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Linear polysialoside outperforms dendritic analogues for inhibition of influenza virus infection *in vitro* and *in vivo*

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ABSTRACT

Inhibition of influenza A virus infection by multivalent sialic acid inhibitors preventing viral hemagglutinin binding to host cells of the respiratory tract is a promising strategy. However, optimal geometry and optimal ligand presentation on multivalent scaffolds for efficient inhibition both *in vitro* and *in vivo* application are still unclear. Here, by comparing linear and dendritic polyglycerol sialosides (LPGSA and dPGSA) we identified architectural requirements and optimal ligand densities for an efficient multivalent inhibitor of influenza virus A/X31/1 (H3N2). Due to its large volume, the LPGSA at optimal ligand density sterically shielded the virus significantly better than the dendritic analog. A statistical mechanics model rationalizes the relevance of ligand density, morphology, and the size of multivalent scaffolds for the potential to inhibit virus-cell binding. Optimized LPGSA inhibited virus infection at IC₅₀ in the low nanomolar nanoparticle concentration range and also showed potent antiviral activity against two avian influenza strains A/Mallard/439/2004 (H3N2) and A/turkey/Italy/472/1999 (H7N1) post infection. *In vivo* application of inhibitors clearly confirmed the higher inhibition potential of linear multivalent scaffolds to prevent infection. The optimized LPGSA did not show any acute toxicity, and was much more potent than the neuraminidase inhibitor oseltamivir carboxylate *in vivo*. Combined application of the LPGSA and oseltamivir carboxylate revealed a synergistic inhibitory effect and successfully prevented influenza virus infection in mice.

KEYWORDS multivalent inhibitor, influenza virus, polyglycerol scaffolds, ligand density, steric shielding

Introduction

Seasonal influenza A infections cause ~500,000 deaths and 3-5 million cases of severe illness annually worldwide [1, 2]. The high mutation frequency of viral proteins limits both the use of vaccination [3] and of approved therapeutics against viral proteins that mediate uncoating (e.g. amantadine) or release (e.g. oseltamivir) of the virus [4]. However, new approaches targeting conserved domains of the major viral spike protein hemagglutinin (HA) are still of great interest, since HA is required for early steps of the infection: binding to the sialic acid (SA) residues on the cell surface, and upon endocytosis, fusion with the endosomal membrane [5]. Recent developments of antibodies binding to the stem domain or to the SA binding pocket of HA are promising candidates with a broad spectrum activity [6, 7].

Mimicking the host cell surface by multivalent presentation of SA residues on suitable polymeric scaffolds is an alternative to prevent infection by inhibiting the virus binding [8-12]. E.g., linear sialylated polyacrylamides (PAA) have been shown to efficiently compete for virus binding to the host cells [8]. However, typically they are cytotoxic [13], and of low biocompatibility [14]. Only a few sialylated multivalent inhibitors of influenza virus have been tested *in vivo*, however, without any promising perspective for application [15, 16]. Major concerns are the geometrical requirements for multivalent ligand presentation optimal for *in vivo* application. Dendritic scaffolds can present a rather structurally defined set of ligands compared to its linear more flexible counterparts. In contrast, depending on their properties carbohydrate-conjugated linear polymers may offer a highly hydrated water swollen coiled scaffold with enhanced steric shielding properties in comparison to those of dendritic scaffolds. Likewise, the optimal ligand density varies depending on the architecture type [17]. The importance of optimal ligand density has been addressed recently using spherical 6'-sialyllactose-PAMAM conjugates for efficient influenza virus inhibition [18].

Finally, a low molecular weight of the polymer (<40 kDa) for systemic clearance and a low toxicity are required for *in vivo* use [19].

Given the significance of scaffold geometries for contributions of steric shielding to inhibition of virus binding, a preference for linear or dendritic polymeric scaffolds is not *a priori* given [17]. We have now undertaken a systematic study on a series of linear and dendritic polyglycerols (LPG and dPG) with a similar linker strategy. Due to its low toxicity and good *in vivo* clearance, PGs are a promising scaffold for a multivalent ligand display [20-22]. Setting the hypothetical models by Whitesides and coworkers [23] on a quantitative basis, our recent studies have emphasized that apart from multivalent interaction size dependent steric shielding is indeed a key factor [24]. Hence, both affinity as well as steric shielding determine the efficacy of a multivalent inhibitor to block virus particles. The highly water soluble SA-conjugated linear PGs possess a dynamic scaffold structure due to their flexibility amplifying steric shielding effects, as suggested by theoretical considerations showing that the linear scaffolds inhibit virus binding more efficiently than the dendritic scaffolds. Taken together, we here present the design and synthesis of a non-toxic, low molecular weight multivalent inhibitor for influenza A virus that displays promising antiviral efficacy *in vivo*.

Materials and Methods

Synthesis and characterization

All reagents and solvents were purchased from commercial suppliers and used without further purification. Reactions requiring dry or oxygen-free conditions were carried out under argon in Schlenk glassware. ¹H spectra were recorded on Bruker AMX 500 (500 MHz) and Delta Joel Eclipse 700 (700 MHz) spectrometer at 25 °C and calibrated by using the deuterated solvent peak. Infrared (IR) spectra were recorded with a Nicolet AVATAR 320

1 FT-IR 5 SXC (Thermo Fisher Scientific, Waltham, MA, USA) with a DTGS detector from
2 4000 to 650 cm^{-1} . A TSQ 7000 (Finnigan Mat) instrument was used for ESI measurements
3 and a JEOL JMS-SX-102A spectrometer was used for the high-resolution mass spectra.
4 Molecular weight distributions of LPG₁₀ **1**, dPG₁₀ **2**, and the post functionalized polymers
5 were determined by means of GPC coupled to a refractive index detector (RI) for obtaining
6 the complete distribution (M_n , M_p , M_w , dispersity). Measurements were carried out under
7 highly diluted conditions (5 mg/ml) from a GPC consisting of an Agilent 1100 solvent
8 delivery system with pump, manual injector, and an Agilent differential refractometer. Three
9 30 cm columns (PPS: Polymer Standards Service GmbH, Germany; Suprema 100 Å, 1000 Å,
10 3000 Å with 5 and 10 μm particle size) were used to separate aqueous polymer samples using
11 water with 0.1 N NaNO_3 as the mobile phase at a flow rate of 1 ml/min. The columns were
12 operated at room temperature (rt) with the RI detector at 50 °C. The calibration was
13 performed by using certified standards pullulan (linear) and dextran (branched) from PSS.
14 WinGPC Unity from PSS was used for data acquirement and interpretation. The molecular
15 weight of the dPG₅₀₀ **3** was determined by gel permeation chromatography (GPC) using a
16 DAWN-EOS multi-angle laser light scattering (MALLS) (Wyatt technology Inc., Santa
17 Barbara CA) and optilab RI detectors; the details have been described previously [25]. An
18 aqueous 0.1 N NaNO_3 solution (pH = 7.0) was used as the mobile phase and dn/dc value for
19 PG used for the molecular weight calculation was 0.12 ml/g [26]. DLS measurements of the
20 various polymers were conducted by using a NanoDLS particle sizer (Brookhaven
21 Instruments Corp.) at 25 °C. Aqueous samples were filtered through 0.2 μm filters prior to
22 analysis. Water of Millipore quality was used in all experiments. An USHIO super high
23 mercury lamp (USH 102d, 100 W) was used for UV assisted thiol-ene click reaction.
24 Naturally occurring sialic acids constitute a family of more than 50 structurally distinct nine-

carbon 3-deoxy-ulosonic acids, the most widespread derivative being 5-*N*-acetyl-neuraminic acid (Neu5Ac). We used the abbreviation of sialic acid (SA) for Neu5Ac.

Virus material

X31 virus (influenza strain A/Aichi/2/68 H3N2, reassorted with internal segments of A/PuertoRico/8/1934 H1N1), A/Mallard/439/2004 (H3N2) or A/turkey/Italy/472/1999 (H7N1) were harvested from allantoic fluid of embryonated chicken eggs. Virus isolates were clarified upon low speed centrifugation (300 x g, 10 min). For binding (inhibition) experiments clarified allantoic fluid was further concentrated by ultracentrifugation (100,000 x g, 1 h).

Cell culture

MDCK-II (Madin-Darby canine kidney epithelial) cells (ATCC) have been maintained in DMEM (supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 µg/ml streptomycin and 100 units/ml penicillin) at 37 °C and 5% CO₂.

Hemagglutination inhibition assay (HAI)

Inhibitors were twofold serially diluted in PBS. Then, 4 HAU X31 virus containing approximately $4 \cdot 10^7$ virus particles were added to all the wells. The amount of virus particles per volume was estimated as published by Desselberger et al. [27]. After 30 min incubation under slight agitation at rt, 50 µl of a 1 % human erythrocyte solution (German Red Cross, $\sim 2 \cdot 10^6$ cells/µl) was added, gently mixed, and incubated for 60 min at rt. The inhibitor constant $K_{i(\text{HAI})}$, reflects the lowest inhibitor concentration, which is necessary to achieve full inhibition of virus induced hemagglutination. To check for full hemagglutination inhibition, the microtiter plate was tilted by 60° to cause droplet formation from the red blood cell pellet [28].

Erythrocyte binding inhibition assay

Experiments were performed as previously described [29]. Briefly, influenza A virus (X31) was labelled with 20 μ M octadecylrhodamine B chloride (R18) in PBS and the free dye was removed upon virus pelleting. 5 μ g labelled virus was incubated with two-fold serial dilutions of binding inhibitors, and incubated for 30 min at rt (0.5 μ g/ μ l X31, $\approx$ 16 HAU/ μ l). The virus-inhibitor complex was mixed with 40 μ l of a 1% human erythrocyte solution ($\sim 2 \cdot 10^6$ cells/ μ l) and incubated for 30 min at rt. Unbound virus was removed upon centrifugation for 10 min at 1500 x g. The resuspended cell pellet was analyzed for R18 fluorescence by flow cytometry (Beckton Dickinson FACS AriaTM flow cytometer). Data were acquired by using BD CellQuest software and analyzed with FlowJo 10.0 software. From each measurement, a single cell population was gated and median fluorescence was normalized to a PBS-treated virus control. Dose response curves of mean values ($n \geq 3$) were fitted using a four parametric logistic fit to obtain IC₅₀ values. The asymmetric standard error (SE) of the fit is given for logIC₅₀.

Microscale thermophoresis

Measurements have been performed using a Monolith NT.115 instrument (Nanotemper) and standard glass capillaries. For safety reasons X31 was inactivated with UV light for 5 min on ice. Then the envelope of viruses (1 mg/ml protein) was labelled at 20 μ M R18 for 30 min at rt under gentle shaking. Unbound R18 was removed under centrifugation at 20,000 x g for 5 min. The resuspended virus was filtered through a 0.45 μ m filter (Millipore). To quantify the amount of virus, and to check whether the prepared virus was still able to bind, a hemagglutination assay was performed. Each inhibitor was twofold serially diluted and mixed with equal amounts of X31 (4 HAU) for 15 min at rt. Then the inhibitor-virus mix was loaded on glass capillaries and measured at rt for initial fluorescence (5 s) and change in fluorescence over time (30 s) with the thermophoresis measurement (MST power: 80%, green LED power 100%). The T-jump with thermophoresis was used for analysis and gated

as shown in Fig. S9. Data were analyzed by using Affinity Analysis Software (Nanotemper, v2.0) and plotted with Graphpad Prism 5. In order to determine the noise level, triplicates of the same virus dilution were measured leading to $\Delta F \approx 5$ FU (Fig. S9). The change in fluorescence was expressed as ΔF_{norm} (%), and represented values after subtraction of background and correlation to the initial fluorescence before IR laser activation.

Infection inhibition assay

Viral infection inhibition assays were monitored using a Cell Titer 96 Aqueous One Solution Assay (Promega) according to the standard protocol of the manufacturer. The soluble 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide salt (MTS) undergoes a colorimetric change upon reduction at active mitochondrial membranes to formazan (Abs. 490 nm). Being proportional to the number of cells, mitochondrial activity can be monitored by this assay. Cells infected with Influenza A virus cause a cytopathic effect, which goes along with reduced number of cells or cell viability, respectively.

Here, 15,000 MDCK II cells were seeded one day before infection. On the day of infection, X31 were pretreated with sialosides in a twofold dilution series for 30 min at rt under slight agitation. Next, cells were washed once with PBS⁺⁺ (containing 0.5 mM MgCl and 0.9 mM CaCl), and pretreated virus (MOI 0.05) was added for 1 h at rt to allow binding. Unbound virus was removed by washing once with 37 °C infection medium (DMEM, 2 mM glutamine, 0.1% FCS, 0.1% BSA, 2.5 µg/ml TPCK-treated trypsin, Pen/Strep), and subsequently incubated for 24 h at 37 °C. Following, 20 µl MTS solution was added to each well and incubated for 1 h at 37 °C. Finally, absorbance at 490 nm was measured and data were normalized to maximum viral cytopathogenicity.

$$\text{Cell viability (\%)} = \frac{(\text{Infected}_{\text{treated}} - \text{Infected}_{\text{untreated}})}{(\text{Uninfected}_{\text{untreated}} - \text{Infected}_{\text{untreated}})}$$

Dose response curves of mean values ($n \geq 3$) were fitted using a four parametric logistic fit to obtain IC_{50} values. The asymmetric standard error (SE) of the fit is given as $\log IC_{50}$.

