Preparation and application of biologically active fluorescent hyaluronan oligosaccharides

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We report the production of biologically active hyaluronan (HA) oligosaccharides labeled with the fluorophore 2-aminobenzoic acid (2AA). Oligosaccharides from 4 to 40 residues in length were purified to homogeneity by ion exchange chromatography using a logarithmic gradient. Molecular weight and purity characterization of HA oligosaccharides is facilitated by 2AA derivatization because it enhances signals in MALDI-TOF MS and improves FACE (fluorophore-assisted carbohydrate electrophoresis) analysis by avoiding the inverted parabolic migration characteristic of 2-aminoacridone (AMAC)-labeled sugars. The small size and shape of the fluorophore maintains the biological activity of the derivatized oligosaccharides, as demonstrated by their ability to compete for polymeric HA binding to the G1-domain of human recombinant versican (VG1). An electrophoretic mobility shift assay was used to study VG1 binding to labeled HA 8-, 10-, 20-, 30-, and 40-mers, and although no stable VG1 binding was observed to labeled 8-mers, the equilibrium dissociation constant (100 nM) for VG1 with HA10 was estimated from densitometry analysis of the free oligosaccharide. Interactions involving HA 20-, 30-, and 40-mers (proposed to be multivalent) could also be studied using this protocol. Oligosaccharides labeled with 2AA therefore show excellent potential as probes in fluorescence-based assays that investigate protein–carbohydrate interactions.

Key words: 2-aminobenzoic acid/electrophoretic mobility shift assay/fluorophore-assisted carbohydrate electrophoresis/mass spectrometry/versican

Introduction

Hyaluronan (HA) is a high-molecular-weight (10^5 to 10^7 Da) linear glycosaminoglycan (GAG) consisting of repeated disaccharide units of β(1→3)-N-acetyl-d-glucosamine-β(1→4)-d-glucuronic acid. Unlike other members of the GAG family, HA is neither differentially sulfated nor epimerized, rendering its structure rather simple in comparison to that of heparin and chondroitin sulfate (Laurent and Fraser, 1992), and its diverse biological functions therefore probably arise from interactions with HA-binding proteins (Day and Prestwich, 2002). Most proteins bind to HA via Link modules, structural domains of ~100 amino acids, which have been proposed to be related to C-type lectin modules owing to their similar structural topology and function (Blundell et al., 2003; Kohda et al., 1996). Members of the Link module superfamily are thought to bind, capture, and stabilize distinct conformations of HA, forming higher-ordered complexes (Day and Sheehan, 2001). In the extracellular matrix of cartilage, for example, HA plays an essential structural role by forming multimeric aggregates through its interactions with cartilage link protein and the G1-domain of the chondroitin sulfate proteoglycan (CSPG) aggrecan (Hardingham and Muir, 1972; Hascall and Heinegaard, 1974a). Similar complexes, in which other CSPGs (e.g., versican, brevican, or neurocan) substitute for aggrecan along with one of four link proteins (Spicer et al., 2003) are likely to contribute to the structural integrity of many other tissues, including brain and aorta (Wight and Merrilees, 2004; Yamaguchi, 2000).

HA oligosaccharides have diverse and interesting biological functions both in vitro and in vivo, such as induction of angiogenesis (Slevin et al., 2002), regulation of macrophage inflammatory responses (Noble et al., 1996); and suppression of tumor growth (possibly by disrupting endogenous CD44 HA-receptor binding to its ligand; Toole, 2004). In addition to these cellular activities, HA oligosaccharides of defined length are widely used to characterize protein–HA interactions. The octasaccharide (HA8), for example, was determined to be the minimum length even oligosaccharide that can bind optimally to the Link module of TSG-6 (Blundell et al., 2003) and the HA-binding domain of CD44 (Teriete et al., 2004). In contrast, binding of polymeric HA to aggrecan, link protein, and versican, which all have two contiguous Link modules in their HA binding domain, is not competed effectively by even-numbered oligosaccharides of fewer than 10 sugars (Bertrand and Delpech, 1985; Hardingham and Muir, 1973; Hascall and Heinegaard, 1974b; Rosenberg et al., 1988).

Polymeric HA can be digested using testicular hyaluronidase to yield even-numbered saturated oligosaccharides (HA4 being the shortest oligosaccharide produced). The resultant oligomers can then be purified by size exclusion chromatography (Hardingham and Muir, 1973; Hascall and Heinegaard, 1974b; Lesley et al., 2000) or anion-exchange chromatography (Almond et al., 1998; Blundell et al., 2004; Mahoney et al., 2001a; Tawada et al., 2002). Proper characterization of HA oligosaccharides after preparation is essential to ensure purity and homogeneity prior to experimental use, and chromatography, nuclear
magnetic resonance (NMR), and mass spectrometry (MS) have been employed to this end (Blundell et al., 2004; Mahoney et al., 2001a; Tawada et al., 2002). Another highly sensitive technique is fluorophore-assisted carbohydrate electrophoresis (FACE). This procedure introduces a fluorophore to the reducing carbohydrate by reductive amination prior to electrophoresis (Starr et al., 1996); the choice of fluorophore is of particular importance because it may change some of the physical properties of the oligosaccharide and therefore have a direct consequence on its experimental performance.

