Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

One-pot gram-scale synthesis of virucidal heparin-mimicking polymers as HSV1 inhibitors

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1 Experimental

1.1 Materials

Meta-chloroperoxybenzoic acid (*m*CPBA) (≤77%), 1,1,1-trimethylolpropane (TMP) (≥ 98%), glycidol (96%) was purified by vacuum distillation, and sulfur trioxide pyridine complex (97%) were purchased from Sigma Aldrich (Darmstadt, Germany). 4-Penten-1-ol (99 %) and anhydrous dimethylformamide (DMF) purchased from Acros (Part of Thermo Fischer Scientific, Geel – Belgium). 7-Octen-1-ol (96%) was purchased from abcr. GmbH (Karlsruhe, Germany). The hyperbranched polyglycerol (hPG), $\bar{M}_n$ 5 KDa, and $Đ$ = 1.7, was synthesized through ring-opening anionic polymerization followed by sulfation to yield hPGS-C_0 as a control according to literatures.[1, 2]

2 Method and Instrumentations

2.1.1 Gel permeation chromatography (GPC)

GPC measurements were carried out using a Shimadzu Prominence-I LC-2030 equipped with an internal UV absorption detector and a Shimadzu RID-29A refractive index detector. Columns were held at 40 degrees Celsius and flow rates of the mobile phase (DMF) were set to 1 mL/min. 100 µL of a sample with a concentration of 5 mg/mL were injected for each measurement. GPC measurements were conducted using polystyrene as the standard.

2.1.2 Zeta potential

Nano ZSPO (Malvern) was used to measure the zeta potential in PB solution.

2.1.3 Nuclear magnetic resonance (NMR).

A Jeol Eclipse 600 MHz spectrometer was used to record NMR spectra at 300 K. Spectra were recorded in parts per million for ^{1}H and ^{13}C. The coupling constants are expressed in Hz. The solvent peaks were used as a reference for the spectra.

2.1.4 Tangential Flow Filtration (TFF)

TFF was performed using an ultrafiltration cassette (Millipore Pellicon, MWCO 2 kDa) in a cassette holder (Sartorius). Using a peristaltic pump (Gibson), the solution was pumped through the system. Rotor speed was maintained at maximum operating.

2.1.5 Cell viability assay CCK-8

The cell viability assay “Cell Counting Kit 8” (CCK-8) from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany) was used to analyze the effect of the sulfated polymers on three different cell lines: A549 human lung carcinoma cells (DSMZ ACC 107), 16HBE14o- human bronchial epithelial cells (Millipore SCC150) and Vero E6 African green monkey kidney epithelial cells (ATCC CRL-1586). A549 and Vero E6 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) and 16HBE14o- cells in Minimum Essential Media (MEM) supplemented with 10% fetal bovine serum, penicillin/streptomycin and GlutaMAX or Glutamine (all from Gibco BRL, Eggenstein, Germany). The cells were passaged every 3 to 4 days after reaching 70% to 90% confluency. The cell viability assay was done according to the manufactures’ instructions. In short, one day prior to the test cells were seeded in a 96 well plate ($5*10^5$ cells in 90 µl cell culture medium per well) and incubated over night at 37°C and 5% CO_2. 90 µl of only cell culture medium without cells was added to the outer wells of the 96-well plates for later background subtraction. On the next day, serial dilutions of the compounds were prepared with water (MilliQ grade) and each concentration was added to three wells containing cells (each 10 µl) and additionally to one outer well without cells. SDS (1%), solvent treated (MilliQ water) and non-treated cells served as controls. The well plates was incubated for another day at 37 °C before CCK-8 solution was added to each well (10µ l). After approximately 3 hours absorbance was measured at a measurement wavelength of 450 nm and a reference wavelength of 650 nm with a plate reader (Infinite pro200, TECAN-reader Tecan Group Ltd., Männedorf, Switzerland). The assay was repeated three times. For

calculating the cell viability, the corrected absorbance (absorbance at measurement wavelength 450nm subtracted by the absorbance at reference wavelength 650nm) was used. For each control compound and concentration, the background was subtracted by using the respective corrected absorbance values of the wells without cells and subtracted this from the corrected absorbance of each of the three replicates. The cell viability was calculated by regarding the corrected and background subtracted absorbance of the non-treated control to 100% cell viability. The results are represented in a bar chart using the mean cell viability of the three repetitions with standard deviation.

2.1.6 Virus propagation and inhibition study

2.1.6.1 Virus propagation and virus titer determination by plaque assay

The GFP-tagged HSV-1 (provided by the Osterrieder Group, Institut für Virologie, Freie Universität Berlin), was propagated on Vero cells (ATCC CRL-81) and the supernatant containing GFP-tagged HSV-1 virions was collected. The virus titer was assessed using a plaque assay. For the plaque assay, Vero cells were pre-seeded in a 12-well plate for two days until confluency before adding 200 µl of virus containing solution for 45 minutes. Afterwards, 1 ml of 0.5% methylcellulose (Sigma M0262) was added as overlay medium. The infected cells were cultured for 2 days for plaque formation. The number of plaques per well was assessed using an epifluorescence microscope (Zeiss Axiovert 100) using the GFP channel. The titer of the initially added solution was calculated and expressed as plaque forming units (PFU)/mL.