Viral nucleoprotein expression analysis

X31 virus was pretreated in an inhibitor titration experiment with PGs and maintained for 30 min at rt. The day before, 5×10^4 MDCK-II cells were seeded in an 8-well plate (ibidi). On the following day, the cells were washed once with PBS^{++} and infected with inhibitor or PBS treated X31 (MOI 0.1) for 1 hour on ice to synchronize the infection. Unbound virus was removed by aspiration of the supernatant and subsequent washing with infection media (DMEM, 0.1% FCS, 0.1% BSA). Following that, cells were incubated for 24 h at $37^\circ C$ and analyzed for viral nucleoprotein expression by immunostaining as follows:

Cells were washed, fixed with 4% paraformaldehyde and permeabilized with 0.5% (w/v) triton X-100. Furthermore, the cells were blocked with blocking buffer (PBS^{++} , supplemented with 3% BSA and 100 mM glycine) for 2 hours at rt. Immunostaining was performed with an FITC coupled mouse α -NP monoclonal antibody (Millipore, 1:500 in PBS^{++} with 1 % BSA) by incubating overnight at $4^\circ C$. Cellular DNA was stained using DAPI. Confocal microscopy was assessed using an Olympus FV 1000 microscope. Acquired images were analyzed for viral NP expression using an ImageJ software as follows: Cells were automatically identified by an ImageJ script from the DAPI signal of the stained nuclei. Each object representing one cell was further analyzed for the fluorescence intensity coming from the FITC coupled α -NP monoclonal antibody. Median fluorescence intensities of all detected objects ($n > 1000$) were expressed in ratio to the infected, PBS-treated cells. Statistics were performed using a one way ANOVA analysis with a Dunnett's multiple comparison test to the infected, PBS treated control.

Inhibition of multicyclic viral replication

MDCK-II cells were infected with IAV X/31 at a multiplicity of 0.01. After removal of the input virus, the cells were incubated in infection medium complemented with different concentrations of LPG₁₀SA_{0.40} **1b** or LPG₁₀ **1** for 24 hours. Virus titers in the supernatants were then determined by plaque titration on MDCK-II cells and are expressed as plaque forming units per ml (pfu/ml).

***In vivo* infection inhibition study**

Animals were maintained and handled according to the Directive 86/609/EEC of the European Community Council and to the institutional, state and federal guidelines. All animal protocols were approved by the ethics committee of the Landesamt für Gesundheit und Soziales (LAGeSo, Berlin, Germany) with registration number GG0011-15. Animals were housed under standard conditions of 12-h light/dark cycle and given access to food and water ad libitum. Experiments were performed with 8 weeks old BALB/c mice (Charles River) as previously described [30]. First, mice were sedated by an intraperitoneal injection of 200 µl Cepetor (4µg/100 µl) and anesthetized by isofluran inhalation. Then, X31 virus was preincubated with inhibitor for 30 minutes at rt in PBS⁺⁺ before the 25 µl virus-inhibitor mix was distributed to both nostrils. After complete uptake of the virus-inhibitor solution, mice were revitalized, by injecting 200 µl antisedan (2 µg/100 µl) intraperitoneally. Groups of five mice were maintained per cage and experiment for 11 days. The mice were weighed daily and the influenza virus infection related loss in body weight was expressed as percentage of the initial body weight. As soon as animals reached a maximum score of 3 for signs of discomfort and suffering or if a loss in body weight of more than 20 % was observed, mice had to be sacrificed upon cervical dislocation as per regulations. At the end of the experiment (day 11) all mice were sacrificed upon cervical dislocation.

Data were statistically analyzed by comparison of infected, inhibitor treated mice to infected, PBS treated animals, conducting a one-way ANOVA and Dunnett's multiple comparison test.

Mice treated with LPG₁₀SA_{0.40} **1b** or the combination of LPG₁₀SA_{0.40} **1b** and oseltamivir carboxylate (Cayman Chemical, 83 μ M) were compared using a two-tailed student's t-test.

Cryo-TEM imaging

Perforated (1 μ m hole diameter) carbon film-covered microscopical 200 mesh grids (R1/4 batch of Quantifoil, MicroTools GmbH, Jena, Germany) were hydrophilized by a 60 s glow discharging at 8 W in a BALTEC MED 020 device. Then 5 μ l of the aqueous solution of polyglycerol sialosides were pipetted to the hydrophilized grid and the supernatant fluid was immediately removed with a piece of filter paper until an ultrathin layer of the sample solution was obtained spanning the holes of the carbon film. The sample was instantly vitrified by plunging the grids into liquid ethane using a spring-loaded guillotine-like apparatus. The vitrified sample was subsequently transferred under liquid nitrogen into a Tecnai F20 TEM (FEI Company, Hillsboro, Oregon) equipped with field emission gun and operating at 160 kV by the use of a Gatan tomography cryo-holder (Model 914). Microscopy was carried out at a 94 K sample temperature using the low-dose protocol of the microscope. Micrographs were taken with an FEI Eagle 4k $\times$ 4k CCD camera using the two-fold binning mode. The analysis was done using the public domain software ImageJ (U. S. National Institute of Health, Bethesda, Maryland, USA).

Results and Discussions

Design, synthesis, and characterization

To compare different scaffold geometries based on similar chemical constructs, we selected three polyglycerol backbones: 10 kDa linear polyglycerols (LPG₁₀) [31], 10 kDa dendritic polyglycerol (dPG₁₀) [32, 33], and 500 kDa dendritic polyglycerol (dPG₅₀₀) [25]. To determine the optimum ligand density, polyglycerol sialosides (PGSAs) were synthesized

with different degrees of SA ligand functionalization (DF) (Scheme 1) in three to four steps by either thiol-ene coupling, i.e., LPG₁₀SA **1a-d** (DF = 0.10-0.70) or copper-catalyzed Sharpless-Huisgen click reaction, i.e., dPG₁₀SA **2a-d** (DF= 0.05-0.90) and dPG₅₀₀SA **3a-d** (DF = 0.05-0.70) (see Supporting Information). To prove that the linkers do not have an effect on the activity in the HAI assay (Fig. 2a), the control compound LPG₁₀SA_{0.2} **1e** (DF = 0.20) was prepared by a copper-assisted click reaction. All intermediates and final products were characterized by spectroscopic techniques (see Supporting Information). The DF of all polymers with SA ligands was determined by ¹HNMR and the overall molecular weight was calculated based on the parent PG backbone.

The size distribution profiles were analyzed by dynamic light scattering (DLS) in phosphate buffered saline (PBS, pH 7.4) (Table 1, Fig. 1, Fig. S1-S3). The hydrodynamic diameter (D_h) for LPG₁₀ **1** was similar to that of dPG₁₀ **2** but smaller than that of dPG₅₀₀ **3** (Table 1). This confirms an earlier observation that dPG and LPG of the same molecular weight are equally compact [20]. However, we observed a larger increase of D_h with increasing DF for the linear LPG₁₀ **1** backbone in comparison to the dendritic dPG₁₀ **2** backbone (Table 1). This shows the higher swelling capacity of the linear LPGSAs' random coil conformation. In contrast, dPGSAs swell only slightly because of their more restricted dendritic arrangement. In addition, the high molecular weight dPGSAs display only a moderate increase of size compared to the linear analogs. Notably, values for D_h of LPG₁₀SAs were in the same range as observed for the higher molecular weight dPG₅₀₀SAs (Table 1), which has a 50 times higher mass of the PG core.

Morphologies were studied by cryo-TEM (Fig. S4). dPG₁₀SA_{0.15} **2b** and dPG₅₀₀SA_{0.20} **3b** showed particles of spherical morphology with uniform diameters of 5.1 nm and 7.5 nm, respectively (Fig. 1e-f). The somewhat larger sizes observed by DLS measurements are

1 attributed to the fact that DLS measures the hydrodynamic radius, which includes the
2 hydration shell undetectable by TEM.

3 However, the linear polyglycerol sialosides tend to generate very low contrast with
4 the exception of compound $\text{LPG}_{10}\text{SA}_{0.2}$ **1e** where numerous dark spots with a diameter of
5 about 6.3 nm indicate compactly coiled structures of a globular morphology (Fig. 1d and Fig.
6 S4). We surmise that the contrast enhancing factor is due to the presence of electron-dense
7 aromatic ring systems of **1e** not present in compounds **1a -1d**.

Scheme 1. Synthesis of linear and dendritic polyglycerol sialosides of different size and degrees of functionalization. SA residues are randomly distributed on the polyglycerol backbone. See Supporting Information for details for synthetic details.

Fig. 1. Size analysis of the linear and dendritic polyglycerol based inhibitors by DLS and cryo-TEM. The volume distribution profiles of a) linear polyglycerol (LPG₁₀ **1**) and linear polyglycerol sialosides (**1a-e**); b) dendritic polyglycerol (dPG₁₀ **2**) and dendritic polyglycerol sialosides (**2a-e**); c) dendritic polyglycerol (dPG₅₀₀ **3**) and dendritic polyglycerol sialosides

(**3a-d**) as observed by DLS at concentration of 1 mg/ml in aqueous PBS (pH 7.4, 150 mM NaCl) at 25 °C. See Figure S1-S3 for intensity and number distribution profiles of all compounds. Percentage-based frequency distribution of the diameter of selected compounds was determined from cryo-electron micrographs (compared with Figure S4), Feret diameter was determined using the public domain software “ImageJ”, histogram bin size is always 0.5 nm. (d) LPGSA **1e**, (n=420) (e) dPGSA **2b** (n=620) (f) dPGSA **3b**, (n=520).

Optimal SA functionalization for influenza virus binding inhibition

The potential of multivalent scaffolds with varying DF to prevent binding of the seasonal influenza A/X31 virus (subtype H3N2) to cells was studied by hemagglutination inhibition (HAI) and a flow cytometry assisted cell binding competition assay. Table 2 provides a subset of the most active compounds. See Table S7 for the complete list. Non-functionalized scaffolds did not prevent virus-induced hemagglutination at virus quantities equivalent to four hemagglutination units (4 HAU). Among the series of dendritic polyglycerols, dPG₁₀SA_{0.15} **2b** and dPG₅₀₀SA_{0.20} **3b** are the most effective inhibitors, with hemagglutination inhibition constants $K_{iHAI_{NP}}$ of about 358 nM and 16 nM nanoparticle concentrations, respectively (Fig. 2a and Table 2). Supported by cryo-TEM and DLS analysis, we determined a spherical geometry for all three compounds classes, which allowed us to express the average SA surface density of the multivalent inhibitor (Table 1). This rough estimate enables the comparison of SA density on an inhibitor (Table 1, Fig. S11) with that on a host cell surface as well as with binding sites on the virus surface. A typical spherical influenza A virion with a diameter of 120 nm bears around 400-500 HA homotrimers with an intratrimeric binding site spacing of 4-5 nm and a distance of 10-12 nm between two trimer centres [34][35] and 50-200 SA are present per 100 nm² of the host cell surface. Translating these numbers into SA binding sites per virus surface area, one can approximate 0.27-0.40

binding sites per an equilateral triangular HA trimer head surface and 0.5-2.0 binding sites per nm² virus surface [9].

Indeed, ligand density is an important factor of the inhibition potential. For 10 kDa PG based PG sialosides, we found a DF in the range of 0.10-0.70 for dPG₁₀, 0.40-0.80 for LPG₁₀, and about 0.10-0.25 for dPG₅₀₀ to be optimal for inhibition. dPG₅₀₀SA_{0.20} **3b** with ~2.5 SA/nm² is the most effective inhibitor in the dPG500 series with a SA density comparable to the upper range on a cell. Increasing DF to values which are even beyond that on the cell surface, however, reduces the inhibitory potential. The smaller sized dPG₁₀ series showed a much broader range of optimum SA densities between 0.2-0.9 SA/nm² (Fig. S11). The stronger reduction in inhibitory activity with increasing DF of the dPG₅₀₀ **3** scaffold series could be related to steric repulsion, which comes more into effect at larger PG-virus interfaces in comparison to that of the smaller sized dPG₁₀ **2** based scaffolds. Generally, no inhibitory effect could be detected for polymers with a DF lower than 0.15 even if high amounts were applied (2 mM SA) in the HAI assay (Fig. 2a and Fig. S11a). We can exclude any reduction of DF due to enzymatic cleavage of SA monomers from polymers by the viral neuraminidase as the thiol-ene linker attaching SA to the scaffolds is hydrolytically stable.

In the series of linear PG sialosides, LPG₁₀SA_{0.40} **1b** and LPG₁₀SA_{0.70} **1c** were the most effective inhibitors with inhibition constants of 328 nM and 59 nM, respectively (Table 2). The optimum DF was shifted to higher values ranging between 0.40-0.80 (Fig. 2a) as compared to the dPG₅₀₀ **3** based series. This may be due to the coiled conformation of the polymer chain (Supporting Information) that reduces the number of SA residues available for binding to the virus. However, assuming a globular shaped linear PG (Fig. S11b), we were able to deduce an optimal ligand density range between 0.30-0.70 SA/nm². In conclusion, our results clearly indicate that geometry, size, and SA density of the scaffolds are determinants of the inhibition potential.

To illustrate the multivalency effect of inhibitors, which is expressed by the enhancement factor β , KiHAI_{SA} values of the scaffolds were compared with the monovalent α -methylsialoside reported earlier and the experiments with α -2,6-sialyllactose [8, 23] (Table 2 and Table S7). The best binding inhibitors LPG₁₀SA_{0.40} **1b**, LPG₁₀SA_{0.70} **1c**, dPG₁₀SA_{0.15} **2b**, and dPG₅₀₀SA_{0.20} **3b** demonstrated up to 15,000-fold enhanced activity compared to α -2,6-sialyllactose and at least 300-fold enhanced activity compared to the reported hemagglutination inhibition value of α -methylsialoside (Table 2) [23]. All compounds were tested in the single-cell binding inhibition assay by flow cytometry at higher viral target concentrations ($\approx$ 16 HAU/ μ l) to allow for a comparison with our previous studies [29]. (Fig. 2b, Table 2, Table S7).

In comparison to the dendritic and linear 10 kDa scaffolds, we noticed a much higher inhibition potential for the bigger dPG₅₀₀SA_{0.20} **3b**. In line with the results of the HAI assay, dPG₅₀₀SA_{0.20} **3b** demonstrated the best performance in virus-cell binding inhibition, with a half maximal inhibitory concentration (IC₅₀) value of 0.82 μ M among all the dPG₅₀₀ **3**-based variants. According to the steric shielding model of multivalent inhibitors [24], we ascribe this is due to the larger nanoparticle diameter. Interestingly, the optimal inhibitors of the LPGSA series, i.e. LPG₁₀SA_{0.40} **1b** and LPG₁₀SA_{0.70} **1c**, which exhibited IC₅₀ values of 2-4 μ M, are almost similar in size as dPG₅₀₀SA **3b** (Table 1). Whereas the smaller dPG₁₀SA_{0.15} **2b** showed an IC₅₀ value around $\sim$ 59 μ M, which supports the relevance of steric shielding of larger scaffolds, i.e., **1b/c**, **3b** (Table 1)

Table 1. Characterization of LPGSAs (**1a-1e**) and dPGSAs (**2a-2e** and **3a-3d**).

