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Short communication

Molecular weight determination of hyaluronic acid by gel filtration chromatography coupled to matrix-assisted laser desorption ionization mass spectrometry

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Abstract

An analytical approach has been described for the molecular weight characterization of enzymatically degraded hyaluronic acid (HA). The approach involved the combined use of aqueous gel filtration chromatography (GFC) with matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). Microfractions were collected across an eluting peak from the chromatography system, followed by mass spectrometric analysis of these narrow fractions. The molecular mass determined by MALDI-MS and the signal obtained from the chromatography established a calibration curve for other hyaluronic acid samples analyzed by this GFC system. Results of one HA sample were obtained from both the calibration curve and direct fraction-by-fraction MALDI-MS analysis, and comparison of these results showed reasonable agreement. In contrast, molecular weights resulted from external calibration using dextran and pullullan standards showed drastically different numbers. Therefore, the GFC–MALDI-MS approach is a reliable method for the molecular weight characterization of polydisperse polysaccharides for which suitable calibration standards are unavailable for conventional GFC analysis.

Keywords: Hyaluronic acid; Polysaccharides

1. Introduction

Hyaluronic acid (HA) is a naturally occurring linear polysaccharide. It is composed of a repeating disaccharide unit of glucuronic acid and *N*-acetylglucosamine via a β-1,4 linkage, as shown in Fig. 1. HA is involved with numerous biological functions, including the lubrication of joints, the regulation of molecular permeation into various tissues, the proliferation, adhesion and motility of cells, and the development of embryos [1]. It has also been demonstrated to play roles in cancer metastasis, in wound healing, and in inflammation [2]. The molecular mass of HA varies depending on its source and preparations. In certain applications,

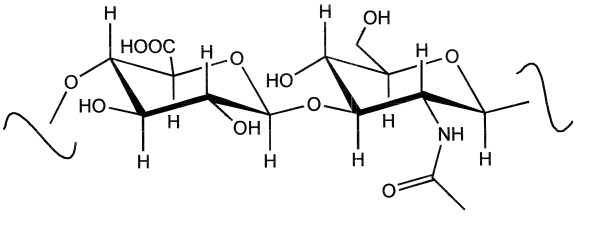

Fig. 1. Structure of a disaccharide unit in HA.

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such as in the cosmetic industry and in ophthalmology, the molecular mass of HA can be in the range of $4 \cdot 10^6$–$8 \cdot 10^6$. Smaller HA sizes from bacterial sources ($1 \cdot 10^6$–$2 \cdot 10^6$) have been used for pharmacuetical applications such as hydrogel formation and drug attachment. Significantly smaller molecular mass resulting from either physical, chemical, or enzymatic degradation have been used for characterization purposes and are presently under investigation for various drug delivery applications.

Characterization of the molecular size of HA can be accomplished by gel permeation or gel filtration chromatography (GPC or GFC), either via external calibration with molecular weight standards, or light scattering analysis [3]. In our experience, light scattering analysis has been impractical due to the need of accurate determination of the refractive index increment, dn/dc, for each HA sample prior to analysis. It is virtually impossible to eliminate all salts and residual organic contaminants from each HA digest preparation so that its true dn/dc value may be determined. In addition, low-molecular-mass HA samples do not produce an intense light scattering signal, which in turn hinders accurate molecular weight determination by this technique. As for GPC or GFC, the lack of suitable molecular weight standards for HA dictates that only relative molecular weight be obtained using this approach. The use of calibration standards of different molecular structure and composition than the analytes has been known to give drastically different results in the GPC analysis of synthetic polymers [4]. The highly polydisperse nature of HA has also precluded its direct molecular mass analysis by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). It has previously been demonstrated that polydisperse polymers yield lower molecular mass by MALDI-MS than by GPC/GFC [5], due to the fact that number fraction vs. m/z are recorded in MS, rather than weight fraction vs. log of molecular weight in GPC/GFC. This difference results in the high mass tail of the polydisperse polymer to disappear earlier into the noise in MS analysis, and the phenomenon worsens as the polydispersity increases. Generally, polymers with a polydispersity higher than 1.2 cannot be mass analyzed by MS directly [4,6–8].

