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Inhibition of Influenza H5N1 invasion by modified heparin derivatives

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Contents:

S1. Chemical modifications of heparin

A. Selective de-N-sulfation was achieved based on the method described by Inoue and Nagasawa [Inoue and Nagasawa, (1976)] using solvolytic de-sulfation by treatment of the pyridinium salt of the relevant heparin derivative with 9/1, v/v, DMSO/MeOH (60 ° C, 30 min).

B. Selective 2-O-desulfation in iduronate residues was using the method described by Jeseja *et al*., (1989). Briefly, a solution of the heparin derivative is dissolved in 0.1 N NaOH, frozen and then freeze dried. The reaction proceeds via an intermediate epoxide, which is opened stereoselectively during the reaction in a concerted fashion to yield 2-de-O-sulfated iduronate residues.

C. Selective 6-O-desulfation was achieved using a modification of the procedure for complete desulfation described by Nagasawa et al., (1977), but employing milder conditions. This is similar to the solvolytic de-sulfation procedure followed for selective de-N-sulfation (Inoue *et al*., (1976)), but was conducted under harsher conditions [65 ° C, 4 h].

D. Complete O- and N - desulfation was achieved following the procedure of Nagasawa *et al*., (1977). This is similar to the solvolytic de-sulfation procedure followed for selective de-N-sulfation (Inoue *et al*., (1976)), but was conducted under harsher conditions [110 ° C, 24 h].

E. Selective re-N-sulfation of heparin derivatives bearing free-amino functions in their glucosamine residues was carried essentially out as described [Embrey *et al*., (1971)] employing excess trimethylamine.sulfurtrioxide complex in a saturated aqueous solution of sodium bicarbonate (55 ° C, 24 h).

F. Selective re-N-acetylation was carried out using standard procedures described previously [Yates et al (1996)] employing excess acetic anhydride on the relevant de-N-sulfated heparin derivative in a saturated aqueous solution of sodium bicarbonate at 4 ° C for 2 h, followed by reaction for a further 16 h at room temperature.

G. Per-sulfation (complete O-sulfation) was achieved essentially as described [Yates et al., (1996) & Yates et al., (2000)] employing excess pyridine.sulfur trioxide complex on the tetramethylammonium salt of heparin in DMF reacted at 55 ° C for 24 h. This reaction resulted in extensive de-N-sulfation and re N-sulfation was then carried out as described [Embrey et al., (1971)], taking care to avoid pH levels above 8, which can result in the formation of an aziridine derivative in 3-O-sulfated glucosamine residues [Yates et al., (1997)].

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S2. Preparation of modified heparins

Chemically modified polysaccharides were prepared by sequential use of the chemical reactions outlined in **S1**.

Polysaccharides were modified as follows:

(1) Heparin – unmodified

(2) N-acetylated heparin

- **A** *Selective de-N-sulfation*
- **F** *Re-N-acetylation*

(3) 2-O-desulfated heparin

- **B** *Selective 2-O-desulfation*

(4) 6-O-desulfated heparin

- **C** *Selective 6-O-desulfation*
- **E** *Re-N-sulfation*

(5) 2 & 6-O-desulfated heparin

- **D** *Complete O and N - desulfation*
- **E** *Re-N-sulfation*

(6) 2-O-desulfated N-acetylated heparin

- **B** *Selective 2-O-desulfation*
- **A** *Selective de-N-sulfation*
- **F** *Re-N-acetylation*

(7) 6-O-desulfated N-acetylated heparin

- **C** *6-O-desulfation and*
- **A** *de-N-sulfation*
- **F** *Re-N-acetylation*

(8) 2 & 6-desulfated N-acetylated heparin

- **D** *Complete O and N - desulfation*
- **F** *Re-N-acetylation*

(9) Per-sulfated heparin

- **G** *Per-sulfation*
- **E** *Re-N-sulfation*

Compounds were characterized by ^{1}H and ^{13}C NMR (see S4) and in terms of disaccharide compositional analysis by exhaustive digestion with heparinase I, II, and III followed by separation using anion exchange chromatography (Propac PA-1; 4 × 250 mm; Dionex, UK) with identification against known standards (see S3). All modified heparin products were desalted, lyophilized, and resuspended in appropriate buffer prior to bioassay.

S3. Chemically modified heparin polysaccharide purity

All chemically modified polysaccharides were purified by gel filtration chromatography (Sephadex G-25, GE Healthcare, UK); recovering exclusively the eluate in excess of the column exclusion limit (molecular weight > 5 kDa). All polysaccharides were subsequently treated with cation exchange resin (Dowex, W- 50, Na^+ form, Alfa Aesar, UK) prior to NMR analysis and bioactivity screening.

