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A microscale double labelling of GAG oligosaccharides compatible both with enzymatic treatment and mass spectrometry

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Electronic Supplementary Information

1. Materials, reagents and instrumentation

1.1 Materials.

1.1.1 Heparin octasaccharide issued from enzymatic depolymerization

Heparin octasaccharide (HPdp8) was obtained as previously described.[1] Briefly, porcine mucosal heparin (10 g) was digested with heparinase I (8 mU/mL) in 150 mL of 0.1 mg/mL BSA, 2 mM $CaCl_2$, 50 mM NaCl, and 5 mM Tris buffer, pH 7.5, for 54 h at 25°C. The enzymatic reaction was stopped by heating the digest at 100°C for 5 min. Digestion products were then size-separated using a Bio-Gel P-10 column (Bio-Rad, Hercules, CA) (4.4 × 150 cm), equilibrated with 0.25 M NaCl, and run at 1 mL/min. Eluted material, detected by absorbance at 232 nm, consisted of a graded series of size-uniform oligosaccharides resolved from disaccharide (dp2) to octadecasaccharide (dp18). To ensure size homogeneity, only the top fractions of each peak were pooled, and each isolated fraction was re-chromatographied on a gel filtration column to further eliminate possible contamination. Samples were dialyzed against distilled water, freeze dried, and quantified by a colorimetric assay[2] or weighted. These oligosaccharides were further purified by strong-anion-exchange HPLC on a 9 x 250 cm preparative ProPac PA1 column, equilibrated in distilled water adjusted to pH 3.5 with HCl and resolved with a gradient of NaCl (0 to 1.4 M over 30 min, then 1.4 to 1.8 M over 60 min) in the same mobile phase. The eluate was monitored on-line for UV absorbance at 232 nm, and the most anionic species were selected.[3]

1.1.2 6-O-endosulfate (HSulf-2).

HSulf-2 was prepared according to a previously described procedure.[4] Briefly, the enzyme was purified from the conditioned medium of HSulf-2 transfected HEK293F cells, using cation exchange and size exclusion chromatography, successively. The purified active enzyme was stored at -80 °C in 50 mM Tris buffer, 300 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$ pH 7.5.

1.2 Reagents.

2-mercaptoethanol (≥99.0%), sodium borodeuteride (98 atom % D), boric acid (≥99.5%), Tris-HCl (≥99.0%), formamide (≥99.5%), dichloromethane (≥99.8%), sodium hydroxide (≥98.0%), sodium chloride (≥99.5%), 1,1,3,3-tetramethylguanidine (≥99.0%) (TMG), 2-(4-hydroxyphenylazo)benzoic acid (HABA) (≥99.5%), were purchased from Sigma-Aldrich Co. (Saint Quentin Fallavier, France). Heparinase I (Heparin lyase I, EC 4.2.2.7) from *Flavobacterium heparinum* were purchased from Sigma-Aldrich Co. (Saint Quentin Fallavier, France). Ultrapure water (Milli-Q, Millipore, Milford, MA) was used. PD MiniTrap G-10 desalting columns were purchased from GE Healthcare (Velizy-Villacoublay, France)

1.3. Instrumentation.

UV-visible measurements were performed using 1µL sample (100µg/50µL) on a nanodrop2000 (Thermo Fisher Scientific, les Ulis, France).

MALDI-TOF MS experiments were performed using an Autoflex III MALDI-TOF/TOF spectrometer (Bruker Daltonics Inc., Bremen, Germany). This instrument was equipped with a Nd: YAG SmartBeam laser (λ = 355 nm) pulsed at a 200 Hz frequency. The mass spectrometer was operated in the negative ion reflector mode with an accelerating potential of -19 kV. Mass spectra were recorded with extraction delay was set to 50 ns. Mass spectra were obtained by accumulation of 400 laser shots and a laser intensity set just above the ionization threshold, to both avoid fragmentation and sulfo group losses and maximize resolution with minimal matrix interference. Mass spectra were processed using Flex Analysis 3.0 software (Bruker Daltonics Inc). The instrument was calibrated using standard peptide and protein mixtures provided by the manufacturer.

Preparation of HABA/TMG$_2$ ionic liquid matrice (ILM). ILMs was prepared as previously described.[4] Briefly, HABA was mixed with TMG at a 1/2 molar ratio in methanol, and the obtained solution was then sonicated for 15 min at 40°C. After removal of methanol by centrifugal evaporation in a SpeedVac for 3 h at room temperature, ILM was left in vacuum overnight. ILMs were then prepared at a concentration of 100 mg/mL in water for use as a matrix. Once prepared, water solutions of ILM could be stored at 4°C up to 1 week and were used without further purification. ILM was further diluted in water at 10mg/mL with addition of 1 µM NaCl to prevent excessive desulfation.

Samples preparation. Samples for MALDI-TOF MS analysis were prepared by mixing 1 µL of modified/unmodified HPdp8 (first solubilised in 50µL aqueous solution) and one volume of HABA/TMG$_2$. 2 µL, corresponding to about 1µg of the mixture was deposited on a mirror polished stainless steel MALDI target and let 10 min at room temperature and atmospheric pressure before analysis.

2. Chemical double labelling procedure

2.1 Synthesis of benzylated Heparin octasaccharide (HPdp8)

HPdp8 sodium salt (100 µg, 37.5 nmol) was dissolved in 400 µL dichloromethane (DCM). Benzyl chloride at 1.1 g/ml (212 µL, 1.7 mmol) was added, and the mixture was stirred at 30℃ for during 24 h. Then, purified by Bio-gel PD-10 column to remove any unreacted reagent, and freeze dried.

