

1 March 23, 2019

2

3 **The degree of polymerization and sulfation patterns in heparan
4 sulfate are critical determinants of cytomegalovirus infectivity**

5

6 Mohammad H. Hasan¹, Rinkuben Parmar¹, Quntao Liang^{2,6}, Hong Qiu³, Vaibhav
7 Tiwari⁴, Joshua Sharp², Lianchun Wang⁵ and Ritesh Tandon^{1*}

8

9 ¹Department of Microbiology and Immunology, University of Mississippi Medical Center,
10 2500 North State Street, Jackson, MS 39216, USA. ²Department of Biomolecular
11 Sciences, School of Pharmacy, University of Mississippi, Oxford, MS 38677, USA.

12 ³Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602.

13 ⁴Department of Microbiology and Immunology, Midwestern University, Downers Grove,
14 IL, 60515, USA. ⁵Department of Molecular Pharmacology and Physiology, University of
15 South Florida, Tampa, FL 33612, USA. ⁶College of Biological Science and Engineering,
16 University of Fuzhou, Fujian, 350108, China.

17

18 ***Corresponding Author:** Ritesh Tandon

19 Phone: 601-984-1705, Fax: 601-984-1708, Email: rtandon@umc.edu

20

21 **Keywords:** CMV, herpesviruses, heparan sulfate, sulfation

22

23 **Running title:** HS as primary CMV attachment receptor

24 **Abstract**

25

26 Herpesviruses attach to host cells by interacting with cell surface heparan sulfate (HS)
27 proteoglycans prior to specific coreceptor engagement which culminates in virus-host
28 membrane fusion and virus entry. Interfering with HS-herpesvirus interactions results in
29 significant reduction in virus infectivity indicating that HS play important roles in initiating
30 virus entry. In this study, we provide convincing evidence that specific sulfations as well
31 as the degree of polymerization (*dp*) of HS govern human cytomegalovirus (CMV)
32 infection and binding by following line of evidences. First, purified CMV extracellular
33 virions preferentially bound to the sulfated longer chain of HS on a glycoarray compared
34 to unsulfated glycosaminoglycans and shorter chain unsulfated HS. Second, the fraction
35 of glycosaminoglycans (GAG) displaying higher *dp* and sulfation had a major impact on
36 CMV infectivity and titers. Finally, cell lines knocked out for specific sulfotransferases
37 Glucosaminyl 3-O-sulfotransferase (3-O-ST-1 and -4 and double -1/4) produced
38 significantly reduced CMV titers compared to wild-type cells. Similarly, a peptide
39 generated against sulfated-HS significantly reduced virus titers compared to the control
40 peptide. Taken together, the above results highlight the significance of the chain length
41 and sulfation patterns of HS in CMV binding and infectivity.

42 **Importance**

43

44 The cell surface heparan sulfates (HS) are exploited by multiple viruses as they provide
45 docking sites during cell entry and therefore are a promising target for the development
46 of novel antivirals. In addition, the molecular diversity in HS chains generates unique
47 binding sites for specific ligands and hence offers preferential binding for one virus over
48 other. In the current study several HS mimics were analyzed for their ability to inhibit
49 cytomegalovirus (CMV) infection. The results were corroborated by parallel studies in
50 mutant mouse cells and virus binding to glycoarrays. Combined together, the data
51 suggests that virus particles preferentially attach to specifically modified HS and thus
52 the process is amenable to targeting by specifically designed HS mimics.

53 **Introduction**

54

55 The heparan sulfate (HS) proteoglycans are present on most cell types and function as
56 primary cellular receptor for medically important viruses, including human
57 immunodeficiency virus (HIV), hepatitis-C virus (HCV), human papillomavirus (HPV),
58 and Dengue virus (DENV)¹⁻⁴. In addition, virtually all human herpesviruses, with the
59 possible exception of Epstein Barr virus, use HS as an initial co-receptor for entry⁵. The
60 interaction between cell surface HS and virus envelope is the primary event in the
61 complex process of virus entry. However, this binding is not sufficient for viral entry and
62 requires fusion between the viral envelope and cell membrane⁶.

63 Herpesviruses including other enveloped viruses enter the host cells using two
64 distinct pathways: 1) A pH-intendent pathway which involves the fusion of the virus
65 envelope with the plasma membrane; and 2) A pH-dependent pathway that involves
66 endocytosis of the virus particle⁷. In cells, where binding of virus to cell surface
67 receptors induces endocytosis, the usual consequence is the acidification of the
68 endosome, which ultimately triggers fusion between the virus envelope and endosomal
69 membrane⁵. Interestingly, HCMV entry follows direct fusion at the cell surface in
70 fibroblasts, while entry into other relevant cell types, such as endothelial cells, follows
71 an endocytic route^{8,9}. In either case, HS functions as the primary attachment receptor.
72 Since the presence of HS receptors are well documented in endosomal membranes it is
73 likely that HS receptors also play a role in intracellular virus trafficking¹⁰⁻¹³.

74 The herpesvirus envelope is a lipid bilayer derived from host cell membranes in
75 which most cellular proteins have been displaced by viral membrane proteins. For

76 human cytomegalovirus (HCMV), at least twenty three different viral glycoproteins have
77 been found to be associated with purified virion preparations ¹⁴. For most herpesviruses,
78 the conserved glycoprotein B (gB) is required for virus entry and binds to cell surface
79 molecules, including HS, which is present not only as a constituent of cell surface
80 proteoglycans but also as a component of the extracellular matrix and basement
81 membranes in organized tissues ^{5,15}. HCMV gB binds to HS resulting in virus
82 attachment ¹⁶ similar to its counterparts in herpes simplex virus (HSV)-1 ^{15,17} and
83 varicella-zoster virus (VZV) ¹⁸. Corroborating this fact, treatment of cells with soluble
84 form of gB inhibits HCMV entry ¹⁹. HCMV binding and infection are reduced by soluble
85 heparin and HS, as well as in cells treated with heparinases or those unable to produce
86 HS ²⁰.

87 The synthesis of HS is a complex process involving multiple specialized enzymes
88 and is initiated from a tetrasaccharide (GlcA-Gal-Gal-Xyl) that is attached to the core
89 protein (Fig 1). HS polymerase is responsible for building the polysaccharide backbone
90 with a repeating unit of -GlcA-GlcNAc- (Fig 2). The backbone is then modified by N-
91 deacetylase/N-sulfotransferase (NDST) responsible for N-deacetylation and N-sulfation
92 of selected glucosamine residues, C₅-epimerase responsible for epimerization of
93 selected glucuronic moieties to iduronic acid, 2-O-sulfotransferase (Hs2st; 2-O-ST)
94 responsible for 2-O-sulfation of selected iduronic acid residues, 6-O-sulfotransferase
95 (H6st; 6-O-ST) for 6-O-sulfation and finally (but rarely) 3-O-sulfotransferases (Hs3st; 3-
96 O-ST) responsible for 3-O-sulfation ^{21,22}. The substrate specificities of these biosynthetic
97 enzymes dictate the structures of HS product, including sulfation levels, the contents of

98 IdoA units and the size of the polysaccharides²¹. The location of the sulfo groups and
99 IdoA in turn play a crucial role in determining the binding and functions of HS.

100 The enzymatic modification of HS chain is known to generate unique binding
101 sites for viral ligands. For example, 3-O-sulfation modification in HS chain generates
102 fusion receptor for HSV glycoprotein D (gD) promoting viral entry and spread²³. The 3-
103 O-S HS is a product of enzymatic modification at C3 position of glucosamine residue,
104 which is relatively rare in comparison to other HS modifications (Fig 2). Expression of
105 Hs3st can make normally resistant Chinese hamster ovary (CHO-K1) cells susceptible
106 to HSV-1 infection²⁴. Studies in clinically relevant primary human corneal fibroblasts
107 have also shown 3-O-S HS as a primary receptor for HSV entry²⁵. Interestingly, both
108 HSV-1 and HSV-2 use HS as an attachment receptor but HSV-1 binds to distinct
109 modification sites on HS that HSV-2 is unable to, which could explain some of the
110 differences in cell tropism exhibited by these two viruses²⁶. For example, while N-
111 sulfation and carboxyl groups are required for both HSV-1 and HSV-2 binding, only
112 HSV-1 is able to bind the specific modification sites generated by 2-O, 6-O, and 3-O-
113 sulfations²⁷. The O-desulfated heparins have little or no inhibitory effect on HSV-1
114 infection but inhibit HSV-2 infection. This susceptibility to O-desulfated heparins can be
115 transferred to HSV-1 by recombinant transfer of the gene for glycoprotein C (gC-2) from
116 HSV-2²⁷. It was recently established that 3-O-S HS are important for HCMV entry in
117 human iris stromal (HIS) cells²⁸. The expression of Hs3st in HIS cells promoted HCMV
118 internalization, while pretreatment of HIS cells with heparinase enzyme or treatment
119 with anti-3-O-S HS (G2) peptide significantly reduced HCMV plaques/foci formation. In
120 addition, co-culture of the HCMV-infected HIS cells with CHO-K1 cells expressing 3-O-S

121 HS significantly enhanced cell fusion. A similar trend of enhanced fusion was observed
122 with cells expressing HCMV glycoproteins (gB, gO, and gH-gL) co-cultured with 3-O-S
123 HS cells. These results highlight the role of 3-O-S HS during HCMV entry.

124 Owing to their inherent structural features, certain sulfated glycans can exert
125 therapeutic effects against infections caused by pathogenic microorganisms. A study by
126 Pomin et al., laid the proof-of-concept by administering sulfated glycans to disrupt the
127 pathogen protein-host glycosaminoglycan (GAG) complex formation causing
128 impairment of microbial binding onto host cells ²⁹. Similarly, sulfated GAG,
129 glycosphingolipids and lectins have been shown to inhibit DENV entry.³⁰ Heparan
130 sulfate mimics, such as suramin, pentosan polysulfate, and PI-88, SPGG ^{31,32} have
131 been reported to be effective against multiple viruses including herpesviruses ^{2,33,34}.
132 The inhibitory activity of HS mimics, including these compounds, is believed to be due
133 to their association with GAG binding sites of the putative receptor-binding domain on
134 the viral protein ^{2,35}. Thus, HS mimics can inhibit virus adsorption and entry.

135 In the current study, we investigated the impact of specific sulfations as well as
136 degree of polymerization (*dp*) in HS chain on both human and mouse CMV infection
137 and binding. Purified CMV extracellular virions preferentially bound strongly to the
138 longer chain sulfated HS but not to the shorter chain unsulfated HS on a glycoarray.
139 Glycosaminoglycans of different *dp* were derivatized from enoxaparin (a low molecular
140 weight heparin) and tested for their ability to inhibit CMV infection in cell culture. The
141 results show that longer glycan chains are more efficient at reducing CMV titers in cells
142 compared to shorter chain glycans. Finally, the cell lines defective in 3-O-ST -1 and -4
143 expression had reduced CMV replication. Moreover, a peptide generated against the

144 sulfated-HS significantly reduced HCMV titers compared to control peptide. Overall,
145 these results indicate that CMV binding to cell surface glycans is dependent on branch
146 length and sulfation pattern of HS.

147

148 **Materials and Methods**

149

150 **Preparation of Glycosaminoglycans (GAGs) oligosaccharides.**

151 Glycosaminoglycans of different *dp* were fractionated from enoxaparin (a low molecular
152 weight heparin) by Bio-Gel P-10 chromatography as previously described³⁶. Briefly, 30
153 mg/2 mL Enoxaparin Sodium derived from porcine intestinal mucosa (Sanofi-Aventis
154 U.S., Bridgewater, NJ) was applied to a Bio-Gel P-10 column (2.5×120 cm, Bio-Rad,
155 Hercules CA) and eluted with 0.2 M NH₄HCO₃ at a flow rate of 14 mL/h. Elution of
156 oligosaccharides was monitored by absorbance at 232 nm. NH₄HCO₃ was removed by
157 heating in oven at 50°C for 24 h.

158

159 **Preparation of the 6-O desulfated Arixtra with MTSTFA.** A detailed procedure on the
160 preparation of 6-O desulfated Arixtra was published previously³⁷. Briefly, 4 mg of Arixtra
161 was added to 10 volumes (w/w) of N-Methy-N-(trimethylsilyl)-trifluoroacetamide
162 (MTSTFA, Sigma, ≥98.5%) and 100 volumes (v/w) of pyridine. The mixture was heated
163 at 100 °C for 30 min, then quickly cooled in an ice-bath, followed by extensive dialysis
164 and freeze-drying. The sample was resuspended in 50% acetonitrile/water at a
165 concentration of 30 μM for later LC-MS/MS analysis.

166

167 **LC-MS/MS Analysis.** The 6-O-desulfated Arixtra (30 µM) was analyzed on a Thermo
168 Orbitrap Fusion Tribrid (Thermo Fisher Scientific) coupled with an Ultimate 3000 Nano
169 LC system (Dionex) using direct infusion. The flow rate was set to 1 µL/min. Mobile-
170 phase was 50% acetonitrile. Nanoelectrospray voltage was set to 2.0 kV in negative ion
171 mode. Full MS scan range was set to 200-2000 m/z at a resolution of 60,000, RF lens
172 was 6%, and the automatic gain control (AGC) target was set to 2.0×10^5 . For the
173 MS/MS scans, the resolution was set to 50,000, the precursor isolation width was 3 m/z
174 units, and ions were fragmented by collision-induced dissociation (CID) at a normalized
175 collision energy of 80%.

176

177 **Cells.** Mouse embryonic fibroblasts (MEF) and human foreskin fibroblasts (HFF) were
178 cultured in Dulbecco's modified Eagle's medium (DMEM, Cellgro, Manassas, VA)
179 containing 4.5 g/ml glucose, 10% fetal bovine serum (SAFC, Lenexa, KS), 1 mM
180 sodium pyruvate, 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin (Cellgro,
181 Manassas, VA) at 37°C with 5% CO₂. Mutant mouse lung endothelial cells (WT, H3st1-
182 knockout, H3st4-knockout, H3st1/4-double-knockout) were obtained from Wang
183 laboratory at University of South Florida and maintained as described earlier ³⁸. Cells
184 were split upon confluence.