2.1.6.2 Plaque reduction assay

The plaque reduction assay was performed according to our former reports.[3] Vero cells were seeded in a 12-well plate until a confluent cell layer was formed. For the incubation, the compounds were diluted in 100 µL DMEM and then incubated with 100 µL of GFP-tagged HSV-1 solution (approx. 2000 PFU/mL) for 45 minutes at 37 °C. Afterwards, the infectivity of the mixture was titrated by a plaque assay using Vero cells as described above. The inhibition of the HSV-1 infection is calculated as follows:

$$Inhibition(\%) = \left(1 - \frac{Plaque\ number\ (sample)}{Plaque\ number\ (virus\ control)}\right) \times 100\% \qquad \text{Equation S 1}$$

The respective half maximal inhibitory concentration (IC_{50}) of each compound was estimated by using the software GraphPad Prism 7 and applying the dose-response model. The tests were done three times with individual experiments.

2.1.6.3 Virucidal assay

The compounds (1 mg/mL) were incubated with HSV-1 suspension containing approximately 1×10^5 PFU for 1 h at 37 °C. Afterwards, the mixture was diluted 5 times 10-fold in DMEM to an endpoint of no active virions. The number of active virions was determined by plaque assay and virus titers were calculated back by the respective dilutions.

2.1.6.4 Pre and post cellular infection assays

Vero cells were seed in a 24 well-plate till 80% confluency. For pre infection assay, the cells were pre-incubated with DMEM containing inhibitors for 45min and then were infected by GFP-tagged HSV-1 at a multiplicity of infection (MOI) of 0.1. For post infection assay, the cells were firstly infected by GFP-tagged HSV-1 at an MOI of 0.1 for 1 hour, and then the compounds were added into the cell culture medium. After 48 hours, the cells were fixed by 2.5% formaldehyde and stained by DAPI to study the cellular infection using an epifluorescence microscope (Zeiss Axiovert 100).

2.1.7 Statistical Analysis

GraphPad 7 was used to analyze cell viability, plaque assay, virucidal assay and cellular infection assays. All values were compared to the control using an ANOVA analysis with Dunnett's multiple comparison correction. The significance value was set to p=0.05.

2.1.8 Fluorescent microscopy image analysis

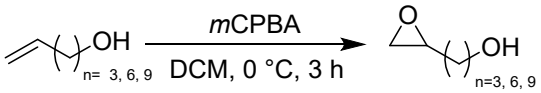
Image analysis was performed using self-written scripts in Fiji [4] and MatLab (MathWorks, Natick, MA, USA). Cells were identified by nuclei staining, segmented by watershed transformation,[5] and infection status was determined by GFP signal. The proportion of infected cells was calculated for four to five images per condition (Figure S4b and c).

2.1.9 Activated partial thromboplastin time (APTT)

The activated partial thromboplastin time (APTT) was determined on a STart Max coagulometer (Stago). Test samples were diluted to different concentrations in H_2O and 2µL of these solutions were added to 50µL of standard human plasma (Siemens Healthcare #ORKL175) mixed with 50µL of Actin FS (Siemens Healthcare #B4218-20) in a cuvette containing a steel ball. After incubation for 180s at 37°C, the cuvette was transferred to the measurement area of the device and the reaction was started by adding 50µL of CaCl2 solution (Siemens Healthcare #ORH0375). The time until clot formation was recorded in seconds and each test was repeated three times. Different concentrations of heparin (Sigma-Aldrich/Merck # H3393-500kU, 201 USP units/ml) diluted in H_2O was used as a reference. Concentrations given refer to the final concentration of the sample in the Plasma+Actin solution (1:50).

3 Synthetic procedures

3.1 Synthesis of comonomers



Scheme S 1. Synthesis of 9-(oxiran-2-yl)nonan-1-ol, 6-(oxiran-2-yl)hexan-1-ol, and 3-(oxiran-2-yl)propan-1-ol through epoxidation using *m*CPBA.