The fluorophore 2-aminouracilone (AMAC) is frequently used to tag HA oligosaccharides (Calabro et al., 2000a,b; Karousou et al., 2004; Mahoney et al., 2001a), but contains bulky aromatic groups ($M_r = 210.24$) and, significantly, has no charge. Therefore, AMAC-labeling does not enhance the migration of sugars during electrophoresis, reducing its efficacy for characterizing heterogeneous HA oligosaccharide mixtures (Mahoney et al., 2001a), and may also contribute to the inverted parabolic separation observed when small AMAC-labeled HA oligosaccharides are electrophoresed (Calabro et al., 2000a; Tawada et al., 2002). Another fluorescent label, 8-amino-1,3,6-naphthalene trisulfonic acid (ANTS) is commonly used to analyze glycans by electrophoresis (Jackson, 1994), including HA and chondroitin oligosaccharides (Tawada et al., 2002; Volpi, 2003). Although ANTS-derivatized HA oligosaccharides (4-mers to 16-mers) display linear separation as a function of length (due to the three negatively charged sulfate moieties on the fluorophore; Tawada et al., 2002), it is rather bulky ($M_r = 381.33$) which may interfere with ligand binding. Moreover, the sulfate moieties may modify the biological activity of the oligosaccharides, because several HA-binding proteins have been reported to also bind sulfated GAGs (Mahoney et al., 2004; Parkar and Day, 1997; Weigel and Weigel, 2003).

In light of these observations, 2-amino benzoic acid (2AA, also referred to as anthranilic acid), was investigated in this study as a suitable alternative because it is small ($M_r = 137.1$), has a similar size and charge to that of D-glucuronic acid, is not sulfated, and is highly fluorescent (Anumula, 1994). In addition, 2AA-labeling has been used to enhance high-performance liquid chromatography, capillary, and gel electrophoreses of glycans isolated from cells, proteins, and lipids (Anumula, 1994; Anumula and Dhume, 1998; Anumula and Du, 1999; Bigge et al., 1995; Huang et al., 2000; Neville et al., 2004; Sato et al., 1997). Labeling with 2AA has also been used for separation and sequencing of heterogeneous heparan sulfate–derived saccharides (Shriver et al., 2000; Turnbull et al., 1999) and for structural profiling of keratan sulfates (Whitham et al., 1999). Significantly, 2AA-labeling does not disrupt biological activity in enzyme-substrate fluorescent assays (Gershkovich and Kholodovych, 1996).

In this study, HA oligosaccharides were derivatized with 2AA and purified by anion-exchange chromatography. Labeled HA oligosaccharides were shown to have correct masses and an increased signal in matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS. In addition, the fluorescent oligosaccharides gave a linear separation under electrophoresis, allowing for the production of defined ladders for molecular weight characterization. More important, the 2AA label was not observed to disrupt the biological activity of the oligosaccharides in several different assays. An electrophoretic mobility shift assay (EMSA), a common tool in the study of DNA–protein interactions (Lane et al., 1992) and recently used in heparin–protein interactions (Lyon et al., 2004; Wu et al., 2002), was used to investigate the binding between 2AA-derivatized oligosaccharides and the human recombinant G1 domain of versican (VG1). In particular, the potentially multivalent interaction of oligosaccharides (HA10–HA40) with VG1 was investigated, and in the case of HA10 an equilibrium dissociation constant was derived. Therefore, purity and molecular weight characterization of HA oligosaccharides can be enhanced by labeling with 2AA. Furthermore, these fluorescent oligosaccharides have potential as probes for studying HA–protein interactions.

Results

HA was digested with ovine testicular hyaluronidase to yield oligosaccharides in the range 4- to 40-mers (Figure 1). These oligosaccharides were derivatized with 2AA and then separated using a logarithmic ionic gradient, which maintains almost equidistant separation of the oligosaccharides up to 40-mers. It was observed that 2AA-labeling gave ~twofold more absorbance at 215 nm compared to an underderivatized HA digest because 2AA has a strong optical absorbance between 200–220 nm (Ito et al., 1998), which greatly facilitated peak detection. The absorbance could also be selectively monitored at 330 nm, a wavelength specific for the fluorophore (Ito et al., 1998), although less sensitive than 215 nm. Thus the combination of 2AA labeling and a logarithmic gradient enhanced both the separation and identification of oligosaccharide peaks.

![Fig. 1](https://academic.oup.com/glycob/article-abstract/15/3/303/574725/1639303/057426)

Fig. 1. Fluorescently labeled HA oligosaccharides were eluted using a logarithmic NaCl concentration gradient (dashed line). The oligosaccharide lengths (calibrated using a labeled 10-mer produced and characterized as described in Blundell et al., 2004) and acetate peak (Ac) are indicated.
After desalting and lyophilization, labeled HA oligosaccharides were analyzed by MALDI-TOF MS in both negative-ion reflectron mode (4–10-mers) and negative-ion linear mode (12–40-mers); each oligosaccharide had an experimental mass within error of its theoretical mass (Figure 2A and 2B; Table I). Observations indicated that 2AA-labeled HA oligosaccharides had a greatly increased signal in MALDI-TOF MS under these particular conditions compared to unlabeled oligosaccharides using the 2,5-dihydroxybenzoic acid (DHB) matrix (Figure 3).

