the FEBS Journal

Isolation of bovine corneal keratan sulfate and its growth factor and morphogen binding

Amanda Weyers[1], Bo Yang[1], Kemal Solakyildirim[1], Vienna Yee[1], Lingyun Li[1], Fuming Zhang[2] and Robert J. Linhardt[1,2,3,4]

1 Department of Chemistry and Chemical Biology, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY, USA
2 Department of Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY, USA
3 Department of Biology, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY, USA
4 Department of Biomedical Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY, USA

Keywords
cornea; fibroblast growth factor; interaction; keratan sulfate; sonic hedgehog

Correspondence
R. J. Linhardt, 4005 Biotech Building, Rensselaer Polytechnic Institute, 110 8th Street, Troy, NY 12180, USA
Fax:1-518-276-3404
Tel:1-518-276-3405
E-mail: linhar@rpi.edu

(Received 2 December 2012, revised 24 January 2013, accepted 28 January 2013)

doi:10.1111/febs.12165

Keratan sulfate (KS) is an important glycosaminoglycan that is found in cartilage, reproductive tissues, and neural tissues. Corneal KS glycosaminoglycan is found N-linked to lumican, keratocan and mimecan proteoglycans, and has been widely studied by investigators interested in corneal development and diseases. Recently, the availability of corneal KS has become severely limited, owing to restrictions on the shipment of bovine central nervous system byproducts across international borders in an effort to prevent additional cases of mad cow disease. We report a simple method for the purification of multi-milligram quantities of bovine corneal KS, and characterize its structural properties. We also examined its protein-binding properties, and discovered that corneal KS bound with high affinity to fibroblast growth factor-2 and sonic hedgehog, a growth factor and a morphogen involved in corneal development and healing.

Introduction

Proteoglycans (PGs), which are proteins modified by the attachment of glycosaminoglycan (GAG) chains, are important in the growth and development of normal tissue [1], and regulate several key biological processes [2]. The structures of the attached GAGs, which are long, highly sulfated, polysaccharide chains, are typically responsible for the signaling properties of PGs. For this reason, the analysis of the change of GAG structures in development and disease states has become a prominent area of research.

Keratan sulfate (KS) represents an important family of GAGs. KS was named after the corneal tissue that it was first isolated from, and different forms of KS are now recognized to be present in several tissues, including articular cartilage, reproductive tissue, and neural tissue [3]. KS is composed of a β-1–4-Gal-β-1–3-GlaNAc disaccharide repeating structure, as shown in Fig. 1 [4–7]. The structure shown in Fig. 1 is based on a composite of several studies, including work by Oeben *et al.* [8] identifying the placement and relative lengths of the disulfated, monosulfated and nonsulfated disaccharide repeating units, work by Stuhlsatz *et al.* [9] in identifying the structure of the linkage region, and work by Tai *et al.* [10] in identifying various end-chain capping structures. KS is classified into three different groups (I–III) on the basis of protein

Abbreviations
FGF, fibroblast growth factor; GAG, glycosaminoglycan; GPC, gel permeation chromatography; HILIC, hydrophilic interaction chromatography; HS, heparan sulfate; KS, keratan sulfate; MWCO, molecular weight cut-off; PG, proteoglycan; RPIP, reverse-phase ion-pairing; SAX, strong anion exchange; SHH, sonic hedgehog; SPR, surface plasmon resonance.

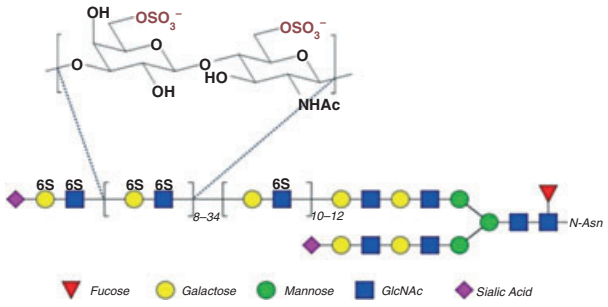

Fig. 1. Typical structure of corneal KS. The repeating Gal-GlcNAc disaccharide structure shown here may be modified with 6-*O*-sulfo groups (as shown). KS chains are known to have a section of low sulfation (a single 6-*O*-sulfo group on the GlcNAc) close to the protein backbone, and a section of higher sulfation (6-*O*-sulfo groups on both GlcNAc and galactose) towards the chain's nonreducing terminus [4–7], and are often capped by sialic acid [10]. In the cornea, KS is N-linked through an asparagine to one of three protein core structures: lumican, keratocan, or mimecan.

linkage structure, and type I, which has an *N*-asparagine linkage (Fig. 1), is found in the cornea [3,11]. Corneal KS is attached to three different core proteins, resulting in three different kinds of KS PG: lumican, keratocan, and mimecan [3,4]. Each core protein typically has two or three chains that are 10–15 kDa in length [4,6].

Several methods for isolating and analyzing KS and KS PGs have been developed as research into KS structure has focused on its influence on corneal health and developmental biology [5,7,12–15]. This research has produced sufficient demand to support the commercial production of a KS isolated from bovine cornea. However, rising concerns about pathological viruses and prions, such as bovine spongiform encephalopathy and mad cow disease, have restricted the shipment of bovine central nervous system byproducts across international borders, making this standardized material inaccessible to many research laboratories.

