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Review

The diverse role of heparan sulfate and other GAGs in SARS-CoV-2 infections and therapeutics

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ABSTRACT

In December 2019, the global coronavirus disease 2019 (COVID-19) pandemic began in Wuhan, China. COVID-19 is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which infects host cells primarily through the angiotensin-converting enzyme 2 (ACE2) receptor. In addition to ACE2, several studies have shown the importance of heparan sulfate (HS) on the host cell surface as a co-receptor for SARS-CoV-2-binding. This insight has driven research into antiviral therapies, aimed at inhibiting the HS co-receptor-binding, e.g., by glycosaminoglycans (GAGs), a family of sulfated polysaccharides that includes HS. Several GAGs, such as heparin (a highly sulfated analog of HS), are used to treat various health indications, including COVID-19. This review is focused on current research on the involvement of HS in SARS-CoV-2 infection, implications of viral mutations, as well as the use of GAGs and other sulfated polysaccharides as antiviral agents.

1. Introduction

The recent coronavirus disease 2019 (COVID-19) pandemic, which began as an outbreak of a novel virus in Wuhan China in late 2019, soon swept the globe. The causative agent is severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a member of the family *Coronaviridae*. SARS-CoV-2 is closely related to the two major coronaviruses that previously caused epidemic outbreaks, i.e., Middle East Respiratory Syndrome (MERS) and SARS-CoV-1 (Chen, Boon, Wang, Chan, & Chan, 2021), however, neither resulted in a pandemic. Coronaviruses are referred to as zoonotic viruses, which are often transmitted by so called spill-over events, when humans and animals are in close

Abbreviations: Ac, acetate; ACE2, angiotensin-converting enzyme 2; ASGR-1, asialoglycoprotein receptor 1; CS, chondroitin sulfate; CD4, cluster of differentiation 4; COVID-19, coronavirus disease 2019; hCoVs, coronavirus strains are infectious to humans; CP, cytoplasm domain; dp, degree of polymerization; DS, dermatan sulfate; E, envelope; EXT1, exostosin glycosyltransferase 1; GalN, galactosamine; GlcN, glucosamine; GlcA, glucuronic acid; GAGs, glycosaminoglycans; HS, heparan sulfate; HP, heparin; HP-1, heptad repeating domain-1; HP-2, heptad repeating domain-2; HSase, heparin lyases; HIV, human immunodeficiency viruses; HA, hyaluronic acid; IdoA, iduronic acid; IL-6, interleukin 6; KS, keratan sulfate; LMWH, low molecular weight heparin; MS, mass spectrometry; M, membrane; MERS, Middle East respiratory syndrome; MD, molecular dynamic; NDST1, *N*-deacetylase/*N*-sulfotransferase 1; NACH, non-anticoagulant low molecular weight heparin; TRiS, non-anticoagulant trisulfated heparin; NTD, N-terminal domain; NMR, nuclear magnetic resonance; N, nucleocapsid; PAMP, pathogen-associated molecular pattern; PGs, proteoglycans; RBD, receptor binding domain; RBM, receptor binding motif; RAHMM, receptor for hyaluronan-mediated motility; SARS-CoV-1, severe acute respiratory syndrome coronavirus 1; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; ss, single stranded; S-protein, spike protein; S, sulfate; SPR, surface plasmon resonance; TM, transmembrane domain; TMPRSS-2, transmembrane protease serine protease-2; AXL, tyrosine-protein kinase receptor; UFH, unfractionated heparin; VOCs, variants of concern; VLP, virus like particle.

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contact (Bauer, Zhang, & Linhardt, 2021; Zhong et al., 2003). The outcome of a spill-over event is determined by a successful zoonotic infection process. Thus, analogous receptors between the species are a prerequisite. One commonly known receptor is heparan sulfate (HS), which is used by many viral agents for binding, e.g., hepatitis virus, human immunodeficiency viruses (HIV), influenza virus, herpes virus (Bauer et al., 2021; Cagno, Tseligka, Jones, & Tapparel, 2019; Kamhi, Joo, Dordick, & Linhardt, 2013). HS co-receptor-binding has also been reported for different coronaviruses (Cagno et al., 2019; Haan et al., 2005; Lang et al., 2011; Madu et al., 2007; Milewska et al., 2014; Vicenzi et al., 2004), including SARS-CoV-2 (Clausen et al., 2020).