Carbohydrate electrophoresis of labeled oligosaccharides was performed on large gels (12 cm × 14 cm) to allow all fractions to be visualized on a single gel (Figure 4A). For each fraction, essentially a single band was visible and, moreover, there was no excess free 2AA (Drummond et al., 2001) that can lead to a high background when imaging the gel. Therefore the labeling and purification protocol developed here results in homogenous preparations and is an improvement on the linear gradient (Mahoney et al., 2001a; Tawada et al., 2002) or molecular sieve (Lesley et al., 2000) approaches used previously, which can result in heterogeneous mixtures of the larger oligosaccharides. Using the techniques described here, defined HA ladders can also be constructed that facilitate the sizing of

Table I. Comparison of theoretical and experimentally derived masses of 2AA-labeled oligosaccharides

<table>
<thead>
<tr>
<th>HA length</th>
<th>Theoretical mass$^a$ [M-H]$^-$</th>
<th>Experimental mass$^b$ [M-H]$^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>896.3</td>
<td>896.2 ± 0.1</td>
</tr>
<tr>
<td>6</td>
<td>1275.5</td>
<td>1275.4 ± 0.1</td>
</tr>
<tr>
<td>8</td>
<td>1654.7</td>
<td>1654.6 ± 0.1</td>
</tr>
<tr>
<td>10</td>
<td>2033.7</td>
<td>2033.7 ± 0.1</td>
</tr>
<tr>
<td>12</td>
<td>2414.0</td>
<td>2414.0 ± 0.6</td>
</tr>
<tr>
<td>14</td>
<td>2793.4</td>
<td>2793.4 ± 0.3</td>
</tr>
<tr>
<td>16</td>
<td>3272.9</td>
<td>3172.4 ± 0.7</td>
</tr>
<tr>
<td>18</td>
<td>3552.0</td>
<td>3551.9 ± 0.9</td>
</tr>
<tr>
<td>20</td>
<td>3931.3</td>
<td>3931.1 ± 1.0</td>
</tr>
<tr>
<td>22</td>
<td>4310.6</td>
<td>4309.9 ± 1.2</td>
</tr>
<tr>
<td>24</td>
<td>4690.0</td>
<td>4689.2 ± 1.6</td>
</tr>
<tr>
<td>26</td>
<td>5069.3</td>
<td>5068.5 ± 2.7</td>
</tr>
<tr>
<td>28</td>
<td>5448.6</td>
<td>5447.8 ± 1.8</td>
</tr>
<tr>
<td>30</td>
<td>5827.9</td>
<td>5827.6 ± 2.0</td>
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<td>32</td>
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<td>6965.4 ± 3.0</td>
</tr>
<tr>
<td>38</td>
<td>7345.2</td>
<td>7344.1 ± 3.4</td>
</tr>
<tr>
<td>40</td>
<td>7724.5</td>
<td>7726.8 ± 3.1</td>
</tr>
</tbody>
</table>

$^a$Based on the mono- and average molecular mass for HA4–HA10 and HA12–HA40, respectively.

$^b$Errors are the SD of five measurements.

Fig. 2. Mixtures of (A) short (4–10-mers) and (B) long (12–36-mers) fluorescently labeled oligosaccharides were analyzed by MALDI-TOF MS in the negative-ion reflectron and linear modes, respectively. Monosodium (+22 Da), disodium (+44 Da), monopotassium (+38 Da), and mixed (sodium and potassium, +60 Da) adducts were also observed in (A).

Fig. 3. Fluorescent HA oligosaccharides gave more intense signals in MALDI-TOF MS than underivatized ones, when equal amounts (62.5 ng) were analyzed simultaneously. Arrows denote HA10 (1912.6 Da) and HA10 2AA (2033.7 Da).
unknown mixtures of HA oligosaccharides, as commonly used for DNA restriction fragments. As an example, ladders with an oligosaccharide separation of four were purified from a digest (Figure 4B), containing either 10-, 14-, ..., 38-mers, or 12-, 16-, ..., 40-mers. 1D NMR was routinely performed on 2AA-labeled 4-, 6-, 8-, and 10-mers to ensure complete derivatization and measure stock solution concentrations prior to experimental use in biological assays (Figure 5). The 1D spectrum of 2AA-labeled HA4 (Figure 5B), for example, has none of the resonances characteristic of free reducing termini (α, β), indicating it was fully derivatized; similar results were obtained for 2AA-labeled 6-, 8-, and 10-mers. Few resonances are clearly resolved in the 1D spectra, however those corresponding to the core of the labeled HA oligosaccharides that could be resolved were essentially unperturbed. For example, the methyl (Me) and amide (ο) protons from the internal GlcNAc ring (only two residues from the 2AA-labeled ring) are found at identical chemical shifts in both free and derivatized HA4 (Figure 5). As expected, differences are observed in the 2AA-labeled ring; the methyl (Me') and amide (S) protons, for instance, are slightly perturbed (Figure 5A). These observations suggest that incorporation of the 2AA-label into HA oligosaccharides produces only minor and local conformational changes.

Binding assays with the human recombinant VG1 were performed to determine whether the 2AA-label affected the biological activity of the oligosaccharides. Both excess HA10 and 2AA-labeled HA10 competed similarly for VG1 binding to biotinylated polymeric HA (Figure 6). This is consistent with HA10 being the shortest length oligosaccharide capable of efficiently competing for the binding of polymeric HA to VG1, also reported for
human hyaluronectin, a proteolytic product of versican (Bertrand and Delpech, 1985). The addition of the 2AA moiety to the reducing end termini of HA does not, therefore, significantly interfere with its protein-binding activity.

An EMSA was used to further investigate binding between VG1 and HA oligosaccharides of defined lengths. In EMSA studies, it is common to use radiolabeled ligands (e.g., $^{35}$S, $^{32}$P) to probe interactions, as seen recently in investigations of the binding between heparin and antithrombin III (Wu et al., 2002). However, in this case, given the lack of sulfate or phosphate groups on HA, a fluorescent EMSA technique was favored, which has also been used to study heparin–protein interactions (Lyon et al., 2004). Careful considerations (low percentage acrylamide gels, low voltage [40 V], and low temperature [$4^\circ$C]) were taken to ensure that electrophoresis conditions did not promote dissociation of the oligosaccharide from the protein.

