Here we report that negatively charged polysulfates can bind to the spike protein of SARS-CoV-2 via electrostatic interactions. Using a plaque reduction assay, we compare inhibition of SARS-CoV-2 by heparin, pentosan sulfate, linear polyglycerol sulfate (LPGS) and hyperbranched polyglycerol sulfate (HPGS). Highly sulfated LPGS is the optimal inhibitor, with a half-maximal inhibitory concentration (IC$_{50}$) of 67 μg/mL (approx. 1.6 μM). This synthetic polysulfates exhibit more than 60-fold higher virus inhibitory activity than heparin (IC$_{50}$: 4084 μg/mL), along with much lower anticoagulant activity. Furthermore, in molecular dynamics simulations, we verified that LPGS can bind stronger to the spike protein than heparin, and that LPGS can interact even more with the spike protein of the new N501Y and E484K variants. Our study demonstrates that the entry of SARS-CoV-2 into host cells can be blocked via electrostatic interaction, therefore LPGS can serve as a blueprint for the design of novel viral inhibitors of SARS-CoV-2.
Polysulfates block SARS-CoV-2 uptake via electrostatic interactions

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Abstract: Here we report that negatively charged polysulfates can bind to the spike protein of SARS-CoV-2 via electrostatic interactions. Using a plaque reduction assay, we compare inhibition of SARS-CoV-2 by heparin, pentosan sulfate, linear polyglycerol sulfate (LPGS) and hyperbranched polyglycerol sulfate (HPGS). Highly sulfated LPGS is the optimal inhibitor, with a half-maximal inhibitory concentration (IC_{50}) of 67 μg/mL (approx. 1.6 μM). This synthetic polysulfates exhibit more than 60-fold higher virus inhibitory activity than heparin (IC_{50}: 4084 μg/mL), along with much lower anticoagulant activity. Furthermore, in molecular dynamics simulations, we verified that LPGS can bind stronger to the spike protein than heparin, and that LPGS can interact even more with the spike protein of the new N501Y and E484K variants. Our study demonstrates that the entry of SARS-CoV-2 into host cells can be blocked via electrostatic interaction, therefore LPGS can serve as a blueprint for the design of novel viral inhibitors of SARS-CoV-2.
Introduction

The severe acute respiratory syndrome corona virus 2 (SARS-CoV-2) poses an ongoing major health problem worldwide.[1] Understanding virus attachment and entry into cells is critical for the development of inhibitors. In a number of viruses, electrostatic interactions are essential for the virion’s adherence to the cell surface.[2-3] Evidence for the importance of this process in viral infection has recently been discussed by Cagno et al.[2], who gathered experimental evidence on the importance of this process in a large number of viruses (see Table 1 of ref.[2]). Figure 1 displays the first steps of virus entry into cells. Virions first attach to the syndecans and glypicans, which are the most important heparan sulfate proteoglycans (HSPGs) located at the cell surface.[3] Each HSPG consists of a protein and a highly charged glycosaminoglycan (GAG) chain. The negatively charged heparan sulfate (HS) moieties of the HSPG interact with basic patches of the viral capsid proteins. As depicted in Figure 1, viruses exploit this nonspecific electrostatic interaction to increase their concentration at the cell surface and to be transferred to a more specific receptor, i.e. the angiotensin-converting enzyme 2 (ACE2).

Figure 1. (left) Binding of SARS-CoV-2 to surface exposed heparan sulfate facilitates virus entry; (right) competitive binding to soluble synthetic polyglycerol sulfates shield the viral surface and therefore finally reduce infectivity.

Recent studies have furnished evidence that electrostatic interactions are important for the infection of cells by SARS-CoV-2.[4] In particular, Kim et al. performed a series of systematic surface plasmon resonance (SPR) studies on the binding of the SARS-CoV-2 spike protein to heparin.[5] This research revealed binding constants as low as 40 pM that could be attributed to electrostatic interactions. Moreover, Kwon et al. found that addition of soluble HS inhibits SARS-CoV-2 cell infectivity, highlighting the importance of HS for the entry of the virus into host cells.[6] Recent work of Clausen et al. showed that the receptor binding domain (RBD) of SARS-CoV-2 exhibits a patch of positive charges on its surface that is considerably larger than it in the RBD of SARS-CoV.[7] Other recent research demonstrated that the attachment of the spike protein to the HSPG is the first step for virus entry into host cells, as shown in Figure 1.[8] In a second step, the attached virion interacts with ACE2, the actual receptor for the entry of SARS-CoV-2.[9-10] The essential role of electrostatic interactions in virus entry provides the principle
mode of action for highly charged anionic inhibitors. Heparin was studied intensively in this regard.[11-12] Moreover, synthetic virus inhibitors based on highly charged anionic dendrimers have been investigated intensively.[13-15]

The work presented here follows our hypothesis that charge-charge interactions are of central importance to inhibit the entry of SARS-CoV-2 into cells. As discussed recently[14, 16-18], charge-charge interactions mainly act through counterion release:[19-20] patches of positive charge on the surface of proteins can become multivalent counterions of highly charged polyelectrolytes such as heparin, thus releasing a concomitant number of counterions condensed to the polyelectrolyte into the bulk phase.[16-17] On the other hand, positively charged patches could be a target for the design of viral-entry inhibitors. Considering that these positively charged residues are located in close vicinity of the ACE2 binding site of the spike protein[7], it is envisioned that inhibitors bound to the positively charged patches can interrupt ACE2 binding, leading to virus entry inhibition.[21]

A systematic study of the interactions of the SARS-CoV-2 virion with the cell surface requires a detailed investigation of the local interaction of HS with the spike proteins. This problem has become even more urgent considering the new variants of the virus that began to appear in late 2020. It seems that these strains can exhibit a much higher infectivity.[22] The N501Y variant is reported to be more infectious than the wild-type virus, and a virus carrying this mutation was adapted to infect mice, which cannot be infected by wild-type SARS-CoV-2.[22] The E484K substitution is reported to enable the virus to escape from neutralizing antibodies.[23] Docking studies and MD simulations require only the more easily retrieved data from the spike protein, offering a powerful and accessible tool for assessing these mutations through quantitative computer simulations.