The epoxidation of the substrates was carried out with *meta*-chloroperbenzoic acid (*m*CPBA) using Prilezhaev reaction with some modifications. Briefly, *m*CPBA (77%) was dissolved in DCM, dried over MgSO4, and then filtered before it was used in the reactions. Then, 1.5 eq of the dried *m*CPBA solution in DCM (21.38 g, 123.8 mmol, 6.86 g, 39.78 mmol, and 10 g, 58.16 mmol) was added dropwise to the solutions of undec-10-en-1-ol (14 g, 82.58 mmol, 1 eq.), oct-7-en-1-ol (3.4 g, 26.52 mmol, 1 eq.), and pent-4-en-1-ol (3.34 g, 38.78 mmol, 1 eq.) in DCM, respectively. The reactions were stirred in 0 °C until the disappearance of the starting material (monitored by TLC). Afterward, the reactions were treated with 10 % aqueous sodium bisulfite followed by extraction from water (3 times), and finally brine. The combined organic phases were then dried over MgSO4, filtered, and concentrated under reduced pressure. The crude products were finally purified using column chromatography on silica gel (5-30 % EtOAC-Cy). 9-(oxiran-2-yl)nonan-1-ol (11.8 g, 77 %), 6-(oxiran-2-yl)hexan-1-ol (3.1 g, 81 %), and 3-(oxiran-2-yl)propan-1-ol (1.9 g, 48 %) were obtained as colorless oils.

3.1.1 9-(oxiran-2-yl)nonan-1-ol

^{1}H NMR (500 MHz, $CDCl_3$) δ 3.65 – 3.55 (t, *J* = 6.6 Hz, 2H), 2.91 – 2.83 (p, *J* = 5.6, 3.0 Hz, 1H), 2.74 – 2.67 (t, *J* = 4.5 Hz, 1H), 2.46 – 2.39 (dd, *J* = 5.1, 2.7 Hz, 1H), 1.76 – 1.67 (s, 1H), 1.56 – 1.47 (m, 4H), 1.46 – 1.37 (dtd, *J* = 18.5, 7.2, 2.1 Hz, 2H), 1.35 – 1.23 (m, 10H). ^{13}C NMR (126 MHz, CDCl3) δ 62.88, 47.02, 32.75, 32.41, 29.42, 29.40, 29.35, 29.33, 25.89, 25.70. MS (ESI$^+$): $C_{11}H_{22}O_2$ [Na^+]: Calculated: 209.1492 *m/z*. Measured: 209.1516 *m/z*.

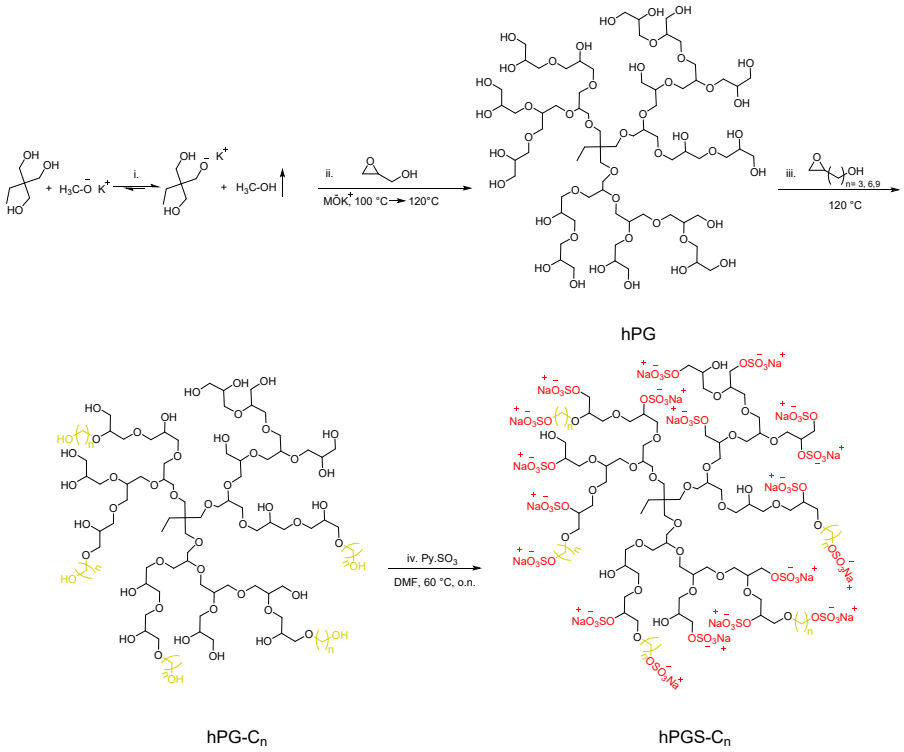
3.1.2 6-(oxiran-2-yl)hexan-1-ol

^{1}H NMR (600 MHz, $CDCl_3$) δ 3.53 – 3.41 (t, *J* = 6.7 Hz, 2H), 3.07 – 2.92 (s, 1H), 2.84 – 2.75 (p, *J* = 4.3 Hz, 1H), 2.68 – 2.59 (t, *J* = 4.6 Hz, 1H), 2.41 – 2.30 (m, 1H), 1.51 – 1.30 (m, 6H), 1.29 – 1.20 (m, 4H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 62.44, 52.44, 47.09, 32.53, 32.32, 29.17, 25.90, 25.66. MS (ESI$^+$): $C_8H_{16}O_2$ [Na^+]: Calculated: 167.1092 *m/z*. Measured: 167.1052 *m/z*.