In EMSA experiments, VG1 retards the fluorescent oligosaccharide when bound to it, resulting in a band shift from the free sugar position; the charge on the fluorophore ensures that a good separation was achieved. As shown in Figure 7A, an increasing amount of VG1 (0–10.7 μM) was added to 2AA-labeled HA 8-, 10-, 20-, 30-, and 40-mers (all at 2.2 μM). For labeled HA8, no band shift is seen in the presence of 0.2–3.6 μM VG1 relative to the control lacking VG1. However, smearing of the free oligosaccharide toward the top of the gel at higher concentrations of VG1 (5.4–10.7 μM) suggests the sugar is binding very weakly to the protein under the conditions analyzed here. In contrast, a clear band shift indicating stable binding is observed with labeled HA10 in the presence of >1.8 μM VG1. Therefore, both free and bound fluorescent bands were visible; moreover, Coomassie blue staining of the gel

![Fig. 6.](https://academic.oup.com/glycob/article-abstract/15/3/303/574725)

**Fig. 6.** The biological activity of labeled HA oligosaccharides was assessed by the binding of biotinylated polymeric HA (12.5 ng/well) to the VG1-coated plates in the presence or absence of competing unlabeled polymeric HA (pHA), HA10, and 2AA-derivatized HA10 (HA10/2AA) oligosaccharides. Values are plotted as mean percentages of binding in absence of competing HA for VG1 after a 10-min development time (error corresponding to the SD of four measurements).

**Fig. 7.** (A) Increasing concentrations of VG1 were mixed with a constant concentration (2.2 μM) of fluorescently labeled HA 8-, 10-, 20-, 30-, and 40-mers and analyzed after EMSA. (B) In the presence of labeled HA10, VG1 protein bands (detected with Coomassie blue staining) colocalized with the bound fluorescent HA10 bands observed in (A). The fraction of the free oligosaccharide ($\theta$) for labeled 10-, 20-, 30-, and 40-mers was calculated by densitometry analysis after EMSA and plotted against the total VG1 concentration (C). Each fluorescent band was measured three independent times to estimate the standard error. A theoretical curve (Theory) representing a $K_d$ of 100 nM for binding of VG1 to HA10 is also shown. Linear interpolation allowed the values of total protein corresponding to a $\theta$ value of 0.5 to be calculated. The values of 1.2, 2.3, 3.8, and 4.9 μM were recorded for HA 10-, 20-, 30-, and 40-mers, respectively.
well established for VG1. Stoichiometry and cooperativity, which are presently not performed here, and is quadratic in the variable equation can be used to model the EMSA experiment as Equation 4, which can now be used to relate the amount of bound protein is calculated from the total protein concentration when equimolar amounts of labeled HA10 and VG1 were mixed compared to controls lacking protein (see Data Supplement online). As a consequence of VG1 quenching the bound HA, only the free oligosaccharide could be taken into consideration on gel densitometry analysis. Between 0.9 and 1.8 μM of total VG1, the concentration of free HA dropped to around 50% of its maximum value, indicating that the interaction between protein and sugar was near equilibrium.

Assuming a 1:1 stoichiometry between VG1 and HA10 and equilibrium kinetics (i.e., $H + P \leftrightarrow HP$, where $H$ represents the HA oligosaccharide and $P$ the protein), Equation 1 can be derived from the standard equations for the equilibrium dissociation constant $K_d$ (Price and Dwek, 1979). If $\theta$ is the fraction of free HA, as obtained from the EMSA experiment by densitometry (Figures 7A and 7C), then $r$ can be defined as the fraction of bound HA, as shown in Equation 2, where $H_t$ is the total concentration of HA.

$$r = \frac{[P]}{K_d + [P]}$$

$$\theta = (1 - 0) \equiv \frac{[HP]}{H_t}$$

Use of Equation 2 allows the amount of free protein to be calculated from the total protein concentration $P_t$, Equation 3. Substitution of Equation 3 into Equation 1 results in Equation 4, which can now be used to relate the amount of free HA, total protein, and the $K_d$ to each other. Thus, the equation can be used to model the EMSA experiment as performed here, and is quadratic in the variable $r$.

$$[P] = P_t - rH_t$$

$$r = \frac{(P_t - rH_t)}{(K_d + P_t - rH_t)}$$

Equation 4 was fitted to the experimentally derived EMSA data for HA10 to determine the $K_d$, because $P_t$, $H_t$, and $r$ are known at each point. It can be seen that the experimental and theoretical curves are in good agreement with one another (Figure 7C) and yield a $K_d$ value of 100 nM for the interaction of HA10 with VG1 under the conditions used here.

Experiments with longer HA oligosaccharides (i.e., HA 20-, 30-, and 40-mers), which are likely to bind multiple VG1 molecules (Courel et al., 2002), also gave rise to clear shifts in HA mobility, as shown in Figures 7A and 7C. However, interpretation of this data (and hence calculation of $K_d$ values) is difficult without some knowledge of the stoichiometry and cooperativity, which are presently not well established for VG1.