In this study, we systemically assess the potential of different polysulfates as entry inhibitors against SARS-CoV-2. Our work combines experimental studies supported by MD simulations: i) Using a plaque reduction assay, we determine the ICso of various highly sulfated polyelectrolytes.[24-25] We compare two natural polysulfates, namely heparin and pentosan sulfate, with highly sulfated polyglycerols, which present a new class of synthetic inhibitors. By comparing linear polyglycerol sulfate (LPGS) to hyperbranched polyglycerol sulfate (HPGS) at fully sulfations, we investigate the influence of molecular weight on inhibitory interaction, as well as the role of architecture in this interaction. ii) MD simulations are used to investigate the binding of mutated spike proteins to LPGS. Here we explore the details of the interaction of HS with the spike protein in order to rationalize our experimental results on inhibition. Moreover, we investigate the consequences of the N501Y and E484K mutations in the spike protein for the virus binding to HS. These investigations aim for a fully quantitative understanding of the inhibition of SARS-CoV-2 by polyanions, and further study potential changes of this inhibition that may be caused by novel mutations of the virus’s genome.

**Results and Discussion**

**Electrostatic interactions of SARS-CoV-2 with host cells.** Previous studies have revealed the essential role of electrostatic interactions for SARS-CoV-2 infection. In the RBD of SARS-CoV-2, five positively charged amino acids are localized next to the ACE2 binding site: R346, R355, K444, R466, and R509. These amino acids form a positively charged patch located at the exterior of the RBD (shown blue in Figure 2b), which is reported to improve the virus binding
affinity to the ACE2 receptor.[26-27] For the new E484K variant, the K484 adds another positive charge to the RBD and is therefore expected to further strengthen viral binding to HS.[23] Recent studies have shown that the positively charged patch contributes to virus binding to cell-surface HS by facilitating virus docking on the cell surface.[3, 5] Based on the finding that cleavage of cell-surface HS inhibits SARS-CoV-2 infection, a two-step process of SARS-CoV-2 was proposed as shown in Figure 1.[7-8] The binding to HS was reported to facilitate the ‘opening’ of the RBD for the binding with ACE2.[7]

The presence of electrostatic interactions with cell-surface HS inspired us to test the polysulfates shown in Figure 2c for SARS-CoV-2 inhibition. Two types of polysulfates have been tested: sulfated polysaccharides (heparin and pentosan sulfate) and synthetic polyglycerol sulfates (LPGS and HPGS). Heparin has been successfully used as a therapeutic for COVID-19 patients.[28-29] In particular, evidence has shown that ACE2 binding can be disrupted by the addition of heparin.[21] As a therapeutic, however, the usage of heparin is very limited due to its very strong anticoagulation activity. Patients may face the risk of bleeding when being treated with heparin.[30] Heparin-mimetic polymers with higher virus inhibitory activity and lower anticoagulant activity than heparin are therefore needed as clinical substitutes.

Synthetic polyglycerol sulfates exhibit a similar charge density as heparin but have a lower anticoagulant activity.[16, 31] Here we studied polyglycerol sulfates with different architectures and molecular weights in order to investigate structural influences on virus binding. The solution structure of HPGS in aqueous solution can be approximated by a sphere with negative surface charges.[16] LPGS is a linear polymer that can attain multiple conformations and may span larger distances, and can hence conform to larger basic patches on the surface of proteins.

![Figure 2](image.png)

**Figure 2.** (a) Crystal structure of the SARS-CoV-2 spike protein RBD (PDB ID: 6M0J)[27] with a few important cationic residues that interact with polyanionic ligands. (b) The electrostatic potential map of RBD. (c) Schematic illustrations of polyglycerol sulfates in linear and hyperbranched architectures, and of the natural polysulfates, respectively. The negatively charged groups are marked red.
Inhibition of SARS-CoV-2. The inhibition of virus binding was studied by plaque reduction assays with authentic SARS-CoV-2 (SARS-CoV2M; BetaCoV/Germany/BavPat1/2020).[32] In our study, SARS-CoV-2 was pre-treated with the inhibitors and then incubated with Vero E6 cells to assess virus binding. The cells were washed with phosphate buffered saline (PBS) to remove unbound virions. Afterwards, the cells were cultured for 48 hours with overlay medium for plaque formation as shown in Figure S1, Supporting Information. Since binding and entry are prerequisite for plaque formation, a plaque reduction assay is an informative way to measure inhibition of virus binding and entry.

Figure 3a and Table 1 show dose-dependent virus inhibition curves for the different samples. We first compared virus inhibition between the synthetic polysulfates, the natural polysulfates heparin and pentosan sulfate. As expected, heparin and pentosan sulfate inhibit infection, although the observed inhibitory activity is rather low. The half-maximal inhibition concentrations ($IC_{50}$) for heparin and pentosan sulfate are $4084.0 \pm 396.3 \text{ and } 1310 \pm 292.8 \mu g/mL$, respectively. It should also be noted that heparin can completely inhibit blood clotting at levels as low as 5 $\mu g/mL$. LPGS$_{20kDa}$ shows an $IC_{50}$ of $66.9 \pm 32.0 \mu g/mL$ (approx. 1.6 $\mu M$) and thus a much higher virus inhibitory activity than heparin and pentosan sulfate.