In the current article, we describe a convenient method for the isolation of multi-milligram quantities of KS from bovine cornea, and the characterization of the structure of this KS by NMR spectroscopy, HPLC-MS, and of its chain length by gel permeation chromatography (GPC) and PAGE. This corneal KS was identical to a commercially obtained KS standard. In addition, protein binding of KS was measured by surface plasmon resonance (SPR) to assess its bioactivity. We describe the interaction of corneal KS GAG with sonic hedgehog (SHH), fibroblast growth factor (FGF)1, and FGF2. These are critically important proteins for corneal development and healing, and these interactions should shed light on the possible involvement of KS PGs in the growth and development of corneal tissue.

Results

Approximately 180 mg of KS GAG was isolated from 50 bovine corneas, each having a dry weight of 17.5 g. In these studies, commercial bovine corneal KS from Seikagaku Kogyo (no longer available outside of Japan) was used as a standard for comparison of structural properties and bioactivities.

The chain length distributions of KS GAGs were first analyzed by PAGE and by GPC. Both KS samples showed similar profiles when analyzed by PAGE, with the commercial KS having a slightly larger distribution of chain lengths and a lower average chain length (Fig. 2A,B). Chain length analysis by GPC also showed similar chain length profiles for both KS samples (Fig. 2C). The calculated weight average and number average molecular masses of the KS sample prepared in this study, 14.3 and 11.6 kDa, were within ~ 15% of those obtained for the commercial KS sample (Table 1), and both KS samples showed similar polydispersities.

Next, KS samples were analyzed by ^{1}H-NMR and ^{13}C-NMR (Fig. 3). The NMR profiles of both KS

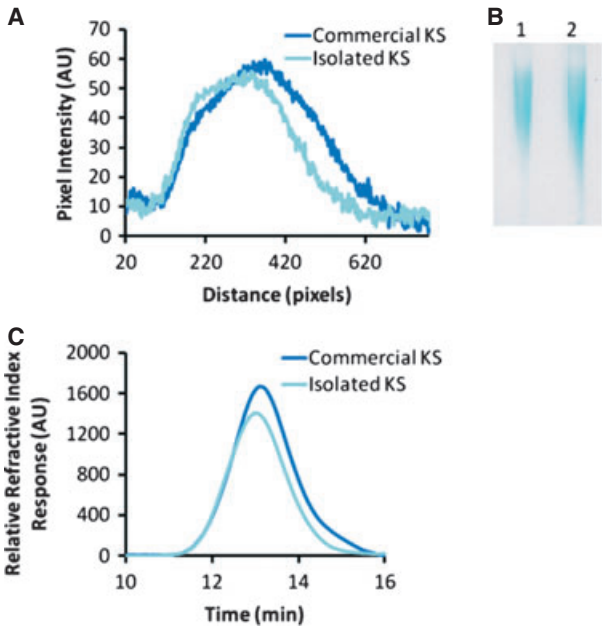

Fig. 2. Molecular mass analysis of isolated and commercial KS. (A) Plot of KS chain profile distributions determined by PAGE. (B) Alcian blue-stained gel obtained on PAGE analysis of isolated (lane 1) and commercial (lane 2) KS GAGs. (C) Plot of KS chain profile distributions of the isolated and commercial KS measured by GPC-HPLC.

Table 1. Relative molecular mass profiles of KS including number average molecular weight ($M_{r,N}$), weight average molecular weight ($M_{r,W}$) and polydispersity as determined with GPC

	$M_{r,N}$	$M_{r,W}$	Polydispersity
Commercial KS	9800	12 900	1.23
Isolated KS	11 600	14 300	1.32

samples were identical and consistent with the known structure of KS. The KS sample prepared in the current study was subjected to digestion with keratanase 2, an endo-β-galactosidase, and then analyzed by both reverse-phase ion-pairing (RPIP)-HPLC-MS and hydrophilic interaction chromatography (HILIC)-MS [16]. Previous studies have used keratanase 2 and an endo-β-galactosidase to study the structures of cartilage and corneal KS oligosaccharides [17–19]. RPIP-HPLC-MS confirmed that the major disaccharide formed on keratinase 2 treatment of the isolated KS was GlcNAc6S-Gal6S (Fig. S1), consistent with the known structure of corneal KS. HILIC-MS revealed a consistent motif of undigestible, highly sulfated (nearly 2-*O*-sulfo groups per disaccharide repeating unit) oligosaccharide domains that was clearly (Figs 4 and S2) also consistent with the known structure of corneal KS. Highly sulfated sialic acid-capped KS oligomers with one sialic acid per chain (Fig. 1) were also detected (Fig. 4), and are similar to structures found in KS articular cartilage [18]. Moreover, the linkage region (Fig. 1) was confirmed by MS analysis (Fig. S3).

Finally, the bioactivities of commercial and isolated KS samples were compared by measuring their protein-binding responses with SPR. The responses of the two KS samples were compared for three proteins: the morphogen SHH, and the growth factors FGF1 and FGF2. These proteins are known to be important in corneal development and healing. The binding responses of the two KS samples were compared with that of a commercial heparin sulfate (HS) standard that is known to interact with all three proteins, and thus served as a positive control (Fig. 5).

The isolated and commercial KS showed similar, but not identical, binding curves for all three proteins, which were markedly different from the HS protein binding interactions (Fig. 5). By use of the accompanying BIAEVALUATION software, each series of protein-binding sensograms was fitted to establish binding models, which were used to quantify the parameters of the GAG–protein interactions (Table 2). Both the observed protein-binding sensograms and their accompanying modeled binding interactions were remarkably similar for the two KS samples. The isolated and commercial KS showed weak but similar binding responses to SHH (K_D values of 6.5×10^{-5} and 5.0×10^{-5}, respectively), and had similar binding responses to FGF2 (K_D values of 7.4×10^{-7} and 9.7×10^{-7}, respectively); neither bound to FGF1. In contrast, HS showed strong binding responses to all three proteins.