Discussion

Labeling of HA oligosaccharides with the small fluorescent tag 2AA not only maintained their biological activity but also greatly facilitated their purification by enhancing the UV absorbance between 200 and 220 nm. In combination with a logarithmic gradient, as used previously for the purification of oligonucleotides (Tashlitskil and Oretskaya, 1997), this allowed the separation of homogeneous even-numbered HA oligosaccharides at least up to 40-mers and perhaps longer. In addition, 2AA-labeled HA oligosaccharides gave more intense signals in MALDI-TOF MS, as has been reported previously for 2AA-labeled chicken ovalbumin oligosaccharides (Anumula and Dhume, 1998). This enhancement is to be expected because the MALDI apparatus used here employs a UV laser to cause ionization, and matrix compounds themselves are selected to be good UV absorbers. Therefore, 2AA, which has been used as a MALDI comatrix for DNA (Zhang and Gross, 2000) in its own right, is a UV absorber and may be directly ionized. Various other fluorophores, including AMAC, have also been reported to increase glycan ionization in MALDI-TOF experiments following derivatization (Harvey, 2003).

The negative charge on 2AA facilitates separation of the HA oligosaccharides as a function of their length and charge during electrophoresis. Consequently, labeled oligosaccharides were observed to migrate a distance inversely proportional to their length, without any borate in the running buffer or a stacking gel, as previously used for HA oligosaccharides derivatized with the neutral fluorophore AMAC (Calabro et al., 2000a; Karousou et al., 2004). Therefore, like ANTS, labeling HA oligosaccharides with 2AA avoids problematic migration (parabolic inversion) under electrophoresis (Tawada et al., 2002; Volpi, 2003).

Crucially, the 2AA label was not observed to disrupt the biological activity of HA oligosaccharides in the assays performed, which is interesting considering that reductive amination breaks the integrity of the reducing terminal GlcNAc ring. This is likely to be due to the fact that 2AA has a 3D structure very similar to D-glucuronic acid, and has a carboxylic acid in the same relative position as in HA, compensating for the breakage of the terminal sugar ring. However, it may also be that only nine sugars are required for the interaction of HA with VG1. Thus, 2AA-labeled HA oligosaccharide can be used in fluorescence-based assays for investigation of HA–protein interactions, as we have demonstrated with EMSA.

A drawback of the EMSA approach used in this study is the inability to measure the free protein concentration, or the amount of bound oligosaccharide because of fluorescence quenching (in contrast to radiolabeled experiments; Wu et al., 2002). Recent structural work has shown that the binding groove of the Link module of TSG-6 contains three tyrosine residues, and sequence alignments indicate this is also likely to be the case for other Link modules, including those of versican (Blundell et al., 2003). It is proposed that these amino acids, with delocalized electrons, may be responsible for quenching the fluorescence of 2AA. Therefore, it is apparent that fluorescence quenching could have a wide application for studying the kinetics of HA–protein interactions.
A $K_d$ of 100 nM was derived for the interaction of VG1 to HA$_{40}$ by theoretical analysis of EMSA results. This is six times lower than the $K_d$ (~17 nM) reported for VG1 binding to polymeric HA by surface plasmon resonance (Matsumoto et al., 2003; Shi et al., 2004). Although these techniques are dissimilar and the two measurements were performed under different conditions (salt strength, pH, and temperature), this may nevertheless explain observations from microtiter plate assays, where HA$_{10}$ is a less efficient inhibitor than polymeric HA for binding to VG1 (Figure 6), suggesting that the affinity of VG1 for HA may increase with molecular weight of the sugar. It has also not escaped our attention that for HA 20-, 30-, and 40-mers, two, three, and four times the respective amount of total VG1 was needed to retard 50% of the oligosaccharide compared to HA$_{40}$. Figure 7C (the minimum size even oligosaccharide that can accommodate the protein). However, only a single bound protein is required per HA molecule to retard it in the EMSA assay. Therefore, if HA$_{20}$ has more independent protein binding sites than HA$_{40}$ (Courel et al., 2002), and the oligosaccharides are at the same molar concentration, then HA$_{20}$ would require less protein to cause an equilibrium dissociation. Thus, our data appear inconsistent with site-independent protein binding to a multivalent HA oligosaccharide. Although it is concluded that binding is nonindependent, and hence displays some form of cooperativity, further work is required to establish the exact nature of this. Comparison with EMSA experiments using other members of the Link module superfamily, where the binding is better characterized (such as TSG-6 or aggrecan) may be very revealing in this regard.

In conclusion, we have developed a facile protocol for fluorescent labeling and separation of HA oligosaccharides that does not perturb their biological function. This methodology will allow a range of fluorescence-based assays to be developed for the analysis of HA–protein interactions. This technique may also be used to label other members of the GAG family and, in combination with EMSA, provide further insight into protein–carbohydrate interactions.

Materials and methods

Materials

Medical-grade HA was purchased from Genzyme (Cambridge, MA). Human recombinant VG1 was expressed in Drosophila S2 cells and purified to homogeneity by a combination of ion-exchange chromatography and reverse phase high-performance liquid chromatography (Seyfried et al., in preparation) and total concentrations determined by amino acid analysis as described previously (Mahoney et al., 2001b). Ovine testicular hyaluronidase was purchased from Calbiochem (Nottingham, U.K.), where 1 U is defined as the amount of enzyme that will cause the same turbidity reduction as 1.0 U of International Standard preparation. The 2AA and sodium cyanoborohydride were purchased from Sigma Aldrich (Poole, U.K.), and the ammonium acetate from Fluka Chemicals (Poole, U.K.). Polyacrylamide gels were made by dilution of ProtoFlowGel stock solution (30% w/v acrylamide, 0.8% w/v methylene bisacrylamide), purchased from Flowgen (Nottingham, U.K.).