185

186 **Virus.** MCMV (strain K181) was grown in MEF cells, while HCMV (Towne strain) was
187 grown on HFF cells. Virus stock was prepared in 3X autoclaved milk, sonicated 3 times
188 and stored at -80°C. During infection, media was removed from the wells of cell culture
189 plates and appropriately diluted virus stock was absorbed onto the cells in raw DMEM.

190 Cells were incubated for 1 hour with gentle shaking every 10 mins followed by washing
191 3X with PBS. Fresh complete medium was added and cells were incubated until the end
192 point. For extracellular virus (ECV) purification, HFF were seeded in roller bottles, grown
193 to confluence and infected with HCMV (Towne strain) at MOI of 0.01. Two days after
194 100% cytopathic effect was observed, infected cell medium was collected and
195 centrifuged at low speed to pellet cellular debris, and the supernatant was transferred to
196 new tubes and centrifuged at 20,000 g for 1 hour to pellet the ECV. This ECV pellet was
197 re-suspended in phosphate buffer, sonicated to eliminate any aggregates, loaded over
198 15-50% continuous sucrose gradients and centrifuged in a SW-41 rotor at 39,000 RPM
199 for 20 min. ECV bands were visualized in incandescent light and harvested by
200 puncturing the sides of the centrifuge tubes. These bands were washed once with
201 phosphate buffer, spun again and the final pellet resuspended in low salt phosphate
202 buffer. An aliquot of the sample was used for assessment of initial quality of ECV by
203 negative staining and transmission electron microscopy. Purified ECV were shipped on
204 ice to Z biotech (Aurora, CO) for glycoarray binding analysis.

205
206 **Cell Viability Assay.** Cells plated in 12 well tissue culture plates were grown to
207 confluence and pretreated for 1h with 10 μ M concentration of candidate HS and then
208 infected with HCMV (Towne strain) at a multiplicity of infection (MOI) of 3.0 or mock-
209 infected. Five hundred μ l of fresh complete medium was added to the wells on day 3
210 and day 6. At the designated time points, media was removed and cells were harvested
211 by trypsinization. Cell viability was determined using trypan blue exclusion on TC20
212 automated cell counter (BioRad Laboratories, Hercules, CA) following manufacturer's

213 protocol.

214

215 **Virus Titers.** Infected or mock-infected samples were harvested within the medium at
216 the designated end points and stored at -80°C before titration. In some experiments,
217 media and cells were separated by low-speed (< 1000 X g) centrifugation and viral
218 loads in supernatant and cells were quantified by titering on wild-type cells. Titers were
219 performed as described earlier ³⁹ with some modifications. In brief, monolayers of
220 fibroblasts grown in 12 well plates and serial dilutions of sonicated samples were
221 absorbed onto them for 1 h, followed by 3X washing with PBS. Carboxymethylcellulose
222 (CMC) (Catalog No. 217274, EMD Millipore Corp., Billerica, MA) overlay with complete
223 DMEM media (1-part autoclaved CMC and 3 parts media) was added and cells were
224 incubated for 5 days. At end point, overlay was removed and cells were washed 2X
225 with PBS. Infected monolayers were fixed in 100% methanol for 7 min, washed once
226 with PBS and stained with 1% crystal violet (Catalog No. C581-25, Fisher Chemicals,
227 Fair Lawn, NJ) for 15 min. Plates were finally washed with tap water, air dried and
228 plaques with clear zone were quantified.

229

230 **Glycoarrays.** A dilution series of purified HCMV virions were incubated on two different
231 custom glycoarrays (Table 1 and Table 2, Z-Biotech) using established protocols ⁴⁰ and
232 the arrays were analyzed to assess specific virus binding. Briefly, 10⁵ to 10⁸ pfu/ml of
233 purified virions were incubated for an hour on glycoarrays containing six replicates of
234 each glycosaminoglycan. After incubation, staining with primary antibody (mouse anti
235 gB (clone 2F12, Virusys Inc, Taneytown, MD) was done at 100 µg/ml and secondary

236 antibody (Goat anti mouse IgG AlexaFlour555) was done at 1 μ g/ml. Maximum strength
237 fluorescent signal was obtained for 10⁸ pfu/ml concentration of the virus, therefore, only
238 this concentration is represented in the final data obtained for plotting the graphs.

239

240 **Effect of anti-HS and anti-3OS HS peptide on CMV entry.** HFF cells were pre-treated
241 with the phage display derived peptides (1 mg/ml) generated against wild-type HS
242 (LRSRTKIIIRRH), and 3-O-S HS (MPRRRRIRRRQK)⁴¹ or left mock treated for 4 hours
243 before the cells were infected with β -galactosidase expressing CMV (ATCC) for 9 days.
244 β -Galactosidase assay were performed using X-gal (Sigma). The effect of entry-
245 blocking activity of peptide was examined by counting number of virus foci. Results are
246 representative of three independent experiments.

247

248 **Results**

249

250 **Purified HCMV extracellular virions preferentially bind to sulfated
251 glycosaminoglycans with increased degree of polymerization.**

252

253 HCMV extracellular virions were purified as described above and incubated with custom
254 glycoarrays containing increasing molecular weight species of hyaluronic acid, heparin,
255 chondroitin sulfate, and dermatan sulfate (Table 1). As indicated in Fig. 3 HCMV binding
256 to non-sulfated hyaluronic acid (HA10 to HA20 and HA93 polymer) was negligible but
257 significant binding to all heparin species was detected with a trend of increased binding
258 to heparins as their *dp* increased. HCMV also showed binding to large size chondroitin

259 sulfate D (CS-D 20), and dermatan sulfate oligosaccharides (DS16-20) but not to
260 Chondroitin Sulphate AC (CS-AC). It is important to note that while the CS-A is sulfated
261 at C4 of the GalNAc, and the CS-C is sulfated at the C6 of the GalNAc only, the CS-D is
262 sulfated at C2 of the glucuronic acid as well as the C6 of the GalNAc sugar and hence
263 has double the amount of sulfation compared to CS-A and CS-C. Dermatan sulfate,
264 formerly referred to as CS-B, is formed from the polymer backbone of chondroitin
265 sulfate by the action of chondroitin-glucuronate C5 epimerase, which epimerizes
266 individual d-glucuronic acid residues to l-iduronic acid. The binding affinity to DS was
267 also size-dependent increasing from DS16 to DS20. Heparin (*dp*30) was the best binder
268 in this assay.

269 On a second HS specific array (Table 2), HCMV showed decent specific binding
270 to sulfated HS with stronger binding to the HS with longer disaccharide chains (HS007
271 to HS024) (Fig 4). HCMV showed minimal binding to unsulfated glycans (HS001-
272 HS006). The maximum binding was observed for HS014, HS015 and HS016, which are
273 all 6-O-S 9-mers with moderate amount of sulfation (1.3-1.8 sulfate group per
274 disaccharide). Also, significant amount of binding was observed for 2-O-S (HS17-
275 HS19), 6-O-S/2-O-S (HS20-22) and 2-O-S/6-O-S/3-O-S (HS23-24) HS that had high
276 amount of sulfation (1.3-2.7 sulfate group per disaccharide) and 6-8 disaccharide per
277 chain. Overall the data from these experiments indicate that the *dp* of HS as well as
278 sulfation is important for HCMV binding.

279

280 **The degree of polymerization of GAG chains impacts CMV infectivity.**

281

282 Glycosaminoglycans of different *dp* were fractionated from enoxaparin (a low molecular
283 weight heparin). All of these GAGs are based on a HS backbone and differ in either *dp*
284 or degree/place of sulfation or both (Fig 5, S1-S3). These GAGs, along with heparin and
285 Arixtra (fondaparinux sodium), were first screened in a GFP-based virus focus reduction
286 assay using GFP tagged HCMV (Towne strain). The viral GFP expression was most
287 efficiently reduced by heparin salt (PIHSS; Heparin sodium salt from porcine intestinal
288 mucosa) whereas arixtra, 6-O-desulfated arixtra and Enoxaparin had little to no impact
289 on GFP expression (Fig 5). In general, enoxaparin derived GAGs with higher *dp* were
290 more efficient in reducing viral GFP compared to low *dp* derivatives. This screening
291 assay when performed at a range of GAG concentrations (10nM to 100 μ M) determined
292 10 μ M as the most effective concentration at reducing viral titers with no additional
293 reduction seen at >10 μ M concentrations (data not shown). To follow up on this primary
294 GFP based screening, we performed viral titer assay using HCMV (Towne strain) that
295 measures total virus yields at 5 days post infection. Most reduction in viral titers was
296 observed for heparin (PIHSS) followed by enoxaparin derivative with >20 *dp* (Fig 6A).
297 Plotting of viral titer reduction as a function of *dp* revealed a general trend where higher
298 *dp* derivatives lead to higher reduction in viral titers (Fig 6B). Thus, this experiment
299 indicated that longer HS chains are more efficient at reducing HCMV titers in cells. To
300 investigate whether this inhibitory effect was due to an increase in avidity of longer
301 chain GAGs towards virus particles, the experiments were repeated at 0.05 g/L
302 concentrations of GAGs (Fig 7A). Similar trend of inhibitory results leaning towards
303 efficacy of higher *dp* against HCMV infection were obtained at 0.05 g/L indicating that
304 this effect is dictated by the molecular composition of GAG and is not a mere effect of

305 increased avidity. A line graph for each concentration of GAGs was generated that
306 demonstrates the relationship of viral titer and degree of polymerization (Fig 7B). To
307 investigate whether some of these effects on virus titers could be attributed to cell
308 death, we performed cell viability assays in both uninfected and infected settings. Cell
309 viability was not affected at the treated concentrations of any of our test GAGs (Fig 8A).
310 Moreover, heparin (PIHSS) and enoxaparin derivatives (*dp* 12 or greater) efficiently
311 protected cells from virus induced lytic death (Fig 8B). These results corroborate the
312 results of our glycoarray experiments that showed that GAG with higher *dp* have higher
313 CMV binding compared to GAG with lower *dp* (Fig 4).

314

315 **Cell lines defective in expression of specific sulfation enzymes have reduced
316 CMV titers.**

317

318 Due to species specificity of HCMV, animal models are frequently used to study CMV
319 pathogenesis ^{42,43}. Studies of murine CMV (MCMV) infections of mice have served a
320 major role as a model of CMV biology and pathogenesis ⁴⁴. Mutant mouse lung
321 endothelial cell lines were from adult mice were mutated for specific sulfotransferase
322 enzymes by a CRISPR-Cas9 based gene editing system ^{38 45 46}. Since previous studies
323 showed that 3-O-S HS is important for HCMV entry in human iris stromal cells ²⁸, we
324 analyzed virus replication in Hs3st1 and Hs3st4 (Glucosaminyl 3-O-sulfotransferase 1
325 and 4, respectively) knockout cell lines as well as the Hs3st1/4 double knockout cell
326 line. At high (5.0) as well as low (0.01) multiplicity of infection (MOI), MCMV growth was
327 significantly reduced in the single Hs3st1 and Hs3st4 knockouts as well as in the double

328 Hs3st1/4 knockouts, indicating that 3-O-sulfation of HS is important for HCMV infection
329 (Fig 9).

330

331 **Peptide generated against 3-O-S HS blocks HCMV infection.**

332

333 In order to examine the effect of sulfated HS on HCMV infection, we utilized phage
334 display derived anti-HS and anti-3-O-S HS peptide ⁴¹. The HFF cells were pre-treated
335 either with anti-HS peptide or anti-3-O-S HS peptide. The mock treated cells were
336 considered as a positive control. As indicated in Fig. 9, the anti-3-O-S HS peptide
337 treatment resulted in a significant reduction of HCMV titers in HFF cells compared to an
338 anti-HS peptide or the mock-treated cells.

339

340 **Discussion**

341

342 In this study, we utilized multiple tools such as glycoarray binding analysis, HS mimics,
343 HS mutant cell lines, and anti-HS/3-OS HS peptides to provide convincing evidence that
344 specifically sulfated HS with higher degree of polymerization determine CMV infection
345 and binding. We first screened several GAGs that are sulfated or unsulfated and have
346 complex sugar structure to investigate which GAGs are more efficient at binding to
347 HCMV virions. This glycoarray analysis indicated that HCMV bound with heparins with
348 strong affinity and showed increased binding for longer chain length (Fig. 3). To further
349 investigate this binding, we utilized another glycoarray consisting of HS of varied
350 polymerization and sulphation levels. The results from this glycoarray indicated that

351 HCMV binds strongly with HS having both longer sugar residues and a moderate level
352 of sulphation. Thus, sulfated HS with more complex branches and sulfation patterns
353 preferentially bind to HCMV. Next, we fractioned HS by length (2-20) from enoxaparin
354 and tested their ability to inhibit HCMV growth in cell culture by competing with HCMV
355 binding. In the preliminary experiment, GFP tagged HCMV was used and the number of
356 GFP+ foci was quantified. The data from this experiment indicated that viral GFP was
357 more effectively reduced when cells were pretreated with GAGs having a higher dp (Fig
358 5). For a deeper understanding of this reduction, we performed a similar experiment
359 where HCMV towne strain was used and viral load was quantified at 5 days post
360 infection. Significant reduction in virus titers was observed in samples treated with
361 higher *dp* of GAG but not with lower *dp* corroborating the results from glycoarray
362 experiments that chain length of GAG is an important factor in determining HCMV
363 binding. Also, this effect was not due to combined affinities of multiple binding sites on
364 GAGs as evidenced by similar trend of inhibition obtained when treating cells with
365 equivalent μM or $\mu\text{g/ml}$ concentrations of GAGs. Treatment of cells with these GAGs did
366 not affect their viability for the duration of treatment (Fig 8A) confirming that the
367 observed reduction in virus titer was not due to the cell death. Moreover, cells
368 pretreated with GAGs associated with longer dp resisted infection induced cell death at
369 late time post infection (Fig 8B). We also tested the impact of specific HS sulfation
370 mutants on MCMV infection. As 3-O sulfation has been reported to be critical for
371 herpesvirus entry^{25,28}, we tested MCMV growth in Hs3st1, Hs3st4 and dual Hs3st1/4
372 knockout cells. For both high and low MOI, virus titer was significantly reduced in

373 Hs3st1, Hs3st4 and dual Hs3st1/4 knockout cells (Fig 9). Additional data from anti-3-O-
374 S HS peptide confirmed the significance of sulfation in HCMV infectivity.