Compound ^a (PG _{MW} SA _{DF})	SA/ Polymer ^b	DF ^c (%)	D_h^d (nm)	PDI ^d	SA/nm ² ^e
LPG ₁₀ 1	-	0	6.95±0.16	0.49±0.04	-
LPG ₁₀ SA _{0.10} 1a	14	10	9.81±2.13	0.45±0.06	0.09
LPG ₁₀ SA _{0.40} 1b	60	44	10.29±1.10	0.59±0.01	0.39
LPG ₁₀ SA _{0.70} 1c	99	73	10.24±1.05	0.32±0.04	0.65
LPG ₁₀ SA _{1.00} 1d	135	100	11.26±0.77	0.42±0.07	0.89
LPG ₁₀ SA _{0.2} 1e	27	20	7.17±0.35	0.68±0.01	0.17
dPG ₁₀ 2	-	0	5.99±0.37	0.37±0.02	-
dPG ₁₀ SA _{0.05} 2a	8	5	6.38±0.23	0.31±0.02	0.07
dPG ₁₀ SA _{0.15} 2b	26	15	7.52±0.17	0.63±0.01	0.23
dPG ₁₀ SA _{0.30} 2c	51	31	7.74±0.58	0.47±0.04	0.45
dPG ₁₀ SA _{0.65} 2d	106	64	9.17±0.58	0.54±0.06	0.94
dPG ₁₀ SA _{0.90} 2e	121	90	9.27±0.41	0.59±0.16	1.07
dPG ₅₀₀ 3	-	0	12.46±0.15	0.05±0.01	-
dPG ₅₀₀ SA _{0.05} 3a	358	5	11.76±0.40	0.15±0.01	0.73
dPG ₅₀₀ SA _{0.20} 3b	1235	19	14.20±0.27	0.07±0.01	2.53
dPG ₅₀₀ SA _{0.40} 3c	2470	38	14.69±0.26	0.12±0.01	5.06
dPG ₅₀₀ SA _{0.70} 3d	4550	70	15.77±1.54	0.50±0.01	9.32

[a] Postfunctionalized M_n calculated by using the M_n of the polyglycerol core and the experimental degree of functionalization (DF) (see Supporting Information for details). [b] The number of sialic acid (SA) units per polymer was calculated from DF by ¹HNMR. [c] Determined by ¹HNMR analysis. [d] Determined from DLS in aqueous buffer (PBS, pH 7.4), the values represent the means of at least three measurements ± standard deviation of the volume distribution profile. [e] The average SA densities on the different scaffolds were calculated assuming the spherical morphologies of PGs, thus dividing the number of SA units with the surface area of the corresponding backbone [LPG₁₀ (A_{nm}^2) = 151, dPG₁₀ (A_{nm}^2) = 113, dPG₅₀₀ (A_{nm}^2) = 488].

Fig. 2. Virus binding and inhibition assays: a) Hemagglutination inhibition constants per nanoparticle concentration ($KiHAI_{NP}$) of LPG₁₀ **1**, dPG₁₀ **2**, and dPG₅₀₀ **3** with different degree of SA functionalization were studied. Individual inhibitor constants of the most effective inhibitors are summarized in Table 2. The entire list of inhibition values of all inhibitors are shown in Table S7. b) Binding inhibition of R18 labelled A/X31 virus to human erythrocytes with the most potent inhibitors. Mean values ($n \geq 3$) were fitted with a four parametric logistic fit to obtain IC_{50} values (Table 2). Error bars: SEM. See Fig. S10 and Table S7 for the IC_{50} values of all inhibitors. c, d) Microscale thermophoresis: Change in fluorescence ΔF_{norm} upon binding of LPGSAs (c) and dPGSAs (d) at different concentrations to fluorescently labelled virus at the steady state. Non-functionalized particles dPG₁₀ **2** and LPG₁₀ **1** served as a control. Binding constants are described as apparent dissociation constants ($Kdapp_{NP}$) assuming a 1:1 binding stoichiometry for viruses and scaffolds. For biphasic dependencies

(see d) each phase was fitted separately. Determined values are summarized in Table 2. Error bars refer: SEM ($n \geq 3$).

Determination of binding affinity of polyglycerol sialosides to intact virus

To assess affinity constants we conducted microscale thermophoresis (MST) measurements of the most potent multivalent compounds, i.e. $\text{LPG}_{10}\text{SA}_{0.40}$ **1b**, $\text{LPG}_{10}\text{SA}_{0.70}$ **1c**, $\text{dPG}_{10}\text{SA}_{0.15}$ **2b**, and $\text{dPG}_{500}\text{SA}_{0.20}$ **3b** titrated against free fluorescent labelled virus (4 HAU). (Fig. 2c, d).

For LPGSAs, K_{dappNP} was 10 μM and 0.6 μM for $\text{LPG}_{10}\text{SA}_{0.40}$ **1b** and $\text{LPG}_{10}\text{SA}_{0.70}$ **1c**, respectively. In contrast to LPGSAs, both $\text{dPG}_{10}\text{SA}_{0.15}$ **2b** and $\text{dPG}_{500}\text{SA}_{0.20}$ **3b** bound in a biphasic manner to the virus. For $\text{dPG}_{500}\text{SA}_{0.20}$ **3b** K_{dappNP} was 12.7 nM and 1.3 μM , while for $\text{dPG}_{10}\text{SA}_{0.15}$ **2b** K_{dappNP} was 1.5 and 82.5 μM (Fig. 2c, d and Table 2). The origin of the second weaker binding site has still to be identified. Sauter et al. [36] have provided evidence for a second SA binding site on HA. The viral neuraminidase may also provide a potential target for multivalent sialosides [37].

Comparing the inhibition constant values from the HAI assay with dissociation constants from MST we noticed that the $K_{\text{IHAI}_{\text{NP}}}$ values of the dendritic dPGSAs are close to the first, i.e. the lower dissociation constant K_{dappNP} determined with MST. In contrast, $K_{\text{IHAI}_{\text{NP}}}$ values of LPGSAs are 10-30 times lower than their K_{dappNP} values. We surmise that this could be attributed to steric shielding contributions by LPGSAs that only become fully effective in the virus-cell competition. Apart from the high affinity, steric shielding is an important determinant for effective virus-cell binding inhibition. Indeed, MST showed that the linear **1c** and dendritic **2b** inhibitors exhibited affinity values in the same range but the hemagglutination inhibition constants for **1c** was 6 fold lower compared to **2b**. Thus, the affinity to the virus is similar, but the inhibition potential of the water swollen linear polymer

1c is higher due to the steric shielding effect. Such an additional steric shielding character of SA conjugated linear scaffolds was also discussed by Whitesides and coworkers, suggesting that the water swollen sugar-coated linear polymer could sterically restrict the virus from binding to the cell surface receptors [10].

Table 2 Inhibitor constants from the hemagglutination and human erythrocyte binding inhibition assay of the most potent functionalized scaffolds and monomeric sialosides. Values are expressed in terms of SA concentration or nanoparticle concentration with its SEM ($n \geq 3$). For binding inhibition experiments the SE of $\log IC_{50}$ is given. For all compounds see Table S7. For α -methylsialoside the inhibition constant $KiHAI_{SA}$ as well as the dissociation constant $Kdapp_{NP}$ is shown as reported earlier [23, 38].

Compound ($PG_{MW}SA_{DF}$)*	$KiHAI_{SA}$ [μM]	$KiHAI_{NP}$ [nM]	Binding inhibition $IC_{50 SA}$ [mM]	Binding inhibition $IC_{50 NP}$ [μM]	$Kdapp_{NP}$ [μM]
α -methylsialoside	2000	-	-	-	2800 $\pm$ 300
α -2,6-sialyllactose	100000	-	-	-	-
$LPG_{10}SA_{0.40}$ 1b	19.5 $\pm$ 5.9	328 $\pm$ 98	0.22 $\pm$ 0.03	3.74 $\pm$ 0.50	10.10 $\pm$ 2.90
$LPG_{10}SA_{0.70}$ 1c	5.9 $\pm$ 2.7	59 $\pm$ 27	0.22 $\pm$ 0.02	2.20 $\pm$ 0.20	0.60 $\pm$ 0.32
$dPG_{10}SA_{0.15}$ 2b	9.3 $\pm$ 1.7	358 $\pm$ 66	1.54 $\pm$ 0.08	59.1 $\pm$ 3.1	1.46 $\pm$ 0.65 and 82.5 $\pm$ 24.0
$dPG_{500}SA_{0.20}$ 3b	20.3 $\pm$ 4.7	16 $\pm$ 4	1.01 $\pm$ 0.05	0.82 $\pm$ 0.05	0.013 $\pm$ 0.006 and 1.35 $\pm$ 0.43

Polyglycerol sialosides interfere with infection and multicyclic virus propagation

Influenza A/X31, at a multiplicity of infection (MOI) of 0.05, was pretreated with inhibitors (most potent inhibitors and controls) for 30 minutes and used to infect MDCK-II

cells. Cell viability was determined 24 h p.i. in order to determine protection from infection at the virus entry step. While inhibitors without SA did not inhibit a virus-induced decrease in cell viability, the multivalent sialoside inhibitors showed a protective effect in a dose dependent manner with IC_{50} values in the submicro- to low nanomolar range (Fig. 3a-c, Table 3) (see Table S8 and Fig. S6 for the entire list of results). The values derived from the infection inhibition assay are essentially consistent with those from the above binding inhibition experiments. A DF at 0.15-0.20 for the two dPG₁₀ **2**- and dPG₅₀₀ **3**-based series and 0.40-0.70 for the LPG₁₀ **1** series turned out to be most efficient in protecting cells from infection. For compounds with DF lower than the above mentioned optimum of each scaffold class (Fig. 2a), inhibition was very low (Fig. 3a-c). Increasing DF far beyond the optimal range reduced the infection inhibition potencies (Fig. 3a-c). Thus, only the favored candidates from binding inhibition experiments, i.e. the selected inhibitor **1b**, **1c**, **2b**, and **3b**, were able to preserve maximum cell viability after challenge with the influenza virus. The half maximal inhibitory concentrations IC_{50NP} were in the range of 1.6 to 215 nM for these four compounds. The most potent linear low molecular weight inhibitor LPG₁₀SA_{0.40} **1b** protected >80% MDCK-II cells from initial infection. Importantly, several significantly active compounds in different series did not affect MDCK-II cells viability at inhibitor concentrations up to 500 μ M SA equivalents for 24 h. The cell viability data of the most potent candidates is given in the Supporting Information (Fig. S5).

In a complementary approach, we tested the potential to inhibit infection by monitoring expression of virus nucleoprotein in infected cells 24 h p.i. by immunofluorescence microscopy (Fig. S7). We selected those candidates from each series that combined the lowest determined IC_{50} value with the lowest molecular weight that is LPG₁₀SA_{0.40} **1b**, dPG₁₀SA_{0.15} **2b**, and dPG₅₀₀SA_{0.20} **3b**, respectively. Again, A/X31 was preincubated with different concentrations of inhibitors from 10 -300 μ M SA, for 30 min

1 before infection of MDCK-II cells at MOI 0.1. Whereas upto 80% of cells exposed to PBS or
2 control inhibitor (unfunctionalized scaffold) treated virus were infected, the linear
3 polyglycerol $\text{LPG}_{10}\text{SA}_{0.40}$ **1b** was able to significantly reduce infection. The $\text{LPG}_{10}\text{SA}_{0.40}$ **1b**
4 was found to be significantly better than $\text{dPG}_{10}\text{SA}_{0.15}$ **2b** at the concentration of 30 μM SA
5 and above, and throughout all tested concentrations better than $\text{dPG}_{500}\text{SA}_{0.20}$ **3b** (Fig. 4a).

Fig. 3. Inhibition of infection of MDCK II cells by influenza virus A/X31 (MOI 0.05) pretreated with sialosides based on a) LPG_{10} b) dPG_{10} and c) dPG_{500} scaffolds. Inhibition of infection was measured 24 h p.i. Influenza virus induced cytopathicity was monitored by a

- 1 CellTiter Aqueous One Solution (Promega) based viability assay. IC₅₀ values were obtained
 2 by fitting plots by a four-parametric sigmoidal function (Table S8). Error bars: SEM (n≥3).

Fig. 4. Inhibition of virus infection: a) Inhibition by 10 to 300 μ M SA of the most potent inhibitors 24 h p.i. with A/X31 virus at MOI 0.1. Non-sialylated control inhibitors were used at 300 μ M nanoparticle concentrations (indicated by striped columns). A/X31 was pretreated with the respective inhibitor for 30 min, then added to cells for 1 h, followed by a washing step to remove unbound virus. Viral nucleoprotein expression was imaged by immunostaining against viral nucleoprotein (Fig. S7). Nuclei were stained with DAPI. Fraction of cells expressing viral nucleoprotein is shown. No inhibition was found for the

non-functionalized scaffold LPG₁₀ **1**. Error bars: SEM (n≥3). b) MDCK II cells were infected for 1 h with A/X31 (H3N2), A/Mallard/439/2004 (H3N2) or A/turkey/Italy/472/1999 (H7N1) virus at an MOI 0.01. Subsequently, cells were washed and incubated in the infection medium supplemented with control substance or inhibitor at indicated concentrations (corresponding to 10 μM SA equivalents for LPG₁₀SA_{0.40} **1b** and dPG₁₀SA_{0.15} **2b**), respectively. 24 h p.i. the viral titer was determined by a plaque assay. Error bars: SEM (n≥3). Data were statistically analyzed using a two-tailed Mann-Whitney t-test (*p<0.05, **p<0.01, *** p<0.001, **** p<0.0001).