Gel filtration chromatographic analysis of the modified polysaccharides was performed using a TSK gel G2000SWXL column (7.8 mm x 300 mm, 0.5 µm bead; Sigma-Aldrich, UK) eluting isocratically with HPLC grade H_2O at 1 $mL.min^{-1}$; detection at λ = 190 nm. All polysaccharide samples exhibited a single major peak (> 95 %), with highly comparable retention times (mean, 6.07 minutes, $\sigma n^{-1} = 0.05$).

Furthermore, all polysaccharides were exhaustively digested with a mixture of bacterial lyase enzymes, heparinase I, II and III, to yield their constituent Δ-disaccharides (denoted ΔD1 to ΔD8). Disaccharides were separated by strong-anion exchange chromatography (Dionex Propac PA-1, UK) and quantified (λ_{Abs} = 232 nm) with reference to authentic standards (Dextra Laboratories, UK) and showed the following composition (%). In all spectra, unidentified peaks were < 5 % of the total area of the constituents.

Polysaccharide	ΔD1	ΔD2	ΔD3	ΔD4	ΔD5	ΔD6	ΔD7	ΔD8
1. Heparin	6.8	-	3.4	13.4	7.0	67.4	-	2.0
2. N-acetylated heparin	14.2	7.0	-	-	-	3.5	-	75.3
3. 2-O-desulfated heparin	7.1	-	13.7	79.2	-	-	-	-
4. 6-O-desulfated heparin	15.1	-	43.2	7.0	34.3	-	-	-
5. 2 & 6-O-desulfated heparin	19.1	-	79.1	-	-	1.8	-	-
6. 2-O-desulfated N-acetylated heparin	14.4	92.9	3.4	19.3	-	-	-	-
7. 6-O-desulfated N-acetylated heparin	55.8	7.7	-	-	-	-	34.8	1.7
8. 2 & 6-O-desulfated N-acetylated heparin	99.0	-	-	-	-	-	-	1.0

ΔD1, ΔUA-GlcNAc; ΔD2, ΔUA-GlcNAc(6S); ΔD3, ΔUA-GlcNS; ΔD4, ΔUA-GlcNS(6S); ΔD5, ΔUA-GlcNS; ΔD6, ΔUA(2S)-GlcNS(6S); ΔD7, ΔUA(2S)-GlcNAc; ΔD8, ΔUA(2S)-GlcNAc(6S)

S4. NMR characterisation of chemically modified heparin polysaccharides

^{13}C and ^{1}H NMR characterisation of all chemically modified polysaccharides was performed on a Bruker 400 MHz instrument at 40 °C in D_2O. Structures were confirmed by assignment using a combination of COSY, TOCSY and HMBC 2D spectra. ^{13}C spectra were obtained using 150 mg of polysaccharide sample. Chemical shift values were obtained relative to a reference standard of trimethylsilylpropionate at 40 °C.