3.1.3 3-(oxiran-2-yl)propan-1-ol

^{1}H NMR (600 MHz, DCM-d_2) δ 3.97 – 3.89 (qd, *J* = 6.8, 3.3 Hz, 2H), 2.83 – 2.76 (dd, 1H), 2.75 – 2.66 (dd, 1H), 3.44 – 3.37 (dd, *J* = 11.6, 6.3 Hz, 1H), 1.92 – 1.53 (m, 4H). ^{13}C NMR (151 MHz, DCM-d_2) δ 59.62, 48.15, 44.85, 27.15, 26.00. MS (ESI$^+$): $C_5H_{10}O_2$ [Na^+]: Calculated: 125.0592 *m/z*. Measured: 125.0581 *m/z*.

3.2 One-pot synthesis approach to hPGS-C_n



Scheme S 2. Synthesis approach of hPGS-C_n by a one-pot, four-step procedure

Table S 1. Overview properties of the polymerizations.

	Glycidol [M1]		[M1]/ [I]	Theoretical M	Experimental M* (hPG)		Glycidol derivative [M2]		[M2]/ [I]	Experimental M* (hPG-C_n)		Overall Yield	Final M ** (hPGS-C_n)
	[g]	[mmol]	[-]	[kDa]	M_n [kDa]	Đ [-]	[g]	[mmol]	[-]	M_n [kDa]	Đ [-]	[g]	M [kDa]
hPGS-C_9	7.31	98.4	70	5.0	3.8	1.7	11.0	59.10	42	9.7	1.5	13.8	12.1
hPGS-C_6	1.32	17.9	70	5.0	3.9	1.8	1.55	10.74	42	6.6	1.8	4.1	11.5
hPGS-C_3	0.36	4.9	70	5.0	3.9	1.8	0.30	3.0	42	6.7	1.7	0.9	10.6
hPGS-C_0	0.37	4.9	70	5.0	4.6	1.7	-	-	-	-	-	0.7	11.3

** Measured by GPC in DMF (without any purifications). ** Calculated using ^{1}H NMR and Elemental analysis (See 3.2.3).*

The polymerizations were all performed solvent-free in round-bottom flasks equipped with a mechanical stirrer under argon atmosphere according to our previously reported literature[6] with some modifications. Briefly for hPGS-C_9, the flask was first heated to 140 °C under reduced pressure over night to ensure that water residues were completely removed. Following that, the flask was filled with argon and the temperature lowered to 60 °C. TMP (188.63 mg, 1.4 mmol, 1.0 eq.) was added at this point under argon circulation. The TMP was then melted under reduced pressure at 65 °C to eliminate any residual water content. To partially deprotonate TMP (~15 % of the OH groups), the temperature was lowered to 55 °C and 0.1 mL of methanolic potassium hydroxide solution (31.5 mg, 0.56 mmol, 0.4 eq.) was added and stirred for 1 h. Then, the pressure was reduced, and the temperature increased to 100 °C for an hour in order to evaporate methanol. The flask was next flushed with argon and distilled glycidol (7.3 g, 98.4 mmol, 70.0 eq.) was slowly added to the reaction over a period of three hours using a syringe pump. In order to assure that no unreacted monomer remains, the reaction condition was maintained for 6 hours followed by another 2 hours at 120 °C. Then, the distilled comonomer, 9-(oxiran-2-yl)nonan-1-ol, (11 g, 59.1 mmol, 42 eq.)

was added slowly to the reaction over a period of 3 hours and stirred for another 3 hours at 120°C. Half of the resulting product, namely hPG-C_9, was taken and placed in the fridge for later uses. As for the rest of the obtained product (7.21 g, 97.30 mmol OH groups) in the flask, it was diluted in anhydrous DMF, lowered to 60 °C, and sulfated *in situ* by adding sulfur trioxide pyridine complex in anhydrous DMF (23.2 g, 145.9 mmol, 1.5 eq. of mol of OH groups). The reaction was stirred overnight at 60 °C. At the end of the reaction, the mixture was diluted with water, followed by neutralization with 1 M NaOH to pH 11. To purify the product, an aqueous polymer solution was circulated over an ultrafiltration membrane (2 kDa MWCO) for 72 hours using TFF. Small polymers fractions and impurities were removed during the filtration *via* permeation and replaced with fresh brine continuously. Gradually, brine solution was replaced with deionized water. In the end, the product hPGS-C_9 was obtained by freeze-drying (13.8 g). Similar procedure was applied to synthesis hPGS-C_6, and hPGS-C_3. The properties of all

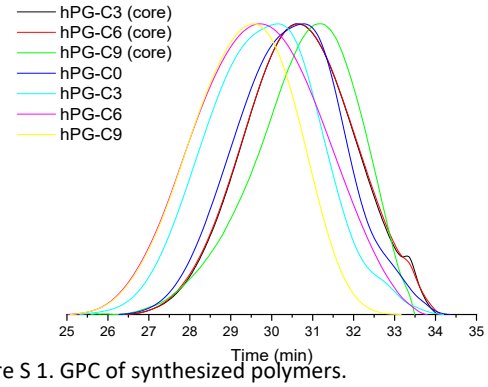


Figure S 1. GPC of synthesized polymers.

polymerizations are listed in Table S1.

