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Heparan sulfate phage display antibodies recognise epitopes defined by a combination of sugar sequence and cation binding

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Phage display antibodies are widely used to follow heparan sulfate (HS) expression in tissues and cells. We demonstrate by ELISA, that cations alter phage display antibody binding profiles to HS and this is mediated by changes in polysaccharide conformation, demonstrated by circular dichroism spectroscopy. Native HS structures, expressed on the cell surfaces of neuroblastoma and fibroblast cells, also exhibited altered antibody binding profiles following exposure to low mM concentrations of these cations. Phage display antibodies recognise conformationally-defined HS epitopes, rather than sequence alone, as has been assumed, and resemble proteins in being sensitive to changes in both charge distribution and conformation following binding of cations to HS polysaccharides.

1. Introduction

Heparan sulfate (HS) is a complex, naturally occurring, anionic linear polysaccharide expressed on the cell surface of practically all mammalian cells and in the extracellular matrix (ECM). It has been implicated in a wide range of cellular processes, ranging from the promotion of cell signalling through the fibroblast growth factor-receptor (FGF-FGFR) tyrosine kinase pathway[1] and Wnt signalling, to interactions with proteins such as β-secretase-1 (BACE-1) and amyloid precursor protein (APP), thought to be involved in Alzheimer's disease,[2,3] as well as in the attachment of pathogenic microorganisms to host cells.[4–10]

HS is composed of 1,4 linked uronic acid residues (either β-D-GlcA or its C-5 epimer, α-L-IdoA) alternating with α-D-GlcN. The uronic acid can be 2-*O*-sulfated, while the glucosamine bears a variety of substituents, which include 6-*O*-sulfate, *N*-acetyl, *N*-sulfate and, more rarely, 3-*O*-sulfate groups. It was first thought that the activities of HS would correlate with the linear sequences of sugar residues but, the technical difficulties associated with their extraction from tissue samples and subsequent separation made direct exploration of this possibility infeasible. Owing to the poor antigenicity of HS, a library of semi-synthetic phage display antibodies was then applied, with the aim of binding HS selectively.[11] At its inception, the selectivity of the antibodies for HS was demonstrated among a series of related glycosaminoglycans (GAGs), which included HS as well as chondroitin sulfate (CS), hyaluronic acid (HA) and the K-5 polysaccharide from *E.coli*. The utility of the phage display library was demonstrated by labelling native HS structures in kidney[11] and, since then, the library has become a widely used tool in glycobiology, amply demonstrating its ability to reveal HS expression patterns[12] in many tissues, including rat spleen,[13] human kidney[14] and developing rat lungs.[15]

Several studies employing a range of both naturally occurring and chemically modified HS structures and analogues have attempted to delineate those HS sequences required for binding to individual phage display antibodies and some broad correlations have been determined. For example, the antibody HS4C3 has been reported as requiring *O*- and *N*-sulfate groups.[12] It has been assumed generally, either tacitly or explicitly, that the selectivity of the phage display antibodies arises predominantly through direct recognition by the peptide in the variable region, of negatively charged *O*-, *N*-sulfates and carboxylic acid groups of the HS, which amounts to recognition of a particular sequence. However, it has recently been realised that binding may be more complex; individual antibodies recognising multiple HS structures.[16] This is consistent with the suggestion that a high degree of conformation-

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al redundancy exists in HS sequences, in terms of the glycosidic linkage and uronate geometry.[17] The consequences of this are that it is possible for several distinct sequences to provide conformational and charge features capable of being recognised and bound. Although HS binding to cations is a well-known phenomenon,[18–20] it is only relatively recently that the potential for cation binding by HS polysaccharides to drastically alter the biological activity of the polysaccharide, for example in cell signaling,[21,22] has become apparent. Following these observations, which demonstrated that cation binding by HS alters both the conformation and protein recognition characteristics of the polysaccharide, the possibility that the binding of cations to HS structures can also alter phage display antibody binding was investigated. Phage display antibodies HS3B7V[12] and HS4E4V[23] were both raised originally against bovine kidney HS and were selected for several reasons. They both recognised HS but by distinct epitopes, and their CDR-3 sequences, considered to be the major determinants of specificity, were very different, comprising SRKTRKPFMRK (6 basic residues out of 11) and HAPLRNTRTNT (2 basic residues plus histidine out of 11), respectively.[16]