^{13}C NMR

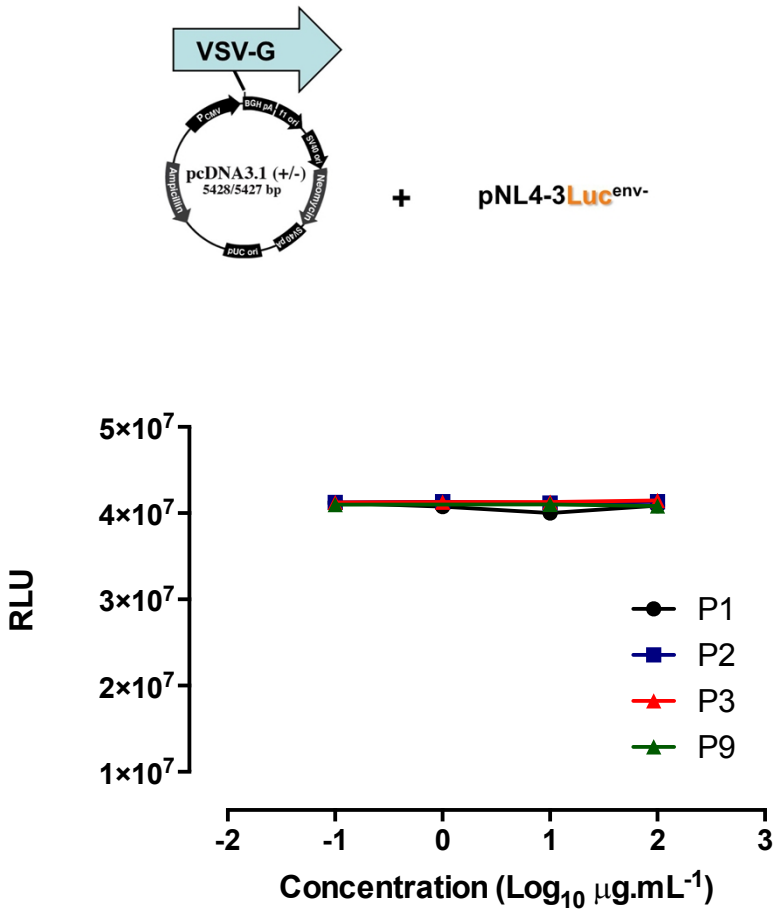
	A1	A2	A3	A4	A5	A6	I1	I2	I3	I4	I5
1. Heparin	99.5	60.7	72.5	78.8	72	69.2	102.1	78.9	72.1	79.0	72.3
2. N-acetylated heparin	96.6	56.2	73.0	79.3	72.3	69.6	102.2	76.8	67.3	74.2	70.8
3. 2-O-desulfated heparin	98.1	60.3	72.4	80.1	71.5	68.7	104.6	71.1	70.4	77.2	71.2
4. 6-O-desulfated heparin	100	60.8	72.4	80.5	73.8	62.6	102.0	77.6	70.7	78.7	71.4
5. 2 & 6-O-desulfated heparin	98.2	60.5	72.5	80.2	73.5	62.4	104.3	72.2	71.5	77.8	72.2
6. 2-O-desulfated N-acetylated heparin	97.1	56.2	72.5	79.6	71.8	68.8	104.6	72.0	71.4	77.0	71.9
7. 6-O-desulfated N-acetylated heparin	96.8	56.6	72.9	80.6	74.2	62.9	102.3	76.6	67.1	74.1	70.6
8. 2 & 6-O-desulfated N-acetylated heparin	97.1	56.2	72.3	79.6	73.7	62.3	104.3	72.5	72.2	77.3	72.6
9. Per-sulfated heparin (N-acetylated; see note)	98.2	55.4	79.6	77.2	72.4	69.3	102.0	74.4	72.7	73.2	70.6

^{1}H NMR

Polysaccharide	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**	**I1**	**I2**	**I3**	**I4**	**I5**
1. Heparin	5.42	3.31	3.69	3.79	4.05	4.30-4.42	5.23	4.37	4.22	4.14	4.82
2. N-acetylated heparin	5.15	4.03	3.76	3.78	4.04	4.31-4.37	5.2	4.37	4.31	4.08	4.91
3. 2-O-desulfated heparin	5.34	3.24	3.65	3.71	4.02	4.36-4.23	5.04	3.78	4.12	4.08	4.84
4. 6-O-desulfated heparin	5.31	3.27	3.71	3.70	3.89	3.86-3.88	5.26	4.35	4.25	4.06	4.84
5. 2 & 6-O-desulfated heparin	5.39	3.26	3.67	3.72	3.87	3.84-3.88	4.95	3.74	4.11	4.08	4.77
6. 2-O-desulfated N-acetylated heparin	5.18	4.00	3.78	3.79	4.08	4.37-4.26	5.01	3.75	3.42	4.10	4.78
7. 6-O-desulfated N-acetylated heparin	5.14	4.03	3.79	3.76	3.91	3.87-3.92	5.26	4.37	4.28	4.07	4.91
8. 2 & 6-O-desulfated N-acetylated heparin	5.18	3.97	3.76	3.74	3.89	3.85-3.88	4.92	3.69	3.89	4.07	4.73
9. Per-sulfated heparin (N-acetylated; see note)	5.14	4.26	4.61	4.04	4.06	4.35-4.40	5.38	4.56	4.79	4.35	5.14

Note: ^{1}H interval chemical shift values are stated for the A6 position of glucosamine. Iduronate carbonyl groups and N-acetyl containing glucosamine derivatives CH_3 groups are not shown.

S5. Heparin derivatives do not neutralize the VSVg-pseudotyped lentiviral vectors



To check that the heparin derivatives are specific for H5-pseudotyped viruses, we tested these compounds at 3 different concentrations against VSVg-pseudotyped viruses, which only differs from H5-VLPs by the composition of the envelope. VSVg-pseudotyped virus was pre-incubated 1:1 with 100 µg/ml, 10 µg/ml, 1 µg/ml and 0.1 µg/ml modified heparin derivatives **P1** (IdoA(2S)-GlcNS(6S)), **P2** (IdoA(2S)-GlcNAc(6S)), **P3** (IdoA-GlcNS(6S)) and **P9** (IdoA(2,3S)-GlcNS(3,6S)) for 30 min at room temperature and added on 293T cells in duplicates.