HS is an anionic polysaccharide, belonging to the group of glycosaminoglycans (GAGs). GAGs are ubiquitously present in all animal cells, particularly in the outer membrane and extracellular matrix of tissues. Here, a complex arrangement of fibrous proteins with proteoglycans (PGs) containing GAG chains, gives support and functionality to the cells themselves (Kovensky, Grand, & Uhrig, 2017). At the cell surface, GAGs interact with a variety of proteins, playing an important role in many biological processes (Nakato, Desai, & Balagurunathan, 2015). This property and their availability from natural sources have made GAGs a target for pharmaceutical applications for decades. Heparin (HP) is the best-known GAG therapeutic, and along with its mimetics, HP shows activity against coagulation, thrombosis, cancer, and inflammatory diseases (Linhardt, 2003; Mohamed & Coombe, 2017; Silverman, Santucci, & Sekeyova, 1991). GAGs can serve as portals of viral entry as well as antiviral agents, they are involved in complex processes throughout virus replication and cell metabolism, and they can serve as inhibitors as well as amplifiers of enzymes and marker molecules for controlling the symptoms of the infection, including the disease itself (Shi, Sheng, & Chi, 2021). Thus, the role of GAGs in viral diseases is highly intricate.

Although SARS-CoV-2 may now be the most thoroughly studied virus in history, there are still few specific therapeutics and vaccines available against human coronaviruses. Vaccines developed against SARS-CoV-2 need to be updated on a regular basis. Development of effective and targeted treatments is necessary and might be facilitated by: (1) in-depth knowledge of the involvement of GAGs in the infection process; (2) the accompanying immune response; and (3) the potential of HP, HP analogs and other sulfated polysaccharides as inhibitory substances. This review is focused on these three topics. We examine the interactions of GAGs with SARS-CoV-2, suggest putative GAG-binding sites on the SARS-CoV-2 spike protein, and describe the potential influence of viral mutations on these interactions, the HS-dependent infection process, anti-SARS-CoV-2 activities of different GAGs and GAG analogs, and a study on the use of GAGs as therapeutics in COVID-19 patients. The significance of this review lies in the comprehensive summary of the complex involvement of HS and other GAGs with the SARS-CoV-2 virus. Based on the reviewed literature, we propose that the infection process of SARS-CoV-2 depends strongly on HS-binding, and that the mutations of SARS-CoV-2 so far increased the affinity towards HS-binding. Additionally, we anticipate GAG-based therapeutics to be effective antiviral agents against SARS-CoV-2 and hope to inspire other researchers to further explore this field.

1.1. Structures of GAGs

GAGS are linear negatively charged polysaccharides that have similar simple backbones of repeating disaccharides units (Yan et al., 2021). However, their overall structure can be extremely complex due to an extraordinary number of combinations of residue types, sulfation levels and positions, glycosyl bonds, and variable chain lengths (Shi et al., 2021). These patterns divide GAGs into four main types: HP/HS, chondroitin sulfate (CS)/dermatan sulfate (DS), keratan sulfate (KS), and hyaluronic acid (HA). HA is linked to cell surface proteins through non-covalent bonding to the cluster of differentiation 4 (CD4) receptor or receptor for hyaluronan-mediated motility (RAHMM), while the other GAGs in Fig. 1 are covalently bound to cell membrane core proteins (Misra, Hascall, Markwald, & Ghatak, 2015). The structure of HP and HS share high similarity, differing in their level of sulfation and epimerization. HP is composed of repeating α-L-iduronic acid or β-glucuronic acid residues, which are linked to glucosamine. Typical sulfation occurs at *N*-, 6- and 3-*O*-positions on the α-glucosamine residue and at 2-*O* on the hexuronic acid-residue. The 3-*O*-sulfo group is essential for the anticoagulant activity (Capila & Linhardt, 2002; Linhardt, 2003).