2. Materials and methods

2.1 Biotinylation of chemically modified heparins and enzyme-linked immunosorbent assay (ELISA)

50 μL of 50 mM *N*-hydroxysuccinimide amino caproate (LC) biotin (NHS-LC biotin in DMSO) was added to 5 mg modified heparin polysaccharide in water [bearing a small proportion of free amine groups] and allowed to react overnight at room temperature. Biotinylated polysaccharides were then separated from the unconjugated biotin by dialysis (membrane, 3 kDa cut-off), freeze-dried and re-suspended in dH_2O. Maxisorp 96-well microtiter plates (Nunc; Roskilde, Denmark) were coated (18 h, 4 °C) with 3 μg ml^{-1} streptavidin (Promega Ltd, Southampton, UK) in 0.1 M Na_2CO_3/HCO_3 (pH 9.6) and blocked with 1% (w/v) bovine serum albumin (BSA) in PBS (2 h, RT). After washing with PBS, plates were incubated with the NHS-biotinylated chemically modified heparin for 2 h. These were HS polysaccharide analogues A and B containing both natural and unnatural residues with predominant repeating disaccharides IdoA2OH-GlcNS,6S and IdoA2S,3S-GlcNS,3S,6S respectively. This was followed by an overnight incubation with various cation preparations ($CaCl_2$, $CuCl_2$, $ZnCl_2$, $MgCl_2$, KCl and NaCl at 100 mM, 10 mM and 1 mM; diluted in dH_2O). Plates were washed in PBS and incubated with the relevant phage display antibody. The anti-VSV-G IgG, clone P5D4 (Sigma-Aldrich, UK; 1 : 500 dilution) was applied (1 h, RT) after washing. HRP conjugated sheep anti-mouse IgG (1 : 1000) was added after PBS washing (1 h). The reaction was visualized by the addition of 50 μL *O*-phenylenediamine dihydrochloride (OPD), the reaction stopped with 100 μl H_2SO_4 (0.5 M) and the absorption measured at 490 nm. Biotinylated porcine mucosal heparan sulfate and the omission of cations, omission of the primary antibody and the anti-VSV tag antibody were used as controls and all behaved as expected (results not shown). The presence of cations on the polysaccharides prior to the experiment or, introduced at any subsequent steps, and any possible residual biotin in the polysaccharide preparations are not responsible for differences in phage display antibody binding because, if they are present, they are common to all experimental cases.

2.2 Cell culture and immunofluorescence

The neuroblastoma cell line SH-SY5Y (#CRL 2266; ATCC, Rockville, USA) was cultured in RPMI-1640 medium with 10% (v/v) heat-inactivated foetal calf serum (FCS) and 1% non-essential amino acids (NEAA) in a 37 °C incubator containing 5% CO_2/95% humidified air. Cells were plated at approximately 10^5 cells ml^{-1} on a 24 well cell culture plate, each containing an 8 mm round glass cover slip and grown for 24 h. Cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 min, rinsed in PBS and incubated overnight at 4 °C with various cation solutions (section 2.1). The control cells were incubated with PBS. After washing, cells were incubated in 10% (v/v) goat serum in PBS (1 h) followed by an overnight incubation at 4 °C with the relevant phage display antibody diluted 1/10 (v/v). Bound antibody was detected using rabbit VSV-G tag antibody (Sigma-Aldrich, UK) diluted 1 : 200 in 1% (v/v) goat serum in PBS. Alexa Fluor-488 conjugated goat anti-rabbit IgG (Invitrogen, UK; 1 : 500 dilution) was incubated for 1 h. Cells were washed with PBS and mounted in a fluorescent mounting medium (Dako, Ely, UK). Negative controls included omission of the phage display antibody against HS and digestion of HS with heparinase I, II and III to remove the HS epitopes prior to the antibody incubation, which were as expected (results not shown). The samples were examined under a confocal laser scanning microscope (TCS SPE, Leica, UK) with excitation at 488 nm and UV for DAPI. As in the ELISA experiment above (section 2.1), any cations that may have been introduced during washing and incubation stages were not responsible for any differences observed because they were common to all experiments.