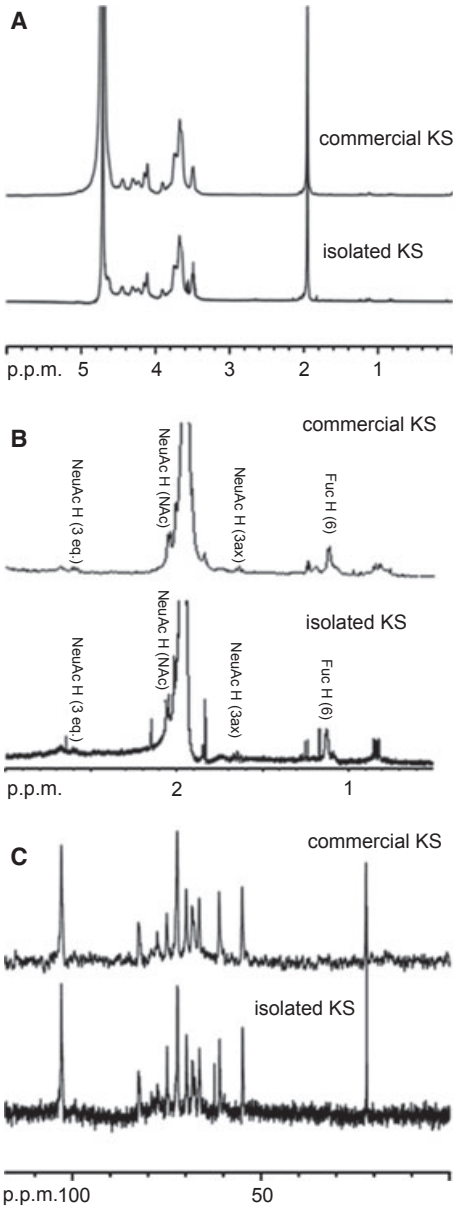

Fig. 3. NMR analysis of commercial and isolated KS. (A) Full ^{1}H-NMR spectrum (0–6 p.p.m.). (B) Expanded ^{1}H-NMR spectrum (0.5–3 p.p.m.). Minor peaks labeled correspond to signals assignable to fucose (Fuc) and neuraminic acid (NeuAc) including the N-acetyl (NAc), the 3-axial (3 ax) and the 3-equatorial (3eq) protons; other minor peaks observed in both commercial and isolated KS were impurities that could not be assigned. (C) ^{13}C-NMR. Peaks at 3.55 p.p.m. in the ^{1}H-NMR and at 63 p.p.m. in the ^{13}C-NMR in the isolated KS sample that are not seen in the commercial KS correspond to a small glycerol impurity.

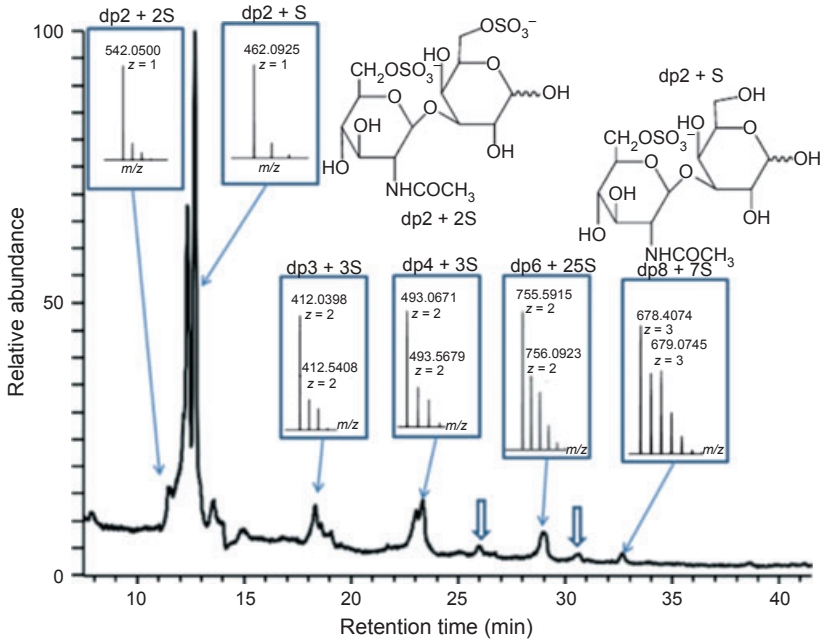

Fig. 4. HILIC-HPLC-FT-MS profiling of fully digested KS domain structures. Some of the oligomers contain highly sulfated domains (approximately one sulfo group per saccharide), which are not digested by keratanase. On the basis of the accurate FT-MS (spectra inserted), we can assign the major compositions of the oligomers eluted at specific retention times. The minor component of sialic acid-capped oligomers was also detected [hollow arrows indicate degree of polymerization (dp)6 + 3S and dp8 + 5S with one sialic acid residue at the nonreducing end].