Next, we studied the effect of the architecture of polyglycerol sulfate on virus inhibition. Previously studies revealed that the degree of sulfation is important for the inhibitors that work based on electrostatic interactions.[13, 33] Therefore, we compared the activities of almost fully sulfated inhibitors (>80%). For LPGS, we found that only the polysulfates with a molecular weight of 20kDa can inhibit the viruses effectively. LPGS with lower molecular weight showed no virus inhibition, highlighting the importance of inhibitor size for binding the spike protein. Comparing the activity between LPGS and HPGS with the same molecular weight, we conclude that LPGS can inhibit infection more effectively than the hyperbranched polymer. Due to its greater backbone flexibility, LPGS can adapt its conformation more easily to the positively charged pockets, resulting in strong binding. HPGS, on the other hand, is a rigid spherical structure and cannot adapt its conformation to the binding pocket. Similar results have been obtained in the study of influenza virus inhibitors, where LPG-sialic acid outperformed HPG-sialic acid for virus binding and inhibition.[34] For HPGS, we see maximum inhibition for the 500kDa molecular weight compound. HPGS with higher (2.6MDa) and lower (20kDa) molecular weights show only poor virus inhibition, highlighting again the importance of molecular weight for virus binding and inhibition. While HPGS$_{500kDa}$ exhibits a lower $IC_{50}$ in molar concentration than LPGS$_{20kDa}$, LPGS$_{20kDa}$ has a lower $IC_{50}$ in mass concentration. Considering that drugs are typically dosed in mass concentration, we consider LPGS$_{20kDa}$ a better inhibitor than HPGS$_{500kDa}$.

We also compared the performance of LPGS$_{20kDa}$ with different degrees of sulfation (94% and 47%) with respect to virus inhibition. Here, increasing the degree of sulfation increases the inhibitory activity of LPGS to a remarkable extent: LPGS$_{20kDa}$ with 47% sulfation shows an $IC_{50}$ of $679.7 \pm 175.7 \text{ g/mL}$, which is 10-fold lower than the activity of almost fully sulfated LPGS$_{20kDa}$. It is surprising that a two-fold increase in sulfation caused a ten-fold improvement in the inhibitory potential. This strong influence of sulfation on inhibition highlights the importance of the charge density of the inhibitor for virus binding.
**Competition of virus binding to host cell.** After identification of LPGS_20kDa as the most potent inhibitor, we used this compound for further investigations, where we will refer to it simply as LPGS. To demonstrate that polysulfates can compete with cells for binding viruses, we acquired fluorescent images of virions binding to Vero E6 cells in the presence of LPGS, as shown in Figure 3(b-c). SARS-CoV-2 virions were prelabelled with DiOC18(3) (DiO) and then incubated with the inhibitors at 1 mg/mL for 45 min at room temperature. The treated virions were incubated on Vero E6 cells for another 45 min on ice, which can block the uptake of virions into the cells. After washing with PBS, the cells were labelled with 4′,6-diamidino-2-phenylindole (DAPI) and visualized by confocal laser scanning microscopy (CLSM) to determine the extent of virus binding to the cells. Without the inhibitor, the viruses bind to cells notably (Figure 3b). LPGS effectively blocks SARS-CoV-2 binding to Vero E6 cells. Automatic image analysis by ImageJ (Figure 3c) revealed that LPGS caused a >87.5% inhibition of virus binding. These results confirm the finding of the plaque reduction assays that LPGS can outperform the cell surface for viral binding and can therefore work as a binding decoy to inhibit SARS-CoV-2.

**Figure 3.** (a) Plaque reduction ratios for the samples at different inhibitor doses. Values are expressed as mean ±SD, n=4. Mw shown here refers to the unsulfated LPG. After sulfation, the Mw for LPGS_20kDa_94% and LPGS_20kDa_47% are 41kDa and 30kDa, respectively. (b) CLSM image for the virus binding to Vero E6 cells in presence of LPGS. Scale bar: 10 μm. (c) Analysis of virus binding to Vero E6 cells from CLSM images for the number of virions per cells. Values are expressed as mean ±SD, n=4. **p<0.01 from Student t-test. More detailed images are shown in Figure S2, Supporting Information.
Table 1. Summary of virus inhibition activity of polysulfates.

| Sample                  | Sulfation degree (%)b | Hydrodynamic size (nm)c | ζ-potential (mV) | IC50 (µg/mL)d | IC50 (µM) |
|-------------------------|-----------------------|-------------------------|-----------------|---------------|-----------|
| LPGS_20kDaa             | 94                    | 6.7 ± 3.3               | -26.1 ± 0.7     | 66.9 ± 32.0   | 1.6 ± 0.8 |
| LPGS_20kDa_47%          | 47                    | 6.5 ± 3.8               | -18.3 ± 0.6     | 679.7 ± 175.7 | 22.7 ± 5.9 |
| LPGS_7kDa               | 81                    | n. d. e                 | -29.1 ± 0.7     | >10000        | --        |
| HPGS_20kDa              | 91                    | 5.1 ± 2.3               | -24.9 ± 3.5     | 1909.0 ± 342.3| 47.7 ± 8.6 |
| HPGS_500kDa             | 85                    | 14.3 ± 7.7              | -21.2 ± 2.3     | 658.5 ± 492.5 | 0.7 ± 0.5 |
| HPGS_2.6MDa             | 82                    | 34.8 ± 12.2             | -17.0 ± 1.8     | >10000        | --        |
| Heparin                 | --                    | 10.9 ± 5.3              | -31.2 ± 1.9     | 4084.0 ± 396.3| 272.3 ± 26.4|
| Pentosan sulfate        | --                    | n. d. e                 | -29.8 ± 2.3     | 1310.0 ± 292.8| --        |

a The Mw refers to unsulfated LPG. b via elemental analysis. c via DLS. d plaque reduction assay. e not detectable.