In this paper, we report on the combined use of GFC with MALDI-MS for the molecular weight characterization of enzymatically degraded HA. A HA digest was run on the aqueous GFC system as a calibration standard, and its eluent was microfractionated for direct MALDI-MS analysis. Using the molecular mass obtained by MS, a calibration curve was constructed for the GFC system to allow the molecular weight characterization of other HA samples. This combined approach has previously been shown to be effective for synthetic organic-soluble polymers [9–11]. In this work, we demonstrate the general utility of this approach towards the molecular weight analysis of polysaccharides on an aqueous GFC system.

2. Experimental

2.1. Chemicals

Native HA was obtained from Collaborative Labs. (East Setauket, NY, USA). Dextran calibration standards (weight-average molecular weight, M_w 10 000 to 600 000, polydispersity 1.50 to 2.0) were purchased from Polysciences (Warrington, PA, USA). Pullulan calibration standards (M_w 5900 to 788 000, polydispersity 1.00–1.23) were obtained from Polymer Labs. (Amherst, MA, USA). Ammonium phosphate and sodium phosphate were obtained from Mallinckrodt (Chesterfield, MO, USA), and 2,5-dihydroxybenzoic acid (DHB) and dextran of molecular mass 10 000 were from Sigma (St. Louis, MO, USA).

2.2. HA digestion

Enzymic digestions were carried out in a fashion similar to that of Vercruysse et al. [12]. In brief, alternating portions of 23 mM sodium phosphate–140 mM NaCl buffer (pH 6.4) and native HA were added to a 2-l beaker to final amounts of 980 ml and 10.0 g, respectively. The resulting ‘‘layered’’ mixture was stirred with a smooth magnetic stir bar at ca. 500 rpm, at room temperature overnight. The clear solution thus formed was warmed with stirring to 37°C over a 4-h period and subjected to 16 mg hyaluronidase (5120 units, type I-S from bovine testes; Sigma) as three portions in 15 ml total of the

above buffer. The mixture was physically swirled for 10–15 min until the viscosity drop allowed the stir bar to effect efficient mixing, then the reaction was continued for 16–20 h at 37°C. Termination of the reaction was done by precipitation of the HA in ice-cold isopropanol (alcohol–water, 10:1, v/v). Product was collected by centrifugation (10 min, 2440 *g*, 5°C), air dried, then dissolved in Milli-Q water and dialyzed exhaustively (8000 M_w cut-off membrane) against water before freeze drying. Slight variations in batch size, digestion time, and reagent concentrations resulted in the different molecular mass distributions observed.

2.3. Fast protein liquid chromatography (FPLC)

Size-exclusion chromatography was performed using a modified method of Cowman et al. [13]. Pyridinium acetate (0.5 *M*, pH 6.5, 5°C) was used to elute across 50 fractions a digested sample of HA from a HiPrep Sephacryl S-300 HR 26/60 column (Amersham Pharmacia Biotech, Piscataway, NJ, USA), at a flow-rate of 0.8 ml/min. Selected fractions (3.5 ml each) were desalted on PD-10 disposable columns (Pharmacia), lyophilized and then analyzed by GFC and MALDI-MS.

2.4. Gel filtration chromatography (GFC)

The system used consisted of an Alliance solvent delivery system controlled by Millennium 32 software, an in-line vacuum degasser, and a column temperature module set to 30°C (Waters, Milford, MA, USA). PL-GFC columns of 1000 Å and 300 Å pore sizes (8 μm particles, 300×7.5 mm; Polymer Labs.) were connected in series. The running buffer was either 20 m*M* ammonium phosphate (pH 4.5) or 20 m*M* sodium phosphate (pH 6.9), at a flow-rate of 1.0 ml/min. Detection was carried out using an Optilab DSP refractive index (RI) detector (Wyatt Technology, Santa Barbara, CA, USA) set to a constant temperature of 40°C. Calibration with dextran and pullullan standards was performed using the Millennium 32 software. HA samples were dissolved in ammonium phosphate at concentrations of 1.0 to 1.5 mg/ml, and were allowed to completely dissolve at room temperature for at least 1 h prior to injection. Injections of up to 100 μl (150 μg) were made.