2.3 BaF assay of cell signalling through FGFR1c

BaF cells (a murine, IL-3 dependent mouse pro-B cell line) are a useful model system with which to investigate the competence of an exogenous polysaccharide to support signalling through a single fibroblast growth factor receptor (FGFR) isoform and a single exogenously added fibroblast growth factor (FGF).[24] BaF cells (10^4 per well), transfected with a chosen FGFR (FGFR1c) were incubated as described previously[21,22] with polysaccharides (1 ng ml^{-1} to 10 μg ml^{-1}) in the chosen cation form. Briefly, cells were incubated (37 °C, 72 h), after which, 5 μL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (5 mg ml^{-1} in PBS) was added followed by a further 4 h incubation. Cells were then solubilized [10% SDS, 0.1 N HCl] and the absorbance at 570 nm was measured.

2.4 Detection of conformational change in polysaccharides using circular dichroism spectroscopy

Circular dichroism spectroscopy (CD) is a technique that is sensitive to conformational changes (particularly the environment of the carboxylate groups of the uronic acids) in GAG polysaccharides in solution and measures the differential absorbance of right and left-circularly polarised light. CD spectra were recorded on a purpose-built synchrotron CD beam line employing a quartz sample cell of 0.02 cm path length and 1 nm resolution. Spectra are presented as molar circular dichroism (per mole per cm) and were corrected for differences in molecular weight according to composition analysis.[25,26]

2.5 Characterisation of polysaccharides A and B

Polysaccharides A and B were prepared as described (ref. 28 and 27 respectively) from porcine mucosal heparin (Celsus Labs, Cincinnati, OH, USA) and purified by size-exclusion chromatography (Sephadex G-25, recovering only the exclusion limit, M_w > 5 kDa). Major disaccharides in polysaccharide A (%) were detected following exhaustive digestion, fluorescence labelling with BODIPY hydrazide and HPAEC analysis with reference to authentic standards (ProPac PA-1 column, Dionex UK) as ΔUA-GlcNAc 31.7, ΔUA-GlcNS 23.7, ΔUA-GlcNS(6S) 44.6.[26] Polysaccharides A and B were also characterised by NMR spectroscopy (D_2O, 40 °C, chemical shift values recorded relative to trimethylsilyl propionate as standard.[27,28] Polysaccharide A: ^{1}H NMR(ppm): (glucosamine) H-1 5.34, H-2 3.26, H-3 3.65, H-4 3.71 H-5 4.02, H-6,6′ 4.36–4.23 (iduronate) H-1 5.04, H-2 3.78, H-3 4.12, H-4 4.08, H-5 4.84. 13C NMR (ppm): (glucosamine) C-1 98.1, C-2 60.3, C-3 72.4, C-4 80.1, C-5 71.5, C-6 68.7 (iduronate) C-1 104.6, C-2 71.1, C-3 70.4, C-4 77.2, C-5 71.2. Polysaccharide B: ^{1}H NMR (ppm): (glucosamine) H-1 5.32, H-2 3.50, H-3 4.48, H-4 4.04, H-5 4.05, H-6,6′ 4.27–4.41 (iduronate) H-1 5.32, H-2 4.55, H-3 4.72, H-4 4.39, H-5 5.05. ^{13}C NMR(ppm): (glucosamine) C-1 99.6, C-2 59.3, C-3 82.9, C-4 76.8, C-5 72.1, C-6 68.7 (iduronate) C-1100.8, C-2 73.6, C-3 72.9, C-4 73.3, C-5 68.7. Changes in conformation and rigidity in polysaccharide A were corroborated by altered NOE values between protons in the structure and by altered T_2 relaxation rates following cation exchange. For example, comparing Na and Ca forms of polysaccharide A, NOE values were as follows: A-1 to I-3; 12.5 (Na) and 10.5 (Ca); I-1 to A-6; 7.3 (Na) and 3.8 (Ca); I-1 to A-6′ 4.0 (Na) and 2.9 (Ca); I-5 to I-2; 2.8 (Na) and 0.8 (Ca). T_2 relaxation times (ms) at selected positions: A-1 130 (Na) and 94 (Ca), A-2 135 (Na) and 105 (Ca), I-1 113 (Na) and 52 (Ca), I-2 95 (Na) and 59 (Ca).[22]