375 Overall, the data from these studies indicate that *dp* of GAGs as well as specific
376 sulfation patterns govern HCMV infection of cells. These studies show the promise that
377 highly polymerized sulfated-HS targeted to develop effective anti-CMV agents. Future
378 studies would be aimed at confirming the CMV glycoproteins that specifically bind to HS
379 on cell surface and their possible structural illustrations. It would also be interesting to
380 pursue specifically designed glycomimetics that inhibit these specific virus-host
381 interactions for their effectiveness in a mouse model of CMV infection as well as in
382 future clinical trials.

383

384 **Acknowledgments**

385

386 The research was supported by American Heart Association (Award 14SDG20390009,
387 PI: Tandon) and NIH (Award R21HL131553, PI: L.W.). QL, JSS and LW acknowledge
388 funding from the National Institute of General Medical Sciences through the Research
389 Resource for Integrated Glycotechnology (P41GM103390).

390

391 **Author Contributions**

392

393 RT, JSS, and LW designed the experiments; MHH, RBP, QL, JSS, VT and RT
394 performed the experiments and analyzed the data. RT and MHH wrote and edited the
395 manuscript.

396 **References**

- 397
- 398 1 Barth, H. *et al.* Cellular binding of hepatitis C virus envelope glycoprotein E2
399 requires cell surface heparan sulfate. *J Biol Chem* **278**, 41003-41012,
400 doi:10.1074/jbc.M302267200 (2003).
- 401 2 Chen, Y. *et al.* Dengue virus infectivity depends on envelope protein binding to
402 target cell heparan sulfate. *Nat Med* **3**, 866-871 (1997).
- 403 3 Giroglou, T., Florin, L., Schafer, F., Streeck, R. E. & Sapp, M. Human
404 papillomavirus infection requires cell surface heparan sulfate. *J Virol* **75**, 1565-
405 1570, doi:10.1128/JVI.75.3.1565-1570.2001 (2001).
- 406 4 Tyagi, M., Rusnati, M., Presta, M. & Giacca, M. Internalization of HIV-1 tat
407 requires cell surface heparan sulfate proteoglycans. *J Biol Chem* **276**, 3254-
408 3261, doi:10.1074/jbc.M006701200 (2001).
- 409 5 Shukla, D. & Spear, P. G. Herpesviruses and heparan sulfate: an intimate
410 relationship in aid of viral entry. *J Clin Invest* **108**, 503-510,
411 doi:10.1172/JCI13799 (2001).
- 412 6 Shukla, D. *et al.* A novel role for 3-O-sulfated heparan sulfate in herpes simplex
413 virus 1 entry. *Cell* **99**, 13-22 (1999).
- 414 7 Blanchard, E. *et al.* Hepatitis C virus entry depends on clathrin-mediated
415 endocytosis. *J Virol* **80**, 6964-6972, doi:10.1128/JVI.00024-06 (2006).
- 416 8 Compton, T., Fiore, A. Early events in human cytomegalovirus infection., p. 229–
417 238. In A. M. Arvin, E. S. Mocarski, P. Moore, R. Whitley, K. Yamanishi, G.

- 418 *Campadelli-Fiume, and B. Roizman (ed.), Human Herpesviruses: Biology,*
419 *Therapy and Immunoprophylaxis. Cambridge Press, Cambridge.* (2007).
- 420 9 Ryckman, B. J., Jarvis, M. A., Drummond, D. D., Nelson, J. A. & Johnson, D. C.
421 Human cytomegalovirus entry into epithelial and endothelial cells depends on
422 genes UL128 to UL150 and occurs by endocytosis and low-pH fusion. *J Virol* **80**,
423 710-722, doi:10.1128/JVI.80.2.710-722.2006 (2006).
- 424 10 Podyma-Inoue, K. A., Moriwaki, T., Rajapakshe, A. R., Terasawa, K. & Hara-
425 Yokoyama, M. Characterization of Heparan Sulfate Proteoglycan-positive
426 Recycling Endosomes Isolated from Glioma Cells. *Cancer Genomics Proteomics*
427 **13**, 443-452 (2016).
- 428 11 Park, H. *et al.* Heparan sulfate proteoglycans (HSPGs) and chondroitin sulfate
429 proteoglycans (CSPGs) function as endocytic receptors for an internalizing anti-
430 nucleic acid antibody. *Sci Rep* **7**, 14373, doi:10.1038/s41598-017-14793-z
431 (2017).
- 432 12 Christianson, H. C. & Belting, M. Heparan sulfate proteoglycan as a cell-surface
433 endocytosis receptor. *Matrix Biol* **35**, 51-55, doi:10.1016/j.matbio.2013.10.004
434 (2014).
- 435 13 Sarrazin, S., Lamanna, W. C. & Esko, J. D. Heparan sulfate proteoglycans. *Cold*
436 *Spring Harb Perspect Biol* **3**, doi:10.1101/cshperspect.a004952 (2011).
- 437 14 Varnum, S. M. *et al.* Identification of proteins in human cytomegalovirus (HCMV)
438 particles: the HCMV proteome. *J Virol* **78**, 10960-10966,
439 doi:10.1128/JVI.78.20.10960-10966.2004 (2004).

- 440 15 Spear, P. G., Shieh, M. T., Herold, B. C., WuDunn, D. & Koshy, T. I. Heparan
441 sulfate glycosaminoglycans as primary cell surface receptors for herpes simplex
442 virus. *Adv Exp Med Biol* **313**, 341-353 (1992).
- 443 16 Compton, T., Nowlin, D. M. & Cooper, N. R. Initiation of human cytomegalovirus
444 infection requires initial interaction with cell surface heparan sulfate. *Virology*
445 **193**, 834-841, doi:10.1006/viro.1993.1192 (1993).
- 446 17 Laquerre, S. *et al.* Heparan sulfate proteoglycan binding by herpes simplex virus
447 type 1 glycoproteins B and C, which differ in their contributions to virus
448 attachment, penetration, and cell-to-cell spread. *J Virol* **72**, 6119-6130 (1998).
- 449 18 Jacquet, A. *et al.* The varicella zoster virus glycoprotein B (gB) plays a role in
450 virus binding to cell surface heparan sulfate proteoglycans. *Virus Res* **53**, 197-
451 207 (1998).
- 452 19 Boyle, K. A. & Compton, T. Receptor-binding properties of a soluble form of
453 human cytomegalovirus glycoprotein B. *J Virol* **72**, 1826-1833 (1998).
- 454 20 Song, B. H., Lee, G. C., Moon, M. S., Cho, Y. H. & Lee, C. H. Human
455 cytomegalovirus binding to heparan sulfate proteoglycans on the cell surface
456 and/or entry stimulates the expression of human leukocyte antigen class I. *J Gen*
457 *Virol* **82**, 2405-2413, doi:10.1099/0022-1317-82-10-2405 (2001).
- 458 21 Esko, J. D. & Selleck, S. B. Order out of chaos: assembly of ligand binding sites
459 in heparan sulfate. *Annu Rev Biochem* **71**, 435-471,
460 doi:10.1146/annurev.biochem.71.110601.135458 (2002).

- 461 22 Multhaupt, H. A. & Couchman, J. R. Heparan sulfate biosynthesis: methods for
462 investigation of the heparanosome. *J Histochem Cytochem* **60**, 908-915,
463 doi:10.1369/0022155412460056 (2012).
- 464 23 Tiwari, V., Tarbutton, M. S. & Shukla, D. Diversity of heparan sulfate and HSV
465 entry: basic understanding and treatment strategies. *Molecules* **20**, 2707-2727,
466 doi:10.3390/molecules20022707 (2015).
- 467 24 Tiwari, V. *et al.* Soluble 3-O-sulfated heparan sulfate can trigger herpes simplex
468 virus type 1 entry into resistant Chinese hamster ovary (CHO-K1) cells. *J Gen
469 Virol* **88**, 1075-1079, doi:10.1099/vir.0.82476-0 (2007).
- 470 25 Tiwari, V. *et al.* Role for 3-O-sulfated heparan sulfate as the receptor for herpes
471 simplex virus type 1 entry into primary human corneal fibroblasts. *J Virol* **80**,
472 8970-8980, doi:10.1128/JVI.00296-06 (2006).
- 473 26 O'Donnell, C. D., Kovacs, M., Akhtar, J., Valyi-Nagy, T. & Shukla, D. Expanding
474 the role of 3-O sulfated heparan sulfate in herpes simplex virus type-1 entry.
475 *Virology* **397**, 389-398, doi:10.1016/j.virol.2009.11.011 (2010).
- 476 27 Herold, B. C., Gerber, S. I., Belval, B. J., Siston, A. M. & Shulman, N. Differences
477 in the susceptibility of herpes simplex virus types 1 and 2 to modified heparin
478 compounds suggest serotype differences in viral entry. *J Virol* **70**, 3461-3469
479 (1996).
- 480 28 Baldwin, J. *et al.* A role for 3-O-sulfated heparan sulfate in promoting human
481 cytomegalovirus infection in human iris cells. *J Virol* **89**, 5185-5192,
482 doi:10.1128/JVI.00109-15 (2015).

- 483 29 Pomin, V. H. Antimicrobial Sulfated Glycans: Structure and Function. *Curr Top
484 Med Chem* **17**, 319-330 (2017).
- 485 30 Hidari, K. I., Abe, T. & Suzuki, T. Carbohydrate-related inhibitors of dengue virus
486 entry. *Viruses* **5**, 605-618, doi:10.3390/v5020605 (2013).
- 487 31 Gangji, R. N. *et al.* Inhibition of Herpes Simplex Virus-1 Entry into Human Cells
488 by Nonsaccharide Glycosaminoglycan Mimetics. *ACS Med Chem Lett* **9**, 797-
489 802, doi:10.1021/acsmedchemlett.7b00364 (2018).
- 490 32 Majmudar, H. *et al.* A synthetic glycosaminoglycan mimetic blocks HSV-1
491 infection in human iris stromal cells. *Antiviral Res* **161**, 154-162,
492 doi:10.1016/j.antiviral.2018.11.007 (2019).
- 493 33 Lee, E., Pavv, M., Young, N., Freeman, C. & Lobigs, M. Antiviral effect of the
494 heparan sulfate mimetic, PI-88, against dengue and encephalitic flaviviruses.
495 *Antiviral Res* **69**, 31-38, doi:10.1016/j.antiviral.2005.08.006 (2006).
- 496 34 Marks, R. M. *et al.* Probing the interaction of dengue virus envelope protein with
497 heparin: assessment of glycosaminoglycan-derived inhibitors. *J Med Chem* **44**,
498 2178-2187 (2001).
- 499 35 Modis, Y., Ogata, S., Clements, D. & Harrison, S. C. A ligand-binding pocket in
500 the dengue virus envelope glycoprotein. *Proc Natl Acad Sci U S A* **100**, 6986-
501 6991, doi:10.1073/pnas.0832193100 (2003).
- 502 36 Wei, Z., Lyon, M. & Gallagher, J. T. Distinct substrate specificities of bacterial
503 heparinases against N-unsubstituted glucosamine residues in heparan sulfate.
504 *The Journal of biological chemistry* **280**, 15742-15748,
505 doi:10.1074/jbc.M501102200 (2005).

- 506 37 Kariya, Y. *et al.* Preparation of completely 6-O-desulfated heparin and its ability
507 to enhance activity of basic fibroblast growth factor. *The Journal of biological*
508 *chemistry* **275**, 25949-25958, doi:10.1074/jbc.M004140200 (2000).
- 509 38 Qiu, H. *et al.* A mutant-cell library for systematic analysis of heparan sulfate
510 structure-function relationships. *Nat Methods* **15**, 889-899, doi:10.1038/s41592-
511 018-0189-6 (2018).
- 512 39 Zurbach, K. A., Moghbeli, T. & Snyder, C. M. Resolving the titer of murine
513 cytomegalovirus by plaque assay using the M2-10B4 cell line and a low viscosity
514 overlay. *Virology journal* **11**, 71, doi:10.1186/1743-422X-11-71 (2014).
- 515 40 Alam, S. M. *et al.* Mimicry of an HIV broadly neutralizing antibody epitope with a
516 synthetic glycopeptide. *Sci Transl Med* **9**, doi:10.1126/scitranslmed.aai7521
517 (2017).
- 518 41 Tiwari, V., Liu, J., Valyi-Nagy, T. & Shukla, D. Anti-heparan sulfate peptides that
519 block herpes simplex virus infection in vivo. *J Biol Chem* **286**, 25406-25415,
520 doi:10.1074/jbc.M110.201103 (2011).
- 521 42 Reddehase, M. J. & Lemmermann, N. A. W. Mouse Model of Cytomegalovirus
522 Disease and Immunotherapy in the Immunocompromised Host: Predictions for
523 Medical Translation that Survived the "Test of Time". *Viruses* **10**,
524 doi:10.3390/v10120693 (2018).
- 525 43 Brune, W., Hengel, H. & Koszinowski, U. H. A mouse model for cytomegalovirus
526 infection. *Curr Protoc Immunol Chapter* **19**, Unit 19 17,
527 doi:10.1002/0471142735.im1907s43 (2001).