Binding with RBD of SARS-CoV-2. In order to confirm direct interaction of the inhibitors LPGS and HPGS with the spike protein, we conducted affinity measurements against the RBD using microscale thermophoresis (MST) (Figure 4a and Table 2). In initial titration experiments against human ACE2, we determined a dissociation constant (Kd) of 359 nM. For the synthetic polysulfates LPGS and HPGS, we detected Kd values of 5 µM and 141 µM towards the RBD of SARS-CoV2. Heparin showed an affinity of 191 µM. In comparison to the difference in IC50 values for LPGS and heparin, similar Kd values for these ligands suggest that the occupation of the HS binding site of the RBD is the inhibitory mechanism. A three times lower affinity of HPGS compared to the IC50 value can be explained by an additional steric contribution of the rather inflexible HPGS sphere.

It should be noted that LPGS binds to the RBD in close vicinity to the ACE2 binding site. Even though some positively charged amino acids were noticed at the ACE2 binding site, our simulation shows that LPGS binds mostly to the highly positively charged pockets on the side of the RBD, i.e. HS binding site (Figure 4b and Figure S3, Supporting Information).

With LPGS established as the best ligand among the polysulfates tested here, analysis on compound-RBD binding were further conducted using mass spectrometry. Figure 4c shows the results of the mass spectrometry experiments with different amounts of heparin or LPGS added to the RBD solution.

The mass spectrum of the pure RBD exhibits two distinct groups of peaks: the first group in the 2500-3600 m/z range corresponds to the protein monomer in the 10-13+ charge states, while the group in the 3600-4600 m/z range corresponds to the RBD dimer in the 16-17+ charge states. The dimer signals generally exhibit a much lower intensity relative to the region assigned to the RBD monomer (Figure 4c, bottom spectrum). Furthermore, all peaks are broad and poorly resolved, which suggests heterogeneity that is most probably the result of post-translational modification. The molecular weight of the pure RBD was calculated to be ~34 kDa.

As compared to the pure RBD protein, the addition of unfractionated heparin to the RBD solution did not lead to a substantially different mass spectrum. (In a previous native mass spectrometry study, heparin-RBD binding was observed, though not with unfractionated heparin.
as used here, but rather with a much less heterogeneous, isolated 20mer.[21]) However, when we added LPGS to the RBD solution, the overall intensity in the 3600-4600 m/z region increased substantially with increasing LPGS:RBD ratios (Figure 4c; dark blue, green, and red traces). Given that the addition of LPGS solution lowered the absolute concentration of RBD in the sample, the increased signal intensity in the 3600-4600 m/z region was unlikely the result of increased RBD dimerization. Furthermore, we obtained one large and poorly resolved signal, instead of several at least partially resolved peaks as expected for oligomers. This suggests a high molecular heterogeneity in the species assigned to the same spectral region. The mean molecular weights of LPGS and RBD are ~40 kDa and ~34 kDa, respectively, and both are highly heterogeneous in weight. The increasing signal intensity in the 3600-4600 m/z region with increasing RBD:LPGS ratios therefore likely arises from the binding of the RBD to LPGS molecules.

Figure 4. (a) Affinity measurements of RBD of wild-type SARS-CoV-2 with LPGS, HPGS, heparin and ACE2 using MST. Each data point represents mean values with N ≥ 4 experiments, and the error bars show the standard deviation. Data points were fitted according to the mass-action law function to obtain Kd values (see Table 1). The differences in the slopes of the dose-response curves depend on changes of the hydration shell areas and effective charges, but do not affect the determinations of Kd-values from the inflection points of the curves. (b) Crystal structure of RBD bound with ACE2 (PDB ID: 6MOJ). ACE2 is shown in secondary structure representation (red), while RBD is shown in surface representation (green). The amino acid residues of RBD (R346, A348, A352, N354, R355, K356, R357, S359, Y396, K444, N450, R466, I468) found in MD simulations to form contacts with the polysulfates are highlighted in VDW representation (blue), denoting the putative HS-binding site. More detailed images are shown in Figure S3, Supporting Information. (c) Mass spectra of 4.0 µL RBD solution mixed with 0, 0.4,
0.8, and 1.2 µL heparin (light traces) or LPGS (dark trace). The charge states are marked with a single dot for the RBD monomer and with a double dot for the RBD dimer, while the calculated m/z for the 10-13+ charge states of the 34 kDa RBD are marked with orange lines.

Table 2. Summary of dissociation constants (Kd) are shown, together with the confidence values (±), indicating with 68% certainty the range where Kd falls.

| Sample         | Kd      |
|----------------|---------|
| ACE2           | 359 ± 49 nM |
| HPGS_20kDa     | 141.9 ± 33.3 µM |
| LPGS_20kDa     | 5.2 ± 3.6 µM  |
| Heparin        | 191.5 ± 57.7 µM |

MD simulations for studying interactions of LPGS and heparin with wild-type RBD. To test the hypothesis that polysulfates inhibit viral infection by electrostatics-mediated binding to the SARS-CoV-2 spike protein, we performed all-atom MD simulations of the RBD of the spike protein and LPGS/heparin in explicit water (see Figure 5 and Methods in Supporting Information for further details). We found that both LPGS and heparin form complexes with RBD (Figure 5a,b), the former being completely bound to RBD, whereas a part of the latter is free in solution. The plot of the number of contacts per amino acid residue reveals that both anionic polymers primarily interact with the cationic residues of RBD: namely R355, K356, R357, and R466 (Figure 5c). Further, we found for LPGS that, the total number of contacts with RBD per polymer’s molecular weight is 1.6 times the value for heparin (Figure 5c inset). Normalized to the charge unit, the value for LPGS is 3.2 times that of heparin. The absolute value of the total protein-polymer interaction energy is also larger for LPGS as compared to heparin (Figure 5e). This stronger binding of LPGS to RBD correlates well with its superior virus inhibition efficacy as observed experimentally.