1.2. The nature of GAG-protein interactions

The poly-anionic nature of GAGs originates from their sulfate and carboxylate groups. Clusters of positively charged amino acid residues, e.g., lysine and arginine, are often observed in binding domains of HS, interacting with the negatively charged sulfate and carboxylate groups (Hileman, Fromm, Weiler, & Linhardt, 1998). GAG-binding is favored in regions of positive charge within a protein. However, the binding is not simple to predict. Arginine residues bind more tightly than lysine residues although their net charge is identical (Hileman et al., 1998). Histidine can also be positively charged, particularly in low pH environments such as lysosomes. Furthermore, a critical spacing between the basic residues has been postulated. GAG-binding is expected at amino acid sequences of 'XBBXBX' and 'XBBBXXBX', which are

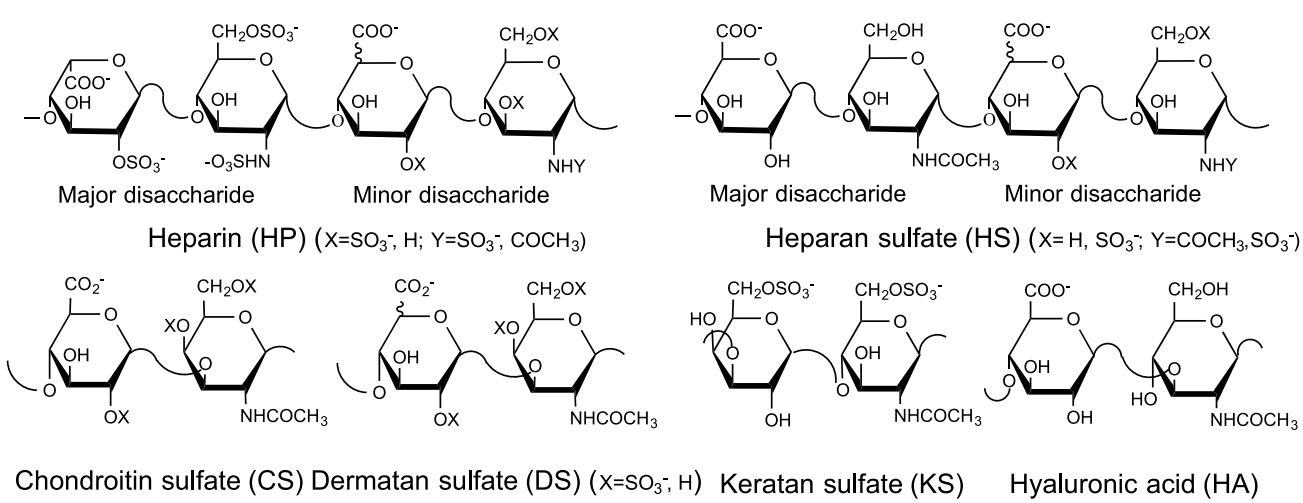

Fig. 1. Structures of GAGs. HP, HS, and HA contain a glucosamine (GlcN) residue while CS and DS contain a galactosamine (GalN) residue. The hexosamine residue of HA, KS, CS and DS are *N*-acetylated (Ac) while the hexosamine residue of HP and HS can be *N*-Ac or *N*-sulfated (S). The uronic acid residues of HA and CS are glucuronic acid (GlcA) while the uronic acid of HP, HS, and DS can be GlcA or iduronic acid (IdoA). All GAGs except for HA can be *O*-*S*.

referred to as Cardin-Weintraub motifs (Cardin & Weintraub, 1989). Due to the size of GAGs, secondary structures within GAG-binding proteins can also correspond to Cardin-Weintraub motifs. One possible consensus sequence is 'TXXBXXTBXXXTBB', which brings the basic residues in proximity due to turns (Hileman et al., 1998). The arrays consist of hydrophatic (X) residues, basic (B) residues, and turns (T). In addition to the binding of consensus sequences, non-ionic binding occurs through hydrogen bonding, hydrophobic interactions or van der Waals interactions (Capila & Linhardt, 2002).

For some GAG-protein interactions, specific requirements of the carbohydrate sequences or sulfation patterns have been observed, e.g., for antithrombin binding by HP, where the 3-*O*-sulfo group is indispensable (Sarrazin, Lamanna, & Esko, 2011). Thus, such specific arrangements of sulfated sugars are worth investigating for ligand binding. However, currently for most binding of GAGs to proteins it is assumed that both specificity, e.g., domains on GAGs, and non-specificity, e.g., electrostatic interactions (Yu et al., 2020) play a combined role.