3. Results

3.1 Cations alter the binding of phage display antibodies to heparan sulfate

To examine the effects of cations on the binding of phage display antibodies to HS, a modification of an ELISA assay previously reported[16] was employed, in which the polysaccharides were first exposed to various cations before application of the phage display antibodies. Briefly, the polysaccharides were converted to the appropriate cation form by use of excess cation exchange resin (Dowex W-50) that had been converted from the acidic form to the appropriate cation form using a solution of the chloride of the metal ion concerned.[21,22] Any differences observed cannot have arisen from residual cations or those that might have been introduced by subsequent washing or incubation steps because these procedures were common to all the experiments. The exposure to physiologically relevant divalent cations such as Mg, Ca, Cu(II) and Zn altered the binding of the phage display antibody HS3B7V to model HS polysaccharide analogues A and B predominantly containing, respectively, the natural occurring disaccharide repeat IdoA-GlcNS,6S [Fig. 1A] and the unnatural oversulfated residues IdoA2S,3S-GlcNS,3S,6S [Fig. 1B]. Polysaccharide B, was included because it has been shown recently that such structures can mimic HS sequences in protein binding and activity.[29] Their recognition by phage display antibodies supports the assertion that their conformation and charge characteristics can be similar to HS.

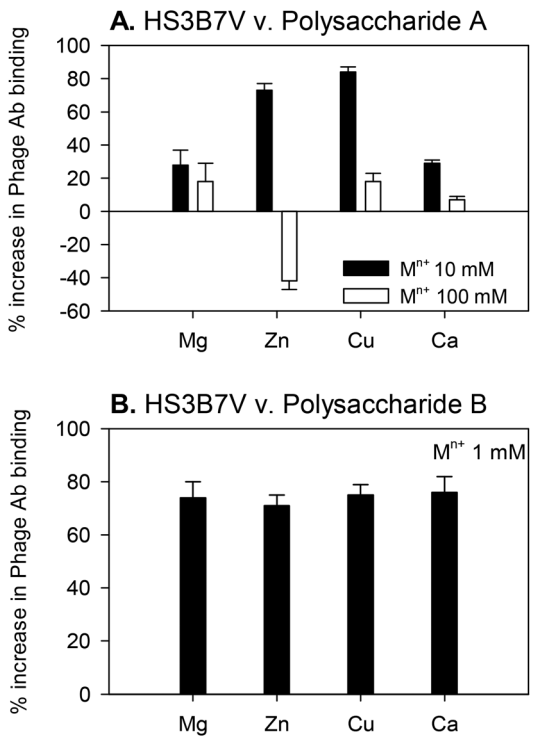

Fig. 1 ELISA establishing that exposure to cations alters the binding of phage display antibody HS3B7V to model GAG polysaccharides containing the naturally occurring disaccharide residue [IdoA-GlcNS,6S – polysaccharide A] (A) and over-sulfated residues [IdoA2S,3S-GlcNS,3S,6S – polysaccharide B], (B) respectively. In accord with the higher conformational flexibility of analogue A, the effect of cation binding is complex and varies with concentration but, is more uniformly increased and shows fewer subtleties with the more highly sulfated and rigid analogue B.