- 528 44 Cekinovic, D., Lisnic, V. J. & Jonjic, S. Rodent models of congenital
529 cytomegalovirus infection. *Methods Mol Biol* **1119**, 289-310, doi:10.1007/978-1-
530 62703-788-4_16 (2014).
- 531 45 Sauer, B. Functional expression of the cre-lox site-specific recombination system
532 in the yeast *Saccharomyces cerevisiae*. *Mol Cell Biol* **7**, 2087-2096 (1987).
- 533 46 Zhang, F., Wen, Y. & Guo, X. CRISPR/Cas9 for genome editing: progress,
534 implications and challenges. *Hum Mol Genet* **23**, R40-46,
535 doi:10.1093/hmg/ddu125 (2014).
- 536 47 Poulain, F. E. & Yost, H. J. Heparan sulfate proteoglycans: a sugar code for
537 vertebrate development? *Development* **142**, 3456-3467, doi:10.1242/dev.098178
538 (2015).
- 539

540 **Figure 1. Structural features of heparan sulfate.** HS is a linear polysaccharide
541 composed of repeating uronic acid [D-glucuronic acid (GlcA) or L-iduronic acid (IdoA)]
542 and D-glucosamine (GlcN) disaccharide subunits. Synthesized chain of HS represents
543 assembly of the tetrasaccharide linker region (GlcA-Gal-Gal-Xyl) at reducing end on
544 serine residues of the protein core followed by the addition of alternating GlcA and
545 GlcNAc residues. The chain extension is also accompanied by a series of modifications,
546 which include 6-O, 3-O sulfations on GlcN and the 2-O sulfation on GlcA. The arrow
547 shows the 3-O position of the GlcN where sulfation is important for herpesvirus binding
548 ^{13,23}.

549

550 **Figure 2. Heparan sulfate modifications.** Heparan sulfate chains are initially
551 synthesized as repeating disaccharide units of N-acetylated glucosamine and
552 glucuronic acid. HS can then be modified by a series of enzymatic reactions, including
553 N-deacetylation and N-sulfation of N-acetylated glucosamine converting it to N-sulfo-
554 glucosamine, C5 epimerization of glucuronic acid to iduronic acid, and O-sulfation at the
555 2-OH, 6-OH, and 3-OH positions. Among sulfations, first is 2-O-sulfation of iduronic acid
556 and glucuronic acid, followed by 6-O-sulfation of N-acetylated glucosamine and N-sulfo-
557 glucosamine units, and finally 3-O-sulfation of glucosamine residues ^{26,47}.

558

559 **Figure 3. Binding of purified extracellular CMV virions on a custom synthesized**
560 **glycosaminoglycan glycoarray.** Relative fluorescence units (RFU), which are directly
561 proportional to the amount of virus binding, are plotted on the Y-axis in the graph.
562 Ligand descriptions and chain structures are provided in Table 1. Six replicates for each

563 GAG were used in the assay. NC: Negative control (print buffer), PC1: positive control
564 (Biotinylated Glycan), PC2: human IgG (0.1 mg/ml), PC3: mouse IgG (0.1 mg/ml), PC4:
565 rabbit IgG (0.1 mg/ml).

566

567 **Figure 4. Binding of purified extracellular CMV on a custom synthesized heparan
568 sulfate glycoarray.** Relative fluorescence units (RFU), which are directly proportional
569 to the amount of virus binding, are plotted on the Y-axis in the graph. Ligand
570 descriptions and chain structures are provided in table 2. Twelve replicates for each
571 ligand were used. NC: negative control (print buffer) PC1: positive control (biotinylated
572 glycan), PC2: human IgG (0.1 mg/ml), PC3: mouse IgG (0.1 mg/ml), PC4: rabbit IgG
573 (0.1 mg/ml).

574

575 **Figure 5. Inhibition of HCMV growth by glycosaminoglycan derivatives.** Primary
576 human foreskin fibroblasts (HFF) grown in 96 well plate were pretreated for one hour
577 with 10 μ M of 1) 6-O-desulfated Arixtra, 2) Regular Arixtra, 3) Heparin sodium salt from
578 porcine intestinal mucosa (PIHSS), 4) Enoxaparin, or series of heparin oligosaccharide
579 from enoxaparin: 5) dp2, 6) dp4, 7) dp6, 8) dp8, 9) dp10, 10) dp12, 11) dp14, 12) dp16,
580 13) dp18, 14) dp20 15) $>$ dp20 or control (dH₂O). Cells were infected with GFP tagged
581 HCMV (Towne strain) virus at an MOI of 3.0. At 5 days post infection, cells were fixed
582 and number of foci (GFP) was counted under an epifluorescent microscope. Percent of
583 viral GFP was calculated compared to virus only infected control (100% GFP
584 expression).

585

586 **Figure 6. Effect of glycosaminoglycan derivatives on HCMV growth.** Primary
587 human foreskin fibroblasts (HFF) were pretreated for one hour with 10 µM of 1) 6-O-
588 desulfated Arixtra, 2) Regular Arixtra, 3) Heparin sodium salt from porcine intestinal
589 mucosa (PIHSS), 4) Enoxaparin, or series of heparin oligosaccharide from enoxaparin:
590 5) *dp*2, 6) *dp*4, 7) *dp*6, 8) *dp*8, 9) *dp*10, 10) *dp*12, 11) *dp*14, 12) *dp*16, 13) *dp*18, 14)
591 *dp*20 15) >*dp*20 or control (dH₂O). Cells were infected with HCMV (Towne strain) virus
592 at an MOI of 3.0. Cells and media were harvested at 5 days post infection and titered for
593 HCMV plaque forming units (pfu) on fresh fibroblasts in tissue culture dishes. Individual
594 samples (3 replicates each) were quantified and displayed as total pfu/ml on Y-axis. (B)
595 Virus titer is plotted (Y-axis) against degree of polymerization (X-axis). Data points
596 ahead of the broken line is for a mixture of GAGs (*dp*>20).

597

598 **Figure 7. Effect of glycosaminoglycan derivatives on HCMV growth.** Primary
599 human foreskin fibroblasts (HFF) were pretreated for one hour with 0.05 g/L (B) of 1) 6-
600 O-desulfated Arixtra, 2) Regular Arixtra, 3) Heparin sodium salt from porcine intestinal
601 mucosa (PIHSS), 4) Enoxaparin, or series of heparin oligosaccharide from enoxaparin:
602 5) *dp*2, 6) *dp*4, 7) *dp*6, 8) *dp*8, 9) *dp*10, 10) *dp*12, 11) *dp*14, 12) *dp*16, 13) *dp*18, 14)
603 *dp*20 15) >*dp*20 or control (dH₂O). Cells were infected with HCMV (Towne strain) virus
604 at an MOI of 3.0. Cells and media were harvested at 5 days post infection and titered for
605 HCMV plaque forming units (pfu) on fresh fibroblasts in tissue culture dishes. Individual
606 samples (3 replicates each) were quantified and displayed as total pfu/ml on Y-axis. At
607 the bottom of each panel, titer is plotted (Y-axis) against degree of polymerization (X-
608 axis). Data points after the broken line is for a mixture of GAGs (*dp*>20).

609

610 **Figure 8. Effect of GAG treatment on cell viability of HFF cells.** Primary HFF were
611 pretreated for one hour with 10 μ M of 1) 6-O-desulfated Arixtra, 2) Regular Arixtra, 3)
612 Heparin sodium salt from porcine intestinal mucosa (PIHSS), 4) Enoxaparin, or series of
613 heparin oligosaccharide from enoxaparin: 5) dp2, 6) dp4, 7) dp6, 8) dp8, 9) dp10, 10)
614 dp12, 11) dp14, 12) dp16, 13) dp18, 14) dp20, 15) $>$ dp20 or control (dH₂O). Cells were
615 either mock infected (A) or infected with HCMV (Towne strain) virus at an MOI of 3.0
616 (B). Cells were harvested at 5 days post infection and cell viability was assessed using
617 Trypan Blue exclusion assay.

618

619 **Figure 9. Mouse CMV replication in sulfotransferase knockout cell lines.** Cells
620 were grown to 90% confluence and infected with wild-type MCMV (strain K181) at low
621 (0.01, 6A, B) and high (5.0, 6C, D) MOI. Cells and the medium were harvested at 3- and
622 5-days post infection, sonicated to release the virus and diluted for plating on to wild-
623 type MEF in tissue culture dishes in order to enumerate total MCMV pfu/ml. Hs3st1 and
624 Hs3st4: Glucosaminyl 3-O-sulfotransferase 1 and 4, respectively. WT: wild-type; KO:
625 knockout. P values of <0.05 were considered significant (*) compared to WT cells.

626

627 **Figure 10. Effect of anti-3-O-S HS peptide on CMV infectivity in HFF cells.** HFF
628 cells were pre-treated with wild-type HS peptide, anti 3-OS HS peptide for 4 hrs. The
629 mock treated cells were used as a positive control. The cells were infected with β -
630 galactosidase expressing CMV for 9 days. β -Galactosidase assay were performed

631 using X-gal (Sigma). The effect of entry-blocking activity of peptide was examined by
632 counting number of foci. Results are representative of three independent experiments.

633

634 **Supplementary Figures**

635

636 **Figure S1. Bio-Gel P10 size exclusion column chromatogram of enoxaparin**
637 **separation.** Fractions were collected and UV readings at 232 nm were taken for each
638 fraction to reconstruct the chromatogram. Samples were pooled to obtain the
639 oligosaccharide fractions of the desired size.

640

641 **Figure S2. ESI-MS of 6-O desulfated Arixtra.** The most abundant MS masses were
642 consistent with the loss of the three 6-O sulfates from Arixtra.

643

644 **Figure S3. MS/MS analysis of the -4 charge state of Arixtra-3SO₃.** Glycosidic bond
645 cleavages isolate desulfation to one desulfation event in the two non-reducing end
646 residues; one desulfation event in the two reducing end residues, and one desulfation
647 event in the central GlcNS. This pattern is consistent with 6-O desulfation.