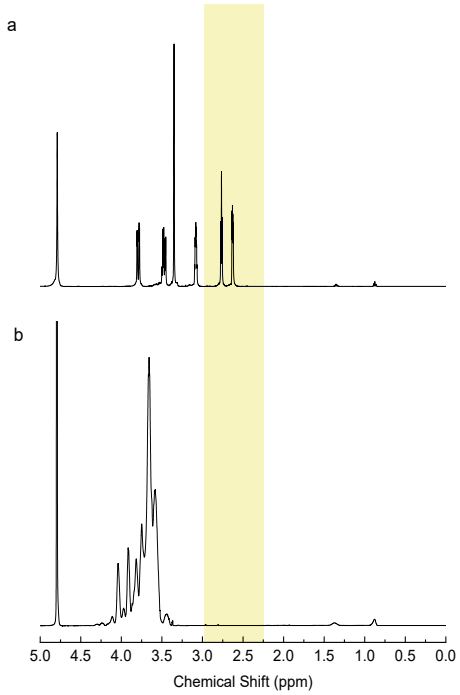


Figure S 2. ^{1}H NMR spectra of the reaction a) at time 0 h and b) upon adding the comonomer.

3.2.1 Degree of functionalization (DF)

The degree of functionalization was calculated using the integrals measured by ^{1}H-NMR in Figure S6 and Equation S1-S3 for hPGS-C_9, hPGS-C_6, and hPGS-C_3 respectively.

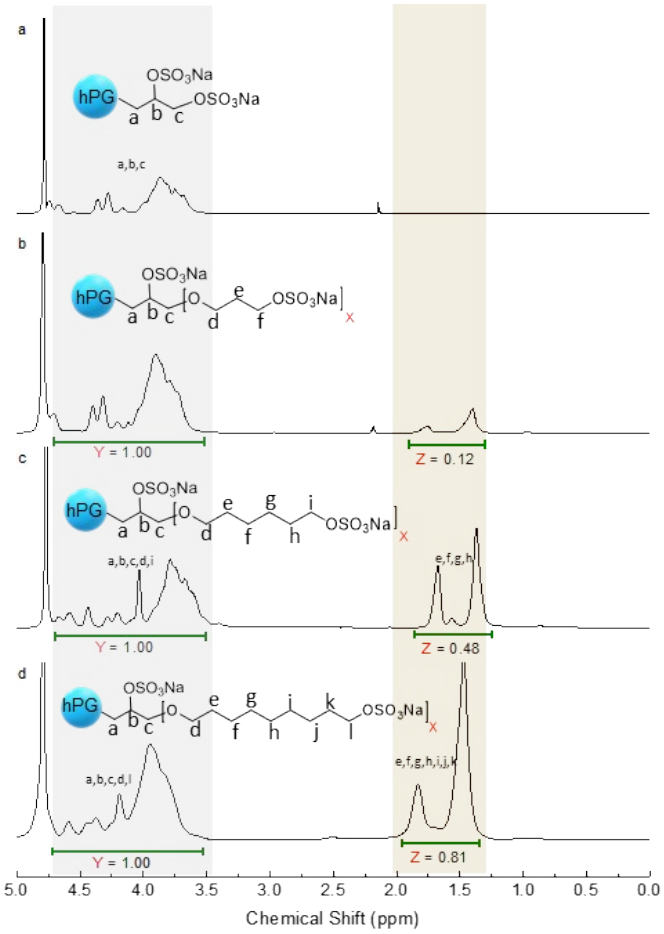


Figure S 3. ^{1}H NMR spectra of a) hPGS-$C_{0,}$ b) hPGS-C_3, c) hPGS-$C_{6,}$ and d) hPGS-C_9 in D2O.

Y=5+4x
Z=14x
Z/Y= 0.81

→ **DF** $_{hPGS\text{-}C_9}$ **= 38%**

Equation S 2

Y=5+4x
Z=8x

→ **DF** $_{hPGS\text{-}C_6}$ **= 39%**

Equation S 3

Z/Y= 0.48

Y=5+4x
Z=2x → **DF $_{hPGS\text{-}C_3}$ = 39%** Equation S 4
Z/Y= 0.12

3.2.2 Degree of sulfation (DS)

On the basis of the sulfur content obtained by elemental analysis (Table S 2), equation S4-S7 were used to calculate the DS of hPGS-C_9, hPGS-C_6, hPGS-C_3, and hPGS-C_0, respectively.