The effect of the cations on antibody binding were complex, particularly to polysaccharide A, with both increases and decreases in binding being observed. This is because subtle changes in conformation and charge distribution result from interactions with varying levels of cations. For instance, a Ca concentration of 100 mM resulted in the lowest binding of analogue A to antibody HS3B7V [Fig. 1A], whereas a reduced cation concentration of 1 mM showed more uniform but increased binding to analogue B [Fig. 1B]. A high Zn concentration (100 mM) also caused decreased antibody binding to analogue A [Fig. 1A].

The binding of cations by HS and potential changes in recognition profiles by phage display antibodies is of considerable importance because the concentration of common cations (Na, K, Mg and Ca) and some transition metal ions (*e.g.* Cu(II) and Zn) are known to vary considerably between tissues and under different physiological conditions (see Discussion below).

Having demonstrated altered phage display antibody binding to different cation forms of HS analogue structures, the binding profiles of the antibodies were examined to determine whether cations can also modify their binding to heparan sulfate on cell surfaces. Immunofluorescence, employing the antibodies applied to neuroblastoma cells, that had been exposed following fixing to a variety of concentrations of cations, was employed [Fig. 2]. Immunostaining revealed higher expression of the antibodies HS3B7V and HS4E4V after divalent cation exposure compared to exposure to PBS (divalent ion free) alone. A variety of binding patterns were evident; for example, diffuse cell surface binding of HS3B7V to cells treated with Ca and Zn [Fig. 2F and H], binding (including punctate binding) of HS4E4V in Zn treated cells to filopodia [Fig. 2D] and localisation, including to cell–cell contacts with HS3B7V for Cu and Zn treated cells [Fig. 2G and H]. In some cases, the results of treatment with two cations were similar, for example, Ca and Zn [Fig. 2F and H] at 10 mM. Controls included the omission of the primary HS antibodies (no staining: images not shown). These data support the notion that the binding of cations by HS can alter the binding of cell surface HS by phage display antibodies.

3.2 The binding of cations to polysaccharide A with the predominant repeating unit [IdoA-GlcNS,6S] alters both its conformation and biological activity

The results above suggested that the propensity for cations to alter conformation and/or binding properties and potentially alter biological activity is a general characteristic of these polysaccharides. That the binding of cations to HS polysaccharides can result in altered conformation was demonstrated by the change in circular dichroism (CD) spectrum of analogue A [Fig. 3A]. CD in the far UV (170–250 nm) is sensitive to the conformational environment of the chromophores (n–π* and π–π* transitions in the C═O bonds of carboxylic acids and *N*-acetyl groups of GAGs[22]). A change in CD on addition of cations demonstrates a change in the conformation around these chromophores, which could comprise, for example, altered glycosidic geometry, a change in the sugar ring (chair or skew-boat) or sulfate conformation, or a combination of these