Fig. 1

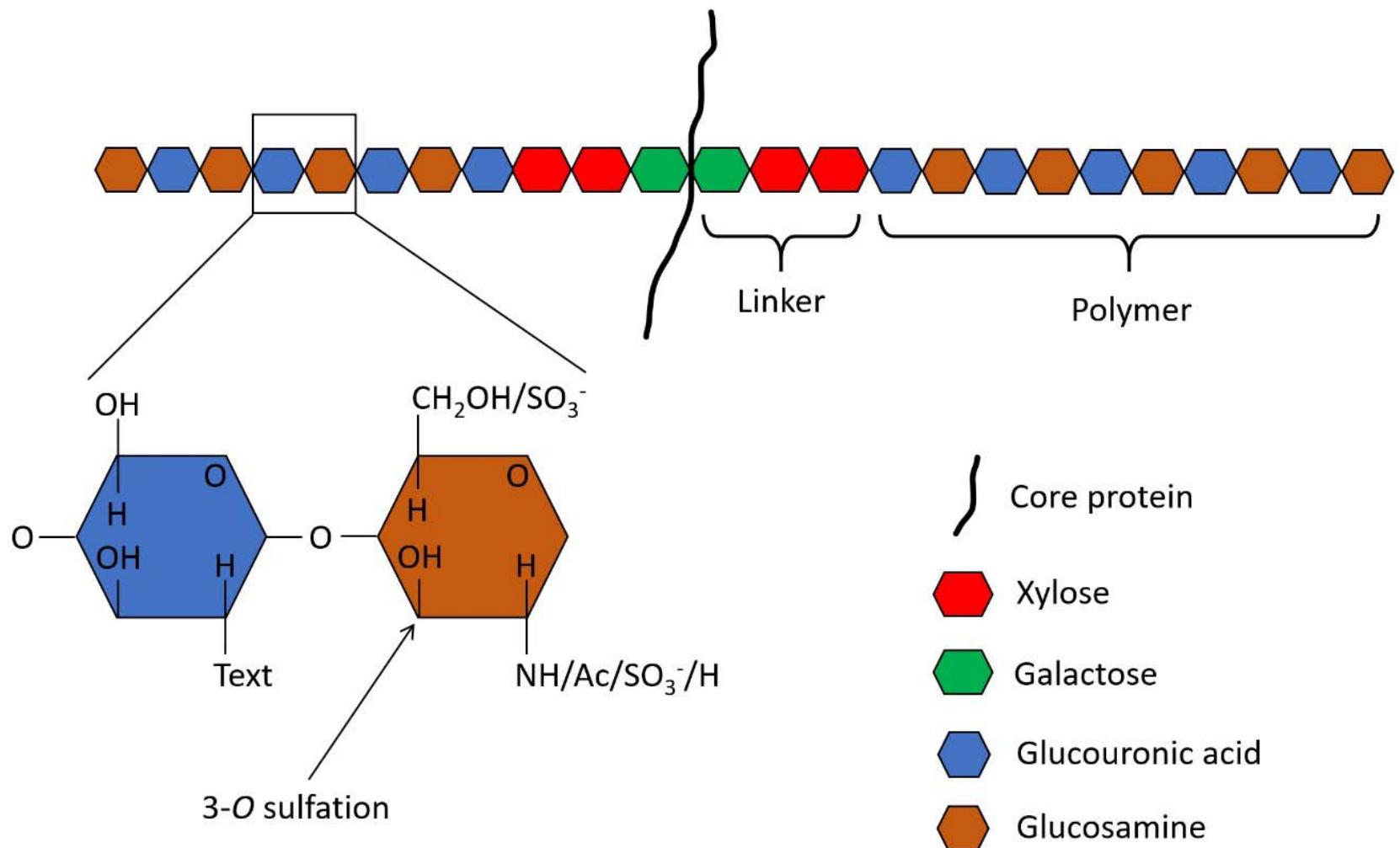


Fig. 2

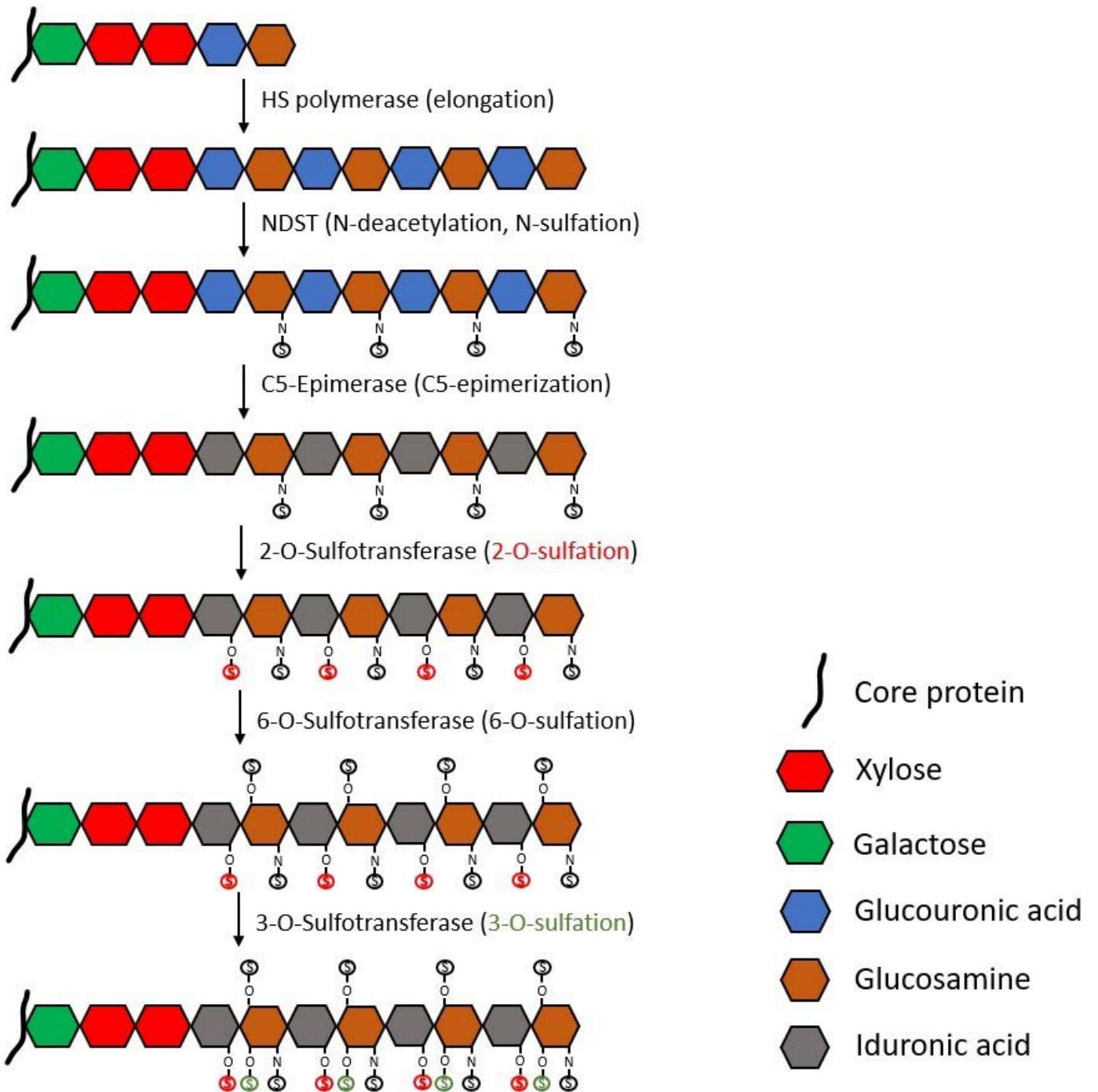


Fig. 3

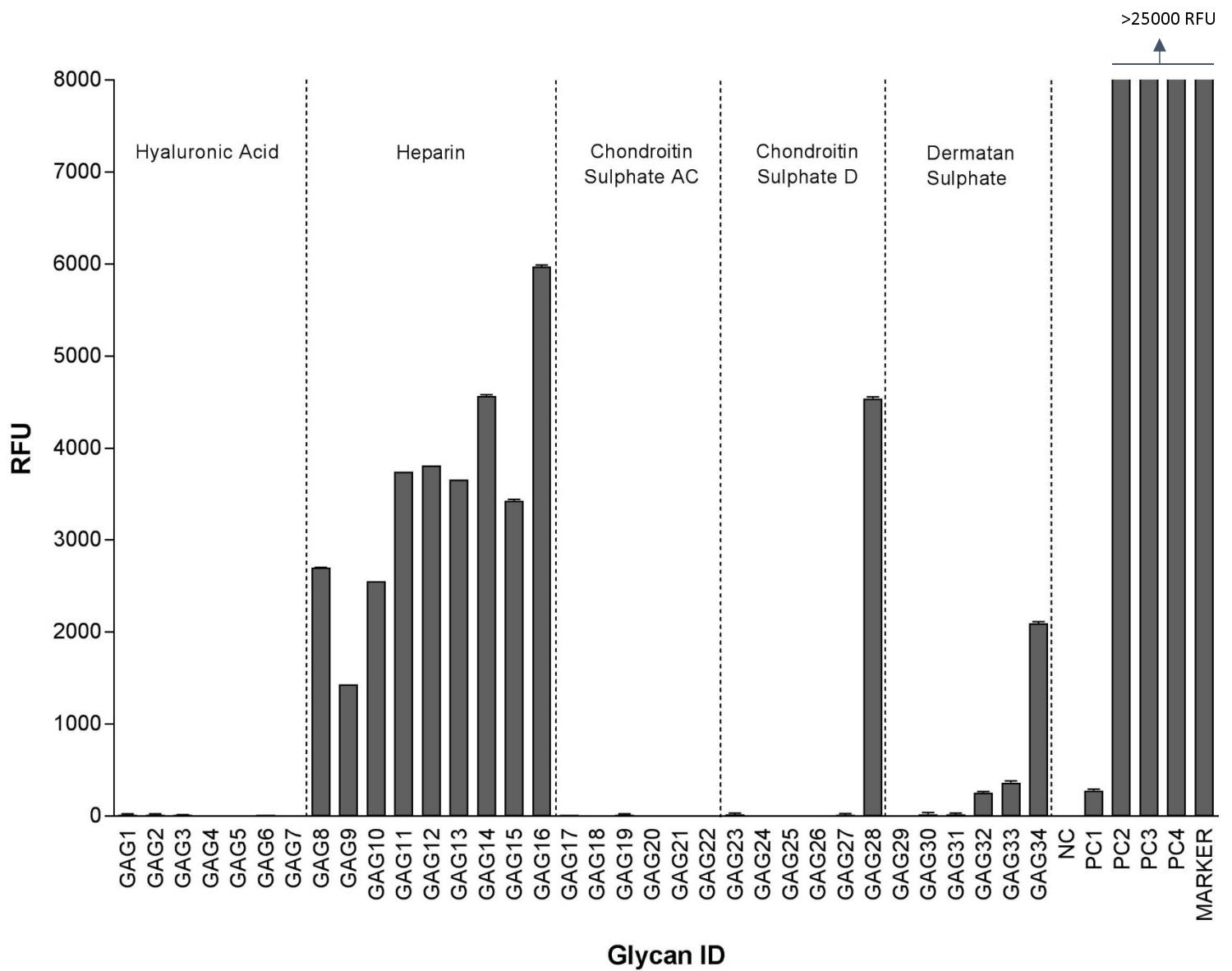


Fig. 4

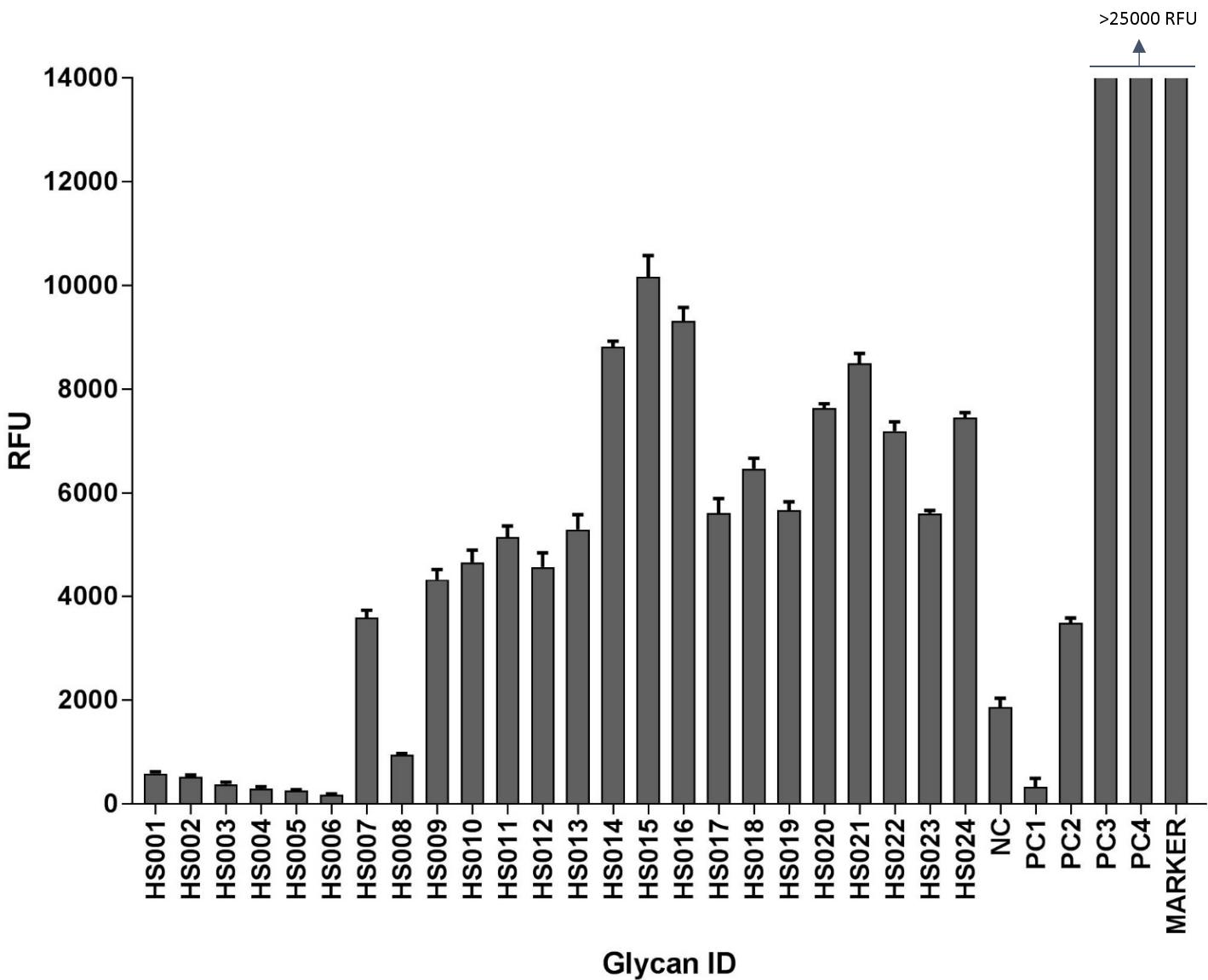


Fig. 5