To test whether the most potent candidates LPG₁₀SA_{0.40} **1b** and dPG₁₀SA_{0.15} **2b** can inhibit propagation of A/X31 (H3N2) virus in a multicyclic setting, we infected MDCK-II cells at MOI 0.01 for 45 min. In order to test for broad antiviral activity, we probed LPG₁₀SA_{0.40} **1b** and dPG₁₀SA_{0.15} **2b** against the two avian influenza strains A/Mallard/439/2004 (H3N2) or A/turkey/Italy/472/1999 (H7N1), too. Nonbound virus was washed off before the cells were overlaid with LPG₁₀SA_{0.40} **1b** and dPG₁₀SA_{0.15} **2b** diluted in the infection medium for 24 h. Subsequently, the virus titer was determined by plaque titration. LPG₁₀SA_{0.40} **1b** and dPG₁₀SA_{0.15} **2b** reduced the virus titer of X31, by 4 and 3 orders of magnitude (log scale), respectively, at an inhibitor concentration of 10 μM SA equivalents demonstrating a potent antiviral activity that inhibit also virus propagation. Further, MDCK-II cells infected with the two avian influenza strains A/Mallard/439/2004 (H3N2) and A/turkey/Italy/472/1999 (H7N1) showed a significantly reduced viral titer by 4 or 1 log scales, respectively, in the presence of the inhibitor LPG₁₀SA_{0.40} **1b** and only 1 log scale in the presence of dPG₁₀SA_{0.15} **2b**. The LPG₁₀SA_{0.40} **1b** was found to be significantly better than the dPG₁₀SA_{0.15} **2b** against A/X31 (H3N2) and A/Mallard/439/2004 (H3N2) strains. The control inhibitors LPG₁₀ **1** and dPG₁₀ **2** did not substantially inhibit the virus titer (Fig. 4b).

Table 3 Mean values ($n \geq 3$) of the half maximal inhibitory concentration (IC_{50}) of selected inhibitors after infection of MDCK-II cells at an MOI of 0.05 for 24 h. For infection inhibition experiments the SE of $\log IC_{50}$ is given. For a list of all compounds see Table S8.

Compound (PG _{MW} SA _{DF})	Infection inhibition $IC_{50\ SA} [\mu M]$	Infection inhibition $IC_{50\ NP} [nM]$
LPG ₁₀ 1	-	500000.00
LPG ₁₀ SA _{0.40} 1b	0.14+0.05	2.35+0.83
LPG ₁₀ SA _{0.70} 1c	0.37+0.09	3.69+0.91
dPG ₁₀ SA _{0.15} 2b	5.59+0.18	215.15+6.92
dPG ₅₀₀ SA _{0.20} 3b	1.98+0.08	1.60+0.06

Antiviral activity of polyglycerol sialosides in mice

Influenza viruses infect the respiratory tract of mammals. To investigate the antiviral activity in *in vivo*, we tested the selected PG sialosides **1b**, **2b**, and **3b** in a murine model of influenza (Fig. 5). 8-10 weeks old BALB/c mice were infected by intranasal application of inhibitor or control treated A/X31 (100 pfu) and daily controlled in body weight. Mice infected with A/X31 lost up to 10 % body weight 4 days post infection, while uninfected mice maintained their body weight in an 11 days experiment (Fig. S8). According to the time course, the body weights were compared on day 4, being the infection peak, for several inhibitor treatments (Fig. 5a). Remarkably, treatments with the most potent inhibitors within each PG series caused a significant lower body weight loss in comparison to non-infected animals, reflecting potent protection from infection. LPG₁₀SA_{0.40} **1b** was outstanding among the tested sialosides, causing an almost complete block of infection by A/X31 in comparison to the non-infected control (Fig. S8). Also along the 11 days experiment, LPG₁₀SA_{0.40} **1b** treated virus at

5 mM or 1 mM SA equivalents ($m_{NP} = 3.3$ or 0.7 mg/kg, $m_{SA} = 1.9$ or 0.4 mg/kg, $n_{SA} = 6.25$ or 1.25 μ mol/kg), which corresponds to 83 μ M or 17 μ M tailored sialic acid, both showed on several days post infection a significantly smaller loss in body weight (Fig. 5b) compared to the infected groups treated with PBS only. The non-functionalized compound LPG₁₀ **1** at equal molar concentrations did not cause any significant inhibitory effect (Fig. 5b).

To further evaluate the anti-infective efficacy of LPG₁₀SA_{0.40} **1b**, we compared its performance to the approved antiviral medication oseltamivir carboxylate (OC), which is equivalent to the active form of the compound generated by digestive hydrolysis of the ethylester. This drug is designed to block viral neuraminidase, which is responsible for virus release from the host cell. Using OC at equimolar concentrations as LPG₁₀SA_{0.40} **1b** for preincubation with virus inoculate, we observed a much weaker protective effect from the infection (Fig. 5a and c). Mice treated with 83 μ M (32.8 μ g/kg) OC still encountered 8% loss in body weight on day 4. Although this concentration is typically used in mice experiments [39], we probed also a 10-fold higher concentration (Fig. S8). Here, although protection against infection was also present at day 3, the inhibitory effect faded at day 4 to 5 as shown by a substantial loss in body weight. Mice infected with virus pretreated with both inhibitors, i.e., OC phosphate and LPG₁₀SA_{0.40} **1b**, retained their body weight just as well as uninfected mice (Fig. S8) and thus the combination therapy boosted the infection inhibition (Fig. 5a and c). Similar synergistic effects were observed earlier *in vitro* by the Whitesides' group who reported that the efficacy of SA-conjugated polyacrylamide increased the inhibition of hemagglutination in the presence of monomeric neuraminidase inhibitors [37].

The potential to prevent infection of mice was also assessed for the low molecular weight dPG₁₀SA_{0.15} **2b** and high molecular weight dPG₅₀₀SA_{0.20} **3b** at 5 mM SA concentrations (Fig. 5d and Fig. S8). Although we observed a significantly reduced body weight loss on day 3 and 4 for dPG₁₀SA_{0.15} **2b** and on day 4 for dPG₅₀₀SA_{0.20} with respect to

the untreated, infected animals, the preventive effect in the mice treated with the dendritic inhibitors were still lower compared to those treated with linear inhibitor **1b**. While the polysialic acid derivatives of LPG₁₀ and dPG₅₀₀ performed similarly well *in vitro*, the latter despite its higher valency and similar size shows a significantly lower activity *in vivo*. Interactions with the respiratory epithelium, such as cellular uptake, or physicochemical parameters (e.g. diffusion in the mucus layer) are known factors that modify the performance of these inhibitors *in vivo*. For example, published studies have proven that soft nanoparticles do have longer lifetimes *in vivo* and are taken up by endocytic pathways to a significantly lower extent than the more rigid ones.[40, 41] Furthermore, a detailed *in vivo* investigation by Ul-haq et. al has revealed differences in the circulations, biodistribution, and clearance profile of LPG₁₀₀ and dPG₁₀₀ which might be attributed to the differences in their deformability and topology.[20]

Fig. 5. Inhibition of influenza A/X31 virus infection of BALB/c mice by SA functionalized LPG and dPG. Virus was pre-mixed with inhibitor, and the mixture applied intranasally to the sedated mice. a) Loss of mice body weight 4 days post infection, reflecting the peak of infection caused body weight loss (Fig. 5b-d and Fig. S8). For comparison, influenza virus was pretreated for 30 min at rt, either with PBS, 104 nmol OC/kg, LPG₁₀SA_{0.40} **1b** at 6.25 μmol SA/kg, the combination of the latter two compounds (Fig. 5c), dPG₁₀SA_{0.15} **2b** at 6.25 μmol SA/kg or dPG₅₀₀SA_{0.20} **3b** at 6.25 μmol SA/kg, and then applied intranasally to the mice. Error bars: SEM (n≥10). b) Infection related loss in body weight was daily controlled for 11 days. LPG₁₀SA_{0.40} **1b** at 6.25 μmol SA/kg and 1.25 μmol SA/kg concentrations and the control inhibitor LPG₁₀ **1** (104 nmol /kg) were compared to an infected, PBS treated group. Error bars: SEM (n≥10). c) Comparison of LPG₁₀SA_{0.40} **1b** to its combination with equimolar nanoparticle concentrations of the activated neuraminidase inhibitor oseltamivir carboxylate (OC). Treatment with OC (104 nmol OC/kg) (light blue) served as a comparison. Even the difference between LPG₁₀SA_{0.40} **1b** and LPG₁₀SA_{0.40} **1b** + OC was statistically significant (two-tailed student's t-test). Error bars: SEM (n≥10). d) dPG₁₀SA_{0.15} **2b** at 6.25 μmol SA/kg concentrations and the control inhibitor dPG₁₀ **2** were compared to an infected, PBS-treated group. Error bars: SEM (n≥5). The data of Fig. 5a, 5b, and 5d were subjected to statistical analysis by using a one-way ANOVA, and a Dunnett's multiple comparison test of all the inhibitor-treated groups to the infected, PBS treated group (*p<0.05, **p<0.01, ***p<0.001). All animal experiments were repeated in parallel, but presented in separate figures for clear comprehensibility.

Model for binding of LPGSA and dPGSA with the influenza virus

To rationalize the relevance of the degree of functionalization (*DF*), the geometry and size of the PGSA for the potential to inhibit virus-cell binding, we outline here the main

assumptions and results of a simple model, based on the statistical mechanics of multivalent binding applied to our specific system (for details see Supporting Information). The typical hemagglutination inhibition that is due to multivalent constructs is proportional to the construct dissociation constant, which in turn is proportional to the exponential of the binding free-energy ΔG_{bind} . Naively, one could think that a larger DF would always correspond to a lower ΔG_{bind} since more binding configurations can form, but this is not always the case. For the described systems, the distance between possible binding sites and the length of the ligands makes it impossible for a ligand to be within the binding distance of two different receptors at the same time. In this case, binding occurs always with an "indifferent topology,"[42] and the number of binding configurations W is just equal to the number of ligands N within binding distance from the viral receptor. In this case, ΔG_{bind} only decreases logarithmically with N , and hence with DF , since the two are directly proportional to each other. However, the steric repulsion between a construct and the binding surface [43] grows linearly with N , since each of the unbound ligands will feel some repulsion from the surface due to excluded volume effects. Based on these restrictions and assumptions, the full calculation of ΔG_{bind} is given by:

$$\begin{aligned} \frac{\Delta G_{\text{bind}}}{k_B T} &= N \frac{\Delta G_{\text{steric}}}{k_B T} - \log N + \frac{\Delta G_{\text{bond}}}{k_B T} \\ &= N \frac{\Delta G_{\text{steric}}}{k_B T} - \Delta S_{\text{avidity}}/k_B + \frac{\Delta G_{\text{bond}}}{k_B T}, \end{aligned} \quad (1)$$

where k_B is Boltzmann's constant and T the temperature. ΔG_{steric} measures the repulsive free-energy due to steric interactions between a single ligand and the virus surface when the multivalent construct is at binding distance from the surface. ΔG_{bond} is the energy of formation of a single bond. $\Delta S_{\text{avidity}} > 0$ is the further attractive contribution that arises in the multivalent binding coming from the various achievable distinct possible binding configurations [42]. Given Eq. 1, G_{bind} has a minimum at a particular value of N (and hence

1 DF). Given $N_{\text{opt}} = 1 / (\Delta G_{\text{steric}} / k_B T)$, a minimum is also observed in the experimental data of
 2 Figure 2a.

3 Two further experimental observations can be explained when calculating the
 4 repulsive contribution ΔG_{steric} by modeling the ligands as rigid rods (for details see
 5 Supporting Information). Meanwhile the leading term in ΔG_{steric} does not depend on the
 6 nanoparticle curvature, according to the experimental data, which shows very close values for
 7 the optimal DF for both large and small nanoparticles. Furthermore, within the same
 8 description, $\Delta G_{\text{steric}} / k_B T$ is more negative for a ligand grafted onto a linear polymer compared
 9 to grafting it to an impenetrable surface of a nanoparticle. This also provides a further reason,
 10 besides the ligands' inaccessibility in a coiled structure, why the minimum for the LPGSA
 11 shifts to higher DF values. Whereas the exact value of N_{opt} depends on the specific model
 12 used to describe the ligand, and an accurate calculation might require detailed molecular
 13 simulations, the possible presence of a minimum of K_i (Fig. 2a) arises from a very general
 14 mechanism of competition between binding entropy and steric repulsion and is thus expected
 15 to be a general aspect of our system. This simple statistical model rationalizes the relevance
 16 of DF , as well as the morphology and the size of PGSA for the potential to inhibit virus-cell
 17 binding (for details see Supporting Information). In particular, it explains (i) the non-
 18 monotonic behavior and the presence of the experimentally observed optimum of DF , and
 19 why that optimum is (ii) similar for large and small nanoparticles, (iii) but shifted to higher
 20 values for linear polymers.

21 Based on our finding that the scaffold geometry affects multivalent interaction, we
 22 here provide a hypothesis on the scaffold topology of the virus bound SA polyglycerols. By
 23 means of the determined diameters of the compounds and on the basis of the spatial
 24 distribution of HA trimers on the virus surface, an estimation of the possible number of
 25 multivalent interactions was made. It could be shown by X-ray crystallographic studies of the

1 HA ectodomain complexed with SA that the SA binding sites were located in shallow
 2 pockets on the globular head domain of HA trimmers [44]. However, the three binding sites
 3 were not orientated in the most distal direction but instead radially outwards. Although both
 4 intra-trimeric and inter-trimeric binding of inhibitors were conceivable due to this spatial
 5 arrangement of the binding sites, inter-trimeric interactions were more likely. The situation is
 6 fitted to the scale to illustrate that the LPG₁₀SA and the dPG₅₀₀SA which are similar in size,
 7 caught up to three binding sites on different trimers (Fig. 6a and 6c), whereas the smaller
 8 dP₁₀GSA series could reach only two of them (Fig. 6b). The change in shape of PGSAs upon
 9 binding with the virus particles will demand a high entropic cost and might be unlikely in this
 10 particular case.

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 12
 13 **Fig. 6.** Multivalent interactions of three different polyglycerol sialosides with trimeric HA on
 14 the virus surface. HA trimers (cyan) are depicted at around 10-12 nm apart from each other as
 15 usually found on the virus surface. Yellow patches on the trimers represent the three binding
 16 sites on the globular head domain. Optimal compounds, i.e., LPG₁₀SA_{0.4} **1b**, dPG₁₀SA_{0.15} **2b**,
 17 and dPG₅₀₀SA_{0.2} **3b**, are shown by compact coiled structure and grey balls respectively. The
 18 size of the grey balls and the compact coiled structure representing dPGs and LPG are as
 19 observed by cryo-TEM. A red mesh surrounding the inhibitors represents the hydrodynamic
 20 shell around the PG scaffold formed by SA. The images **a**, **b**, and **c** show the top view. The

cartoon illustrates the possible number of multivalent interactions of the different sized inhibitors with binding sites on the HA trimers on the virus surface.