2.5. Mass spectrometry

MALDI was carried out using a Voyager DE RP time-of-flight instrument (PerSeptive Biosystems, Framingham, MA, USA) equipped with a high current detector. Typically, 1 μl of sample was mixed with 1 μl of a 10 mg/ml solution of DHB in 50% aqueous methanol and then allowed to dry at room temperature. Mass analysis was done in linear negative mode with delay extraction. When analyzing HA microfractions, the grid voltage and delay time varied depending on the mass range of interest, whereas the acceleration and guide wire voltages were kept constant at 20 kV and 0.3%, respectively. A low mass gate was applied and varied from m/z 500 to 7000 depending on the mass region of interest. A total of 64 mass spectra were acquired and summed for each sample spot. Mass calibrations were performed over several m/z ranges, using protein standards such as angiotensin, adrenocorticotropin (ACTH) fragments, myoglobin and bovine serum albumin with the DHB matrix.

2.6. Microfractionation

For microfraction collection, 20 m*M* ammonium phosphate was used as the running buffer in GFC. Typically, up to 150 μg of HA was injected onto the GFC system. The outlet flow at 1.0 ml/min from GFC was split such that a 12 μl/min flow was obtained at the capillary outlet of the InterPlate fraction collector (PerSeptive Biosystems). Fractions were collected every 5 s directly onto a 100-well MALDI sample plate, which resulted in sample spots of ~1 μl each. DHB matrix was added manually to each sample spot and crystallization took place at room temperature. Generally 50 to 70 fractions were collected across a GFC eluting peak for analysis by MALDI-MS.

3. Results and discussion

Mass analysis of polydisperse polymers by MS has long been a challenge. Typically, as the polydis-

persity of the analyte increases to above 1.2, ionization of high mass species becomes more difficult and the mass spectra obtained tend to contain only peaks in the low m/z range [9,11]. This is illustrated in Fig. 2a, where an HA digest (HA 9) was analyzed by MALDI-MS. As seen in Fig. 2a, most peaks are observed at below m/z 10 000, with virtually no signal in the higher m/z range. This is due to the aforementioned problem with highly polydisperse samples, as well as the suppression by lower m/z ions in general.

The GFC analysis of the same sample using dextran and pullullan calibration standards yielded drastically different molecular weight information, as listed in Table 1. Since the elution in GFC depends on the hydrodynamic size of the analyte molecule rather than actual molecular mass, the use of calibration standards with molecular structure different than that of the analyte will only produce a relative molecular weight. Therefore, while GFC can provide some information on the size of polymers, true molecular weight information can only be obtained if well characterized standards of the same type as the analyte are used for calibration.

In order to obtain meaningful molecular weight information on such polydisperse polymers, microfractionation of the samples eluting off sizing columns has been shown to be beneficial [10,11]. Fig. 2b shows the MALDI-MS spectrum of an FPLC fraction of an HA digest (HA 7). Although the sample had been crudely fractionated prior to analysis, it was clear that high mass signal was still severely suppressed. However, in comparison to Fig. 2a, some benefits of a crude fractionation could be observed here as an envelope of peaks emerged with a maximum at m/z~2000. Fig. 3 shows the effect of