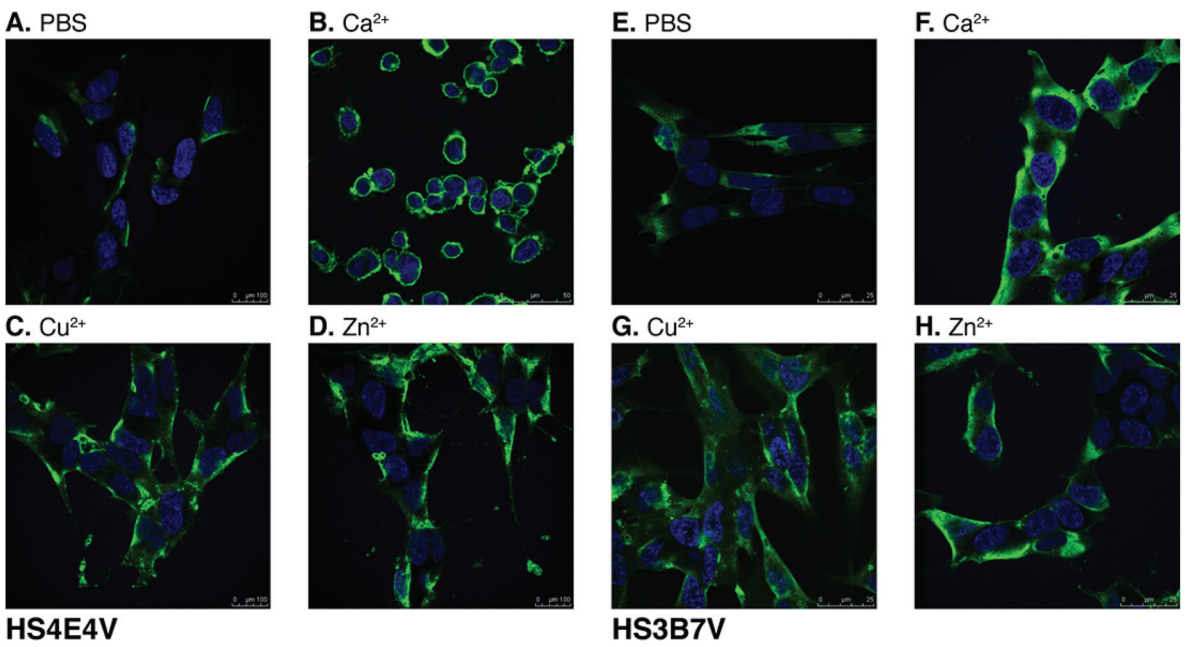

Fig. 2 Exposure of native cell surface HS structures to a variety of physiologically relevant cations alter phage display antibody binding profiles. The binding of phage display antibodies HS4E4V [A to D] and HS3B7V [E to H] to HS on the surface of fixed, cultured SH-SY5Y neuroblastoma cells was altered by exposure to divalent cations at 10 mM. A and E, show HS expression following exposure to PBS free of Ca and Mg ions (divalent ion free); B and F, following exposure to Ca ions; C and G to Cu ions (10 mM). D and H are after exposure to Zn ions (10 mM). Original magnification ×40.

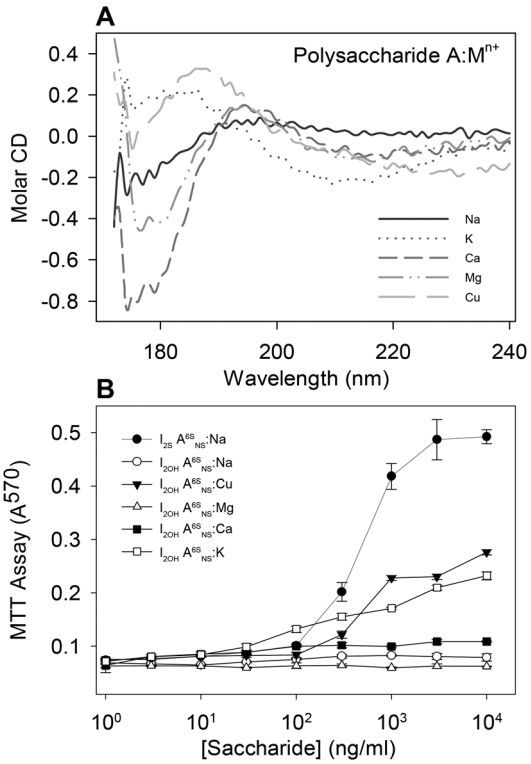

Fig. 3 A. Circular dichroism spectrum demonstrating changes in conformation of analogue A following exposure to cations [Na, K, Mg, Ca, Cu(II)]. CD reports the differential absorbance of right and left circularly polarized light in the UV region and is highly sensitive to changes in the chiral conformation of the chromophores. B. In a BaF cell assay, in which the only available route for cell signaling is *via* the transfected FGFR1c and exogenously added FGF-1 and polysaccharide, the ability of analogue A to support FGF signaling changes and depends on the cation form of the polysaccharide. The polysaccharide A:cation complexes are compared to sodium heparin [I_{2S} $A^{6S}{}_{NS}$:Na].

factors. Furthermore, any changes observed by CD are chiral in nature and are likely to result in altered protein binding.