To understand this surprising, stronger binding affinity of LPGS despite its smaller linear charge density relative to heparin, we characterized the flexibility of both polymers in terms of their end-to-end distances (R) and persistence lengths (P). As shown in Figure 5d, the R distribution for LPGS is wider than that for heparin, implying a higher flexibility of LPGS. From the values of R and contour length (L0) of the polymers, we estimated P of LPGS to be 3 times smaller than that of heparin (see Figure 5d inset for all the values and see Supporting Information for the calculation details). The higher flexibility of LPGS helps adjust its conformation to the heterogeneous surface topography of RBD, which in turn leads to its enhanced binding. Therefore, in the future design of polymers that binds to SARS-CoV-2 spike protein, both the backbone flexibility and the charge density of polymers should be simultaneously optimized for effective binding to spike proteins.
MD simulations of interactions of LPGS with RBD mutants. The successful comparison between experimental and simulated results for wild-type RBD-LPGS interactions encouraged us to indirectly test the effectiveness of LPGS in inhibiting SARS-CoV-2 mutants via simulations: specifically those exhibiting the E484K and N501Y mutations in the RBD.[22] We found that LPGS forms complexes with both RBD mutants (Figure 5f). As in the case of wild-type RBD, LPGS interacts mostly with the mutants’ cationic residues, as indicated in the per-residue contacts plot (Figure 5f). From the total number of contacts (Figure 5f inset) and interaction energies (Figure 5e), we found that LPGS binds to N501Y RBD as effectively as to wild-type RBD, but more tightly to E484K RBD, which is consistent with the presence of an extra cationic residue on this mutant surface. The results of our MD simulations thus suggest that LPGS could also work successfully in inhibiting SARS-CoV-2 mutants.

Biosafety evaluations. To further exclude the side effects of cellular toxicity for virus inhibition, we tested the inhibitors with three different cell lines, including Vero E6, A549, and human
bronchial epithelial (HBE) cells. An evaluation of LPGS’s toxicity to A549 and HBE cells can reveal its safety for potential clinical applications. As shown in Figure S4, Supporting Information, LPGS did not show any cellular toxicity up to a dose of 10 mg/mL, revealing a half-maximal cytotoxicity concentration (CC₅₀) value higher than 10 mg/mL. Selectivity index was calculated by comparing IC₅₀ with CC₅₀. LPGS yielded a selectivity index higher than 150, affirming the potential of LPGS for preclinical testing.

Furthermore, the anticoagulation activity of LPGS was investigated by activated partial thromboplastin time (aPTT), as shown in Figure S5, Supporting Information. With similar charge density, LPGS shows much lower anticoagulation activity than heparin. At a concentration of 5 μg/mL, heparin leads to complete anticoagulation of plasma, while a concentration of 25 μg/mL of LPGS is required to yield similar effect. This variance is caused by different core structures. Heparin can bind specifically and strongly to antithrombin and inhibit blood coagulation.[35-36] Earlier studies of heparin-mimicking polymers indicated an important role of saccharide units in anticoagulation activities.[37-38] Glycerol-based polymers therefore have a weaker anticoagulant effect than heparin.

Conclusion

In this study we investigated the inhibition of SARS-CoV-2 by polysulfates of different sources (natural and synthetic), different architectures (linear and hyperbranched), different molecular weights (7 kDa to 2.6 MDa) and different degrees of sulfation (~100% and ~50%) by authentic SARS-CoV-2 plaque reduction assays. Using MD simulations, we demonstrated that the positively charged patch near the RBD of SARS-CoV-2 is responsible for the binding of the spike protein to the HSPG located on the cell surface. LPGS_20kDa with 94% sulfation stands out as the most promising SARS-CoV-2 inhibitor, with an IC₅₀ of 67 ± 32 μg/mL. Its inhibitory activity is roughly 61-fold higher than heparin.

We also showed that architecture, molecular weight, molecular flexibility, and sulfation can influence SARS-CoV-2 binding and inhibition. For future design of SARS-CoV-2 inhibitors, these factors should be carefully considered and evaluated for the rational design. With the MD simulations, we were further able to demonstrate that LPGS can bind to the RBD of virus variants, and conclude that LPGS might inhibit infection by variants carrying the E484K and N501Y mutations. Further experiments are needed to elucidate the structural details in RBD variations and their impact on infectivity and inhibitor binding.

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**Keywords:** polysulfates • SARS-CoV-2 • inhibition • virus binding • electrostatic interactions

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Supporting Information

Polysulfates block SARS-CoV-2 uptake via electrostatic interactions

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Experimental Procedures

Materials. Anhydrous solvents (dimethylformamide and toluene), benzoylated cellulose dialysis tubes (2000Da, 32 mm width) and heparin (sodium salt from porcine intestinal mucosa, H3393-500KU) were purchased from Merck (Darmstadt, Germany). Pentosan sulfate is obtained from Bene Arzneimittel GmbH, Munich, Germany. All other chemicals were bought from Merck (Darmstadt, Germany) unless stated otherwise.

Methods. Elemental composition determination was performed on a Vario EL CHNS element analyzer by Elementar Analysensysteme GmbH (Langselbold, Germany). 1H NMR spectra were recorded on a Bruker AMX 500 (Bruker Corporation) or Jeol ECP 500 (JEOL GmbH). Chemical shifts (δ) are reported in ppm via the deuterated solvent peak as the standard. GPC measurements in water were performed with an Agilent 1100 equipped with an automatic injector, isopump, and Agilent 1100 differential refractometer (Agilent Technologies, Santa Clara,
The PSS Suprema (pre-column, 1x with pore size of 30 Å, 2x with pore size of 1000 Å (all of them with a particle size of 10 µm) column, was calibrated against Pullulan standards prior to measurements. The GPC measurements in THF were done with an Agilent Security (1200 Serie), equipped with automatic injector, isopump, and UV and RI detector. The separation was done via a PL gel from Agilent (1x pre-column, 3x Mixed-C with a particle size of 5 µm), which was calibrated against polystyrene standards. The GPC measurements in water were performed on an Agilent 1100 from Agilent technologies equipped with isopump. The column was calibrated with pullulan standards prior to measurements.