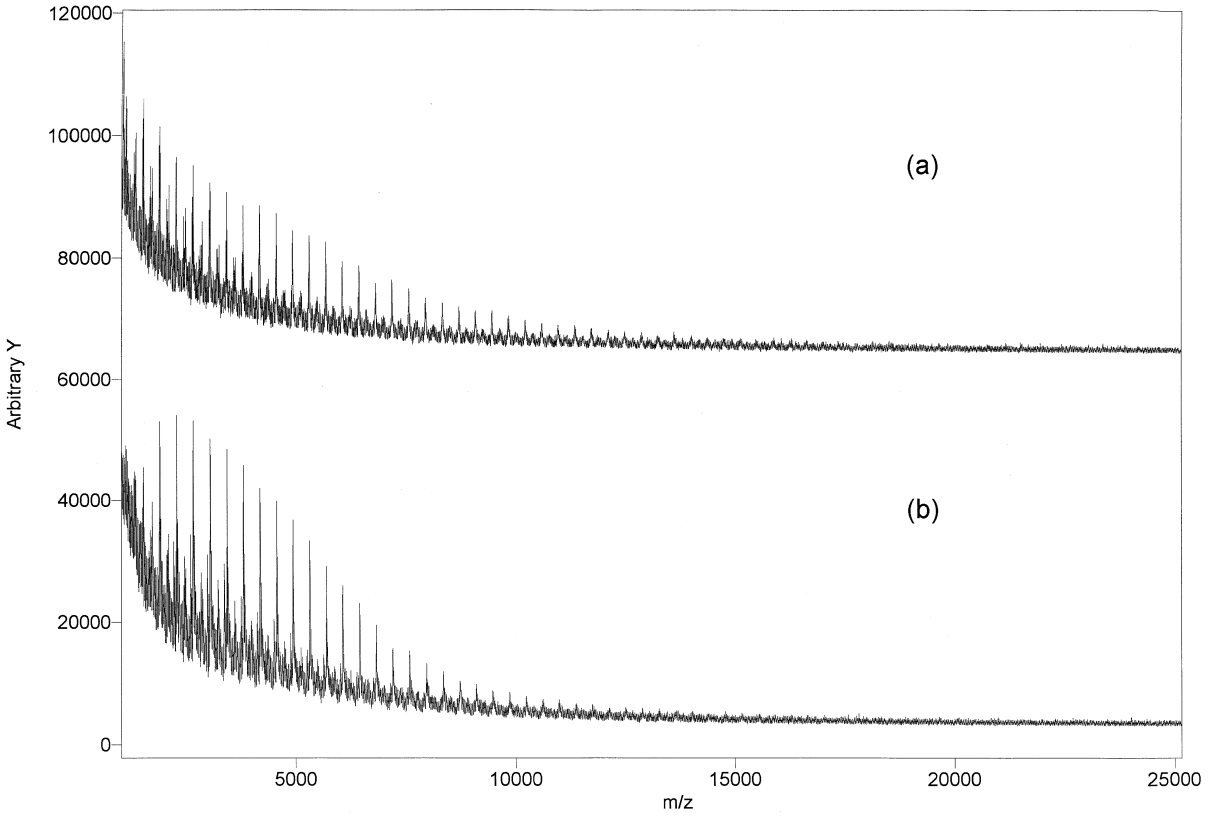

Fig. 2. MALDI-MS spectra of two HA digest mixtures: (a) HA 9, 1.3 mg/ml, without any fractionation; (b) HA 7, 1.0 mg/ml, fractionated on an FPLC system. Both samples were dissolved in 20 m*M* $NH_4H_2PO_4$ (pH 4.5), and this was diluted 100-fold with the DHB matrix for MALDI-MS analysis. See Section 2.5 for MS conditions.

Table 1
Molecular weight values for HA digests obtained by different methods ($n=3$ in each measurement)[a]

Sample	Method	M_n	M_w	M_z	M_p	PD
HA 8	GFC (pullulan standards)	131 209	265 319	439 931	226 561	2.02
	GFC (dextran standards)	144 813	685 774	1 601 891	470 275	4.74
	GFC–MALDI-MS	100 762	293 991	599 722	60 054	2.91
HA 9	GFC (pullulan standards)	47 504	90 820	140 760	75 670	1.91
	GFC (dextran standards)	40 072	130 411	347 875	73 303	3.26
	GFC–MALDI-MS	22 729	38 639	64 178	17 788	1.70
HA 10	GFC (pullulan standards)	40 972	71 431	110 979	61 128	1.74
	GFC (dextran standards)	33 471	88 061	261 530	53 815	2.63
	GFC–MALDI-MS	17 637	28 924	53 124	14 660	1.64

[a] M_n, M_w, M_z, M_p = number-average, weight-average, z-average and peak top molecular weights, respectively. PD = polydispersity. See text for description of these terms.

microfractionation of this sample on its MALDI data, where peaks in a Gaussian shaped envelope are clearly seen to be shifting from low to high m/z with decreasing elution time in GFC. This differs from the data in Fig. 2b where the sample was only crudely fractionated on an FPLC system. The selected fractions in Fig. 3 show the vast differences in the types of data obtained at different points of the GFC eluting peak. Half of the fractions (total=14) collected from the top 50% of the GFC peak height were analyzed by MALDI-MS, and molecular mass from the most intense ion in each fraction was used to construct a GFC–MALDI-MS calibration curve for the columns used (third-order fit: $y=-0.01767x^3+0.69781x^2-9.48476x+48.52291$, $r^2=0.99780$). Based on this curve, and using the RI signal exported from the Millennium software, various molecular weights and polydispersity were calculated with the equations listed below:

$$M_n = \sum n_i \cdot M_i / \sum n_i$$

$$M_w = \sum n_i \cdot M_i^2 / \sum n_i \cdot M_i$$

$$M_z = \sum n_i \cdot M_i^3 / \sum n_i M_i^2$$

$$PD = M_w / M_n$$

where M_n = number-average molecular weight, M_w = weight-average molecular weight, M_z = z-average molecular weight, PD = polydispersity. M_i and n_i are the molecular weight and RI response, respectively, at time point i. Using these equations, the FPLC fractionated HA 7 was found to have $M_n=14\,587$, $M_w=20\,725$, $M_z=28\,239$ and PD = 1.42.