Proteins and their GAG binding partners, need to be extensively characterized to study the interaction of GAGs and proteins. While it is a straightforward process to determine the structure of proteins, elucidating GAG structure continues to be challenging. Commonly applied methods for structural determination are nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), and X-ray crystallography (Yang & Chi, 2017). Oligosaccharides with structurally defined sequences are often used in a microarray high throughput format to study specific interactions with proteins (Hao et al., 2021; Liu et al., 2021; Watanabe, Takeda, Hiemori, Minamisawa, & Tateno, 2021; Yu et al., 2020). Other popular methods, used in affinity studies of protein-GAG-interactions, are surface plasmon resonance spectrometry (SPR), affinity chromatography or isothermal calorimetry (Shi et al., 2021). In addition to lab-based binding assays, putative protein-GAG-binding can be determined by the comparison of amino acid sequences to other proteins known to bind GAGs. One method is the identification of so-called Levenshtein distances, which encodes the similarity of the amino acid residues. Furthermore, computational predictions, such as homology modeling, electrostatic potential energy determination, MD, and molecular docking, of binding sites can aid in the determination of GAG-protein interactions. Additionally, novel binding sites can be identified (Yu et al., 2020). Which computational method is most appropriate and in which setting, has been previously discussed (Sankaranarayanan, Nagarajan, & Desai, 2018).

1.3. GAGs as receptors in the human body

GAGs are commonly found in both the cell interior and in the glycocalyx surrounding most cells (Shi et al., 2021) in all vertebrates and invertebrates (Cagno et al., 2019). Heparan sulfate proteoglycans (HSPG), comprise most of the GAGs in the extracellular matrix and influence membrane organization and cell adhesion. HSPGs further bind to many proteins, e.g., cytokines, chemokines, and growth factors, influencing a variety of signaling pathways (Shi et al., 2021).

The concentration of the different GAGs in the human body varies among the different tissue types, which emphasizes their specific role in physiology and pathophysiology (Linhardt & Toida, 2004). Furthermore, HS composition varies with age and among individuals. Mutations in the HSPGs are associated with many diseases and can impact susceptibility to infectious diseases (Sarrazin et al., 2011). Moreover, the cell specific GAG pattern is known to be a portal of binding and entry for many pathogens like viruses (Bauer et al., 2021; Kamhi et al., 2013; Kim, Li, & Linhardt, 2017; Kovensky et al., 2017). The negative charge of the HSPGs interacts with basic residues of viral surface glycoproteins or capsid proteins. Several viral pathogens use these receptors to facilitate entry (Bauer et al., 2021; Cagno et al., 2019). The presence of HSPGs on most human cells could abet a broad cell tropism of HS-receptor-binding viruses. However, there is controversy on HS promotion of virus dissemination and virulence (Cagno et al., 2019).

Due to the key role in viral infection processes, GAGs can serve as antiviral agents. There has been an increase in examining HP as an antiviral agent. Indeed, as early as the 1960s the first antiviral studies were conducted on HP (Park, 2016). GAG analogs include the charged polysaccharides, carrageenan and cellulose sulfate (Cagno et al., 2019). Their anti-infective effects, however, have often been negligible when applied as systemic drugs. For further reading, we refer to these comprehensive reviews (Kovensky et al., 2017; Mohamed & Coombe, 2017; Wang et al., 2022).

2. SARS-CoV-2 – host cell interaction

Currently, seven coronavirus strains are infectious to humans (hCoVs), causing respiratory disease; two Alpha-coronaviruses and five Beta-coronaviruses. However, three of the Beta-coronavirus strains are the causative agents of severe endemic and pandemic diseases, i.e., MERS-CoV, SARS-CoV-1, and SARS-CoV-2. Their genome shares >80 % sequence identity, particularly the encoded four structural proteins; spike (S), envelope (E), membrane (M), and nucleocapsid (N) (Fig. 2), and these genomes have high similarities (Naqvi et al., 2020). Like all coronaviruses, SARS-CoV-2 is an enveloped virus with a positive-sense ssRNA (single stranded) genome (V'kovski, Kratzel, Steiner, Stalder, & Thiel, 2021). The SARS-CoV-2 genome measures about 29 kb (Vankadari, 2020).