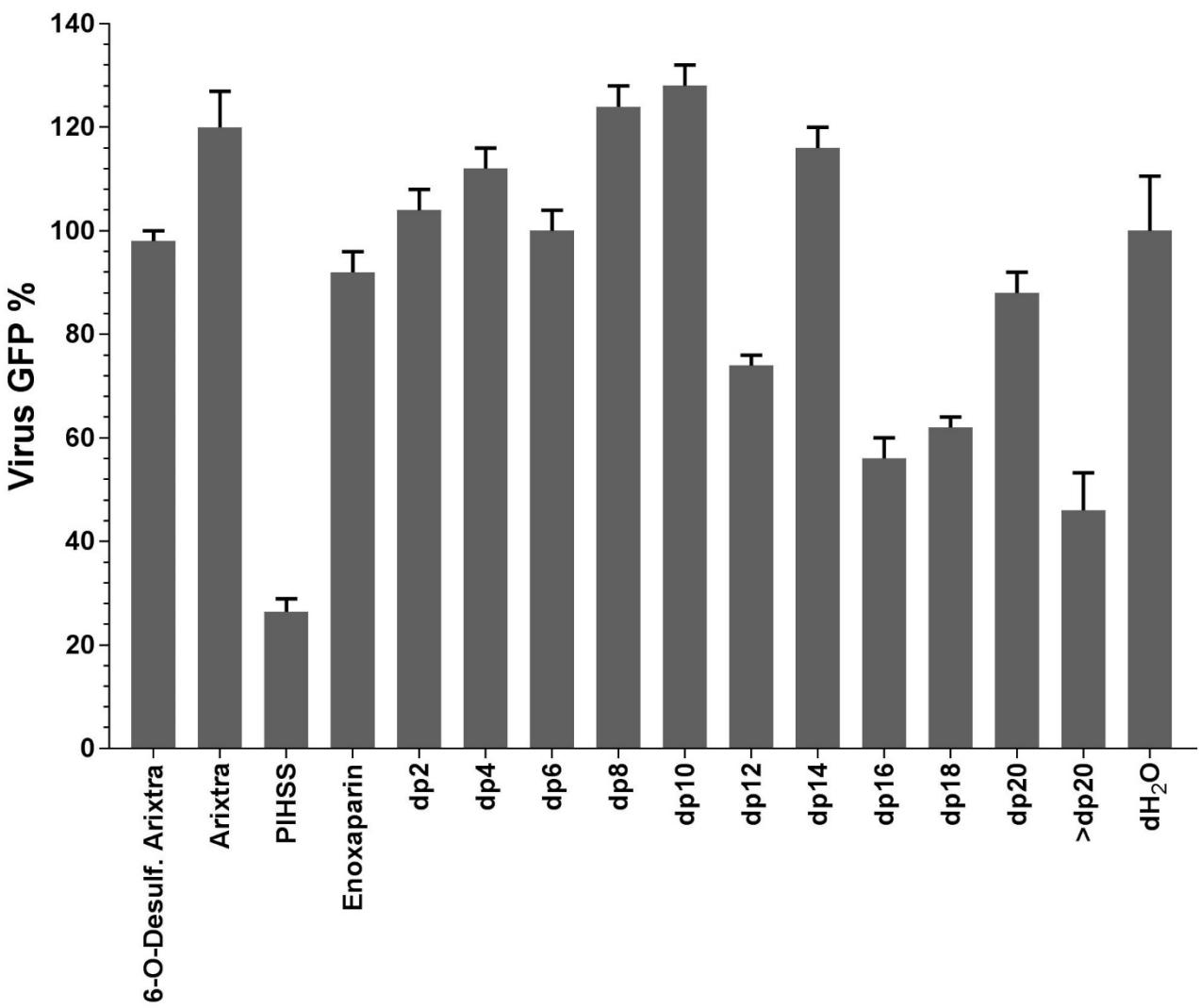


Fig. 6

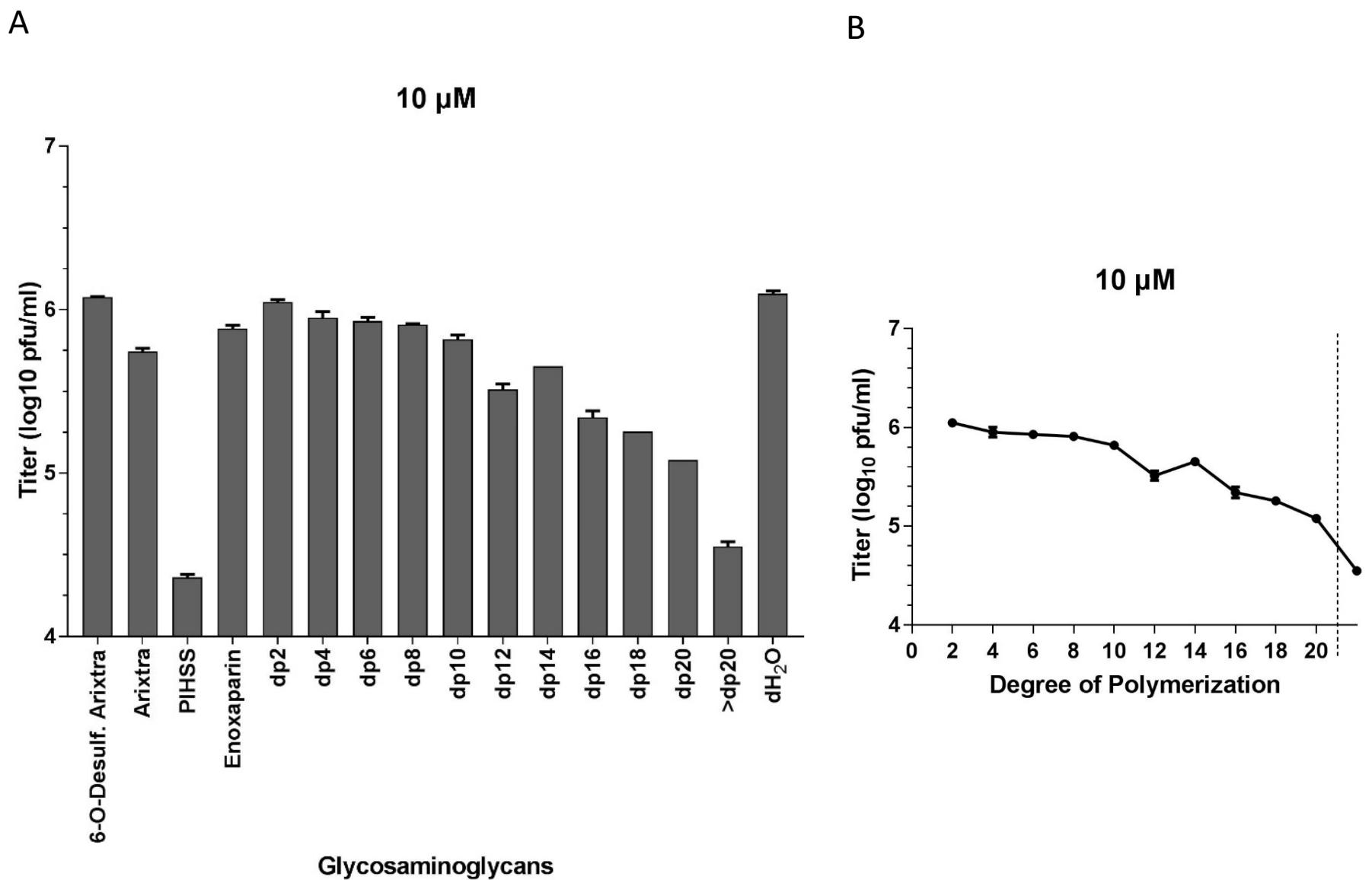
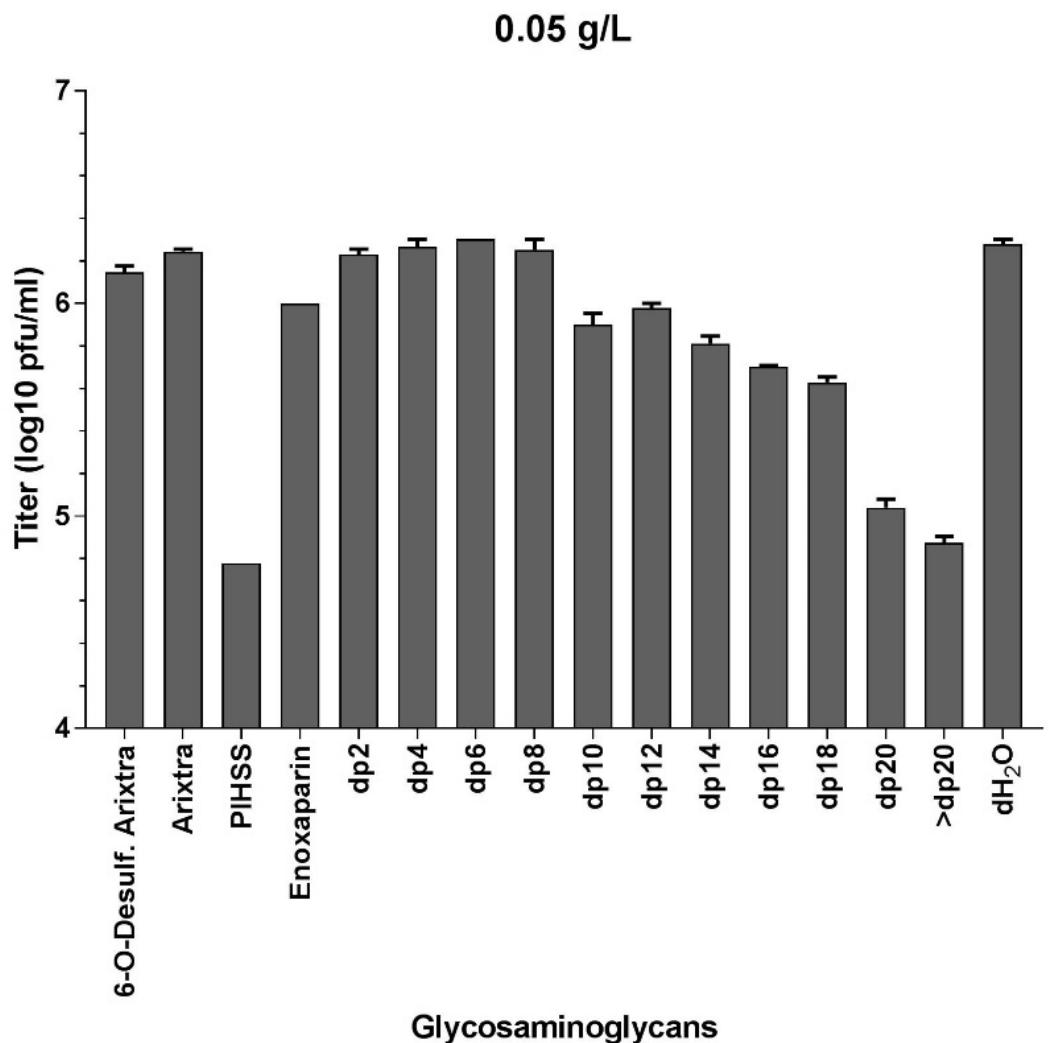


Fig. 7

A



B

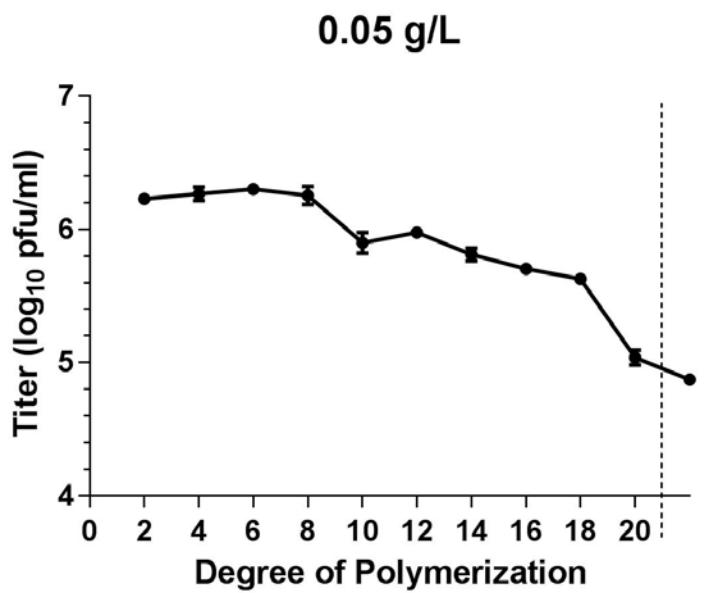
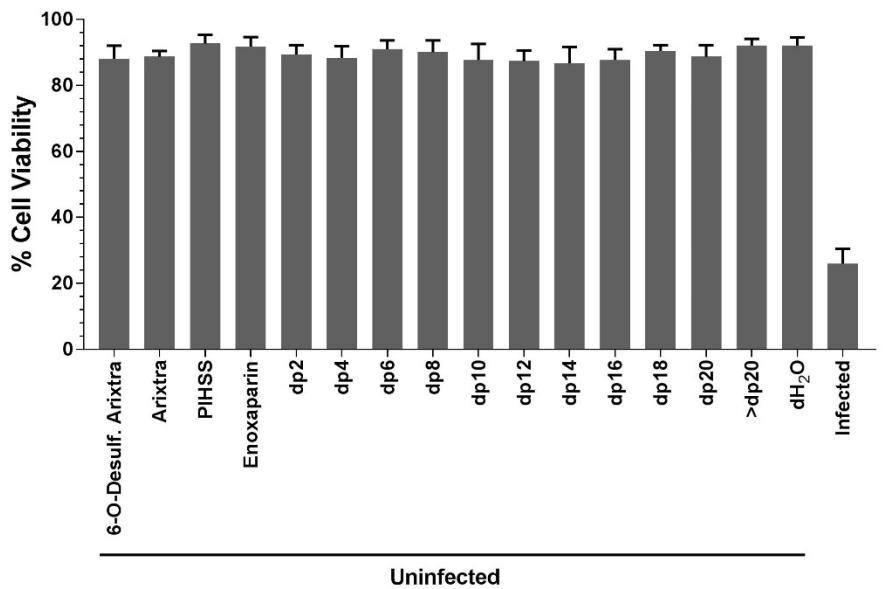