Next, another HA digest (HA 9) was run on the GFC system, microfractionated and then analyzed by MALDI-MS in the same manner described for the HA 7 FPLC fraction. Microfractions of this sample also showed increasing M_r in their mass spectra with decreasing GFC elution time (data not shown). Using the calibration curve generated with HA 7, the molecular weights for HA 9 were calculated and are shown in Table 1. For comparison, the most intense molecular mass obtained at various time points by direct MALDI-MS analysis and by GFC–MALDI-MS calibration are listed in Table 2. As can be seen, an average deviation of ~6% was obtained between the results from both methods, with the best correlation seen near the peak top of the GFC peak where the analyte was more abundant. The comparison shows the calibration curve from GFC–MALDI-MS to be applicable to the analysis of HA samples on the same GFC system. In contrast, calibration using dextran and pullulan standards yielded drastically larger molecular weights and polydispersity for HA 9, as seen in Table 1. Two other HA samples digested to different extent have also been analyzed using this calibration curve, and the results are also shown in Table 1, along with results from dextran and pullulan calibration for comparison. Based on the results in Table 1, it would appear that, for the most part, HA occupies two- to three-times larger hydrodynamic volume than dextran or pullulan in the

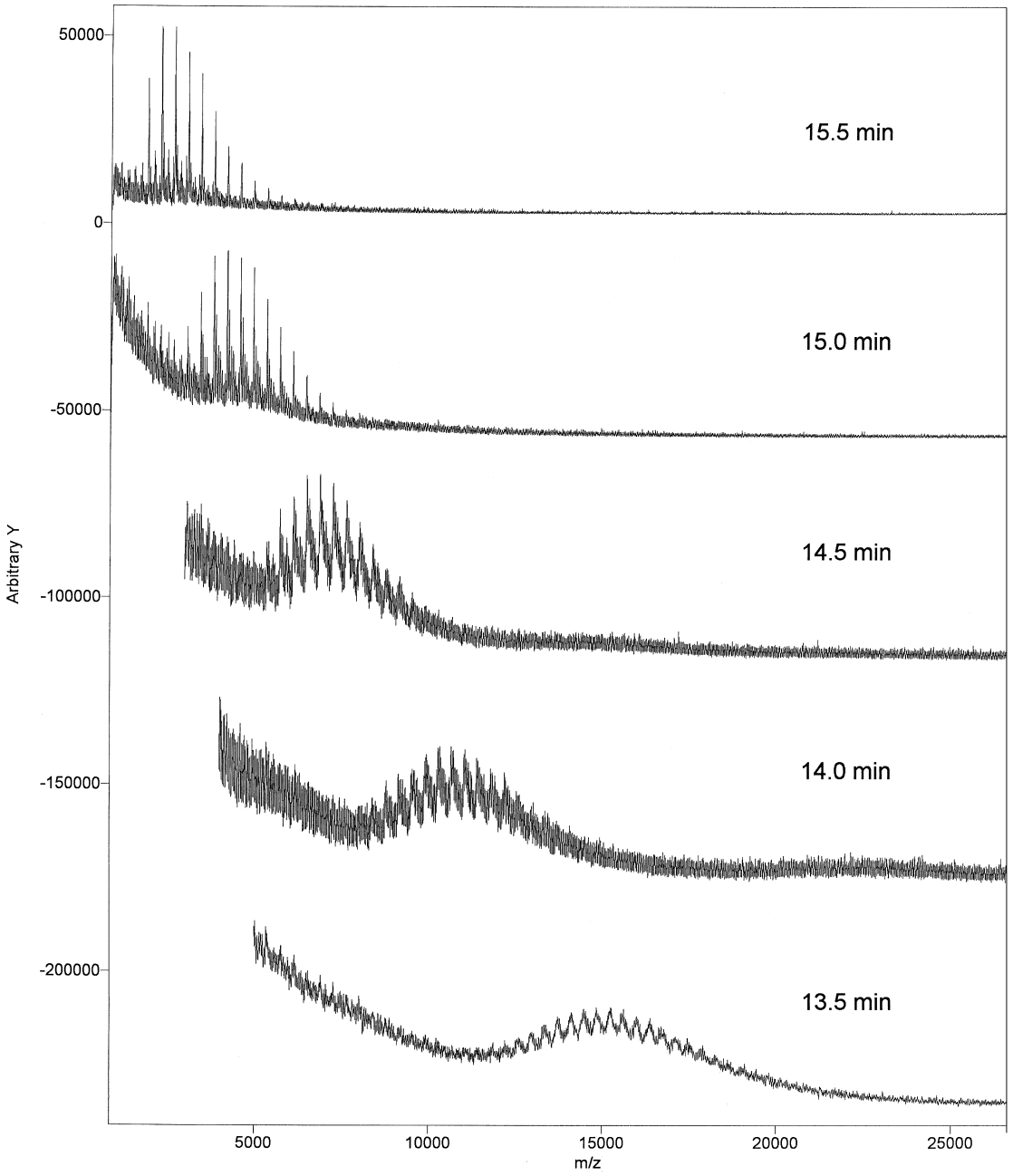

Fig. 3. GFC–MALDI-MS analysis of an FPLC fraction of HA 7, through microfractionation: 1-μl fractions were collected every 5 s across the GFC eluting peak, with a total of 55 fractions collected. Sample concentration was 1.0 mg/ml, and 60 μg was injected for this analysis. Selected fractions are shown in this figure, and the fraction at 14.0 min corresponds to the peak maximum in GFC. For all spectra acquired, the accelerating voltage = 20 kV and guide wire = 0.3%, whereas the grid voltage and delay time varied from 95% and 1000 ns, respectively, for the fraction at 15.5 min, to 90% and 1200 ns, for the fraction at 13.5 min.