HA digestion and fluorescent labeling

HA was dissolved at a concentration of 1 mg/ml in digest buffer (100 mM sodium acetate, 150 mM NaCl, adjusted to pH 5.2 with glacial acetic acid) and preincubated for 10 min at 37°C. To this, ovine testicular hyaluronidase (1 U/µl in reaction buffer) was added to give a final concentration of 10 U enzyme per mg HA and the mixture was incubated for 1 h at 37°C. The reaction was terminated by boiling for 10 min and centrifugation to remove denatured enzyme. Oligosaccharides in the resultant digest mixture were then derivatized with 2AA by reductive amination at the reducing end sugar essentially as described by Neville et al. (2004). Briefly, a 5 ml stock solution of 2AA (150 mg) and sodium cyanoborohydride (225 mg) dissolved in 2% (v/v) acetic acid in methanol was prepared and added to the HA oligosaccharides at a 3:1 (v/v) ratio in microcentrifuge tubes. Samples were heated (80°C) for 45 min to complete the reaction. Excess 2AA was removed by running samples over a Sephadex G-10 desalting column (Amersham Biosciences, Little Chalfont, U.K.) on an AKTA 900 FPLC system (Amersham Biosciences) in 50 mM ammonium acetate and then lyophilized repeatedly to remove residual acetyl buffer. Purified HA 4-, 6-, 8-, and 10-mers, prepared as described in Blundell et al. (2004), were also individually labeled.

Anion-exchange chromatography

Lyophilized, labeled HA digests were resuspended in water and purified on a Mono-Q 5/50 GL anion-exchange column (Amersham Biosciences) using an AKTA 950 FPLC system (Amersham Biosciences) with UNICORN software. The column was equilibrated in Milli-Q water (buffer A) at a flow rate of 2 ml/min. Labeled HA oligosaccharides (2 ml at ~1 mg/ml in water) were loaded onto the column and the initial conditions (100% buffer A) were maintained for 5 min. Oligosaccharides were then eluted with buffer B (500 mM NaCl) using a logarithmic gradient described by the equation %B = 8.4 * log$_t$(1.0 + 0.45t), where $t$ represents time in min. The fractions were monitored at both 215 nm and 330 nm, respectively, the latter being a wavelength specific for 2AA (Ito et al. 1998). A 2AA-labeled 10-mer (produced and characterized as described in Blundell et al., 2004) was used to calibrate peak elution positions. Fractions of each length oligosaccharide were pooled and desalted into 50 mM ammonium acetate over a G-10 Sephadex column, lyophilized, and resuspended in Milli-Q water. An additional anion-exchange chromatography step was performed to ensure homogeneity prior to EMSA. Each peak fraction was then collected, lyophilized and desalted as described. Oligosaccharide fractions were finally lyophilized repeatedly from Milli-Q water to remove any residual ammonium acetate.

MALDI-TOF MS

Purified 2AA-labeled HA oligosaccharides were analyzed using an Etta MALDI-TOF Pro Version 2.0 mass spectrometer (Amersham Biosciences). Shorter oligosaccharides (4–10-mers) were analyzed in negative-ion reflectron mode with a 20 kV potential. Briefly, equal mass amounts of labeled oligosaccharide sample solutions...
in water were diluted 1:100 (v/v) with water and mixed 1:1 (v/v) with 2.5 mg/ml 2,5-DHB as described previously by Blundell et al. (2004). Solutions for external calibration were composed of a mixture of adrenocorticotropic hormone fragment 18–39 ([M-H]− = 2463.20 Da) and angiotensin II ([M-H]− = 1044.54 Da) in 50% [v/v] acetonitrile, 0.5% [v/v] trifluoracetic acid. A ladder of longer oligosaccharides (12–36-mers) was analyzed in negative-ion linear mode with a 20 kV potential and a low mass rejection of 2000 m/z. Oligosaccharide samples were mixed from water 1:1 (v/v) with saturated 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) in 50% (v/v) acetonitrile, 0.5% (v/v) trifluoracetic acid. Solutions for external calibration were composed of a mixture of adrenocorticotropic hormone and oxidized insulin ([M-H]− = 3492.65). Samples of labeled HA 38- and 40-mers were analyzed individually.

To assess the sensitivity on MALDI-TOF MS for 2AA-labeled HA 38- and 40-mers were analyzed individually. A ladder of longer oligosaccharides, a mixture of unlabeled HA10 and HA10-2AA. Plates were washed three times; 200 µl of a 1:10,000 dilution of ExtraAvidin alkaline phosphatase (Sigma Aldrich) was added and incubation for 30 min at room temperature was allowed. After three more washes, wells were incubated for 10 min with 200 µl of a 1 mg/ml solution of disodium p-nitrophenyl phosphate (Sigma Aldrich) in 100 mM Tris–HCl, 100 mM NaCl, 5 mM MgCl2, pH 9.3. The absorbance at 405 nm was determined and corrected by subtracting values from the uncoated control wells.

**FACE**

Purified 2AA-labeled oligosaccharides were diluted from water with 50% (v/v) glycerol to give a final concentration of 16% (v/v) glycerol. Native polyacrylamide, 27% (w/v), 0.72% (w/v) methylene bisacrylamide gels (without stacking gel) were made up in the running buffer: 192 mM glycine, 25 mM Tris, pH 8.4. Approximately equimolar amounts of each labeled oligosaccharide and the digested ladder were loaded and run at 300 V, 4°C for 3 h using a Protean gel apparatus (BioRad, Hemel Hempstead, U.K.) employing a constant cooled water pump system.