Table S 2. Elemental analysis of the synthesized polymers.

Compound	C (%)	H (%)	N (%)	S (%)
hPGS-C_9	27.61	5.06	0.03	15.07
hPGS-C_6	26.59	4.61	0.03	15.10
hPGS-C_3	19.78	4.63	0.01	16.58
hPGS-C_0	20.39	3.21	0.02	16.51

$$\text{DS (hPGS-C}_n\text{)} = \frac{S\%\ (experimental\ (obtained\ from\ EA))}{S\%_t\ (total\ S\%\ in\ case\ of\ 100\%\ sulfation)}$$ Equation S 5

where

$$S\%_t = DF^* \underbrace{(S_n\ \%)}_{\text{Theoretical S\% of } \ce{O(CH2)nOSO3Na}} + (1\text{-}DF)^* \underbrace{(18.2)}_{\text{Theoretical S\% of } \ce{OCH2OSO3Na}}$$ Equation S 6

$$DS_{hPGS\text{-}C_9} = \frac{15.07}{0.38 * 11.12 + 0.62 * 18.2} * 100\% = 97\ \%$$ Equation S 7

$$DS_{hPGS\text{-}C_6} = \frac{15.10}{0.39 * 13.02 + 0.61 * 18.2} * 100\% = 93\ \%$$ Equation S 8

$$DS_{hPGS\text{-}C_3} = \frac{16.58}{0.39 * 15.70 + 0.61 * 18.2} * 100\% = 96\ \%$$ Equation S 9

$$DS_{hPGS\text{-}C_0} = \frac{16.51}{18.2} * 100\% = 91\ \%$$ Equation S 10

3.2.3 Molecular weight

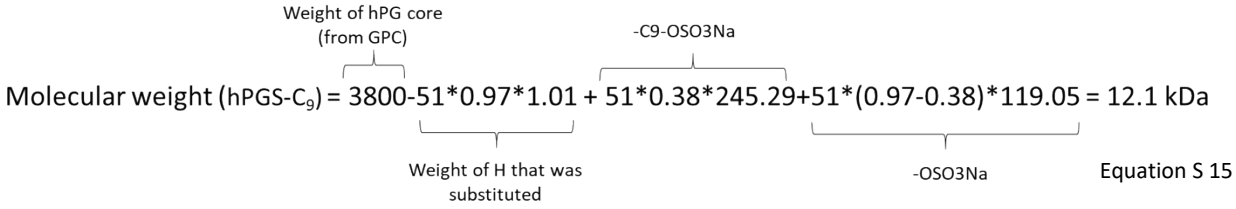
According to Equation S 12-15, the final molecular weight of each compound can be approximately calculated by using the DF, DS, and Mn values.

Total Number of OH $_{hPGS\text{-}C_9}$ **=** $\frac{3800}{74} = 51$ Equation S 11

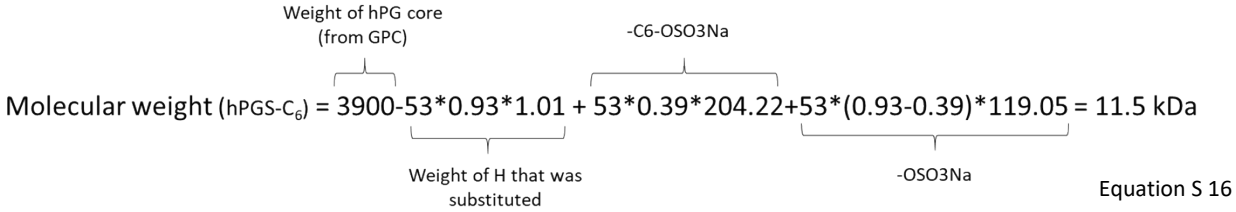
Total Number of OH $_{hPGS\text{-}C_6}$ **=** $\frac{3900}{74} = 53$ Equation S 12

Total Number of OH $_{hPGS\text{-}C_3}$ **=** $\frac{3900}{74} = 53$ Equation S 13

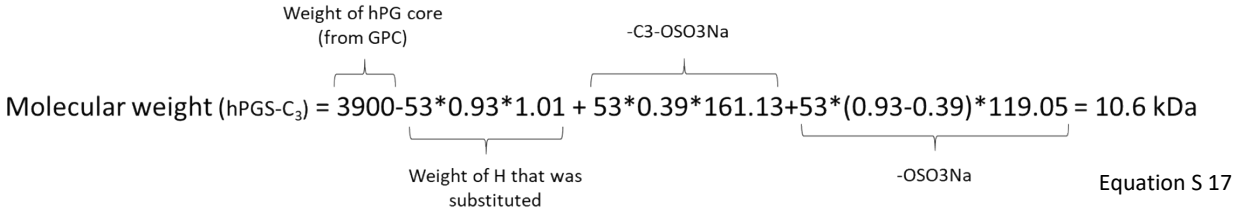
Total Number of OH $_{hPGS\text{-}C_0}$ **=** $\frac{4600}{74} = 62$ Equation S 14