Table 2
Comparison of results of HA 9 obtained by direct MALDI-MS analysis of microfractions and GFC–MALDI-MS calibration[a]

t_R (min)	M_r by direct MALDI-MS of microfractions	M_r by GFC–MALDI-MS calibration	Difference	RSD (%)
12.00	41 213	45 360	−4147	−10.062
12.17	36 695	39 491	−2796	−7.619
12.33	32 345	34 617	−2272	−7.026
12.50	30 178	30 514	−336	−1.114
12.67	27 249	27 019	230	0.844
12.83	25 032	24 006	1026	4.097
13.00	21 695	21 376	319	1.470
13.17	19 516	19 055	461	2.361
13.33	17 345	16 987	358	2.063
13.50	15 581	15 125	456	2.926
13.66	14 384	13 436	948	6.588
13.83	12 502	11 896	606	4.844
14.00	10 615	10 485	130	1.229
14.17	9864	9188	676	6.854
14.33	8347	7998	349	4.184
14.50	7590	6906	684	9.009
14.67	6457	5910	547	8.475
14.83	5329	5006	323	6.055
15.00	4949	4193	756	15.279
15.17	3429	3468	−39	−1.140
15.33	3051	2830	221	7.236
15.50	2674	2276	398	14.896
15.67	2293	1801	492	21.456
15.83	1914	1402	512	26.772
16.00	1536	1071	465	30.273
			Average RSD:	5.998

[a] t_R = Retention time on GFC; M_r(direct MALDI) = M_r detected by MALDI-MS; M_r(GFC–MALDI) = M_r calculated using the GFC–MALDI-MS calibration curve; RSD = relative standard deviation.

buffer system used. Finally, Fig. 4 shows a magnified region of one of the mass spectra, indicating the prominent mass differences to be that of the disaccharide unit of HA, with minor mass differences representative of the losses of the individual monosaccharides.

Similar to HA, the effect of polydispersity on MALDI-MS data of dextran has been observed, namely, the appearance of ions only in the low m/z range. By applying the GFC–MALDI-MS microfractionation methodology, we have been able to obtain more meaningful mass spectra for a M_r 10 000 dextran sample (data not shown). However, for polysaccharides in which no suitable GFC calibration standards are available, such as HA, the GFC–MALDI-MS method described here may be the only viable approach for obtaining reasonably accurate molecular weight information.