CONCLUSIONS

We have shown that the nontoxic low-molecular weight polyglycerol scaffolds decorated with sialic acid as the virus receptor-mimicking ligand may act as efficient multivalent inhibitors of influenza virus activity *in vitro* as well as *in vivo*. We compared two different general geometries, linear and dendritic polyglycerol sialosides, which differ in both molecular weight and the multiplicity of SA units. We have optimized the ligand densities for the linear and dendritic PGs to achieve a high inhibitory potency against influenza virus. This study clearly reveals that high ligand densities on the multivalent architectures are not necessary for effective influenza virus inhibition. By a rational and systematic approach, we have demonstrated that the linear sialic acid-functionalized PGs are more potent multivalent inhibitors of influenza A virus infection than the dendritic PG scaffolds *in vitro* and *in vivo*. Our results indicate that at a comparable binding affinity to the virus, the higher inhibitory potential of linear compounds to prevent virus-cell binding is related to steric shielding of the virus. Comparison of different multivalent scaffolds in a mouse infection model proved that linear sialylated PGs were able to fully prevent from infection at prophylactic, single dose administration. This result stresses the need for *in vivo* experiments as an important step to evaluate multivalent inhibitors. $\text{LPG}_{10}\text{SA}_{0.40}$ **1b**, the most potent influenza inhibitor *in vivo* is small enough (~30 kDa) to pass the kidney threshold for clearance.

Most importantly, functionalized scaffolds did not cause any acute toxicity to mice as judged by the absence of clinical symptoms in the 11-days setup. Combined application of the neuraminidase inhibitor oseltamivir carboxylate (OC) boosted the inhibition, while OC alone did not show beneficial effects in this setting. The combined usage of both compounds is a

promising therapeutic approach that combines multivalent nanomaterials with conventional antinfluenza drugs.

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Supporting Information

Synthetic procedures, characterization data, ¹HNMR spectra, additional figures from DLS, Cryo-TEM, cell viability, FACS, MST, infection assay, and *in vivo*, and details on statistical mechanical model are available online.

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Scheme 1

ACCEPTED MANUSCRIPT

Fig. 1.

Fig. 2.

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1 **Fig. 4.**

Fig. 5.

Fig. 6.

Figure captions

Scheme 1. Synthesis of linear and dendritic polyglycerol sialosides of different size and degrees of functionalization. SA residues are randomly distributed on the polyglycerol backbone. See Supporting Information for details for synthetic details.

Fig. 1. Size analysis of the linear and dendritic polyglycerol based inhibitors by DLS and cryo-TEM. The volume distribution profiles of a) linear polyglycerol (LPG₁₀ **1**) and linear polyglycerol sialosides (**1a-e**); b) dendritic polyglycerol (dPG₁₀ **2**) and dendritic polyglycerol sialosides (**2a-e**); c) dendritic polyglycerol (dPG₅₀₀ **3**) and dendritic polyglycerol sialosides (**3a-d**) as observed by DLS at concentration of 1 mg/ml in aqueous PBS (pH 7.4, 150 mM NaCl) at 25 °C. See Figure S1-S3 for intensity and number distribution profiles of all compounds. Percentage-based frequency distribution of the diameter of selected compounds determined from cryo-electron micrographs (compared with Figure S4), Feret diameter was determined using the public domain software “ImageJ”, histogram bin size is always 0.5 nm. (d) LPGSA **1e**, (n=420) (e) dPGSA **2b** (n=620) (f) dPGSA **3b**, (n=520).

Fig. 2. Virus binding and inhibition assays: a) Hemagglutination inhibition constants per nanoparticle concentration ($K_{\text{HAI}_{\text{NP}}}$) of LPG₁₀ **1**, dPG₁₀ **2**, and dPG₅₀₀ **3** with different degree of sialic acid functionalization were studied. Individual inhibitor constants of the most effective inhibitors are summarized in Table 2. The entire list of inhibition values of all inhibitors are shown in Table S7. b) Binding inhibition of R18 labelled A/X31 virus to human erythrocytes with the most potent inhibitors. Mean values (n≥3) were fitted with a four parametric logistic fit to obtain IC_{50} values (Table 2). Error bars: SEM. See Fig. S10 and Table S7 for the IC_{50} values of all inhibitors. c, d) Microscale thermophoresis: Change in

fluorescence ΔF_{norm} upon binding of LPGSAs (c) and dPGSAs (d) at different concentrations to fluorescently labelled virus at the steady state. Non-functionalized particles dPG₁₀ **2** and LPG₁₀ **1** served as a control. Binding constants are described as apparent dissociation constants (K_{dappNP}) assuming a 1:1 binding stoichiometry for viruses and scaffolds. For biphasic dependencies (see d) each phase was fitted separately. Determined values are summarized in Table 2. Error bars refer: SEM ($n \geq 3$).

Fig. 3. Inhibition of infection of MDCK II cells by influenza virus A/X31 (MOI 0.05) pretreated with sialosides based on a) LPG₁₀ b) dPG₁₀ and c) dPG₅₀₀ scaffolds. Inhibition of infection was measured 24 h p.i. Influenza virus induced cytopathicity was monitored by a CellTiter Aqueous One Solution (Promega) based viability assay. IC₅₀ values were obtained by fitting plots by a four-parametric sigmoidal function (Table S8). Error bars: SEM ($n \geq 3$).

Fig. 4. Inhibition of virus infection: a) Inhibition by 10 to 300 μM SA of the most potent inhibitors 24 h p.i. with A/X31 virus at MOI 0.1. Non-sialylated control inhibitors were used at 300 μM nanoparticle concentrations (indicated by striped columns). A/X31 was pretreated with the respective inhibitor for 30 min, then added to cells for 1 h, followed by a washing step to remove unbound virus. Viral nucleoprotein expression was imaged by immunostaining against viral nucleoprotein (Fig. S7). Nuclei were stained with DAPI. Fraction of cells expressing viral nucleoprotein is shown. No inhibition was found for the non-functionalized scaffold LPG₁₀ **1**. Error bars: SEM ($n \geq 3$). b) MDCK II cells were infected for 1 h with A/X31 (H3N2), A/Mallard/439/2004 (H3N2) or A/turkey/Italy/472/1999 (H7N1) virus at an MOI 0.01. Subsequently, cells were washed and incubated in the infection medium supplemented with control substance or inhibitor at indicated concentrations (corresponding to 10 μM SA equivalents for LPG₁₀SA_{0.40} **1b** and dPG₁₀SA_{0.15} **2b**),

respectively. 24 h p.i. the viral titer was determined by a plaque assay. Error bars: SEM ($n \geq 3$). Data were statistically analyzed using a two-tailed Mann-Whitney t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Fig. 5. Inhibition of influenza A/X31 virus infection of BALB/c mice by SA functionalized LPG and dPG. Virus was pre-mixed with inhibitor, and the mixture applied intranasally to the sedated mice. a) Loss of mice body weight 4 days post infection, reflecting the peak of infection caused body weight loss (Fig. 5b-d and Fig. S8). For comparison, influenza virus was pretreated for 30 min at rt, either with PBS, 104 nmol OC/kg, LPG₁₀SA_{0.40} **1b** at 6.25 μ mol SA/kg, the combination of the latter two compounds (Fig. 5c), dPG₁₀SA_{0.15} **2b** at 6.25 μ mol SA/kg or dPG₅₀₀SA_{0.20} **3b** at 6.25 μ mol SA/kg, and then applied intranasally to the mice. Error bars: SEM ($n \geq 10$). b) Infection related loss in body weight was daily controlled for 11 days. LPG₁₀SA_{0.40} **1b** at 6.25 μ mol SA/kg and 1.25 μ mol SA/kg concentrations and the control inhibitor LPG₁₀ **1** (104 nmol /kg) were compared to an infected, PBS treated group. Error bars: SEM ($n \geq 10$). c) Comparison of LPG₁₀SA_{0.40} **1b** to its combination with equimolar nanoparticle concentrations of the activated neuraminidase inhibitor oseltamivir carboxylate (OC). Treatment with OC (104 nmol OC/kg) (light blue) served as a comparison. Even the difference between LPG₁₀SA_{0.40} **1b** and LPG₁₀SA_{0.40} **1b** + OC was statistically significant (two-tailed student's t-test). Error bars: SEM ($n \geq 10$). d) dPG₁₀SA_{0.15} **2b** at 6.25 μ mol SA/kg concentrations and the control inhibitor dPG₁₀ **2** were compared to an infected, PBS-treated group. Error bars: SEM ($n \geq 5$). The data of Fig. 5a, 5b, and 5d were subjected to statistical analysis by using a one-way ANOVA, and a Dunnett's multiple comparison test of all the inhibitor-treated groups to the infected, PBS treated group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). All animal experiments were repeated in parallel, but presented in separate figures for clear comprehensibility.

Fig. 6. Multivalent interactions of three different polyglycerol sialosides with trimeric hemagglutinin on the virus surface. Hemagglutinin (HA) trimers (cyan) are depicted at around 10-12 nm apart from each other as usually found on the virus surface. Yellow patches on the trimers represent the three binding sites on the globular head domain. Optimal compounds, i.e., $\text{LPG}_{10}\text{SA}_{0.4}$ **1b**, $\text{dPG}_{10}\text{SA}_{0.15}$ **2b**, and $\text{dPG}_{500}\text{SA}_{0.2}$ **3b**, are shown by compact coiled structure and grey balls respectively. The size of the grey balls and the compact coiled structure representing dPGs and LPG are as observed by cryo-TEM. A red mesh surrounding the inhibitors represents the hydrodynamic shell around the PG scaffold formed by SA. The images **a**, **b**, and **c** show the top view. The cartoon illustrates the possible number of multivalent interactions of the different sized inhibitors with binding sites on the HA trimers on the virus surface.

Table 1. Characterization of LPGSAs (**1a-1e**) and dPGSAs (**2a-2e** and **3a-3d**).

Compound ^a (PG _{MW} SA _{DF})	SA/ Polymer ^b	DF ^c (%)	D_h^d (nm)	PDI ^d	SA/nm ² ^e
LPG ₁₀ 1	-	0	6.95±0.16	0.49±0.04	-
LPG ₁₀ SA _{0.10} 1a	14	10	9.81±2.13	0.45±0.06	0.09
LPG ₁₀ SA _{0.40} 1b	60	44	10.29±1.10	0.59±0.01	0.39
LPG ₁₀ SA _{0.70} 1c	99	73	10.24±1.05	0.32±0.04	0.65
LPG ₁₀ SA _{1.00} 1d	135	100	11.26±0.77	0.42±0.07	0.89
LPG ₁₀ SA _{0.2} 1e	27	20	7.17±0.35	0.68±0.01	0.17
dPG ₁₀ 2	-	0	5.99±0.37	0.37±0.02	-
dPG ₁₀ SA _{0.05} 2a	8	5	6.38±0.23	0.31±0.02	0.07
dPG ₁₀ SA _{0.15} 2b	26	15	7.52±0.17	0.63±0.01	0.23
dPG ₁₀ SA _{0.30} 2c	51	31	7.74±0.58	0.47±0.04	0.45
dPG ₁₀ SA _{0.65} 2d	106	64	9.17±0.58	0.54±0.06	0.94
dPG ₁₀ SA _{0.90} 2e	121	90	9.27±0.41	0.59±0.16	1.07
dPG ₅₀₀ 3	-	0	12.46±0.15	0.05±0.01	-
dPG ₅₀₀ SA _{0.05} 3a	358	5	11.76±0.40	0.15±0.01	0.73
dPG ₅₀₀ SA _{0.20} 3b	1235	19	14.20±0.27	0.07±0.01	2.53
dPG ₅₀₀ SA _{0.40} 3c	2470	38	14.69±0.26	0.12±0.01	5.06
dPG ₅₀₀ SA _{0.70} 3d	4550	70	15.77±1.54	0.50±0.01	9.32

[a] Postfunctionalized M_n calculated by using the M_n of the polyglycerol core and the experimental degree of functionalization (DF) (see Supporting Information for details). [b] The number of sialic acid (SA) units per polymer was calculated from DF by ¹HNMR. [c] Determined by ¹HNMR analysis. [d] Determined from DLS in aqueous buffer (PBS, pH 7.4), the values represent the means of at least three measurements ± standard deviation of the volume distribution profile. [e] The average SA densities on the different scaffolds were calculated assuming the spherical morphologies of PGs, thus dividing the number of SA units with the surface area of the corresponding backbone [LPG₁₀ (A_{nm}^2) = 151, dPG₁₀ (A_{nm}^2) = 113, dPG₅₀₀ (A_{nm}^2) = 488].

Table 2 Inhibitor constants from the hemagglutination and erythrocyte binding inhibition assay of the most potent functionalized scaffolds and monomeric sialosides. Values are expressed in terms of SA concentration or nanoparticle concentration with its SEM ($n \geq 3$). For binding inhibition experiments the SE of $\log IC_{50}$ is given. For all compounds see Table S7. For α -methylsialoside the inhibition constant $KiHAI_{SA}$ as well as the dissociation constant $Kdapp_{NP}$ is shown as reported earlier [23, 38].