**NMR spectroscopy**

Samples for NMR (2AA-labeled HA 4-, 6-, 8-, and 10-mers) were prepared from lyophilized material reconstituted in 10% (v/v) D2O and adjusted to pH 6.0 with 0.1 M HCl and NaOH. A 1 mM sample of 2AA was also prepared. Homonuclear 1D spectra were recorded on these samples at 750 MHz, 25°C with an acquisition time of 113.66 ms over 1024 complex points. All spectra were referenced externally to the water resonance at 4.765 ppm downfield of 2,2-dimethyl-2-silapentane-5-sulphonate (DSS). Concentrations of oligosaccharides were measured by comparing the integrated peak intensities from the aromatic ring protons at 7.7 ppm and 7.3 ppm in the 1D spectra from each sample with that of the 1 mM 2AA sample.

**HA binding assays**

The HA-binding properties of VG1 were examined using a microtiter plate-based assay essentially as described in Mahoney et al. (2001b), Maxisorp F96 plates (Nunc, Loughborough, U.K.) were coated overnight with 1.2 pmol VG1 in 200 µl (final volume) of 20 mM Na2CO3, pH 9.6; this concentration was shown to be the minimum that gives maximal binding to (12.5 ng/well) biotinylated polymeric HA. All dilutions, incubations and washes were performed in 50 mM HEPES, 100 mM NaCl, 0.05% (v/v) Tween-20, pH 7.4. The coating solution was removed and the plates washed three times. Nonspecific binding sites were blocked by incubation with 1% (w/v) bovine serum albumin for 90 min at 37°C followed by three more washes. Plates were then incubated at room temperature for 4 h with 12.5 ng/well biotinylated HA (prepared from rooster comb as described in Mahoney et al., 2001b) in the absence or presence of unlabeled polymeric HA (pHA), HA10, or HA10-2AA. Plates were washed three times; 200 µl of a 1:10,000 dilution of ExtraAvidin alkaline phosphatase (Sigma Aldrich) was added and incubation for 30 min at room temperature was allowed. After three more washes, wells were incubated for 10 min with 200 µl of a 1 mg/ml solution of disodium p-nitrophenyl phosphate (Sigma Aldrich) in 100 mM Tris–HCl, 100 mM NaCl, 5 mM MgCl2, pH 9.3. The absorbance at 405 nm was determined and corrected by subtracting values from the uncoated control wells.

**Electrophoretic mobility shift assay (EMSA)**

Samples were made up by adding 18 µl of HA oligosaccharides (of known concentration) in 50 mM sodium acetate, 1 mM ethylenediamine tetra-acetic acid (EDTA), pH 5.8, to 2 µl of VG1 (variable concentrations) in 4 M guanidine-HCl, 50 mM sodium acetate, 1 mM EDTA, pH 5.8, and incubated at room temperature for 1 h at this concentration of guanidine-HCl (i.e., 400 mM), which has been reported to be associative for proteoglycan–HA interactions (Bonnet et al., 1985). Prior to loading, 8 µl 50% (v/v) glycerol was added to reach the final concentrations of 2.2 µM for all HA oligosaccharides and concentrations of VG1 shown in Figure 7A. Native 4.5% (w/v) polyacrylamide, 0.12% (w/v) methylene bisacrylamide gels (7 cm x 8 cm) were prepared without a stacking gel, containing 10% glycerol (v/v) and 1 M Tris (pH 8.45). Samples were loaded into separate lanes and electrophoresed (Miniprotean II, BioRad) for 40 min at 40 V and 4°C, using a 100 mM Tris, 100 mM tricine (pH 8.6) running buffer. Visualization of the gel is described below. Gels were stained with 1% (w/v) Coomassie brilliant blue R (Sigma Aldrich) 40% (v/v) ethanol, and 10% (v/v) acetic acid to detect protein after UV visualization.

**Fluorimetry**

For fluorimetry experiments, samples of 2AA-labeled HA10 (HA10-2AA), alone or mixed with equimolar amounts of VG1 (VG1+HA10-2AA) were diluted into associative buffer conditions as described for the EMSA. The samples were incubated at room temperature for 1 h and emission scans (200–550 nm) recorded using a Jasco FP-777 Spectrofluorometer (excitation 330 nm). Fluorescent intensity between 325–475 nm, spanning the maximum emission range for 2AA (Ito et al., 1998), was calculated by subtracting recorded control values (buffer or VG1 alone) from HA10-2AA and VG1+HA10-2AA values, respectively.

**Gel imaging and densitometry**

Gels were visualized using a UV transilluminator (Uvi Tec, Cambridge, U.K.) fitted with a 430 nm bandpass blue filter (Andover, NH) over the camera. A UG1 glass plate (H.V. Skan, Solihull, U.K.) was placed over the UV lamp and under the gel to block visible light (essentially as described...
by Turnbull et al., 1999; Drummond et al., 2001). The aperture was adjusted to avoid saturating the camera charged coupling device and multiple images were recorded with different exposure times; the negative image is displayed for clarity. Densitometry analysis was performed using the Scion Image software (Scion, MD). The concentration of 2AA-labeled HA 20-, 30-, and 40-mer stocks were determined by densitometry after FACE analysis. Briefly, equivalent volumes (1 µl) of HA10/2AA control (0.125 µg/µl) and labeled oligosaccharide stocks (20-, 30-, and 40- mers) of unknown concentration were run on a 27% (w/v) acrylamide, 0.72% (w/v) methylene bisacrylamide gel (7 cm × 8 cm) for 2 h at 200 V with the same gel and running buffer conditions as those described in FACE. Total fluorescence (Tf) was calculated using the formula

\[ T_f = (\text{band area}) \times (\text{band intensity} – \text{background intensity}) \]

The Tf ratio of 2AA-labeled HA10 compared to labeled 20-, 30-, or 40-mers was then used to quantify and normalize stock solutions.