2.2 Non reducing end labelling of HP-dp8 benzyl ester

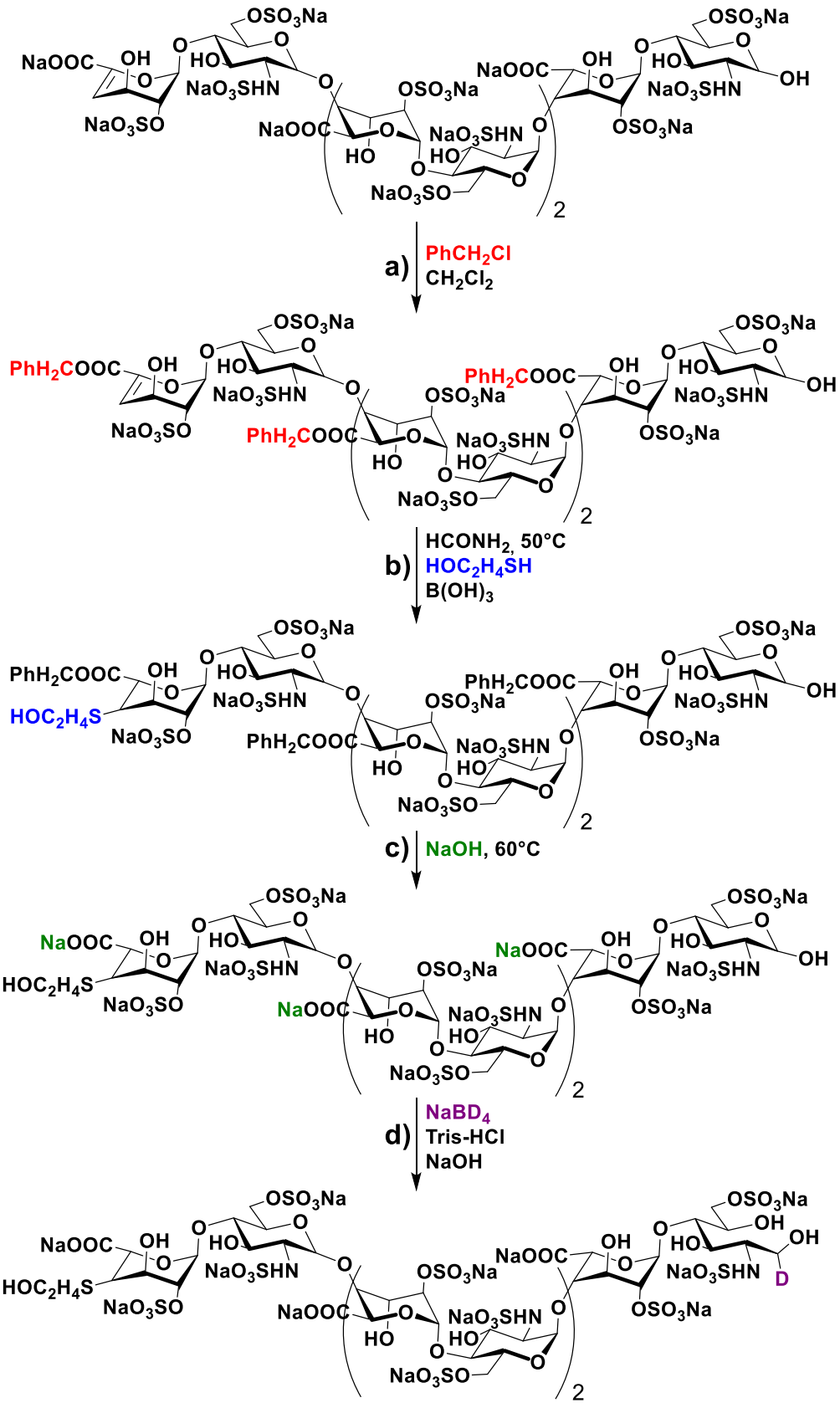
HPdp8 benzyl ester obtained in step 2.1 was dissolved in 1 mL formamide at 50℃. Mercaptoethanol (SE) 2-MEAB **4** (58.8 mg, 0.3 mmol) and boric acid (1.5 mg, 0.02 mmol) were then added. The mixture was stirred at 45℃ for 24 h. Then, the resulting mixture was purified by Bio-gel PD-10 column to remove any unreacted mercaptoethanol and formamide, and then freeze dried.

2.3 Deprotection of mercaptoethanol-HPdp8 benzyl ester

SE-HPdp8 benzyl ester obtained in step 2.2 was dissolved in 200 µl deionized water, heated at 60℃, and then 200 µl of NaOH 0.1 M was added. The mixture was stirred at 60℃ for 1 h, and let under stirring at room temperature during 30 min. Then, 300 µL of 1 µM NaCl aqueous solution was added. Solution was homogenized and purified/desalted by Bio-gel PD-10 column to remove any reagent and salts excess, and then freeze dried.

2.4 Reducing end labelling of SE-HPdp8 with deuterium

SE-HPdp8 obtained in step 2.3 was solubilized in a solution of 200 µL 1.0 M $NaBD_4$, in buffer Tris-HCl 50 mM, pH 8.5, and then incubated overnight at 37°C. Then, 200 µL of acetic acid were added, and the mixture was stirred at room temperature for 20 minutes. Resulting RE labelled oligosaccharides was desalted and purified by Bio-gel PD-10 column, and then freeze dried.



Scheme S1 Chemical route to the double labelling of heparin based octasaccharide (HPdp8). Reagents and conditions: (a) benzyl chloride, DCM, RT; (b) mercaptoethanol, formamide, boric acid, 50°C, 24h; (c) NaOH, 60°C; (d) sodium borodeuteride, Tris-HCl , water, RT.

3. References

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