Discussion

The isolated KS and commercial KS were analyzed by GPC, PAGE, NMR, HPLC-MS and SPR. These KS samples were found to have nearly identical structural properties, suggesting that the simplified method of preparing multi-milligram amounts of KS that was used in the current study gave high-purity bovine corneal KS. Analysis of the protein-binding interactions of the two KS samples by SPR also showed similar responses, suggesting that the bioactivities of these bovine corneal KS samples were identical.

SPR, a highly sensitive analytical technique that is used to measure the strength of molecular binding interactions, showed for the first time that bovine corneal KS bound two biologically important proteins, FGF2 and SHH. Previous binding studies had shown that KS underwent no binding interactions with either FGF1 or FGF2 [16]. We can find no previous reports of KS interaction with SHH. The morphogen SHH and the FGFs are signaling factors that have been shown to play a wide range of roles in tissue development, especially in the nervous system [17,18].

The FGFs encourage and regulate growth and development in many key cellular roles throughout the body. Research into FGFs and their receptors has shown that they have a wide range of developmental roles in the eye, including retinal development [19]. FGF2 has been found in human tears, from where, in the event of corneal damage, it can migrate into the stroma and act on keratocytes, which are fibroblast cells responsible for helping to maintain the optical transparency of the cornea [4,20]. FGF2 has also been shown be dysregulated by the corneal epithelium during corneal wounding, suggesting that it plays a role in repairing tissue damage during wound healing [21].

Similarly, studies on SHH have implicated this morphogen in a wide range of biological functions, including roles in corneal health, development, and wound healing. SHH has been found to be integral to the prevention of myopia [22], essential for normal retinal development [23], and to be unregulated during corneal wound healing [24]. SHH (and FGF) are also implicated in the genesis of eye and retinal development [25–28]. However, SHH's role in upregulating cellular growth may also have unwanted side effects in corneal wound healing, as it has also been implicated in the development of unfavorable corneal vascularization during wound healing [29].

Taken together, these studies indicate that SHH and FGF2 play significant roles in many aspects of eye

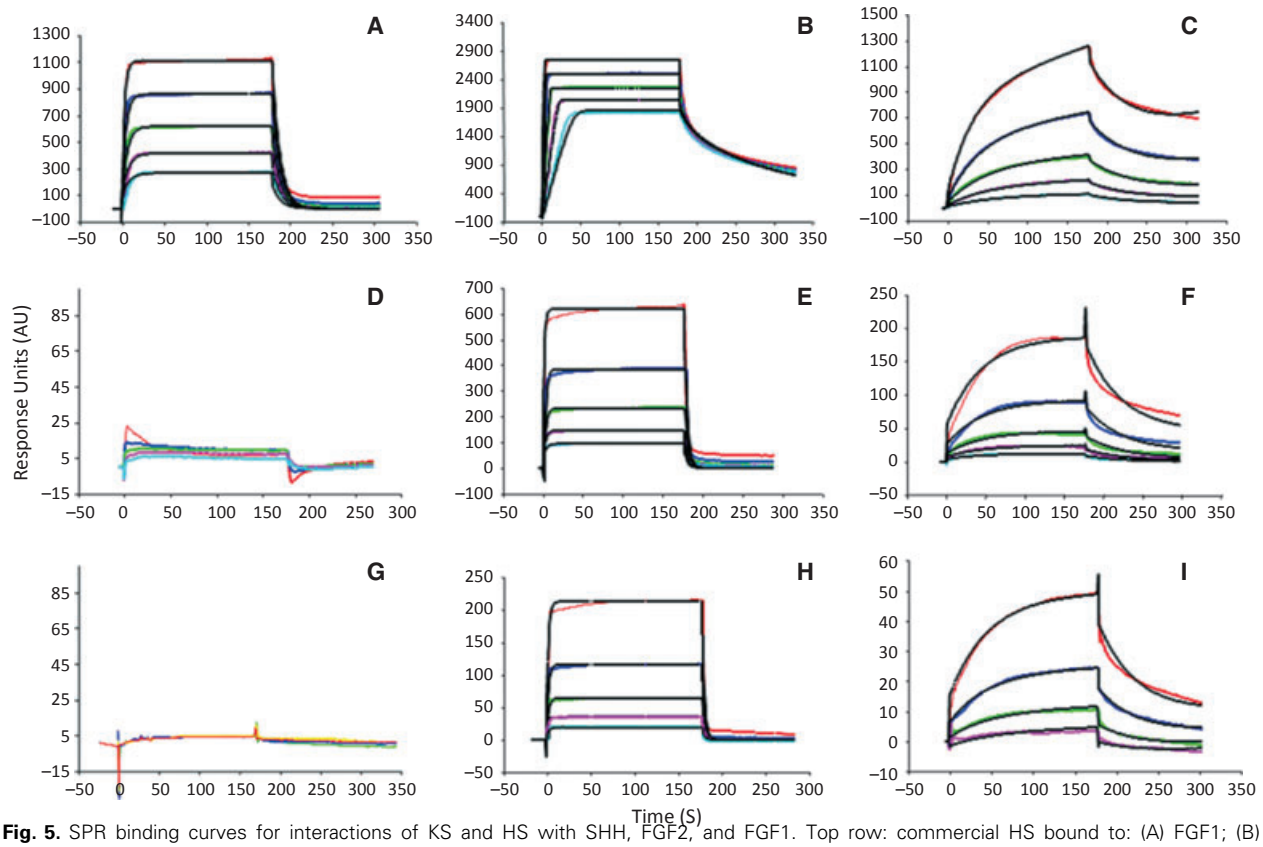

Fig. 5. SPR binding curves for interactions of KS and HS with SHH, FGF2, and FGF1. Top row: commercial HS bound to: (A) FGF1; (B) FGF2; and (C) SHH. Middle row: commercial KS bound to: (D) FGF1; (E) FGF2; and (F) SHH. Bottom row: isolated KS bound to: (G) FGF1; (H) FGF2; and (I) SHH. The concentrations of each protein used are delineated by line color: red, 1000 nM; navy blue, 500 nM; green, 250 nM, pink, 125 nM; light blue, 63 nM.