4. Conclusion

An approach using aqueous GFC in combination with MALDI-MS analysis through microfractionation has been described for the molecular weight analysis of HA digests. Comparison of results from direct MALDI-MS analysis and from the calibration curve showed good correlation. Therefore, a calibration curve generated with an HA sample can be applied to the GFC analysis of other HA digests done on the same system. The GFC–MALDI-MS approach should be applicable for the molecular

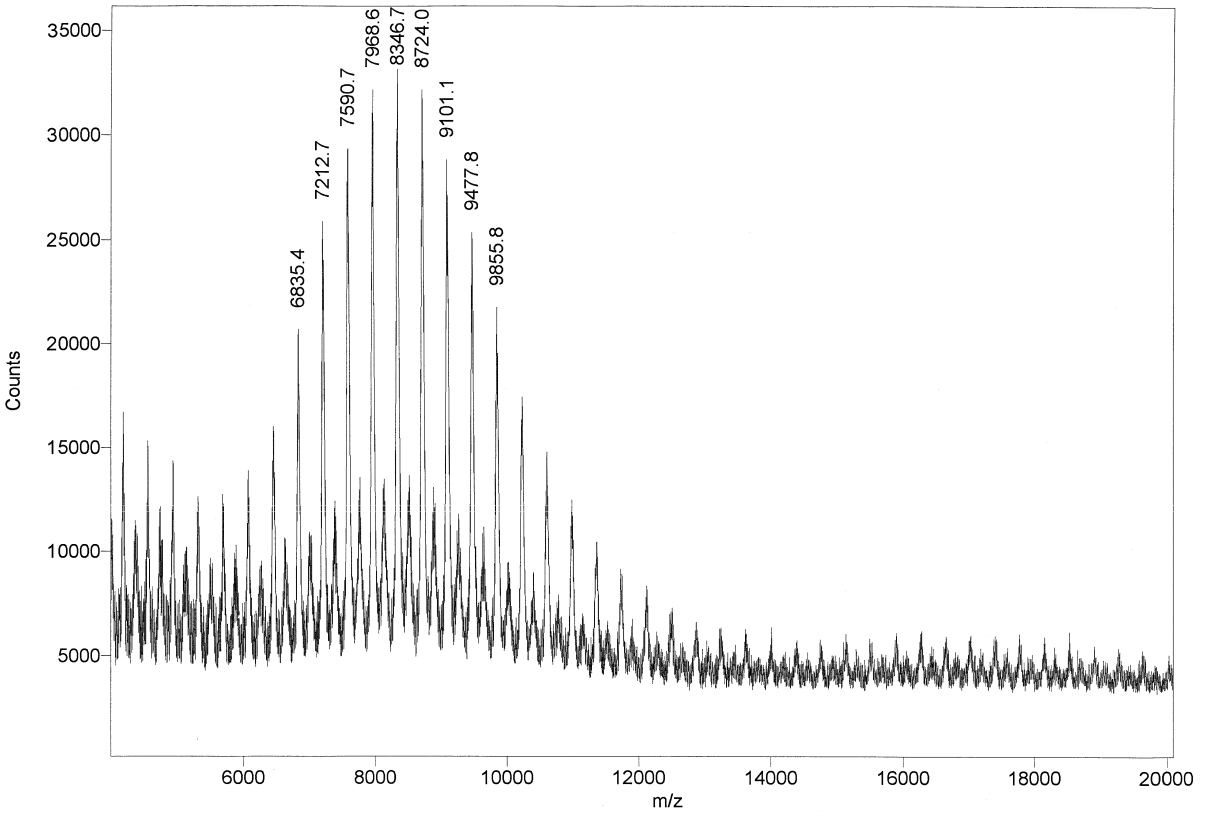

Fig. 4. Expanded view of a mass spectrum of HA 9 from the fraction collected at 14.33 min, showing the major mass differences to be that of the disaccharides (378 mass units) of HA, while the minor peaks correlate to mass differences arising from the monosaccharides (204 and 174 mass units).

weight analysis of other polydisperse polysaccharides, where no suitable calibration standards are available for their GFC experiments. Once a calibration curve has been established on a given GFC system through the GFC–MALDI-MS approach, subsequent GFC analysis of the polysaccharide samples will only involve simple data analysis on a spreadsheet using data exported from the GFC software.

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