4. Discussion

The binding of HS by phage display antibodies (and proteins more generally) has been viewed conventionally as involving interactions between a series of negatively charged groups (carboxylic acids, *N*- and *O*-sulfates on HS) and regions on the protein surface, especially but not exclusively containing positively charged amino acid side chains such as lysine and arginine. However, the binding patterns of phage display antibodies to HS are clearly complex, with previous work showing that multiple HS structures bind the basic surfaces on the variable regions of the antibodies.[16] Here, we have shown that cation binding to several polysaccharides, both to HS analogues in microwells and HS on a cell surface, resulted in altered phage display antibody binding for several phage display antibodies. It is therefore reasonable to conclude that this is a general property of this system. While we show that cation binding can alter the conformation of the polysaccharide, it is also possible that cation binding to the antibody could contribute to a change in recognition characteristics, although the latter possibility is much more difficult to assess experimentally. For example, in ELISA assays for HS3B7V binding to HS analogue A [Fig. 1A], serving as a model of HS and containing the naturally occurring, predominant repeating disaccharide [IdoA-GlcNS,6S], it is noteworthy that binding is modified in a complex manner, increasing or decreasing as a function of cation concentration. This is the consequence of the subtle interplay between cation and polysaccharide conformation, flexibility and charge distribution. The detailed measurement of cation binding rates and affinities are very difficult to address and lie outside the scope of this paper, although some work has been done.[18,19,30] Copper(II) ions exhibit an unusually specific interaction and mode of binding with the highly sulfated residues of heparin, containing the motif IdoA2S-GlcNS,6S binding the ion in a four-fold planar arrangement.[31] The broader applicability of the effect of cations on polysaccharide structure and subsequent phage display antibody binding was then examined for the case of a non-natural, sulfated polysaccharide, containing over-sulfated sequences, analogue B. In this case, the binding of HS3B7V was increased for all cations tested, but, less variation was observed. This polysaccharide, provides a comparison with the more flexible analogue A because, while being active in several assays in which HS and heparin act, it is considered to be relatively rigid compared to other GAGs (and considerably more rigid than uncharged and branched structures) due to steric hindrance.[27] Furthermore, since such analogues have been shown to be capable of inducing changes in protein structure and biological activity,[29] and the phenomenon of phage display antibody binding is also affected by cations, this suggests potential for their use in other recognition roles, including identification of HS mimics.

The effect of cations has also been demonstrated for phage display antibodies HS4E4V and HS3B7V on the cell surface of cultured SH-SY5Y neuroblastoma cells (an aggressive childhood cancer originating in the neural crest and associated with metastasis and poor outcome) following exposure to low mM levels of cations [Fig. 2]. That the binding of cations to HS polysaccharides can alter the conformation, was demonstrated here by the change in CD spectrum of analogue A [Fig. 3A]. This causes both the antibody binding profiles [Fig. 1] and the biological activity to change [Fig. 3B]. There is also the possibility that particular charged groups may be shielded by the presence of particular cations, which would be consistent with the sensitivity of HS4E4 and HS4C3 to changes in 2-*O*-sulfation,[32] although the ability of divalent cations to form bridges between the polysaccharide and the binding peptide region may be an additional factor. These findings suggest that the propensity for cations to alter conformation and/or binding properties and, in certain cases, to subsequently alter biological activity, is a general characteristic of these molecules, but one to which phage display antibodies are sensitive.