Table 2. Protein-binding constants derived from SPR analysis. NB, no binding detected. The on-rate k_{on} is expressed in reciprocal molar seconds, the off-rate k_{off} is expressed in reciprocal seconds, and the dissociation constant K_D is expressed in molar.

GAG	Protein interaction	k_{on} (1/Ms)	k_{off} (1/*s*)	K_D (M)
HS	SHH	1.5×10^4	0.017	1.2×10^{-6}
	FGF2	7.4×10^7	0.53	7.2×10^{-9}
	FGF1	2.2×10^5	0.11	4.9×10^{-7}
Commercial KS	SHH	437	0.022	5.0×10^{-5}
	FGF2	3.0×10^5	0.29	9.7×10^{-7}
	FGF1	NB	NB	NB
Isolated KS	SHH	344	0.022	6.5×10^{-5}
	FGF2	6.2×10^5	0.46	7.4×10^{-7}
	FGF1	NB	NB	NB

health and development. The novel KS binding interactions with SHH and FGF2 reported here suggest that KS GAGs and KS PGs may play important roles in GAG–protein interactions that guide the development, wound healing, and structural properties of the cornea.

Experimental procedures

Bovine corneas were from Pel-freeze Biological (Rodgers, AR, USA). Actinase E was from Kaken Biochemicals (Tokyo, Japan). Commercial KS and keratanase (endo-1,4-β-D-galactohydrolase; EC 3.2.1.103) were from Seikagaku (Tokyo, Japan). The chondroitin lyases ABC and ACII were from Associates of Cape Cod (East Falmouth, MA, USA). Hyaluronan standards were from Caisson LLC (Oklahoma City, OK, USA). Vivapure Maxi and Mini Q-H strong anion exchange (SAX) columns were from Sartorius (Goettingen, Germany). Biotin-poly(ethylene glycol)$_3$-NH_2 was from Sigma Aldrich (St Louis, MO, USA). Amicon centrifugal filter molecular weight cut-off (MWCO) membranes were from Millipore (Billerica, MA, USA). Cloning, *Escherichia coli* expression and purification of the recombinant heparin lyase I (EC 4.2.2.7), heparin lyase II (no EC assigned) and heparin lyase III (EC 4.2.2.8) from *Flavobacterium heparinum* were performed in our laboratory as previously described [30–32]. All other chemicals were of reagent grade.

Isolation of KS from corneal tissue

The opaque corneal tissue was isolated from the excess tissue in the bovine corneas received. The corneal tissue was manually dissected into ~ 3-mm-square pieces, and proteolyzed with actinase E (2% solution, amount per wet tissue weight) at 55 °C for 24–48 h. The resulting solution was centrifuged (5000 ***g*** for 10 min) to remove large particles, and passed through a 0.22-μm cellulose filter. The filtered solution was loaded onto a prewashed (one column volume of 50 mM sodium acetate, 50 mM sodium chloride, pH 4.5, hereafter buffer A) SAX spin column (Vivapure Maxi Q-H). The loaded column was then washed (three column volumes of buffer A), and a high-salt buffer was used to elute the isolated corneal GAGs (one column volume of 16% sodium chloride, w/v). Methanol was added to the eluted GAG solution to bring the total methanol volume to 80% (v/v), and the methanol/water/GAG mixture was allowed to precipitate overnight at 4 °C and then pelleted by centrifugation (5000 ***g*** for 20 min) to give a solid white pellet.

The isolated pellet was then digested with chondrotinase enzymes (chondrotinase ABC and ACII) and heparinase enzymes (heparinase I, II, and III), and digested CS and HS disaccharides were removed by washing through a 3000-kDa MWCO spin column. The GAG solution was lyophilized to yield a fluffy, fibrous KS isolate.

Chain length analysis by PAGE

The isolated and commercial KS samples were analyzed by native PAGE. The KS samples were analyzed on a 0.75 mm × 6.8 cm × 8.6 cm mini-gel cast with 10% (9.36% acrylamide and 0.61% bis-acrylamide) resolving gel monomer solution and 5% stacking gel monomer solution, as previously described [33,34]. KS samples (5 μg) were applied to each lane [5 μL of a 1 $mg{\cdot}mL^{-1}$ solution combined with 5 μL of a 50% (w/v) sucrose solution], and then subjected to electrophoresis at 200 V for 20 min. KS was visualized in the gels with an Alcian blue stain [0.5% (w/v), with 2% (v/v) aqueous acetic acid]. The gel was stained for 30 min, and destained completely with deionized water. The scanned gel image was analyzed with IMAGEJ (http://imagej.nih.gov/ij/) [35]. Pixel intensity was measured along the center of the each gel lane to give a plot of the average distribution of each KS chain.