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Abbreviations

2AA, 2-amino benzoic acid; AMAC, 2-aminoacridone; ANTS, 8-amino-1,3,6-naphthalene trisulfonic acid; CSPG, chondroitin sulfate proteoglycan; DHB, dihydroxybenzoic acid; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; EDTA, ethylenediamine tetra-acetic acid; EMSA, electromophoretic mobility shift assay; FACE, fluorophore-assisted carbohydrate electrophoresis; GAG, glycosaminoglycan; HA, hyaluronan; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; NMR, nuclear magnetic resonance; TSG-6, protein product of tumor necrosis factor stimulated gene-6; VG1, G1 domain of versican.

References


Mahoney, D.J., Aplin, R.T., Calabro, A., Hascall, V.C., and Day, A.J.
Mahoney, D.J., Blundell, C.D., and Day, A.J. (2001b) Mapping the
Kohda, D., Morton, C.J., Parkar, A.A., Hatanaka, H., Inagaki, F.M.,
Shriver, Z., Raman, R., Venkataraman, G., Drummond, K., Turnbull, J.,
Sievie, N.T. Seyfried et al.
Kohda, D., Morton, C.J., Parkar, A.A., Hatanaka, H., Inagaki, F.M.,
module: a hyaluronan-binding domain involved in extracellular matrix
for the analysis of protein-nucleic acid interactions. *Microbiol. Rev.*, 56,
Laurent, T.C. and Fraser, J.R. (1992) Hyaluronan. *FASEB J.*, 6,
2397–2404.
and its NK1 and NK2 variants with glycosaminoglycans using a
modified gel mobility shift assay: elucidation of the minimal size
of binding and activatory oligosaccharides. *J. Biol. Chem.*, 279,
43560–43567.
Mahoney, D.J., Aplin, R.T., Calabro, A., Hascall, V.C., and Day, A.J.
(2001a) Novel methods for the preparation and characterization of
hyaluronan oligosaccharides of defined length. *Glycobiology*, 11,
1025–1033.
Mahoney, D.J., Blundell, C.D., and Day, A.J. (2001b) Mapping the
hyaluronan-binding site on the link module from human tumor
necrosis factor-stimulated gene-6 by site-directed mutagenesis. *J. Biol.
Mahoney, D.J., Whittle, J.D., Milner, C.M., Clark, S.J., Mulloy, B.,
for the non-covalent immobilization of heparin to surfaces. *Anal.
Biochem.*, 330, 123–129.
Matsumoto, K., Shionyu, M., Go, M., Shimizu, K., Shinomura, T.,
with hyaluronan and link protein. *J. Biol. Chem.*, 278,
41205–41212.
Nebille, D.C., Coquard, V., Priestman, D.A., Te Vruchte, D.J.,
Analysis of fluorescently labeled glycosphingolipid-derived oligosac-
charides following ceramide glycanase digestion and anthranilic acid
Hyaluronan fragments activate an NF-kappa B/I-kappa B alpha
autoregulatory loop in murine macrophages. *J. Exp. Med.*, 183,
2373–2378.
of human TSG-6 mediate binding to hyaluronan and chondroitin-4-
Proteoglycans of bovine articular cartilage. Studies of the direct
interaction of link protein with hyaluronate in the absence of
monosaccharides derivatized with 2-aminoenzoic acid by capillary
Shi, S., Groteh, S., Zhang, Y., O’Connor-McCourt, M.D., Poole, A.R.,
Roughley, P.J., and Mort, J.S. (2004) Link protein has greater affinity
Slevin, M., Kumar, S., and Gaffney, J. (2002) Angiogenic oligosaccharides
of hyaluronan induce multiple signaling pathways affecting vascular
endothelial cell mitogenic and wound healing responses. *J. Biol.
link protein gene family whose members are physically adjacent
to chondroitin sulfate proteoglycan core protein genes: the missing
Fluorophore-assisted carbohydrate electrophoresis in the separation,
analysis, and sequencing of carbohydrates. *J. Chromatogr. A*, 720,
295–321.
Tawada, A., Masa, T., Oonuki, Y., Watanabe, A., Matsuizaki, Y., and
Asari, A. (2002) Large-scale preparation, purification, and character-
ization of hyaluronan oligosaccharides from 4-mers to 52-mers.
*Glycobiology*, 12, 421–426.
Terite, P., Banerji, S., Noble, M., Blundell, C.D., Wright, A.J.,
Pickford, A.R., Lowe, E., Mahoney, D.J., Tammi, M.I.,
Kahmann, J.D., and others. (2004) Structure of the regulatory
hyaluronan binding domain in the inflammatory leukocyte homing
Toole, B.P. (2004) Hyaluronan: from extracellular glue to pericellular
rapid sequencing of heparan sulfate and heparin saccharides. *Proc.
Volpi, N. (2003) Milligram-scale preparation and purification of
oligosaccharides of defined length possessing the structure of
chondroitin from defructosylated capsular polysaccharide K4. *Glyco-
rat 175-kDa hyaluronan receptor for endocytosis (HARE). *J. Biol.
Whitham, K.M., Hadley, J.L., Morris, H.G., Andrew, S.M.,
method for the structural profiling of keratan sulfates: analysis
of keratan sulfates from brain and ovarian tumors. *Glycobiology*, 9,
Wight, T.N. and Merrilees, M.J. (2004) Proteoglycans in athero-
sclerosis and restenosis: key roles for versican. *Circ. Res.*, 94,
1158–1167.
(2002) A new strategy for defining critical functional groups on
tion mass spectrometry methods for oligodeoxynucleotides: improve-
ments in matrix, detection limits, quantification, and sequenc-