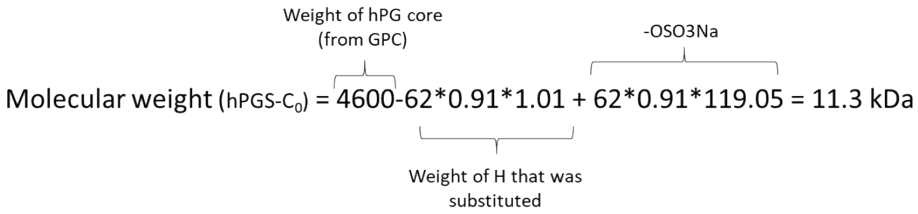
Equation S 15



Equation S 16



Equation S 17



Equation S 18

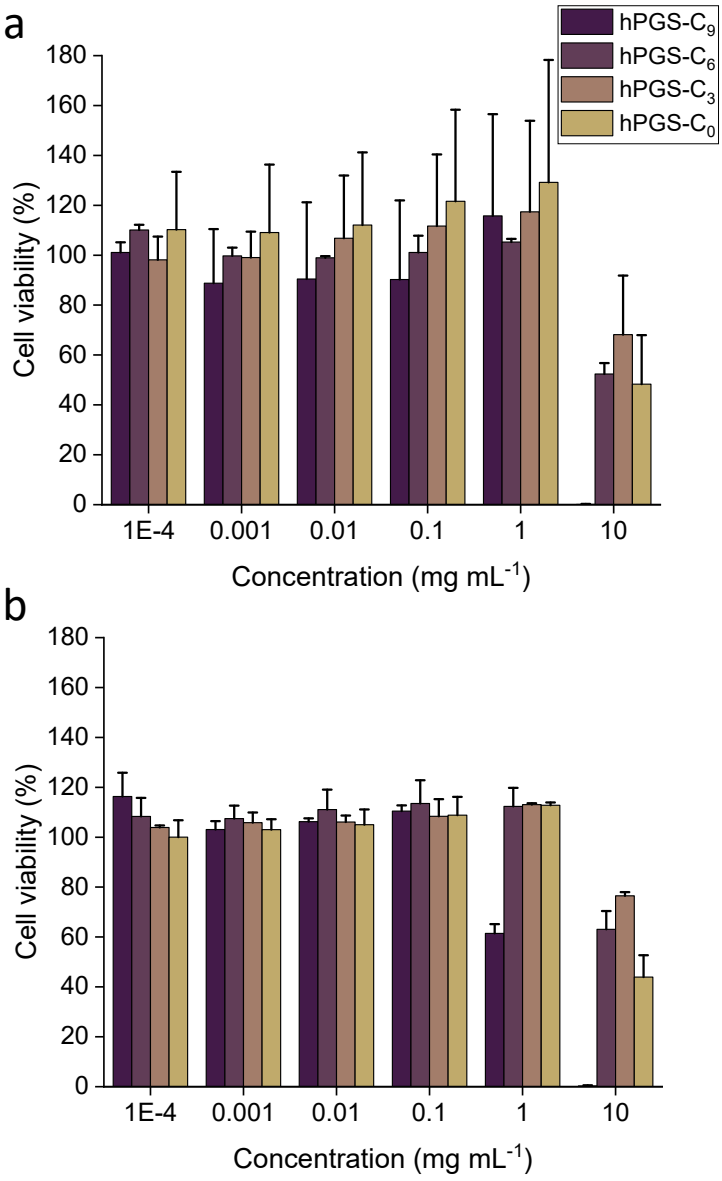


Figure S 4. Cytotoxicity profile of the synthesized inhibitors determined by a CCK-8 assay using (a) A549 and (b) 16HBE14o-. The cell viability of compound treated cells is normalized to the cell viability of non-treated cells that was