Chain length analysis by GPC

The average chain lengths of the isolated and commercial KS samples were measured with GPC performed on an HPLC system. KS chain size was correlated with sample elution time by comparison with a set of known hyaluronan molecular mass standards (30.6, 54, 128, and 262 kDa). Each KS sample was injected (20 μL, containing 20 μg) at a flow rate of 0.6 $mL{\cdot}min^{-1}$ onto an apparatus composed of a Shimadzu LC-10Ai pump, a Shimadzu CBM-20A controller, a TSK-GEL G4000PWxl size exclusion column, and a Shimadzu RID-10A refractive index detector. The mobile phase was 0.1 M $NaNO_3$ and the column was maintained at 40 °C with an Eppendorf column heater. The GPC chromatograms were recorded and analyzed with the LC SOLUTION (Shimadzu Scientific Instruments, Columbia, MD, USA) GPC Postrun function (Version 1.25).

Structural analysis by NMR

The purified KS from bovine cornea and the commercial KS standard were prepared for NMR analysis by removing exchangeable protons. Both samples were dissolved in 0.4 mL of 99.996 atom% deuterium oxide (^2H_2O), and then freeze-dried, replacing exchangeable protons with deuterium. All NMR data were acquired on Bruker Avance II Ultrashield 600 MHz (14.1 T) and 800 MHz (18.8 T) NMR instruments equipped with an ultrasensitive HCN cryoprobe with a *z*-axis gradient. The ^{13}C-NMR spectra were recorded at 150 MHz. The spectra were acquired at a probe temperature of 298 K. A sweep width of 20.5 p.p.m. and an acquisition time of 2.66 s were employed.

Structural analysis by RPIP-HPLC-MS

The isolated and commercial KS samples were digested with keratanase 2 (10 μg of KS and 30 mU of enzyme, in 100 mL of 10 mM Tris/HCL buffer, pH 7.4), and the resulting disaccharides were isolated from the digestion mixture by passing them through a 30-kDa MWCO filter, and analyzed on an Agilent 1200 LC/MSD instrument (Agilent Technologies, Wilmington, DE, USA). The Agilent LC-MS was equipped with a 6300 ion trap and a binary pump followed by a UV detector equipped with a high-pressure cell. The isolated disaccharides were injected onto a Poroshell 120 C18 column (2.1 × 150 mm, 2.6 μm; Agilent) heated to 45 °C. The disaccharides were separated with a gradient of buffers. Solution A was water/acetonitrile at 85 : 15 (v/v), and solution B was water/acetonitrile at 35 : 65 (v/v). Both eluents contained 12 mM tributyl amine and 38 mM NH_4OAc, with the pH adjusted to 6.5 with acetic acid. The buffer gradient used was a 10-min gradient of solution A (100%), followed by a linear gradient (0–50% solution B) from 10 to 40 min, with a 150 $\mu L{\cdot}min^{-1}$ flow rate. The column effluent entered the source of the ESI-MS for continuous detection by MS. To obtain the maximum abundance of ions in a full-scan spectrum (200–1500 Da), the electrospray interface was set in negative ionization mode, with a skimmer potential of – 40.0 V, a capillary exit of −40.0 V, and a source temperature of 350 °C, with nitrogen (8 $L{\cdot}min^{-1}$, 40 p.s.i.) used as a drying and nebulizing gas.

HILIC-ESI-LTQ-Orbitrap-FT-MS analysis of KS

A HILIC-FT-MS-based method was used to analyze the keratinase 2-digested KS disaccharides and oligosaccharides to obtain domain structures. Briefly, the digested KSs were separated with a Luna HILIC column (2.0 × 150 mm, 200 A; Phenomenex, Torrance, CA, USA) and detected with an LTQ-Orbitrap XL FT mass spectrometer (Thermo Fisher Scientific, San-Jose, CA, USA) running in negative-ion mode. An HPLC binary pump was used to deliver the gradient, from 10% mobile phase A to 35% mobile phase A in 40 min at a flow rate of 150 $\mu L \cdot min^{-1}$. Mobile phase A was HPLC-grade water with 5 mM ammonium acetate. Mobile phase B was HPLC-grade 98% acetonitrile with 2% water and 5 mM ammonium acetate. An FT-MS detector operating in negative-ion mode with optimized parameters was used to prevent in-source fragmentation, with a spray voltage of 4.2 kV, a capillary voltage of – 40 V, a tube lens voltage of −50 V, a capillary temperature of 275 °C, a sheath flow rate of 30 units, and an auxiliary gas flow rate of 6. External calibration of mass spectra was used to routinely produce a mass accuracy of better than 3 p.p.m. All FT mass spectra were acquired at a resolution of 60 000 with a 400–2000-Da mass range.