During the original selection of phage display antibodies *e.g.*[11] the cations present during binding to the kidney HS were not determined and, although the HS had been subjected to anion exchange chromatography involving sodium chloride, so was likely to be predominantly in the sodium form initially, subsequent steps in the selection process will have introduced other cations into the system.

There are many instances in which the concentration of common physiological cations may vary in cells, on their surfaces as well as in the extracellular matrix, and imbalances can have serious physiological consequences. Calcium levels are regulated by ion-pumps but, calcium imbalance is known to be involved in several pathological processes including cancer.[33] Less common but, nevertheless, essential cations such as Cu and Zn, also play important roles in normal and disease physiology. Copper in its free form is extremely toxic, in part a consequence of its ability to propagate free radicals *via* the Fenton reaction, and is transported by an elaborate mechanism.[34] Failure to regulate copper levels results in severe illness, for example, Menkes' and Wilson's diseases, during which levels in the liver can reach 250 μg g^{-1}.[35] The liver contains HS that is rich in highly sulfated IdoA2S-GlcNS,6S residues, which are known to bind Cu(II) ions at a specific site[31,33] and also high levels of IdoA-GlcNS,6S (mimicked by HS analogue A in this work).[36] This latter repeating disaccharide has also been reported in diabetic kidney.[37] Altered levels of Zn are a feature of ageing[38] and neurodegenerative disease,[39] while GAG expression is also known to vary[40] and both potassium and calcium levels have been related to ageing in *Drosophila*.[41] The levels of cations and expression of HS structures both vary considerably during normal and disease processes and here, the detection of GAG structures using phage display antibodies is affected by cation levels. Phage display antibody HS4C3 has been shown to bind a sequence comprising predominantly [Ido-GlcNS,6S] residues, in associated with metal ions [Fig. 1A] confirming that antibodies can bind to several distinct combinations of sequence[42] and cation. This is perhaps less surprising when viewed in light of the recently suggested high level of redundancy in HS structures[29] and confirms that a degree of conformational degeneracy is indeed present in interactions between these short peptide sequences and regions of HS. Recognition of negatively charged groups on HS by positively charged amino-acids in the short peptide stretches may also require a compatible overall conformation and binding divalent cations in particular could provide a means by which compatibility can be attained for a variety of sequences. This principle could be relevant to the interactions of full-length proteins with HS and other GAGs.

The influence of cation binding on the conformation of other biological macromolecules has been documented for proteins[43,44] and for DNA.[45] The present findings for HS polysaccharides could have important implications for the interpretation of HS expression profiles in tissues using these antibodies and suggest that phage display antibodies may enable protein binding profiles to HS to be followed more closely than previously realised.

It is also noteworthy that structures which do not occur naturally, such as that with the predominant repeating disaccharide structure of analogue B, [IdoA2S,3S-GlcNS,3S,6S] can, nevertheless, still adopt suitable conformational and charge distribution arrangements to allow binding of phage display antibodies, as they have been shown to do with intact proteins.[29] Such synthetic polysaccharide structures are also susceptible to influence by cation binding and the resulting conformational and binding consequences [Fig. 1A and B]. This supports the idea that complementary surfaces, defined by the combined effects of sequence, cation binding and their conformational consequences, define the recognition element in HS for antibodies and proteins.

5. Conclusions

In any search for characteristics by which different areas of a cell or tissue can be differentiated, it is desirable to follow functional properties, rather than expression based solely on the detection of sequences, especially when the latter requires some form of interpretation dependent on a knowledge of structure–function relationships, which is often absent or, at best, only partial. We suggest that these antibodies may provide a functional read-out in the sense that their binding provides a means of tracking the protein binding profile of HS which, in tissues as elsewhere, exist in association with cations. In conclusion, the phage display antibody library recognises the overall conformation of HS structures with their associated cations and it is this, ultimately, that is relevant to protein binding and function.

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