2.1. Structure of the S-protein

The S-protein controls the virus-host cell interaction, i.e., it mediates receptor recognition, membrane fusion, virus entry, and antibody neutralization (Gallagher & Buchmeier, 2001). The exposure of the S-protein on the viral surface and its significant functions makes it a major target for antiviral drugs, including entry inhibitors, antibodies and vaccines (Yu et al., 2020). Detailed structural descriptions of the SARS-CoV-2 S-protein have been proposed by multiple researchers (Gobeil et al., 2021; Huang, Yang, Xu, Xu, & Liu, 2020; Peng, Wu, Wang, Qi, & Gao, 2021; Tang, Bidon, Jaimes, Whittaker, & Daniel, 2020). The S-protein is a class I fusion protein and can be divided into two subunits; S1, located at the N-terminus, and S2 (Huang et al., 2020) (Fig. 2). Each S-protein consists of a trimer of these subunits (Benton et al., 2020). While the S1 subunit is responsible for binding to host cell receptors, the S2 subunit facilitates membrane fusion. In SARS-CoV-2, the S1 subunit contains a receptor binding domain (RBD), located at the C-terminus. In the RBD, the so-called receptor binding motif (RBM) for ACE2 (angiotensin converting enzyme 2) binding is encoded. The RBD can exist in a closed and an open conformation. In the closed conformation, the RBD is buried and not accessible by the host cell receptors. In Fig. 2, the green monomer is displayed in the open conformation, while the other two are in the closed state.

The S-protein is heavily glycosylated (Casalino et al., 2020), which accounts for one-fifth of its molecular weight, however shields 40 % of its surface (Grant, Montgomery, Ito, & Woods, 2020). At least two *O*- and 22 *N*-glycosylation sites have been identified; however, only 17 of the latter are occupied within the S-protein ectodomain, including three in the RBD (Shajahan, Supekar, Gleinich, & Azadi, 2020) and none in the ACE2 receptor binding domain (Grant et al., 2020). The glycan structures can shield the binding of antibodies or receptors (Grant et al., 2020) and thus evade human immune response (Vankadari & Wilce, 2020; Yang, Du, & Kaltashov, 2020). Additionally, host cell HS-binding with the glycans on the S-protein may improve attachment of the virus to the host cell (Schuurs et al., 2021).

2.2. Identification of the HS-dependent infection of SARS-CoV-2

Coronaviruses use a variety of receptors and co-receptors to infect cells. It was suspected from early on that ACE2 was the major binding receptor of SARS-CoV-2 (Zhou et al., 2020). The binding domain to this

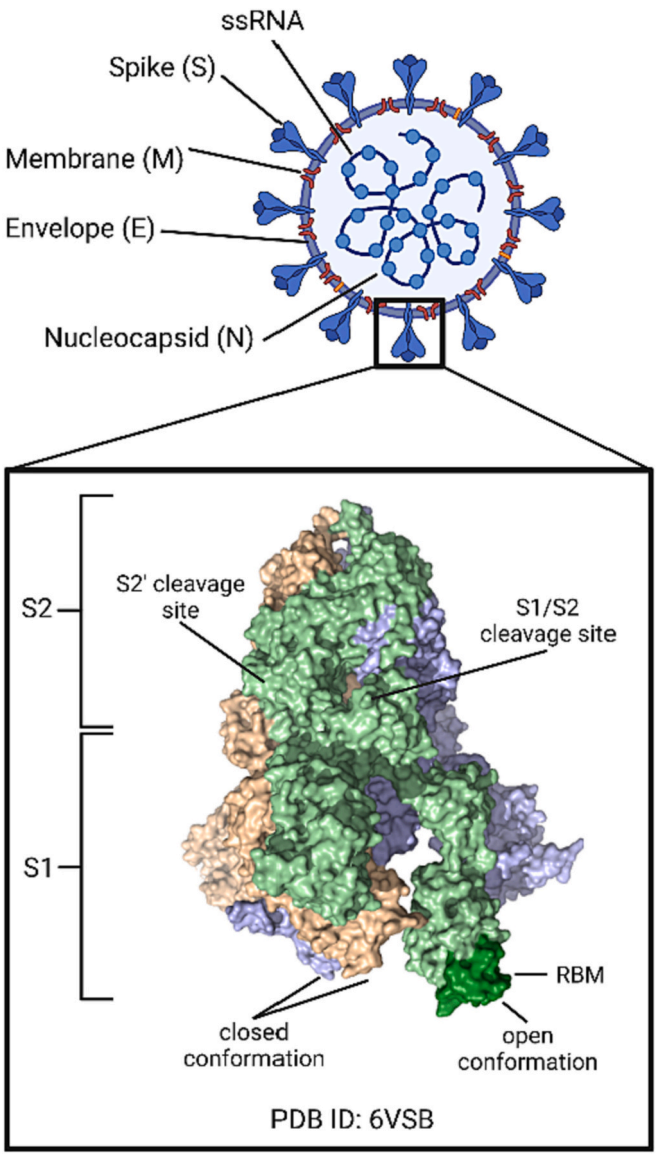

Fig. 2. Schematic representation of SARS-CoV-2-structure and its S-protein. The trimeric S-protein structure was derived from PDB ID 6VSB, and was displayed using *PyMOL* (Schrödinger). The two monomers in closed conformation are wheat and light blue colored, the monomer in the open conformation is displayed in light green, and its receptor binding motif (RBM) in dark green. The positions of the S1/S2 and S2′ cleavage sites were marked according to (Gupta et al., 2021). Figure prepared using biorender.com.