Compound ($PG_{MW}SA_{DF}$)*	$KiHAI_{SA}$ [μM]	$KiHAI_{NP}$ [nM]	Binding inhibition $IC_{50 SA}$ [mM]	Binding inhibition $IC_{50 NP}$ [μM]	$Kdapp_{NP}$ [μM]
α -methylsialoside	2000	-	-	-	2800 $\pm$ 300
α -2,6-sialyllactose	100000	-	-	-	-
LPG ₁₀ SA _{0.40} 1b	19.5 $\pm$ 5.9	328 $\pm$ 98	0.22 $\pm$ 0.03	3.74 $\pm$ 0.50	10.10 $\pm$ 2.90
LPG ₁₀ SA _{0.70} 1c	5.9 $\pm$ 2.7	59 $\pm$ 27	0.22 $\pm$ 0.02	2.20 $\pm$ 0.20	0.60 $\pm$ 0.32
dPG ₁₀ SA _{0.15} 2b	9.3 $\pm$ 1.7	358 $\pm$ 66	1.54 $\pm$ 0.08	59.1 $\pm$ 3.1	1.46 $\pm$ 0.65 and 82.5 $\pm$ 24.0
dPG ₅₀₀ SA _{0.20} 3b	20.3 $\pm$ 4.7	16 $\pm$ 4	1.01 $\pm$ 0.05	0.82 $\pm$ 0.05	0.013 $\pm$ 0.006 and 1.35 $\pm$ 0.43

Table 3 Mean values ($n \geq 3$) of the half maximal inhibitory concentration (IC_{50}) of selected inhibitors after infection of MDCK-II cells at an MOI of 0.05 for 24 h. For infection inhibition experiments the SE of $\log IC_{50}$ is given. For a list of all compounds see Table S8.

Compound ($PG_{MW}SA_{DF}$)	Infection inhibition $IC_{50 SA}$ [μM]	Infection inhibition $IC_{50 NP}$ [nM]
LPG ₁₀ 1	-	500000.00
LPG ₁₀ SA _{0.40} 1b	0.14+0.05	2.35+0.83
LPG ₁₀ SA _{0.70} 1c	0.37+0.09	3.69+0.91
dPG ₁₀ SA _{0.15} 2b	5.59+0.18	215.15+6.92
dPG ₅₀₀ SA _{0.20} 3b	1.98+0.08	1.60+0.06

Supporting Information

Linear polysialoside outperforms dendritic analogs for inhibition of influenza virus infection in vitro and in vivo

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Contents

Synthesis and characterization of compounds	4
1. Experimental section	4
1.1 Synthesis of LPG ₁₀ 1 , dPG ₁₀ 2 and dPG ₅₀₀ 3	4
1.2 Synthesis of LPGSAs 1a-1e (Scheme S1)	4
Synthesis of LPGallyl	5
Synthesis of LPGSA _{protected}	6
Synthesis of LPGSAs 1a-d	7
Synthesis of LPGOMs	8
Synthesis of LPGN ₃	9
Synthesis of LPGSA 1e	9
1.3 Synthesis of dPGSAs 2a-2e and 3a-3d (Scheme S2)	10
Synthesis of dPGOMs	11
Synthesis of dPGN ₃	12
Synthesis of dPGSAs 2a-2e and 3a-3d	13
1.4 Inverse gated (IG) ¹³ CNMR spectra of dPG ₁₀ 2 and dPG ₅₀₀ 3	15
1.5 ¹ H NMR spectra of intermediate compounds LPGallyl, LPGSA _{protected} , LPGOMs, LPGN ₃ , dPGOMs, dPGN ₃	16
1.6 ¹ H NMR spectra of LPGSAs and dPGSAs	19
Supporting figures and tables	22
Number and intensity distribution profiles in DLS (Figure S1-S3)	22
Cryo-TEM images of compounds 1e , 2b , and 3b (Figure S4)	25
Cell viability assay (Figure S5)	26
IC ₅₀ values of infection inhibition experiments (Figure S6)	27
Immunodetection of viral nucleoprotein (Figure S7)	27
In vivo infection inhibition plots (Figure S8)	28

Signal curves for microscale thermophoresis (Figure S9)	29
Erythrocyte binding inhibition experiments (Figure S10)	30
Hemagglutination inhibition in respect to ligand density (Figure S11)	31
Virus binding inhibition experiments (Table S7)	31
IC ₅₀ values from cell viability based infection inhibition experiments (Table S8)	32
Theoretical model for virus binding with LPGSA and hPGSA	33
References	39

Synthesis and characterization of compounds

1. Experimental section

1.1. Synthesis of LPG₁₀ **1**, dPG₁₀ **2**, and dPG₅₀₀ **3**

LPG₁₀ **1** was synthesized by the previously reported procedure [1] and characterized by the ¹H NMR and GPC (H₂O) for the determination of absolute molecular weight and dispersity using a pullulan standard.

GPC (H₂O): M_n = 10.1 kDa, M_w = 13.1 kDa, D = 1.29

dPG₁₀ **2** was synthesized using the previously reported procedure [2] characterized by NMR and GPC (H₂O) for the determination of absolute molecular weights and dispersity. The degree of branching (DB) was calculated using inverse gated (IG) ¹³C NMR as reported in literature.[3]

GPC (H₂O): M_n = 12.3 kDa, M_w = 16.3 kDa, D = 1.32

Degree of branching (DB) as determined by IG ¹³CNMR = 59%

dPG₅₀₀ **3** was synthesized using a different procedure as reported by Imran Ul-Haq et.al [4] and characterized by NMR, GPC, and MALLS.

GPC (DMF): M_n = 481 kDa, M_w = 552 kDa, PDI = 1.15

GPC-MALLS (H₂O): M_n = 436 kDa, M_w = 481 kDa, PDI = 1.10

DB as determined by IG ¹³CNMR = 62%

1.2. Synthesis of LPGSAs **1a-1e**

LPGSAs **1a-1d** were synthesized in three steps starting from LPG₁₀ **1**: allylation, thiol-ene

click reaction with sialic acid thiol intermediate [5], and hydrolysis of sialic acid (SA) conjugate with 2M NaOH. LPGSA **1e** was synthesized in three steps starting from LPG₁₀ **1**: mesylation, substitution by azide, and copper-catalyzed Sharpless/Huisgen click reaction LPG-N₃ with prop-2-ynyl α -thiosialoside [5][6] (**Scheme S1**).

Scheme S1. Synthesis of linear polyglycerol sialosides (LPGSAs **1a-1e**). Sialic acid residues are randomly distributed on the LPG backbone. $(1+n)_m$ indicates the total number of monomeric units in the linear polymer chain. The inset shows the simplified cartoons of compounds **1a-d** and **1e**.

Synthesis of LPG-allyl

LPG₁₀ **1** (0.1 g, 0.14 mmol OH to be functionalized) was dried at 60 °C overnight under high vacuum. Dried LPG was dissolved in dry DMF (3 mL) and cooled to 0 °C. To the stirred solution of LPG in dry DMF at 0 °C, NaH (4.59 mg, 0.18 mmol, 1.3 eq., 95%) was added. After addition ice bath was removed and the temperature of the reaction mixture was allowed to reach room temperature. The reaction mixture was allowed to stir at room temperature for 2 hours and cooled down again to 0 °C. The allyl bromide (24.12 μ L, 0.28

mmol, 2.0 eq.) in dry DMF (1 mL) was added drop wise to the reaction mixture using a syringe at 0° C. The ice bath was removed and after stirring for 16 hours at room temperature the reaction was quenched with water. The DMF was removed in vacuum and the resulting mixture was dialyzed in MeOH to afford 90 mg of LPG-allyl (DF = 0.10). Degree of allylation was quantified by ¹H NMR. Yield = 84%. ¹H NMR (500 MHz, CD₃OD): δ(ppm) = 5.97-5.89 (m, 1H, OCH₂CH=CH₂), 5.23 (dd, 2H, J₁=45Hz, J₂=10 Hz, OCH₂CH=CH₂), 4.01 (d, 1H, J=5Hz, OCH₂CH=CH₂), 3.71-3.49 (m, LPG backbone). IR (CH₃OH): ν (cm⁻¹) = 3338, 2907, 2066, 1645, 1460, 1420, 1349, 1201, 1260, 1100, 997, 923.

The degrees of functionalization (DF) in the different reactions were confirmed by ¹H NMR of the pure product correlating the allyl protons at 5.9 -5.8 ppm with the polyglycerol backbone protons (3.7 - 3.4). Table S1 shows the yields and different degrees of allylation achieved as determined by ¹H NMR.

Table S1 Yield and degree of functionalization of LPG-allyl.

LPG-allyl (DF)	0.10	0.44	0.73	1.00
Yield %	84	80	86	87

Synthesis of LPGSA_{protected}

LPG-allyl (DF=0.10) (0.08 g, 0.102 mmol of allyl group) was dissolved in dry methanol (3 mL). Added freshly prepared sialic acid thiol intermediate⁵ (77.46 mg, 0.153 mmol, 1.5 eq.) and 2,2-dimethoxy-2-phenylacetophenone (DMPA) as radical initiator (26.14 mg, 0.102 mmol, 1.0 eq.). The catalytic amount of tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl) was added to the reaction to avoid oxidation of the thiol intermediate. The solution was degassed by flushing argon through the reaction mixture for 10 minutes. The reaction mixture was stirred and irradiated with UV light using a high

pressure UV lamp at room temperature for 2 hours. The reaction completion was monitored by ^1H NMR by looking for the disappearance of resonance peak corresponding to one of the allyl protons at $\delta = 5.85$ ppm. The reaction mixture was dialyzed to remove DMPA and excess of sialic acid derivative against methanol to afford 107.8 mg $\text{LPGSA}_{\text{protected}}$ (DF=0.10) with a quantitative conversion (determined by ^1H NMR). Yield= 82%. ^1H NMR (500 MHz, CD_3OD): $\delta(\text{ppm}) = 5.35$ (brs, 3H, H-8, NH, H-7, SA), 4.84 (m, 1H, H-4, SA), 4.57-3.49 [m, SA (H-9, H-5, H-6, COOCH_3 , $-\text{SCH}_2\text{CH}_2\text{CH}_2\text{O}-$), and LPG backbone], 2.85-2.61 (m, 3H, SA, H-3e, $-\text{SCH}_2\text{CH}_2\text{CH}_2\text{O}-$), 2.17-1.83 (m, 18H, 8SA, $4\times\text{OCOCH}_3$, H-3a, $-\text{NHCOCH}_3$, $-\text{SCH}_2\text{CH}_2\text{CH}_2\text{O}-$). IR (CH_3OH): 3363, 2932, 2874, 1735, 1662, 1548, 1369, 1216, 1119, 1076, 1030, 951 cm^{-1} . **Table S2** shows the reaction yields for the preparation of $\text{LPGSA}_{\text{protected}}$ with different DFs.

Table S2. Yield and degree of functionalization of $\text{LPGSA}_{\text{protected}}$.

$\text{LPGSA}_{\text{protected}}$, DF	0.10	0.44	0.73	1.00
Yield %	82	77	70	80

Synthesis of LPGSA 1a-1d

2M NaOH (5 mL) was added to the $\text{LPGSA}_{\text{protected}}$ (DF=0.10) (0.09 g, 0.07 mmol of ligands) and stirred at room temperature for 2 hours. The reaction mixture was neutralized by adding 2M HCl solution and dialyzed against water for 3 days. The solvent of the dialysis was changed thrice a day. The aqueous solution obtained after dialysis was lyophilized to afford the pure 72.5 mg LPGSA **1a** with a quantitative conversion (determined by ^1H NMR). Yield= 92%. ^1H NMR (500 MHz, D_2O): $\delta(\text{ppm}) = 3.88$ -3.56 [m, SA (H-8, NH, H-7, H-4, H-9, H-5, H-6, $-\text{SCH}_2\text{CH}_2\text{CH}_2\text{O}-$); LPG backbone], 2.81-2.70 (m, 3H, SA, H-3e, $-\text{SCH}_2\text{CH}_2\text{CH}_2\text{O}-$), 1.75-2.08 (m, 3H, SA, H-3a, $-\text{NHCOCH}_3$, -

SCH₂CH₂CH₂O-). IR (KBr): 3358, 3292, 2926, 2876, 1740, 1660, 1604, 1371, 1223, 1120, 1037 cm⁻¹.

Table S3 shows the yields, DF, and the average molecular weight as determined by ¹H NMR and GPC of the final compounds LPGSA **1a-1d**.

Table S3. Yields and calculated molecular weights of LPGSA 1a-1d.

LPGSA	1a	1b	1c	1d
DF	0.10	0.44	0.73	1.00
Yield %	92	87	76	79
M _w (kDa) (NMR)	15	31.7	46	59
GPC (H ₂ O)				
Mn (kDa)	12.4	39.3	33.7	49.3
Mw (kDa)	16.9	67.6	54.0	63.6
PDI	1.4	1.7	1.6	1.3

Synthesis of LPG-OMs (DF = 0.20)

LPG₁₀ **1a** (0.1 g, 0.27 mmol OH to be functionalized) was dried at 60 °C overnight under high vacuum. Dried LPG was dissolved in dry DMF (3 mL) followed by addition of TEA (0.075 mL, 0.54 mmol, 2.0 eq. with respect to OH group) and cooled to 0 °C. Then methane sulphonyl chloride (MsCl) (27.16 µL, 0.35 mmol, 1.3 eq. with respect to OH group) dry DMF (1 mL) was added drop wise using a syringe at 0 °C. After stirring the solution for 1 hours at 0 °C, the mixture was allowed to reach room temperature and was stirred for 16 h. The dry DMF was removed in vacuo and the resulting mixture was dialyzed in MeOH to afford 96.4 mg of LPG-OMs as a honey-like product. Degree of mesylation was quantified as 20% by ¹H NMR. Yield = 80%. ¹H NMR (500 MHz, D₂O): δ(ppm) = 4.43-3.52 (m, CH₂ and CH, PG scaffold); 3.16 (brs, CH₃ mesyl), IR (film): ν =

3030, 2941, 2361, 1709, 1457, 1362, 1184, 971, 813, 753 cm^{-1} . The Figure S13 shows the ^1H NMR of the LPG-OMs (DF=0.20).

The degrees of functionalization were confirmed by ^1H NMR of the pure products correlating the CH_3 -Ms at 3.16 ppm with the polyglycerol backbone protons at 4.43-3.52 ppm.

Synthesis of LPG-N₃ (DF=0.20)

LPG-OMs with DF = 0.20 (0.09 g, 0.20 mmol OMs) was dissolved in dry DMF (5 mL) in a one-neck flask equipped with a refluxing condenser. NaN_3 (65.52 mg, 1.00 mmol, 5 eq. with respect to OMs group) was added to the reaction mixture and stirred at 60 °C for 1 day. The resulting salts were filtered off. DMF from the reaction mixture was evaporated using a rotary evaporator and the residue was dialyzed in MeOH to obtain 0.081 g of LPG-N₃ with a quantitative conversion (determined by ^1H NMR). Yield= 98%. ^1H NMR (500 MHz, CD_3OD): $\delta(\text{ppm}) = 3.72\text{--}3.38$ (m, CH_2 and CH, LPG scaffold), IR (film): $\nu = 3349$, 2922, 2878, 2098 (N_3), 2034, 1633, 1540, 1160, 1041 cm^{-1} .