Protein-binding comparisons by SPR

Binding interactions between GAGs and proteins were measured on a Biacore 3000 SPR instrument (GE Healthcare, Waukesha, WI, USA). Both KS and HS samples were immobilized on a streptavidin-coated sensor chip (Sensor Chip SA; GE Healthcare, Uppsala, Sweden). Four separate flow cells were constructed: one containing a biotinylated commercial KS, one containing a biotinylated KS isolated from bovine cornea, and two control cells, one containing a biotinylated HS and one containing only biotin. KS and HS samples were biotinylated by reductive amination. The isolated KS was digested with peptide-*N*-glycosidase to remove residual amino acid residues, with a previously published procedure [36]. Briefly, peptide-*N*-glycosidase (5 U) was added to 500 μg of isolated KS in 150 μL of a digestion buffer (fresh 50 mM ammonium bicarbonate, pH 8.3) and digested overnight at 37 °C. The digested KS was then isolated and purified with a SAX spin column (Vivapure Mini Q-H; see isolation of KS from bovine corneas above). The eluted, purified KS was precipitated by methanol precipitation [80% (v/v) methanol] and centrifugation (5000 ***g*** for 10 min). KS and HS were biotinylated by reductive amination. A typical procedure was as follows. Five hundred micrograms of the GAG was dissolved in 1 mL of formamide. To a stirred KS solution, 500 μg of the amine-biotin (100 μL of a 5 $mg \cdot mL^{-1}$ dimethylsulfoxide solution) and 600 μg of $NaBH_3CN$ (50 μL of a 12 $mg \cdot mL^{-1}$ dimethylsulfoxide solution) were added dropwise and allowed to react at 60 °C for 12 h. After 12 h, $NaBH_3CN$ was added a second time (600 μg, 50 μL of a fresh 12 $mg \cdot mL^{-1}$ dimethylsulfoxide solution), and left stirring to react for a further 12 h at 60 °C. After this, 0.5 mL of water was added to the vial to quench any remaining unreacted $NaBH_3CN$. KS was isolated from the reaction mixture with a SAX spin column (Vivapure Mini Q-H), with the previously outlined procedure (see above).

The prepared samples (biotinylated HS, KS, or pure biotin) were then immobilized on a streptavidin-coated CM5 sensor chip (GE Heathcare, Uppsala, Sweden), according to the manufacturer's protocol. In brief, a 20 mL solution of the GAG–biotin conjugate (0.1 $mg \cdot mL^{-1}$) in HBS-EP buffer (10 mM Hepes, 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20, pH 7.4) was injected over the flow cell of the sensor chip at a flow rate of 10 $mL \cdot min^{-1}$. The biotin control flow cell was prepared with a 1-min injection with saturated biotin in HBS-EP buffer.

Different dilutions of protein samples were injected at a flow rate of 40 $\mu L \cdot min^{-1}$ for 3 min. At the end of the sample injection, the same HBS-EP buffer was passed over the sensor surface to facilitate dissociation. After a 2-min dissociation time, the sensor surface was regenerated by injection with 40 μL of 2 M NaCl to obtain a fully regenerated surface. The response was monitored as a function of time (sensorgram) at 25 °C. The parameters of binding kinetics were determined by globally fitting the sensorgram curves to a 1 : 1 Langmuir model from BIAEVALUATION, the accompanying Biacore software (GE Healthcare, Uppsala, Sweden).

Acknowledgements

The authors gratefully acknowledge support from the National Institutes of Health in the form of grant no. GM38060 and GM067545.

References

1 Bülow HE & Hobert O (2006) The molecular diversity of glycosaminoglycans shapes animal development. *Annu Rev Cell Dev Biol* **22**, 375–407.
2 Linhardt RJ & Toida T (2004) Role of glycosaminoglycans in cellular communication. *Acc Chem Res* **37**, 431–438.
3 Funderburgh J (2002) Keratan sulfate biosynthesis. *IUBMB Life* **54**, 187–194.
4 Hassell JR & Birk DE (2010) The molecular basis of corneal transparency. *Exp Eye Res* **91**, 326–335.
5 Meyer K, Linker A, Davidson EA & Weissmann B (1953) The mucopolysaccharides of the bovine cornea. *J Biol Chem* **205**, 611–616.
6 Funderburgh JL (2000) Keratan sulfate: structure, biosynthesis, and function. *Glycobiology* **10**, 951–958.

7 Mccarthy MM & Bakers JR (1979) Isolation and desulfation of keratan sulfates. *Carbohydr Res* **69**, 151–164.

8 Oeben M, Keller R, Stuhlsatz HW & Greiling H (1987) Constant and variable domains of different disaccharide structure in corneal keratan sulphate chains. *Biochem J* **248**, 85–93.

9 Stuhlsatz HW, Keller R, Becker G, Oeben M, Lennartz L, Fischer DC & Greiling H (1989) Structure of keratan sulfate proteoglycans: core proteins, linkage regions, carbohydrate chains. In *Keratan Sulfate: Chemistry, Biology, Chemical Pathology* (Greiling H & Scott JE, eds), pp. 1–11. The Biochemical Society, London.

10 Tai GH, Huckerby TN & Nieduszynski I (1996) Multiple non-reducing chain termini isolated from bovine corneal keratan sulfates. *J Biol Chem* **271**, 23535–23546.

11 Quantock AJ, Young RD & Akama TO (2010) Structural and biochemical aspects of keratan sulphate in the cornea. *CMLS* **67**, 891–906.

12 Young RD, Akama TO, Liskova P, Ebenezer ND, Allan B, Kerr B, Caterson B, Fukuda MN & Quantock AJ (2007) Differential immunogold localisation of sulphated and unsulphated keratan sulphate proteoglycans in normal and macular dystrophy cornea using sulphation motif-specific antibodies. *Histochem Cell Biol* **127**, 115–1120.

13 Dunlevy JR, Beales MP, Berryhill BL, Cornuet PK & Hassell JR (2000) Expression of the keratan sulfate proteoglycans lumican, keratocan and osteoglycin/mimecan during chick corneal development. *Exp Eye Res* **70**, 349–362.

14 Blochberger TC, Cornuet PK & Hassell JR (1992) Isolation and partial characterization of lumican and decorin from adult chicken corneas. *J Biol Chem* **267**, 20613–20619.