**Synthesis of linear polyglycerol sulfate (LPGS).** Linear polyglycerol (LPG) was firstly synthesized by anionic ring opening polymerization of ethoxyethyl glycidyl ether,[1-2] followed by deprotection in slightly acidic media.

**Synthesis of ethoxyethyl glycidyl ether (EEGE).** The acetal protection of glycidol was done slightly modified to a reported protocol.[3] In summary, in an ice bath glycidol (70 mL, 1.052 mol, 1 eq.) of glycidol was mixed under stirring with divinyl ether (403.3 mL, 4.21 mol, 4 eq.) and p-TsOH.H2O (2 g, 0.0105 mol, 0.01 eq.) was slowly added to the mixture. After 4 hours the reaction was quenched and washed with saturated NaHCO3 solution. The organic phase was dried over sodium sulfate and concentrated under reduced pressure. The crude product was dried over CaH2 and distilled under vacuum over preheated molecular sieve and stored under argon in freezer until further use. Due to storage under dry and inert conditions, weighing of the final product was not possible and complete conversion of starting material is assumed.

1H NMR (500 MHz, ACETONE-D6) δ 4.70 (qd, J = 5.3, 2.4 Hz, 1H), 3.77 (dd, J = 11.5, 3.0 Hz, 1H), 3.73 – 3.57 (m, 1H), 3.50 – 3.41 (m, 1H), 3.30 (dd, J = 11.5, 6.4 Hz, 1H), 3.12 – 3.00 (m, 1H), 2.69 (ddd, J = 5.3, 4.1, 2.1 Hz, 1H), 2.52 (ddd, J = 11.9, 5.2, 2.6 Hz, 1H), 1.22 (t, J = 5.3 Hz, 3H), 1.12 (td, J = 7.1, 1.1 Hz, 3H).

**Polymerization of ethoxyethyl glycidyl ether (PEEGE) and deprotection to LPG.** The polymerization was done based on a reported protocol. (Macromolecules 2010, 43 (4), 1778-1784.) In summary for 20 kDa LPG: In a flame-dried Schlenck flask Oct4NBr (172.78 mg, 0.310 mmol, 0.0047 eq.) was dried under high vacuum and dissolved in 60 mL dry toluene. Afterwards EEGE (10 mL, 65.6 mmol, 1 eq.) was added under inert conditions. Then in an ice bath i-Bu3Al (1.4 mL, 1.55 mmol, 0.023 eq.) was added all at once. The reaction was let to proceed overnight, thereafter quenched by addition of 1 mL of EtOH. The excess of activator was precipitated in cold Et2O. The product was dialyzed in acetone (MWCO: 2kDa) for further purification. After
drying, the product was obtained as 8.38 g colorless viscous oil (50%). (GPC THF- \( M_n \): 32.4 kDa, PDI: 1.07). \(^1\)H NMR (500 MHz, ACETONE-D6) \( \delta \) 4.81 – 4.60 (m, 1H), 3.83 – 3.32 (m, 7H, monomer unit), 1.33 – 1.22 (m, 3H), 1.14 (t, 3H). The acetal groups were then deprotected in a solution of 3% HCl (37%) in EtOH. The mixture was stirred overnight and then purified by dialysis against water (MWCO:2 kDa). (GPC water- \( M_n \): 17.7 kDa, PDI: 1.2).

**Synthesis of hyperbranched polyglycerols.** Hyperbranched polyglycerol (hPG) was synthesized by ring-opening multibranching polymerization of glycidol in a heterogeneous reaction mixture in dioxane. This method only allowed the molecular weight of hPG to be increased to only 800-900 kDa. To obtain the high-molecular weight hPG, a two-step polymerization is used as reported in our previous studies.[4]

First, a macroinitiator was synthesized in a heterogeneous reaction mixture in dioxane. Dry trimethylolpropane (120 mg, 0.89 mmol, 1.0 eq.) was partially deprotonated (30 % OH) with potassium methoxide (67 \( \mu \)L, 0.27 mmol, 0.3 eq., 25 % in methanol) in argon atmosphere at 60 °C for 30 min. After the addition of 24 mL dioxane (dry) the turbid mixture was heated to 100 °C. Glycidol (12 mL, 0.18 mol, 201 eq.) was slowly added (0.5 ml h\(^{-1}\)) via syringe pump into the reaction mixture. The polymer was purified by removing the dioxane, precipitation as methanolic solution in acetone and dialysis against water in regenerated cellulose membrane (10 kDa MWCO). The resulted hPG was obtained with a yield of 93.6 %.

In the second step, this hPG was used as macroinitiator to grow the polymer further. 2.5 g (0.034 mol, total OH groups) of the lyophilized polymer was dissolved in dry DMF (35 mL). The polymer was partially deprotonated with the addition of potassium hydride in oil (30 wt.%) (80 \( \mu \)g, 272 \( \mu \)L, 2.0 \( \mu \)mol). The temperature was increased to 100 °C and glycidol (25 mL, 0.37 mol) were added with a rate of 0.9 mL h\(^{-1}\). After precipitation in acetone and dialysis against water in regenerated cellulose membrane (50 kDa MWCO) the resulted molecular weight was 2.6 MDa with a \( D \) of 1.4.

**Sulfation of the polyglycerols.** All the polymers were sulfated according to an already published protocol with slight adjustments[5] The completely dry corresponding polymer was dissolved in dry DMF (10 mL for 1 g). The mixture was then heated up to 60 °C and respective amount of SO\(_3\)/pyridine (1.5 eq. of -OH groups) was added. The mixture was let stir overnight under inert atmosphere. Thereafter the pH was brought to 13 by addition of 1M NaOH to the solution. Then the polymer was dialyzed against saturated solution of NaCl for 2 days and 2
days in water. After drying in high vacuum, the crude product was obtained as white solid powder. The degree of functionalization was determined via elemental analysis.