Synthesis of LPGSA 1e

LPG-N₃ with DF=0.20 (0.07g, 0.17 mmol N_3 groups) was dissolved in 4 mL of THF- H_2O (1:1) solvent mixture followed by addition of solution of prop-2-ynyl α -thiosialoside (120.6 mg, 0.22 mmol, 1.3 eq. with respect to N_3 groups) in THF (1 mL). $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (8.4 mg, 0.034 mmol, 0.2 eq with respect to N_3 groups) was dissolved in 0.2 mL of H_2O and added to the solution of sodium ascorbate (67.3 mg, 0.34 mmol, 2.0 eq with respect to N_3 groups) in 0.4 mL H_2O . The resultant solution of salts in water was added drop wise to the solution containing LPGN_3 and prop-2-ynyl α -thiosialoside. The reaction mixture was degassed thoroughly with argon for 5 minutes and then allowed to stir for 3 days at room temperature under argon atmosphere. The progress of the reaction was monitored by IR.

When the peak at $\sim 2100\text{ cm}^{-1}$ corresponding to azide stretching frequency completely disappeared, solvent of the reaction mixture was evaporated at rotary evaporator. 2M NaOH (8 mL) was added to the residue, and stirred at room temperature for 2 h. The reaction mixture was neutralized by adding 2M HCl solution and dialyzed against water and aqueous EDTA solution for 2 days and again using water for 4 days. The solvent of the dialysis was changed thrice a day. The aqueous solution obtained after dialysis was lyophilized to afford the pure 98.5 mg LPGSA **1e** with a quantitative conversion Yield= 78%. $^1\text{H NMR}$ (500 MHz, D_2O): $\delta(\text{ppm}) = 8.01$ (s, 1H, C=CH), 4.69-3.45 [m, SA (H-8, NH, H-7, H-4, H-9, H-5, H-6); $\text{SCH}_2\text{C}=\text{C}$; and LPG core], 2.81-2.80 (m, 1H, SA, H-3e), 2.01(s, 1H, SA, NHAc), 1.88-1.84 (m, 1H, SA, H-3a). IR (film): $\nu = 3355, 2929, 2879, 1698, 1640, 1558, 1457, 1430, 1374, 1346, 1315, 1276, 1223, 1120, 1060, 955\text{ cm}^{-1}$. $M_{\text{w}}(\text{NMR}) = 31.7\text{ kDa}$, $M_{\text{w}}(\text{GPC}/\text{H}_2\text{O}) = 29.7\text{ kDa}$, $M_{\text{n}}(\text{GPC}/\text{H}_2\text{O}) = 23.1\text{ kDa}$, PDI = 1.28.

1.3 Synthesis of dPGSA derivatives

dPGSAs **2a-2e** and **3a-3d** were synthesized starting from dPG₁₀ **2** and dPG₅₀₀ **3** respectively in three steps: mesylation, substitution by azide, and copper-catalyzed Sharpless/Huisgen click reaction dPG-N₃ with prop-2-ynyl α -thiosialoside (**Scheme S2**). Prop-2-ynyl α -thiosialoside was synthesized using Roy⁵ and a slightly modified procedure of Ogura⁶.

Scheme S2. Synthesis of dPGSAs **2a-2e** and **3a-3d**.

Synthesis of dPG-OMs

dPG₁₀ **2** (0.1g, 0.067 mmol OH to be functionalized) was dried* at 60 °C overnight under high vacuum. Dried dPG was dissolved in dry DMF (3 mL) followed by addition of TEA (0.0187 mL, 0.134 mmol, 2.0 eq. with respect to OH group), and then cooled to 0 °C. Then methane sulphonyl chloride (MsCl) (6.74 µL, 0.087 mmol, 1.3 eq. with respect to OH group) in dry DMF (1 mL) was added dropwise using a syringe at 0 °C. After stirring the solution for 1 hour at 0 °C, the mixture was allowed to reach room temperature and was stirred for 16 hours. The dry DMF was removed in vacuo and the resulting mixture

was dialyzed in MeOH for 2 days to afford 88 mg of pure dPG-OMs with DF=0.05 as a honey-like product. Degree of mesylation was quantified by ^1H NMR. Yield = 84%. ^1H NMR (500 MHz, D_2O): δ (ppm) = 4.33-3.51 (m, CH_2 and CH, PG scaffold); 3.15 (brs, CH_3 mesyl), 1.29 (m, CH_2 core); 0.92 (m, CH_3 core). IR (film): ν = 3030, 2941, 2361, 1709, 1457, 1362, 1184, 971, 813, 753 cm^{-1} . **Table S4** shows the yields and different degree of mesylation achieved in different reactions.

*dPG₅₀₀ **3** was not dried at 60 °C overnight under high vacuum. Instead dPG₅₀₀ **3** was dissolved in pyridine, and pyridine was removed under high vacuum at room temperature. The procedure was repeated thrice to obtain moisture free dPG₅₀₀ **3**.

The degrees of functionalization were confirmed by ^1H NMR of the pure products correlating the CH_3 -Ms at 3.15 ppm with the polyglycerol backbone protons.

Table S4. Yield and degree of functionalization (DF) of dPGOMs.

dPGOH	dPG ₁₀ 2					dPG ₅₀₀ 3			
dPG-OMs, DF	0.05	0.15	0.31	0.64	0.90	0.05	0.19	0.38	0.70
Yield %	84	81	79	86	88	82	76	82	87

Synthesis of dPG-N₃

dPG-OMs, DF = 0.05 (0.08 g, 0.051 mmol OMs) was dissolved in dry DMF (5 mL) in a one-neck flask equipped with a refluxing condenser. NaN₃ (16.25 mg, 0.25 mmol, 5 eq. with respect to OMs group) was added to the reaction mixture and stirred at 60 °C for 1 day. The resulting salts were filtered off. DMF from the reaction mixture was evaporated using rotary evaporator and the residue was dialyzed in MeOH to obtain 0.071 g of dPG-N₃ with a quantitative conversion (determined by ^1H NMR). Yield= 88%. ^1H NMR (500 MHz, D_2O): δ (ppm) = 4.00-3.39 (m, CH_2 and CH, PG scaffold), 1.40 (m, CH_2 core), 0.89

(m, CH₃ core), IR (film): ν = 2873, 2361, 2102 (N₃), 1457, 1273, 1122, 668 cm⁻¹. Table S5 shows the yields and different degree of azidation achieved in different reactions.

Table S5. Yield and degree of functionalization of dPG-N₃.

dPGOH	dPG ₁₀ 2					dPG ₅₀₀ 3			
dPGN ₃ , DF	0.05	0.15	0.31	0.64	0.90	0.05	0.19	0.38	0.70
Yield %	88	85	89	87	86	80	78	80	84

Synthesis of dPGSAs 2a-2e and 3a-3d

dPGSA 2a

dPG-N₃ with DF = 0.05 (0.07 g, 0.045 mmol N₃ groups) was dissolved in 4 mL of THF-H₂O (1:1) solvent mixture followed by addition of solution of prop-2-ynyl α -thiosialoside (32.6 mg, 0.059 mmol, 1.3 eq. with respect to N₃ groups) in THF (1 mL). The CuSO₄·5H₂O (2.3 mg, 0.0092 mmol, 0.2 eq with respect to N₃ groups) was dissolved in 0.1 mL of H₂O and added to the solution of sodium ascorbate (18.3 mg, 0.092 mmol, 2.0 eq with respect to N₃ groups) in 0.2 ml H₂O. The resulting solution was added drop wise to the solution containing dPG-N₃ and prop-2-ynyl α -thiosialoside. The reaction mixture was degassed thoroughly with argon for 5 minutes and then allowed to stir for 3 days at room temperature under argon atmosphere. The progress of the reaction was monitored by IR. When the peak at ~2100cm⁻¹ corresponding to azide stretching frequency completely disappeared, the solvent was evaporated at rotary evaporator. 2M NaOH (5 mL) was added to the residue, and stirred at room temperature for 2 hours. The reaction mixture was neutralized by adding 2M HCl solution and dialyzed first against water and aqueous EDTA solution for 2 days and again using only water for 4 days. The solvent of the dialysis was changed thrice a day. The aqueous solution obtained after dialysis was

lyophilized to afford the pure 72.3 mg dPGSA **3** with a quantitative conversion (determined by ^1H NMR). Yield= 82%. ^1H NMR (500 MHz, D_2O): δ (ppm) = 7.96 (s, 1H, C=CH), 4.60-3.39 [m, SA (H-8, NH, H-7, H-4, H-9, H-5, H-6); $\text{SCH}_2\text{C}=\text{C}$; and nPG core], 2.80-2.77 (m, SA, H-3e), 2.02 (s, SA, NHAc), 1.81 (SA, H-3a), 1.36 (m, $-\text{CH}_2-$ core), 0.88-0.87 (m, $-\text{CH}_3$ core). IR (film): ν = 3355, 2922, 2873, 1609, 1559, 1456, 1388, 1323, 1281, 1250, 1111, 1078, 954 cm^{-1} .

Table S5 and **S6** shows the yields, degree of functionalization, and molecular weight determined by NMR/GPC of the final compounds dPGSA **2a-2e** and **3a-3d**.

Table S5. Yield, DF, and molecular weight of dPGSAs **2a-2e**.

dPGSA	2a	2b	2c	2d	2e*
DF	0.05	0.15	0.31	0.64	0.90
Yield %	82	87	81	90	92
M_w (kDa) (NMR) GPC (H₂O)	15	23	32	55	59
M_n (kDa)	14.6	24.9	24.2	40.2	56.8
M_w kDa)	28.9	35.2	42.1	52.6	86.5
PDI	2.0	1.4	1.7	1.3	1.5

*The compound **2e** was prepared using dPG (M_n = 7.2 kDa, M_w = 10.4 kDa) and analyzed by GPC-MALLS.

Table S6. Yields, DFs, and molecular weights of dPGSAs **3a-3d**.

dPGSA	3a	3b	3c	3d
DF	0.05	0.19	0.38	0.70
Yield %	81	72	78	74
M_w (kDa) (NMR)	621	960	1440	2103

1.4 Inverse-gated (IG) ^{13}C NMR of dPG₁₀ 2 and dPG₅₀₀ 3

IG ^{13}C NMR (175 MHz, D₂O) of dPG₁₀ 2.

1 IG ^{13}C NMR (175 MHz, D_2O) of dPG₅₀₀ **3**.

2

3 1.5 ^1H NMR spectra of intermediate compounds LPGallyl, LPGSA_{protected}, LPG-

4 OMs,

5 LPGN₃, dPGOMs, dPGN₃

6 ^1H NMR (500 MHz, CD_3OD) of LPGallyl (DF=0.10).

7

1 ^1H NMR (500 MHz, CD_3OD) of $\text{LPGSA}_{\text{protected}}$ (DF=0.10).

3 ^1H NMR (500 MHz, D_2O) of LPG-OMs .

1 ^1H NMR (500 MHz, D_2O) of LPG-N_3 , $\text{DF}=0.20$.

2

3 ^1H NMR (500 MHz, D_2O) of $\text{dPG}_{10}\text{OMs}$, $\text{DF}=0.05$.

4

5

1 ^1H NMR (500 MHz, D_2O) of $\text{dPG}_{10}\text{N}_3$, $\text{DF}=0.05$.

2

3 ^1H NMR spectra of the representative compounds from LPGSAs, dPGSAs.

4 ^1H NMR (500 MHz, D_2O) of LPGSA **1b**, $\text{DF}=0.4$.

5

6

1 ^1H NMR (500 MHz, D_2O) of LPGSA **1c**, DF=0.70.

2
3
4 ^1H NMR (500 MHz, D_2O) of LPGSA **1e**, DF = 0.20.

1 ^1H NMR (500 MHz, D_2O) of dPGSA **2b**, DF = 0.15.

2
3 ^1H NMR (500 MHz, D_2O) of dPGSA **3b**, DF = 0.20.
4

Supporting Figures and Tables

Figure S1. The number and intensity distribution profiles of linear polyglycerol (LPG₁₀ 1) and polyglycerolsialosides (1a-e) as observed by dynamic light scattering (DLS). All samples were analyzed at 1mg/ml in aqueous PBS (pH 7.4, 150 mM NaCl) at 25 °C.

Figure S2. The number, and intensity distribution profiles of dendritic polyglycerol (dPG₁₀ 1) and dendritic polyglycerolsialosides (2a-e) as observed by DLS. All samples were analysed at 1 mg/ml in aqueous PBS (pH 7.4, 150 mM NaCl) at 25 °C.

Figure S3. The number, and intensity distribution profiles of dendritic polyglycerol (dPG₅₀₀ **3**) and dendritic polyglycerolsialosides (**3a-d**) as observed by DLS. All samples were analysed at 1mg/ml in aqueous PBS (pH 7.4, 150 mM NaCl) at 25 °C.

Figure S4. Cryo-TEM images of compounds **1e**, **2b**, and **3b**. (a) Cryo-electron micrograph of dPG₁₀SA_{0.15} **2b**, 1 mM sialic acid (corresponding to 38.5 μ M PG particles) in PBS (10 mM, 150 mM NaCl). The determined diameter is 5.1 nm (median of Feret diameter, n=620) (b) Cryo-electron micrograph of dPG₅₀₀SA_{0.20} **3b**, 1mM sialic acid (corresponds to 0.8 μ M PG particles) in PBS (10 mM, 150 mM NaCl). The determined diameter is 7.5 nm (median of

Feret diameter, $n=520$) (c) Cryo-electron micrograph of $\text{LPG}_{10}\text{SA}_{0.20}$ **1e**, 5.4 mM sialic acid (corresponds to 200 μM PG particles). The determined diameter is 6.3 nm (median of Feret diameter, $n=420$).

Figure S5. Cell viability plots. After a 24 h incubation of MDCK II cells with compounds representing the most potent inhibitors of each PG scaffold class, cell viability was determined using a CellTiter AQueous One Solution (Promega). Error bars represent the SEM ($n \geq 3$).