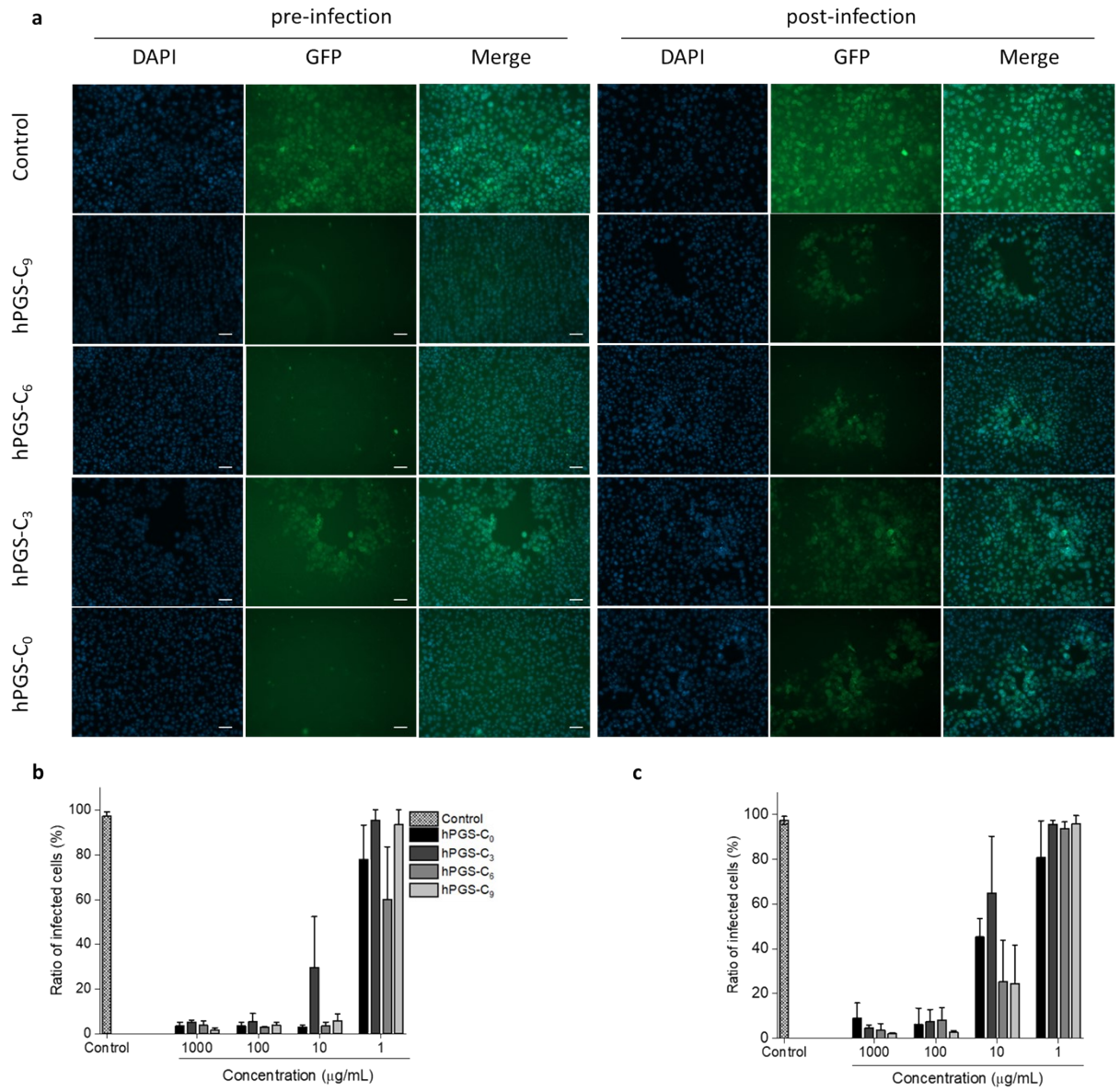


Figure S 5. a) fluorescent microscopy images of infected cells for pre- and post-infection assay for all the compounds with a concentration of 10 µg/mL. Scale bar: 20 µm. Cell nuclei are marked in blue, and infected cells in green. Ratios of infected cells for each compound obtained from b) pre-infection and c) post-infection assay. (Control: DMEM medium). Values are expressed as mean ± SD, n = 4.

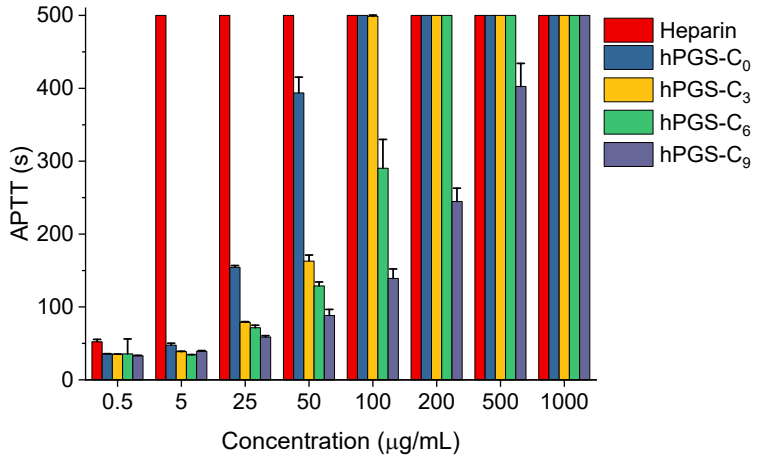


Figure S6. The APTT for synthetic inhibitors and heparin. Untreated human plasma with APTT of 31s was used as a control. Data are expressed as mean ± SD (n=3). x-axis gives concentration in μg mL^{-1}.

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