**Plaque reduction assay.** SARS-CoV-2 München (SARS-CoV2M; BetaCoV/Germany/BavPat1/2020) was propagated on Vero E6 cells and titrated via plaque assay.[6] For a plaque reduction assay, Vero E6 cells were grown in a 12-well plate. The virions (approx. 100PFU) were firstly incubated with the compound at different concentrations for 1 hour, prior to the incubation with Vero E6 cells for 1 hour. The cells were washed with PBS once and then cultured in Avicel overlay medium for 2 days. After being fixed by the addition of 4% formaldehyde, the cells were stained with 0.75% crystal violet (aqueous solution) to count plaques. The experiment was performed in BSL3 laboratory at the Institut für Virologie, Freie Universität Berlin. The plaque reduction ratio was calculated by comparing treated samples with the non-treated virus controls as follows:

$$\text{Plaque reduction (\%)} = (1 - \frac{\text{Plaque number (sample)}}{\text{Plaque number (virus control)}}) \times 100\%$$

The IC50 value was estimated by ‘[inhibitor] vs. response’ model in GraphPad Prism 7.0.

**Virus binding to Vero E6 cells.** The virions were inactivated by 4% formaldehyde for 24 hours in BSL3 under appropriate biosafety conditions. The formaldehyde inactivated SARS-CoV-2 virions were purified and concentrated by ultracentrifugation (100,000 rpm, 2h) with 20% sucrose solution. The pellet was resuspended in PBS and stored at -80 °C before usage.

To study virus attachment, the virions were firstly labelled with 3,3′-Dioctadecyl-oxacarbocyanin-perchlorate (DiO, ThermoFisher Scientific, USA). For the labelling, 100 µL virus solution was incubated with 5 µL 20 µM DiO (ethanol) for 45 min. Then, the free dye was removed by spinning column (Protein A HP SpinTrap™, GE Healthcare, Germany). For the inhibitor binding, 10 µL labelled virions was incubated with 90 µL inhibitor solution (1000 µg/mL) for 45 min at 37 °C. The mixture was incubated with Vero E6 cells for 1 h on ice. The unbound virus was removed by washing with PBS. The cell nucleus was stained with DAPI, and then the cells were visualized by confocal laser scanning microscopy (SP8, Leica, Germany).

**Image analysis for virus binding.** Quantification of bound virus particles per cell was performed by a self-written ImageJ macro. The nuclei were segmented from the background using a machine learning classifier of the Trainable Weka Segmentation plugin of ImageJ. The output probability maps of the nuclei were blurred with a Gaussian filter (radius = 5) and the
touching nuclei were segmented by thresholding the background and finding local maxima (Process > Find Maxima... > Output type: Segmented Particles). The automatically detected nuclei were then manually checked and recounted and corrected if necessary. Images showing the virus particles were scaled up by a factor of 3 using bicubic interpolation to decrease pixel noise. Quantification was also performed by thresholding the background and finding local maxima which correspond to the virus particles. Virus particles per cell were determined for 4 images per sample which were then used to calculate mean and standard deviation. Paired t-tests were used to find statistically significant differences between samples.

**Mass spectrometry experiments.** 1 mg/mL RBD (Thermofisher, Germany), 14.6 mg/mL LPGS, and 7.6 mg/mL heparin solutions were purified by filtering through an Amicon® Ultra 3 kDa molecular weight cut-off filter (Merck, Germany) twice, which also facilitated the buffer exchange into 250 mM pH 7 ammonium acetate solution. After their purification, the LPGS and heparin solutions were diluted five-fold, and 0.4 µL, 0.8 µL, and 1.2 µL of them were mixed respectively with 4.0 µL RBD solution. The samples were loaded into Pd/Pt-coated nano-ESI capillaries produced in-house and introduced in positive ion mode at a capillary voltage of 1000 V into a Synapt G2 S instrument modified with a linear drift tube ion mobility cell. The spectra were recorded in TOF-only mode to provide softer conditions for the formed complexes in the gas phase. At least two data sets were acquired for each sample, and spectra were collected with 1 s acquisition time for 5-20 min depending on the signal intensity. Due to the natural heterogeneity of the LPGS and heparin samples, background-correction was necessary for both the RBD-LPGS and the RBD-heparin data sets. For background correction, the spectra of the pure LPGS and heparin were acquired, mean-smoothed, and normalized for an m/z position where the pure RBD spectrum exhibits low signal intensity, while the pure LPGS or heparin spectrum exhibit high signal intensities; then, the as-normalized LPGS or heparin spectrum was subtracted from the corresponding mean-smoothed RBD-LPGS or RBD-heparin spectra.

**Microscale thermophoresis.** Experiments were conducted with a Monolith NT.115 (NanoTemper) from the laboratory of Prof. Heberle (FU Berlin). For the measurements His-tagged RBD of the SARS-CoV-2 S-protein from recombinant expression in mammalian cells was kindly provided by Dr. Coskun (TU Dresden). For the experiments, twofold dilution series of human ACE2 (novoprotein, kindly provided by Prof. Bader at MDC Berlin), LPGS, HPGS and heparin were mixed with a final concentration of 50 nM NHS-Red (NanoTemper) labeled RBD.
Protein labeling and purification was performed with a 2\textsuperscript{nd} generation NHS-Red labeling kit. The labeling efficiency was analyzed from spectroscopic measurements (Nanodrop) to be approximately 1:1 (protein:dye). The measurements were performed in DPBS and premium capillaries (NanoTemper) at 25°C, and default settings for the measurement runs (Initial fluorescence=5s, thermophoresis=30s, and recovery=5s). Obtained data were analyzed with a gating strategy 1.5 s after the start of the infrared laser, and data were fitted as previously shown.[7]