Figure S6. IC₅₀ values of infection inhibition experiments. Inhibition values were derived from dose response curves (Figure 3) with sialylated polyglycerols against influenza A/X31 virus. Virus was pretreated with linear sialoside LPG₁₀SA_{0.40} **1b** for 30 min, then added to cells for 1 h (MOI of 0.05), followed by a washing step to remove unbound virus. 24 h p.i. the cell viability was monitored by using a CellTiter AQueous One Solution (Promega). Bars with stripes highlight the optimum candidates, i.e., **1b**, **2b**, and **3b** with minimal optimum DF selected for further investigation. Error bars: SE of the logIC₅₀ (n≥3). Data were fitted with a four parametric logistic fit to obtain IC₅₀ values.

Figure S7. Immunodetection of viral nucleoprotein (NP) in MDCK II cells 24 h after infection with X31 virus (MOI 0.1) in the presence of inhibitors (300-10 μ M sialic acid). Non-sialylated control inhibitors were used at 300 μ M nanoparticle concentrations (indicated by *). Cell nuclei were stained with DAPI (upper row) and viral NP was detected with a FITC coupled anti-NP antibody (lower row).

Figure S8. In vivo infection inhibition plots. a) dPG₅₀₀SA_{0.20} **3b** at 5 mM SA concentrations and the control inhibitor dPG₅₀₀ **3** were compared to an infected, PBS treated group. Error bars indicate SEM (n≥5). Data were subjected to statistical analysis by using a one-way ANOVA, and a Dunnett's multiple comparison test of all inhibitor treated groups to the infected, PBS treated group (*p<0.05, **p<0.01, ***p<0.001). b) Body weight of BALB/c mice relative to the initial body weight on the day of infection (Day 0) with 100 pfu X31. Independent groups of mice were challenged X31 virus pretreated with either PBS, 83 μM or 833 μM oseltamivir carboxylate. Error bars indicate the SEM (n≥5). Data were statistically analyzed by conducting a one-way ANOVA with a Dunnett's multiple comparison posttest to the PBS treated group (*p>0.05, **p>0.01, ***p>0.001).

Figure S9. Signal curves from microscale thermophoresis measurements of a) three equally, but independently prepared dilutions of R18 labeled X31 virus in PBS, b) equal amounts of R18 labeled X31 virus combined with different dilutions of LPG₁₀ **1**, c) equal amounts of R18 labeled X31 virus combined with different dilutions of dPG₅₀₀SA_{0.20} **3b**. Vertical lines reflect the gated intervals of the initial fluorescence (blue), and 30 s after the T-jump within the thermophoresis process (red).

Figure S10. Erythrocyte binding inhibition experiments: Dose dependent binding inhibition plots of R18 labeled X31 virus (16 HAU/ μ l) to 1% (v/v) red blood cells in the presence of a) LPG₁₀, b) dPG₁₀, and c) dPG₅₀₀ based inhibitor scaffolds. Error bars represent the SEM ($n \geq 3$). Dashed lines are derived from a four parametric sigmoidal fit to determine IC₅₀ values (Table S7).

Figure S11. Hemagglutination inhibition in respect to ligand density with either LPG₁₀ **1** and dPG₁₀ **2**, (a) or dPG₅₀₀ **3** (b) with different sialic acid (SA) densities. Error bars represent the SEM (n≥3).

Table S7. Virus binding inhibition experiments. Inhibition values (KiHAI) from the hemagglutination inhibition assay and the single erythrocyte binding inhibition assay (IC₅₀) complementing data shown in Table 2. Values are expressed in terms of sialic acid and nanoparticle concentration. For binding inhibition experiments the SE of logIC₅₀ is given. Maximum concentration tested without an inhibitory effect is indicated by (*). n.d. = not determined.

Compound (SA%, scaffold)	KiHAI Sialic acid [μM]	KiHAI Nanoparticle [nM]	Binding inhibition IC _{50SA} [mM]	Binding inhibition IC _{50NP} [μM]
LPG ₁₀ 1	-	2000000.0*	-	4000.00*
LPG ₁₀ SA _{0.10} 1a	2000.0*	139860.0*	2.30+0.05	164.14+3.57
LPG ₁₀ SA _{1.00} 1d	1000.0±0.0	7407.4±0.0	1.00*	7.41*
LPG ₁₀ SA _{0.2} 1e	1860.0±620.0	68888.9±22963.0	4.00*	148.15*
dPG ₁₀ 2	-	2000000.0*	-	4000.00*
dPG ₁₀ SA _{0.05} 2a	2000.0*	250000.0*	2.53+0.15	316.13+18.75
dPG ₁₀ SA _{0.30} 2c	62.5±0.0	1225.5±0.0	1.93+0.05	37.75+0.98
dPG ₁₀ SA _{0.65} 2d	57.3±21.6	540.5±203.8	1.02+0.17	9.65+1.60
dPG ₁₀ SA _{0.90} 2e	25000.0±0.0	206612.6±0.0	n.d.	n.d.
dPG ₅₀₀ 3	-	2000000.0*	-	4000.00*
dPG ₅₀₀ SA _{0.05} 3a	2000.0*	5587.00*	n.d.	n.d.
dPG ₅₀₀ SA _{0.40} 3c	666.7±166.7	269.9±67.5	3.42+0.03	1.39+0.01
dPG ₅₀₀ SA _{0.70} 3d	50000±0.0	10989±0.0	n.d.	n.d.

Table S8. IC₅₀ values from cell viability based infection inhibition experiments. IC₅₀ values are presented either in terms of the sialic acid or nanoparticle concentration indicated by SA or NP respectively. In addition, the IC_{50NP} is also expressed in terms of its corresponding mass. n.d= not determined. No detectable inhibition within the tested concentration range is indicated by (*). For infection inhibition experiments the SE of logIC₅₀ is given. This table complements Table 3.

Compound (PG _{MW} SA _{Dr})	Infection inhibition IC _{50SA} [μM]	Infection inhibition IC _{50NP} [nM]
LPG ₁₀ SA _{0.10} 1a	43.16+0.12	3.08+8.57
LPG ₁₀ SA _{1.00} 1d	16.15+0.74	119.63+5.48
LPG ₁₀ SA _{0.2} 1e	4.85+0.22	179.44+0.22
dPG ₁₀ 2	-	10000.00
dPG ₁₀ SA _{0.05} 2a	500.00*	62.50*
dPG ₁₀ SA _{0.30} 2c	1.84+0.06	36.14+1.17
dPG ₁₀ SA _{0.65} 2d	1.86+0.29	15.40+2.74
dPG ₁₀ SA _{0.90} 2e	n.d.	n.d.
dPG ₅₀₀ 3	-	500000.00*
dPG ₅₀₀ SA _{0.05} 3a	500.00*	1.40*
dPG ₅₀₀ SA _{0.40} 3c	1.07+0.15	0.43+0.06
dPG ₅₀₀ SA _{0.70} 3d	n.d.	n.d.

Theoretical model for virus binding with LPGSA and dPGSAs

The aim of this section is to show how a simple model based on the statistical mechanics of binding can be used to rationalize the observed experimental data regarding the inhibition efficiency of ligands-functionalized nanoparticles and polymers. More precisely, we aim at understanding trends in inhibition efficiency as a function of the degree of functionalization. The starting point to consider is that the hemagglutination inhibition efficiency for multivalent constructs is typically proportional to their dissociation constant [7] or in other words proportional to the exponential of the binding strength, ΔG_{bind} . ΔG_{bind} depends on the trade-off between how much binding free-energy is gained through ligand-receptor bonds formation and how much is lost via steric repulsion, since once brought within binding distance from the virus' surface ligands will also feel a steric repulsion due to excluded volume effects. This trade-off depends on the spatial arrangement of both ligands and receptors, which controls their binding free-energy, and molecular quantities such as the length and rigidity of the ligands [8, 9]. For this reason, in order to understand the binding strength of our multivalent constructs to viruses, it is important to consider the spatial organization of the sialic acid (SA) receptors on the viral surface, as well as that of SA itself on the multivalent construct. On a typical influenza virus with radius 50 nm, around 400 HA proteins are present, each having 3 binding sites for SA. From these values, the average distance between receptors (without accounting for spatial correlations) can be estimated as $d_{\text{RR}} = \frac{4\pi R_{\text{virus}}^2}{3N_{\text{HA}}} \approx 5$ nm, whereas the distance between any pair of receptors on the same HA unit is about 4.5 nm. Since both distances are much larger than the length of a SA ligand, the latter cannot be at the same time within binding distance of two different receptors (a possible realization of the virus-multivalent construct system is schematically depicted in Fig. S12 for clarity).

Figure S12. Schematic representation of a multivalent construct bound to a surface in three different binding configurations. Given that the average distance between receptors is much larger than the ligands length, ligands cannot be within binding distance from two different receptors at once. In this case, an “indifferent topology” of binding occurs [10] where only one bond can be formed at any given time between the $N = 3$ ligands that can interact with the viral surface and the receptor, leading to a binding contribution due to avidity entropy [10] of

$$\Delta G = -T\Delta S_{\text{avidity}} = -k_B T \log N \quad (1)$$

If N is the number of ligands that can interact with the viral surface at any given time, either via forming a bond with a viral receptor, or because they are sterically repelled by the viral surface, there are N binding configurations, each of which have only one of the N ligands bound, and the others $N - 1$ unbound. Note that N is directly proportional to the degree of functionalization DF . This binding scenario is the so called “indifferent topology” described by Kitov and Bundle[10] where binding of a ligand exclude the concurrent binding of others. Under these conditions, and assuming to a first

approximation that the formation energy for a single bond ΔG_{bond} is roughly the same for all ligands-receptor pairs, the interaction free-energy of the multivalent construct is:

$$\begin{aligned} \frac{\Delta G_{\text{bind}}}{k_B T} &= -\log N Z_u^{N-1} Z_b \\ &= -\log N Z_u^N \frac{Z_b}{Z_u} \\ &= -N \log Z_u - \log N - \log \frac{Z_b}{Z_u} \quad (2) \\ &= N \Delta G_{\text{steric}} - \frac{\Delta S_{\text{avidity}}}{k_B} + \Delta G_{\text{bond}} \quad (3) \end{aligned}$$

In Eq. 2 $Z_{u(b)}$ is the partition function for an unbound (bound) ligand with respect to that of an unbound ligand not confined by the virus surface, and as such is a function of the virus-nanoparticle binding distance h , as well as of the length and rigidity (or, more precisely, the molecular structure) of the specific ligand used and the construct to which it is grafted (see **Scheme 1**). For this reason, in Eq. 2, we can identify $-\log Z_u = \Delta G_{\text{steric}}$ and $-\log \frac{Z_b}{Z_u} = \Delta G_{\text{bond}}$ as the (repulsive) steric contribution and the (attractive) bond contribution to the binding free-energy due to a single ligand, respectively, and $\Delta S_{\text{avidity}} = k_B \log N$ is the so-called avidity entropy contribution arising due to multivalent binding, which depends on the number of possible binding configurations the construct can achieve [10].

The microscopic picture behind this model is easily explained. By increasing the number of ligands on the nanoparticle available for binding, one increases the binding free energy by $\Delta G_{\text{bind}} = -T \Delta S_{\text{avidity}} = -k_B T \log(N)$, the entropic contribution due to all possible binding configurations considering that only one of them can bind a receptor at the same time (note that the bonding contribution would be instead proportional to N if each ligand can bind concurrently a different receptor, but this is impossible in our system since the

inter-receptor distance is too large compared to the ligands length, as previously explained). Hence, ΔG_{bind} grows logarithmically with N . However, the number of unbound ligands which can sterically interact with the surface of the virus grows linearly with N , and so does their induced repulsive interaction. Since the binding energy decreases slower than the steric repulsion increases, their sum will attain a minimum at a specific value due to the compromise between these opposite terms. In fact, by taking the derivative of both sides of equation Eq. 2, we find that the lowest attainable free-energy, i.e. the lower dissociation constant and hence the optimal hemagglutination inhibition, is at:

$$N^{\text{opt}} = \frac{k_B T}{\Delta G_{\text{steric}}} \quad (4)$$

clearly, such a minimum will be effectively observed only if $N_{\text{opt}} > N_{\text{max}}$, where N_{max} is obtained at the highest possible degree of functionalization, i.e. $DF = 100\%$. This simple model does not only predict the existence of a minimum, but since ΔG_{steric} depends on molecular parameters of our system it can tell us how this minimum shift as a function of the ligand molecular structure. Let us thus treat ligands as rigid rods of length L , grafted to a point at a binding distance h from the viral surface (clearly $h < L$ for binding to occur). In this case, Z_u can be calculated for ligands grafted on a hard surface of curvature radius R , representing a nanoparticle, or grafted to an (ideal) polymer. Considering for simplicity the surface of the virus as a flat surface (a good approximation given that viruses are much larger than the constructs we study ($R_{\text{virus}} \approx 50 \text{ nm} \gg R_{\text{nanoparticle}} \approx R_{\text{polymer}}$), we obtain the following two expressions:

$$\frac{\Delta G_{\text{steric}}^{\text{nanoparticle}}}{k_B T} = -\log Z_u^{\text{nanoparticle}} \approx -\log \left[\frac{h}{L \left(1 + O\left(\frac{1}{R_{\text{nanoparticle}}}\right) \right)} \right] \quad (5)$$

$$\frac{\Delta G_{\text{steric}}^{\text{polymer}}}{k_B T} = -\log Z_u^{\text{polymer}} \approx -\log \left[0.5 \left(1 + \frac{h}{L} \right) \right] \quad (6)$$

together with Eq. 4, these formulas agree with two other experimentally observed behaviors:

1. Since the repulsive free-energy for ligands coated on a nanoparticle $\Delta G_{\text{steric}}^{\text{nanoparticle}}$, does not depend in its leading term on the nanoparticle radius, the model predicts that both small and large dPGSA have roughly the same optimal degree of functionalization.
2. Given that $\Delta G_{\text{steric}}^{\text{polymer}} < \Delta G_{\text{steric}}^{\text{nanoparticle}}$ (independently on the binding distance), we predict N^{opt} (and hence the optimal degree of functionalization DF) to shift at higher values for the linear constructs when compared to nanoparticles to which the same ligands are grafted.

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