the amount of substitution at each position, the distribution of the cross-linker can be estimated from 1H NMR spectra of the degraded gel. Indeed, comparison of the NMR spectra of the degraded gel with the 1H NMR spectra of the 2-B, 4-B, 2-B4 and 4-B-4 fragments shows that the signals allowing quantification of substitution positions are separated from the resonances of the unsubstituted disaccharide (Fig. 6) (Wende et al., 2016). These NMR signals can therefore be integrated to yield the amount of substitution at C2, C3 of ΔGlcA and C4, C6 of GlcNAc (Fig. 6). For ΔGlcA, these signals are H1 for substitution at C2 and H4 for substitution at C3. For GlcNAc, these signals are H1 of GlcNAc(α) for C4 substitution and H1 of GlcNAc(β) for C4 and C6 substitution.

The relative amount of substitution for the six hydrogels investigated is presented in Fig. 7. The distribution is similar for all six hydrogels with the most common substitution position at GlcNAc-OH4 (>40%) followed by ΔGlcA-OH2 (∼30%) GlcNAc-OH6 (∼15%) and ΔGlcA-OH3 (∼5%).

Some other small peaks that are sometimes observed in the 1D 1H spectra (Fig. 6) belong to (E)-3-dehydroxy-2-en-6-GlcNAc, peeling product arising from the hydrolysis of the linkage between GlcA and GlcNAc at the reducing end in HA and previously described by Blundell & Almond, 2006. The N-acetyl signal in this compound also has a slightly higher chemical shift. All 1H and 13C signals as well as the JHH coupling constants could be determined for the major α-anomer while some resonances could not be assigned unambiguously for the minor β-anomer (SI). Other small signals observed in

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the 1H NMR spectra arise from oligosaccharides with GlcA at the reducing end occurring from the non-enzymatic hydrolysis of the linkage between GlcA and GlcNAc.

4. Conclusion

The method proposed here, degradation of hydrogels by chondroitinase ABC, followed by 1D 1H and 13C NMR analysis, simultaneously provides four types of structural information on cross-linked HA hydrogel that other techniques cannot. The degree of modification, the degree of cross-linking, the position of linker, as well as amount of linker at each position on the hydroxyl groups of HA, can be obtained without prior chromatographic separation steps.

Acknowledgement

The authors would like to thank Morgan Karlsson at Calderma, Uppsala for preparing the experimental hydrogels.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2016.11.029.

References


Fig. 6. 1D 1H NMR spectrum of BDDE cross-linked HA (hydrogel 2) after incubation with chABC: 1: H1 and H3 signals (α and β-forms) of the peeling product reported by Blundell & Almond, 2006; 2: H4 of ΔH4A substituted by BDPE at C3 of ΔGlcA; 3: H1 of ΔH4A substituted by BDPE at C2 of ΔGlcA; 4: H1(α) of ΔH4A substituted by BDPE at C6 of GlcNAc; and 5: H1(α) of ΔH4A substituted by BDPE at C4 of GlcNAc.

Fig. 7. Substitution pattern for the six investigated hydrogels. The bars show mean values and the error bars indicate the calculated standard deviations.

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