Fig. 8

A



B

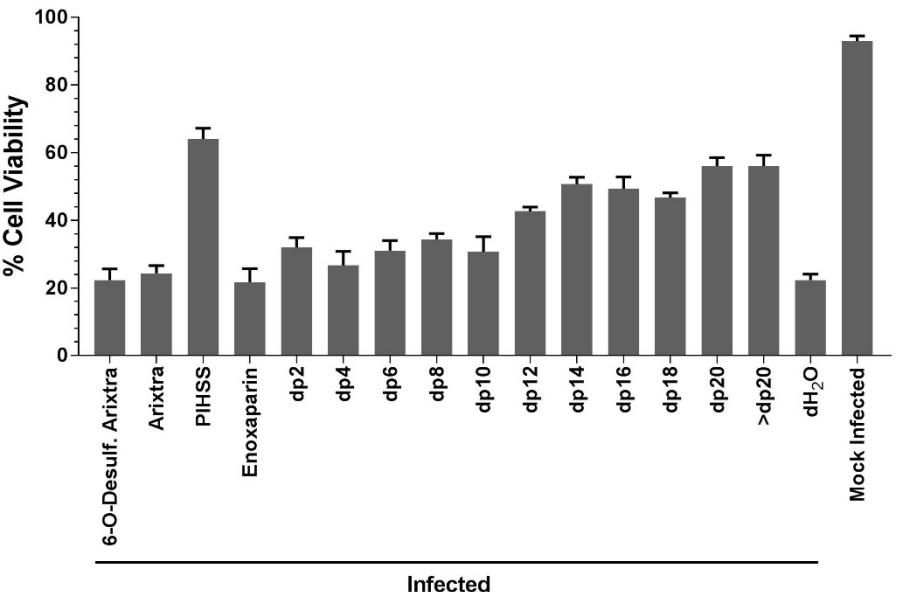


Fig. 9

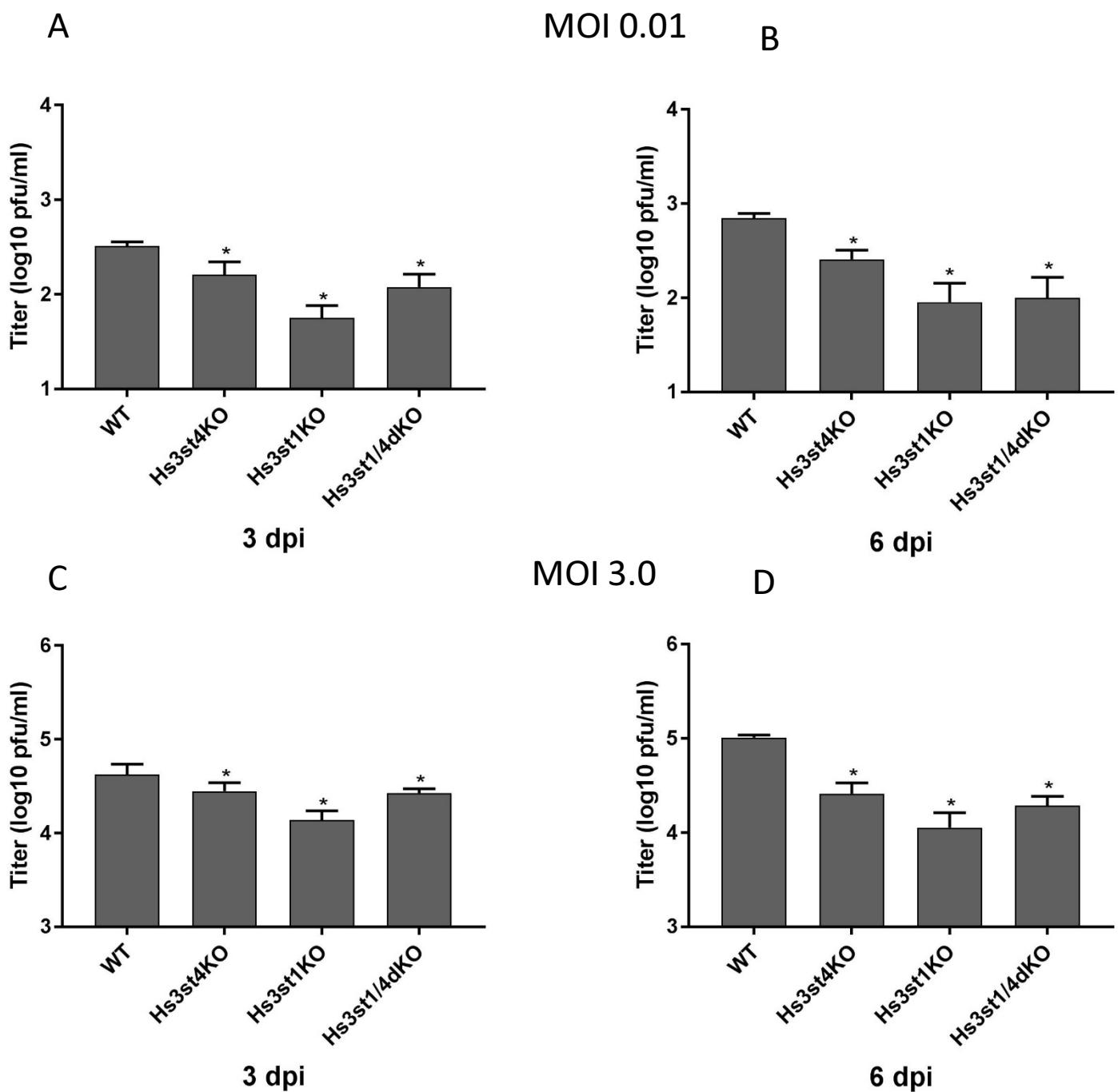


Fig. 10

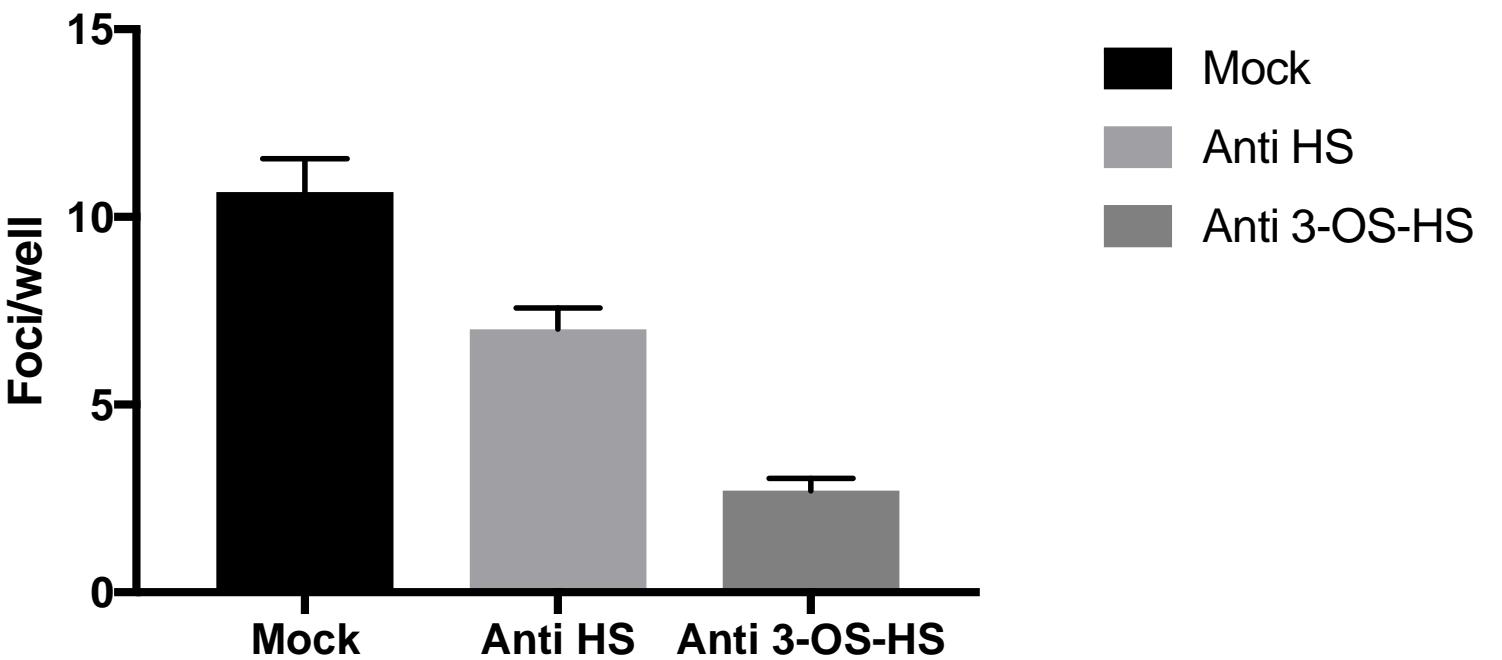


Fig. S1

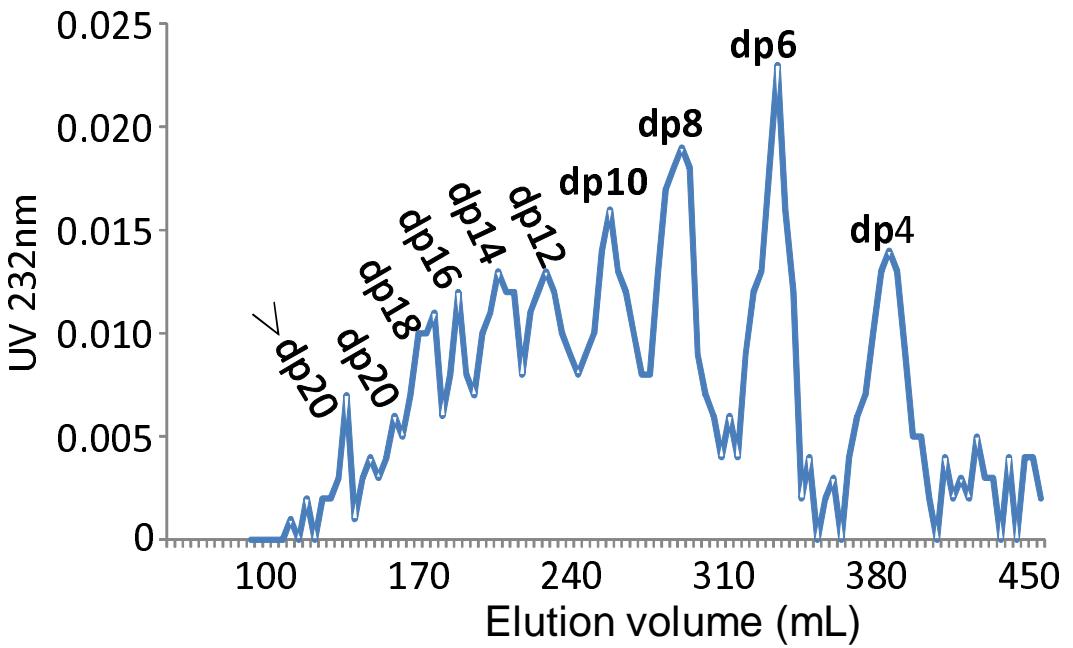


Fig. Gel permeation elution profiles on Bio-gel P-10 of Enoxaparin

Fig. S2

MS analysis of 6-O-desulfated Arixtra

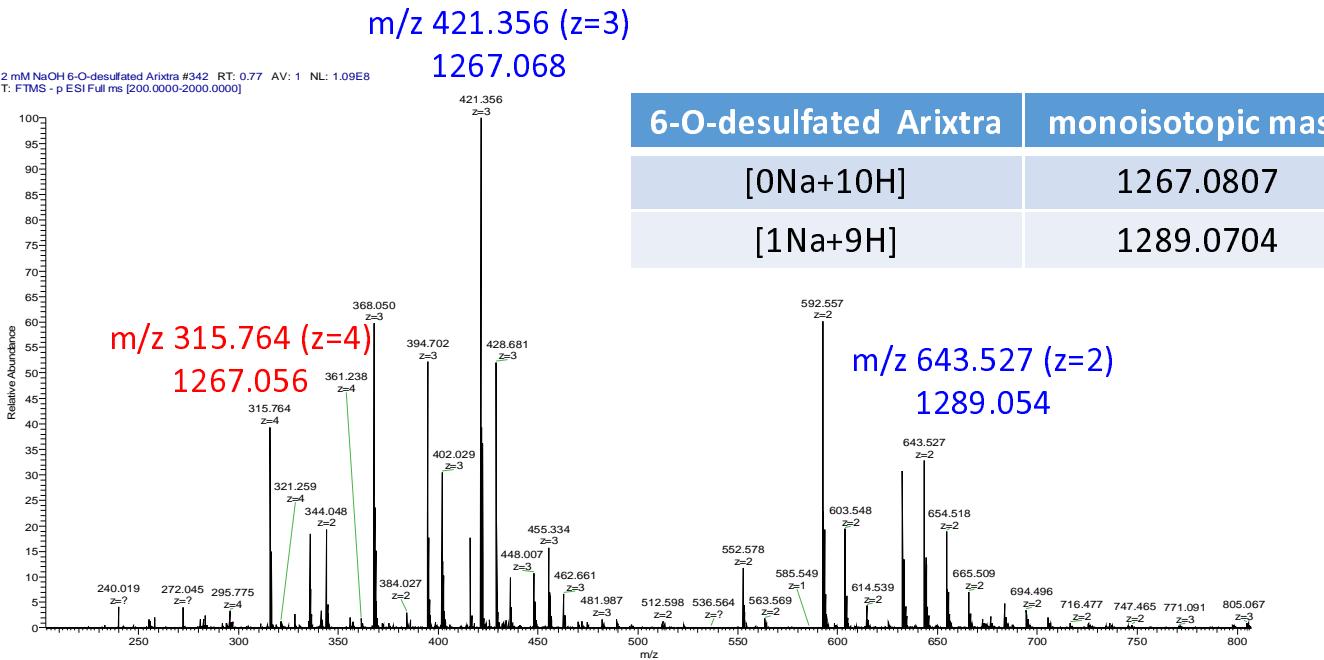


Fig. S3

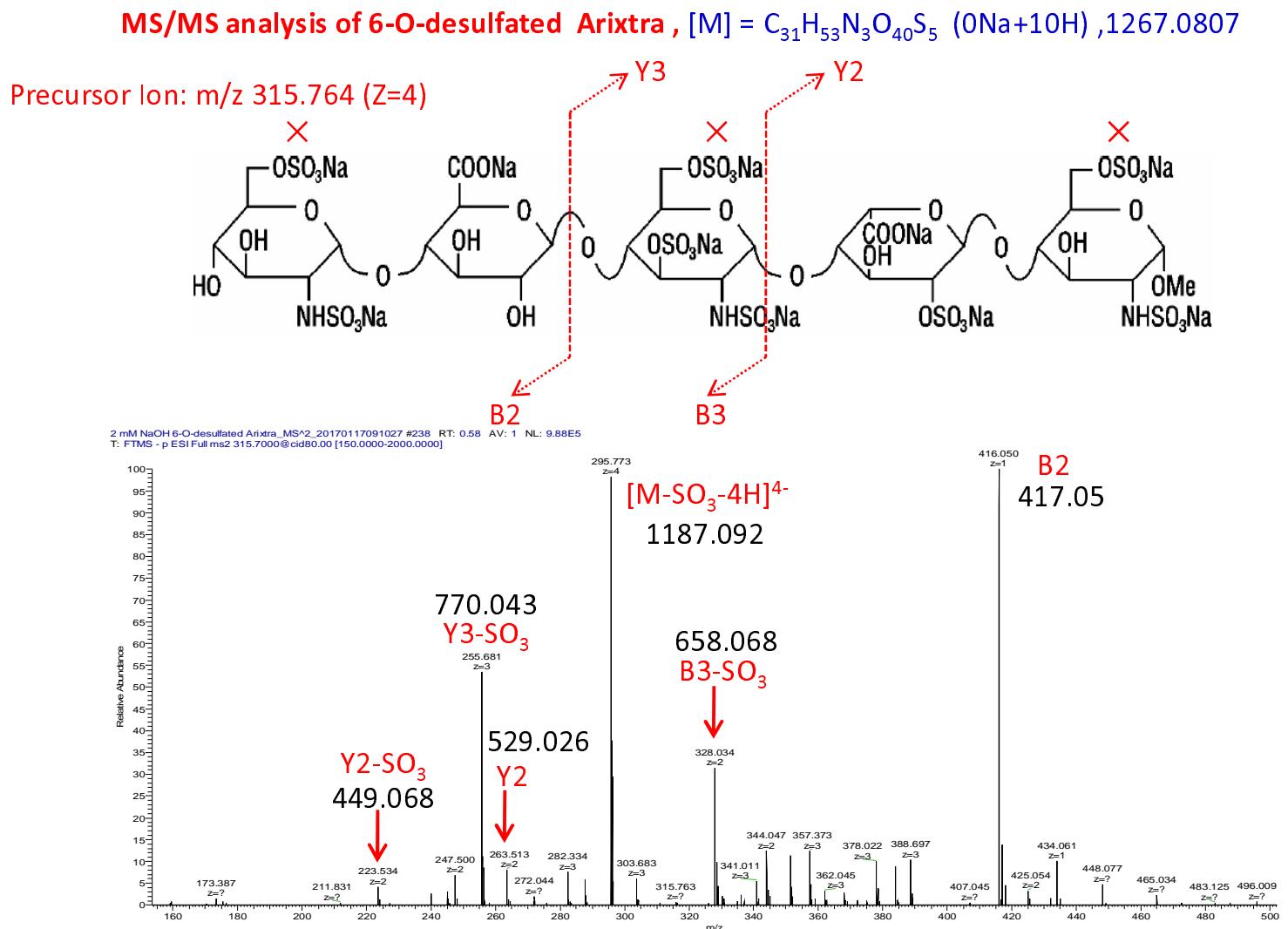


Table 1. A custom designed glycoarray containing hyaluronic acid, heparin, chondroitin sulfate and dermatan sulfate species. The structure, molecular weight, number of sugar residues and sulfate groups per disaccharide for each glycosaminoglycan are listed.