15 Beales MP, Funderburgh JL, Jester JV & Hassell JR (1999) Proteoglycan synthesis by bovine keratocytes and corneal fibroblasts: maintenance of the keratocyte phenotype in culture. *Invest Ophthalmol Vis Sci* **40**, 1658–1663.

16 Li L, Zhang F, Zaia J & Linhardt RJ (2012) Top-down approach for the direct characterization of low molecular weight heparins using LC-FT-MS. *Anal Chem* **84**, 8822–8829.

17 Huckerby TN, Tai GH & Nieduszynski IA (1998) Oligosaccharides derived by endo-beta-galactosidase digestion of bovine corneal keratan sulphate – characterisation of tetrasaccharides with incomplete sulphation and containing unsulphated N-acetylglucosamine residues. *Eur J Biochem* **253**, 499–506.

18 Brown GM, Huckerby TN, Morris HG, Abram BL & Nieduszynski IA (1994) Oligosaccharides derived from bovine articular cartilage keratan sulfates after keratanase II digestion: implications for keratan sulfate structural fingerprinting. *Biochemistry* **33**, 4836–4846.

19 Brown GM, Huckerby TN & Nieduszynski IA (1994) Oligosaccharides derived by keratanase II digestion of bovine articular cartilage keratan sulphates. *Eur J Biochem* **224**, 281–308.

20 Conrad AH, Zhang Y, Tasheva ES & Conrad GW (2010) Proteomic analysis of potential keratan sulfate, chondroitin sulfate A, and hyaluronic acid molecular interactions. *Invest Ophthalmol Vis Sci* **51**, 4500–4515.

21 Gan L, Fagerholm P & Palmblad J (2005) Expression of basic fibroblast growth factor in rabbit corneal alkali wounds in the presence and absence of granulocytes. *Acta Ophthalmol Scand* **83**, 374–378.

22 Qian Y, Chu R, Hu M & Hoffman M (2009) Sonic hedgehog expression and its role in form-deprivation myopia in mice. *Curr Eye Res* **34**, 623–635.

23 Vinothkumar S, Rastegar S, Takamiya M, Ertzer R & Strähle U (2008) Sequential and cooperative action of Fgfs and Shh in the zebrafish retina. *Dev Biol* **314**, 200–214.

24 Saika S, Muragaki Y, Okada Y, Miyamoto T, Ohnishi Y, Ooshima A & Kao WW-Y (2004) Sonic hedgehog expression and role in healing corneal epithelium. *Invest Ophthalmol Vis Sci* **45**, 2577–2585.

25 Martí E & Bovolenta P (2002) Sonic hedgehog in CNS development: one signal, multiple outputs. *Trends Neurosci* **25**, 89–96.

26 Mason I (2007) Initiation to end point: the multiple roles of fibroblast growth factors in neural development. *Nat Rev Neurosci* **8**, 583–596.

27 Esteve P & Bovolenta P (2006) Secreted inducers in vertebrate eye development: more functions for old morphogens. *Curr Opin Neurobiol* **16**, 13–19.

28 Van Setten GB (1996) Basic fibroblast growth factor in human tear fluid: detection of another growth factor. *Graefe's Arch Clin Exp Opthalmol* **234**, 275–277.

29 Fujita K, Miyamoto T & Saika S (2009) Sonic hedgehog: its expression in a healing cornea and its role in neovascularization. *Mol Vis* **15**, 1036–1044.

30 Shaya D, Tocilj A, Li Y, Myette J, Venkataraman G, Sasisekharan R & Cygler M (2006) Crystal structure of heparinase II from Pedobacter heparinus and its complex with a disaccharide product. *J Biol Chem* **281**, 15525–15535.

31 Godavarti R, Davis M, Venkataraman G, Cooney C, Langer R & Sasisekharan R (1996) Heparinase III from Flavobacterium heparinum: cloning and recombinant expression in Escherichia coli. *Biochem Biophys Res Commun* **225**, 751–758.

32 Yoshida E, Arakawa S, Matsunaga T, Toriumi S, Tokuyama S, Morikawa K & Tahara Y (2002) Cloning, sequencing, and expression of the gene from

Bacillus circulans that codes for a heparinase that degrades both heparin and heparan sulfate. *Biosci Biotechnol Biochem* **66**, 1873–1879.
33 Laremore TN, Ly M, Zhang Z, Solakyildirim K, McCallum SA, Owens RT & Linhardt RJ (2010) Domain structure elucidation of human decorin glycosaminoglycans. *Biochem J* **431**, 199–205.
34 Laremore TN, Ly M, Solakyildirim K, Zagorevski DV & Linhardt RJ (2010) High-resolution preparative separation of glycosaminoglycan oligosaccharides by polyacrylamide gel electrophoresis. *Anal Biochem* **401**, 236–241.
35 Abràmoff MDM, Hospitals I & Magalhães PJ (2004) Image processing with ImageJ. *Biophotonics Int* **11**, 36–42.
36 Babu P, North SJ, Jang-Lee J, Chalabi S, Mackerness K, Stowell SR, Cummings RD, Rankin S, Dell A & Haslam SM (2009) Structural characterisation of neutrophil glycans by ultra sensitive mass spectrometric glycomics methodology. *Glycoconj J* **26**, 975–986.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1. KS digest disaccharide analysis by RPIP.

Fig. S2. Fourier transform tandem mass spectrum of KS-derived monosulfated disaccharide.

Fig. S3. Fourier transform mass spectrum of KS-derived linkage region.