**MD simulations.** The coordinates for the wild type RBD of the SARS-CoV-2 spike protein were obtained from the deposited crystal structure (PDB ID: 6M0J). Two different RBD mutants (E484K or N501Y mutations) were built using PyMOL. The structure of the heparin pentamer was built using CHARMM-GUI Glycan Reader & Modeler.[8] The structure of LPGS undecamer was built using Avogadro software.[9] CHARMM36m[10] and CHARMM Carbohydrates[11][12] force field parameters were used to model the protein and Heparin, respectively. Parameters and partial atomic charges for LPGS were obtained using the CGenFF program[13],[14] and CHARMM General force field.[15][16] CHARMM-compatible TIP3P water[17][18] and ion parameters[19] were used. RBD/LPGS and RBD/Heparin were arranged and solvated in boxes of sizes 7×7×9.5 nm\(^3\) and 7×7×10 nm\(^3\), as shown in Figure 5a,b (manuscript), respectively. Enough Na\(^+\) ions were added to charge neutralize each system, then Na\(^+\) / Cl\(^-\) ion pairs were added to obtain a 150 mM NaCl solution estimated from the mole fraction of ion pairs and water. The simulation for each case was performed at least for 500 ns in the \textit{NpT} ensemble at \(T = 300\) K and \(p = 1\) bar with periodic boundary condition in \textit{xyz} directions, using GROMACS 2020.1 package.[20] The stochastic velocity rescaling thermostat[21] with a time constant of \(\tau_T = 0.1\) ps was used to control the temperature, while for the pressure control an isotropic Parrinello-Rahman barostat[22] was used with a time constant of \(\tau_p = 2\) ps and a compressibility of \(\kappa = 4.5\times10^{-5}\) bar\(^{-1}\). The LINCS algorithm[23] was used to constrain the bonds involving H-atoms, allowing a timestep of \(\Delta t = 2\) fs. Electrostatics interactions were computed using the particle mesh Ewald (PME) method[24] with a real-space cutoff distance of 1.2 nm, while van der Waals (VDW) interactions were modeled using Lennard-Jones potentials with a cutoff distance of 1.2 nm where the resulting forces smoothly switch to zero between of 1 nm to 1.2 nm.

**Simulation data analysis.** The electrostatic potential map of the protein was calculated using the APBS tool[25] and visualized using VMD.[26] Simulation snapshots were rendered using VMD as well.
**Number of close contacts:** A contact is defined by an atom of LPGS/Heparin falling within 3 Å of any atom of the protein residue. The total number of contacts averaged over the last 100 ns of simulation data is presented in the main text.

**Persistence length:** The persistence length \( P \) of a polymer can be estimated from the polymer’s Kuhn length \( b \), using the well-known relationship for the *worm like chain* model: \( P = b^2/2 \). The Kuhn length, \( b \), is related to the contour length \( L_0 \) and the end-to-end distance \( R \) of a polymer via the expression: \( b = \langle R^2 \rangle/L_0 \), where \( \langle \cdot \rangle \) represents the time average. So, the persistence length was calculated using \( P = \langle R^2 \rangle/2L_0 \), where \( \langle R^2 \rangle \) and \( L_0 \) were obtained from 200 ns of simulation data where the polymer is not attached to the protein. \( L_0, R, \) and \( P \) for both LPGS and Heparin are given in the inset of Figure 5d in the main text.

**Cell viability.** The cells, including A549 cells, Vero E6 cells and human bronchial epithelial cell (HBE), were seeded in a 96-well plate at a density of \( 1 \times 10^4 \) cells/well in DMEM medium. After 24 hours, the compounds were added to each well at a final concentration of 0.1 μg/mL to 1 mg/mL. The cells were cultured for another 24 hours with compounds dispersed in the medium. Finally, the viabilities of the cells were investigated with a CCK-8 assay according to the manuals, for which the result was revealed by optical absorbance at 450 nm. Cell without any compounds was set as positive control, while 1% SDS solution was set as the negative control. The biocompatibility of the compounds was studied by comparing with the positive and negative controls as shown follows:

\[
\text{Cell viability (\%)} = \left( \frac{\text{ABS450 (compounds)} - \text{ABS450 (negative control)}}{\text{ABS450(positive control)}} \right) \times 100\%
\]

**Activated partial thromboplastin time (aPTT).** The anticoagulating activity for the samples was revealed by activated partial thromboplastin time of the plasma treated with the samples.[27] Briefly, 100 μL plasma (Siemens Healthcare, Erlangen, Germany) and 100 μL Actin FS (Siemens Healthcare, Erlangen, Germany) were mixed and incubated (3 min, 37 °C) with 4 μL samples in different concentrations. H2O was set as the control. The reaction was started by the addition of 100 μL of prewarmed (37 °C) clotting activator CaCl₂.

**Results and Discussion**
Figure S1. Plaque images for SARS-CoV-2 treated with different compounds at 50μg/mL.

Figure S2. (a) CLSM image for the virus binding to Vero E6 cells in presence of the compounds. Scale bar: 10 μm. (b, c) Analysis of virus binding to Vero E6 cells from CLSM images for the number of virions. Values are expressed as mean ±SD, n=4. The addition of concentrated heparin (1 mg/mL) can also reduce the binding of SARS-CoV-2, probably because of steric shielding of virions by heparin at high dose.
Figure S3. Distribution of HS binding site on the RBD of SARS-CoV-2. ACE2 is shown in secondary structure representation (red), whereas RBD is shown in surface representation (green). The amino acid residues of RBD (R346, A348, A352, N354, R355, K356, R357, S359, Y396, K444, N450, R466, I468) found to form contacts with the polysulfates in MD simulations are highlighted in VDW representation (blue), denoting the putative HS-binding site.

Figure S4. Evaluation of cellular toxicity of polyglycerol sulfates, against A549, Vero E6 and HBE cells, respectively. Values are expressed as mean ±SD, n=3.
Figure S5. Activated partial thromboplastin time (aPTT) of plasma treated with polysulfates at different concentrations. Values are expressed as mean ±SD, n=3.

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