ID	Name	Structure	Molecular Weight (Da)	No. of Sugar Residues	Sulfate Groups per Disaccharide
GAG1	Hyaluronic Acid dp10 (HA10)	$\Delta\text{HexA}\beta 1,3 [\text{GlcNAc}\beta 1,4 \text{GlcA}\beta 1,3]_4 \text{GlcNAc}$	1,950	10	0
GAG2	Hyaluronic Acid dp12 (HA12)	$\Delta\text{HexA}\beta 1,3 [\text{GlcNAc}\beta 1,4 \text{GlcA}\beta 1,3]_5 \text{GlcNAc}$	2,350	12	0
GAG3	Hyaluronic Acid dp14 (HA14)	$\Delta\text{HexA}\beta 1,3 [\text{GlcNAc}\beta 1,4 \text{GlcA}\beta 1,3]_6 \text{GlcNAc}$	2,700	14	0
GAG4	Hyaluronic Acid dp16 (HA16)	$\Delta\text{HexA}\beta 1,3 [\text{GlcNAc}\beta 1,4 \text{GlcA}\beta 1,3]_7 \text{GlcNAc}$	3,150	16	0
GAG5	Hyaluronic Acid dp18 (HA18)	$\Delta\text{HexA}\beta 1,3 [\text{GlcNAc}\beta 1,4 \text{GlcA}\beta 1,3]_8 \text{GlcNAc}$	3,650	18	0
GAG6	Hyaluronic Acid dp20 (HA20)	$\Delta\text{HexA}\beta 1,3 [\text{GlcNAc}\beta 1,4 \text{GlcA}\beta 1,3]_9 \text{GlcNAc}$	3,900	20	0
GAG7	Hyaluronic Acid Polymer (HA93)	$\Delta\text{HexA}\beta 1,3 [\text{GlcNAc}\beta 1,4 \text{GlcA}\beta 1,3]_n \text{GlcNAc}$	93,000	462	0
GAG8	Heparin dp10 (H10)	$\Delta\text{HexA},2S-\text{GlcNS},6S-(\text{IdoUA},2S-\text{GlcNS},6S)_4$	3,000	10	3
GAG9	Heparin dp12 (H12)	$\Delta\text{HexA},2S-\text{GlcNS},6S-(\text{IdoUA},2S-\text{GlcNS},6S)_5$	3,550	12	3
GAG10	Heparin dp14 (H14)	$\Delta\text{HexA},2S-\text{GlcNS},6S-(\text{IdoUA},2S-\text{GlcNS},6S)_6$	4,100	14	3
GAG11	Heparin dp16 (H16)	$\Delta\text{HexA},2S-\text{GlcNS},6S-(\text{IdoUA},2S-\text{GlcNS},6S)_7$	4,650	16	3
GAG12	Heparin dp18 (H18)	$\Delta\text{HexA},2S-\text{GlcNS},6S-(\text{IdoUA},2S-\text{GlcNS},6S)_8$	5,200	18	3
GAG13	Heparin dp20 (H20)	$\Delta\text{HexA},2S-\text{GlcNS},6S-(\text{IdoUA},2S-\text{GlcNS},6S)_9$	5,750	20	3
GAG14	Heparin dp22 (H22)	$\Delta\text{HexA},2S-\text{GlcNS},6S-(\text{IdoUA},2S-\text{GlcNS},6S)_{10}$	6,300	22	3
GAG15	Heparin dp24 (H24)	$\Delta\text{HexA},2S-\text{GlcNS},6S-(\text{IdoUA},2S-\text{GlcNS},6S)_{11}$	6,850	24	3
GAG16	Heparin dp30 (H30)	$\Delta\text{HexA},2S-\text{GlcNS},6S-(\text{IdoUA},2S-\text{GlcNS},6S)_{14}$	9,000	30	3
GAG17	Chondroitin Sulphate AC dp10 (CS10)	$\Delta\text{UA} - (\text{GalNAc},6S \text{ or } 4S - \text{GlcA})_4 - \text{GalNAc},6S \text{ or } 4S$	2,480	10	1
GAG18	Chondroitin Sulphate AC dp12 (CS12)	$\Delta\text{UA} - (\text{GalNAc},6S \text{ or } 4S - \text{GlcA})_5 - \text{GalNAc},6S \text{ or } 4S$	2,976	12	1
GAG19	Chondroitin Sulphate AC dp14 (CS14)	$\Delta\text{UA} - (\text{GalNAc},6S \text{ or } 4S - \text{GlcA})_6 - \text{GalNAc},6S \text{ or } 4S$	3,472	14	1
GAG20	Chondroitin Sulphate AC dp16 (CS16)	$\Delta\text{UA} - (\text{GalNAc},6S \text{ or } 4S - \text{GlcA})_7 - \text{GalNAc},6S \text{ or } 4S$	3,968	16	1
GAG21	Chondroitin Sulphate AC dp18 (CSD18)	$\Delta\text{UA} - (\text{GalNAc},6S \text{ or } 4S - \text{GlcA})_8 - \text{GalNAc},6S \text{ or } 4S$	4,464	18	1
GAG22	Chondroitin Sulphate AC dp20 (CSD20)	$\Delta\text{UA} - (\text{GalNAc},6S \text{ or } 4S - \text{GlcA})_9 - \text{GalNAc},6S \text{ or } 4S$	4,960	20	1
GAG23	Chondroitin Sulphate D dp10 (CSD10)	$\Delta\text{UA} - (\text{GalNAc},6S \text{ or } 4S - \text{GlcA} +/- 2S)_4 - \text{GalNAc},6S$	2,480	10	1 or 2
GAG24	Chondroitin Sulphate D dp12 (CSD12)	$\Delta\text{UA} - (\text{GalNAc},6S \text{ or } 4S - \text{GlcA} +/- 2S)_5 - \text{GalNAc},6S$	2,976	12	1 or 2
GAG25	Chondroitin Sulphate D dp14 (CSD14)	$\Delta\text{UA} - (\text{GalNAc},6S \text{ or } 4S - \text{GlcA} +/- 2S)_6 - \text{GalNAc},6S$	3,472	14	1 or 2
GAG26	Chondroitin Sulphate D dp16 (CSD16)	$\Delta\text{UA} - (\text{GalNAc},6S \text{ or } 4S - \text{GlcA} +/- 2S)_7 - \text{GalNAc},6S$	3,968	16	1 or 2
GAG27	Chondroitin Sulphate D dp18 (CSD18)	$\Delta\text{UA} - (\text{GalNAc},6S \text{ or } 4S - \text{GlcA} +/- 2S)_8 - \text{GalNAc},6S$	4,464	18	1 or 2
GAG28	Chondroitin Sulphate D dp20 (CSD20)	$\Delta\text{UA} - (\text{GalNAc},6S \text{ or } 4S - \text{GlcA} +/- 2S)_9 - \text{GalNAc},6S$	4,960	20	1 or 2
GAG29	Dermatan Sulphate dp10 (DS10)	$\Delta\text{HexA} - \text{GalNAc},4S - (\text{IdoA} - \text{GalNAc},4S)_4$	2,480	10	1
GAG30	Dermatan Sulphate dp12 (DS12)	$\Delta\text{HexA} - \text{GalNAc},4S - (\text{IdoA} - \text{GalNAc},4S)_5$	2,976	12	1
GAG31	Dermatan Sulphate dp14 (DS14)	$\Delta\text{HexA} - \text{GalNAc},4S - (\text{IdoA} - \text{GalNAc},4S)_6$	3,472	14	1
GAG32	Dermatan Sulphate dp16 (DS16)	$\Delta\text{HexA} - \text{GalNAc},4S - (\text{IdoA} - \text{GalNAc},4S)_7$	3,968	16	1
GAG33	Dermatan Sulphate dp18 (DS18)	$\Delta\text{HexA} - \text{GalNAc},4S - (\text{IdoA} - \text{GalNAc},4S)_8$	4,464	18	1
GAG34	Dermatan Sulphate dp20 (DS20)	$\Delta\text{HexA} - \text{GalNAc},4S - (\text{IdoA} - \text{GalNAc},4S)_9$	4,960	20	1

Table 2. A custom designed glycoarray containing different heparan sulfate species. The structure, molecular weight, number of sugar residues and sulfate groups per disaccharide for each glycosaminoglycan are listed.

ID	Structure	Molecular Weight (Da)	No. of Sugar Residues	Sulfate Groups per Disaccharides
HS001	GlcNAcα1-4GlcAβ1-4GlcNAcα1-4-GlcA	1000	4	0
HS002	GlcAβ1-4GlcNAcα1-4GlcAβ1-4GlcNAcα1-4GlcA	1,176	5	0
HS003	GlcNAcα1-4GlcAβ1-4GlcNAcα1-4GlcAβ1-4GlcNAcα1-4GlcA	1,379	6	0
HS004	GlcAβ1-4GlcNAcα1-4GlcAβ1-4GlcNAcα1-4GlcAβ1-4GlcNAcα1-4GlcA	1,555	7	0
HS005	GlcNAcα1-4GlcAβ1-4GlcNAcα1-4GlcAβ1-4GlcNAcα1-4GlcAβ1-4GlcNAcα1-4GlcA	1,758	8	0
HS006	GlcAβ1-4GlcNAcα1-4GlcAβ1-4GlcNAcα1-4GlcAβ1-4GlcNAcα1-4GlcAβ1-4GlcNAcα1-4GlcA	1,934	9	0
HS007	GlcNSα1-4GlcAβ1-4GlcNSα1-4GlcA	1,076	4	1
HS008	GlcAβ1-4GlcNSα1-4GlcAβ1-4GlcNSα1-4GlcA	1,252	5	0.8
HS009	GlcNSα1-4GlcAβ1-4GlcNSα1-4GlcAβ1-4GlcNSα1-4GlcA	1,493	6	1
HS010	GlcAβ1-4GlcNSα1-4GlcAβ1-4GlcNSα1-4GlcAβ1-4GlcNSα1-4GlcA	1,669	7	0.9
HS011	GlcNSα1-4GlcAβ1-4GlcNSα1-4GlcAβ1-4GlcNSα1-4GlcAβ1-4GlcNSα1-4GlcA	1,910	8	1
HS012	GlcAβ1-4GlcNSα1-4GlcAβ1-4GlcNSα1-4GlcAβ1-4GlcNSα1-4GlcAβ1-4GlcNSα1-4GlcA	2,087	9	0.9
HS013	GlcAβ1-4GlcNSα1-4GlcAβ1-4GlcNSα1-4GlcAβ1-4GlcNSα1-4GlcAβ1-4GlcNS6Sα1-4GlcA	2,166	9	1.1
HS014	GlcAβ1-4GlcNSα1-4GlcAβ1-4GlcNSα1-4GlcAβ1-4GlcNS6Sα1-4GlcAβ1-4GlcNS6Sα1-4GlcA	2,246	9	1.3
HS015	GlcAβ1-4GlcNSα1-4GlcAβ1-4GlcNS6Sα1-4GlcAβ1-4GlcNS6Sα1-4GlcAβ1-4GlcNS6Sα1-4GlcA	2,327	9	1.6
HS016	GlcAβ1-4GlcNS6Sα1-4GlcAβ1-4GlcNS6Sα1-4GlcAβ1-4GlcNS6Sα1-4GlcAβ1-4GlcNS6Sα1-4GlcA	2,406	9	1.8
HS017	GlcNSα1-4GlcAβ1-4GlcNSα1-4GlcAβ1-4GlcNSα1-4GlcNS6Sα1-4GlcAβ1-4GlcNS6Sα1-4GlcA	1,990	8	1.3
HS018	GlcNSα1-4GlcAβ1-4GlcNSα1-4GlcNS6Sα1-4GlcAβ1-4GlcNS6Sα1-4GlcAβ1-4GlcNS6Sα1-4GlcA	2,070	8	1.5
HS019	GlcNAcα1-4GlcAβ1-4GlcNSα1-4GlcNS6Sα1-4GlcAβ1-4GlcNS6Sα1-4GlcNS6Sα1-4GlcA	2,432	8	1.3
HS020	GlcNS6Sα1-4GlcAβ1-4GlcNS6Sα1-4GlcAβ1-4GlcNS6Sα1-4GlcNS6Sα1-4GlcNS6Sα1-4GlcA	2,310	8	2.3
HS021	GlcNS6Sα1-4GlcAβ1-4GlcNS6Sα1-4GlcNS6Sα1-4GlcNS6Sα1-4GlcNS6Sα1-4GlcA	2,389	8	2.5
HS022	GlcNAc6Sα1-4GlcAβ1-4GlcNS6Sα1-4GlcNS6Sα1-4GlcNS6Sα1-4GlcNS6Sα1-4GlcA	2,353	8	2.3
HS023	GlcNS6Sα1-4GlcAβ1-4GlcNS3S6Sα1-4GlcNS6Sα1-4GlcNS6Sα1-4GlcA	1,893	6	2.7
HS024	GlcNAc6Sα1-4GlcAβ1-4GlcNS3S6Sα1-4GlcNS6Sα1-4GlcNS6Sα1-4GlcNS6Sα1-4GlcA	2,433	8	2.5