receptor is in the S1 subunit, therefore called the RBD, more specifically, the RBM (Fig. 2). However, some neutralizing antibodies do not target the RBM or the RBD, but other regions of the S-protein (Bermejo-Jambrina et al., 2021). Thus, obligatory co-receptors are suspected.

Binding to HS was reported for several coronaviruses (Cagno et al., 2019; Haan et al., 2005; Hippensteel, LaRiviere, Colbert, Langouët-Astrié, & Schmidt, 2020; Lang et al., 2011; Madu et al., 2007; Milewska et al., 2014; Vicenzi et al., 2004), and the dependency of the SARS-CoV-2 virus on HS for cell attachment and virus entry was suspected early into the pandemic (Clausen et al., 2020; Kim et al., 2020; Mycroft-West et al., 2020). The binding of HS to SARS-CoV-2 S-protein was demonstrated in several binding studies. A wide range of S-protein equilibrium binding constants (K_D) to heparin were observed based on surface plasmon resonance (SPR) analysis. Depending on the experimental set-up, the K_D for monomeric and trimeric S-protein showed picomolar interactions (Kim et al., 2020), nanomolar interactions (Clausen et al., 2020; Dwivedi et al., 2021; Liu et al., 2021; Song et al., 2021; Yan et al., 2021; Yue et al., 2021) and micromolar interactions (Hao et al., 2021). It is expected that the binding of the SARS-CoV-2 virus or its compounds will depend on the salt concentration due to the proposed dominance of electrostatic interactions. It is worth mentioning that strong attachment was still observed at 120 mM NaCl, which corresponds to the lung tissue microenvironment (Zhang et al., 2020).

Next to the studies with the trimeric S-protein, the different subunits, i.e., monomeric S, S1 and S2 as well as RBD, were applied in affinity experiments. Here, differences between the full S-protein and its subunits were obtained. The affinity for full S-protein was higher than for the RBD itself (Clausen et al., 2020; Liu et al., 2021), while affinity remained the same for the S2 subunit compared to the RBD and S1 subunit (Hao et al., 2021). This points towards the presence of several GAG-binding sites on the S-protein beyond just the RBD. A study by Watanabe et al. (2021) revealed specific binding patterns of full monomeric S-protein and the S1 and S2 subunits. While S1 almost exclusively bound to heparin, S2 showed high affinities for CS and HS and their fractions. These observations suggest that the S2 subunit significantly binds to a variety of GAGs, complementing the selectivity of the S1 subunit for heparin. In support, a GAG-binding motif in the region of the S1/S2 junction was identified (Hoffmann, Kleine-Weber, & Pöhlmann, 2020), which is essential for furin cleavage and subsequent infection. Evidence for this was that full-length heparin can bind to the S1/S2 junction to inhibit furin cleavage (Paiardi et al., 2022), and that induced mutations in the S-protein, eliminating the polybasic furin region, inhibited the binding of the S-protein to cells (Partridge et al., 2021).

Further investigations revealed the bigger picture of a proposed obligatory HS-binding mechanism for SARS-CoV-2 infection by using modifying the glycosylation, more specifically the HS composition, of the host cells. Zhang et al. (2020) reported that the knockdown of an HS chain initiation enzyme gene (*Xylt2*) caused 80 % entry inhibition of SARS-CoV-2 pseudotyped virus like particles (VLPs) and knockout of an HS chain sulfation enzyme gene (*Slc35b2*) reduced entry by 25 %. A similar study by Clausen et al. (2020) revealed a reduction in S-protein binding on cells with a knockout of a GAG assembly enzyme gene (*B4galt7*), and an inhibition of infection by pseudotyped virus due to mutations in GAG-transferases (exostosin glycosyltransferase 1 (*Ext1*) and *N*-deacetylase/*N*-sulfotransferase 1 (*Ndst1*)). A knockdown of *Ext1* showed the same inhibitory effect (Bermejo-Jambrina et al., 2021). Furthermore, treatment of different cell lines with heparin lyases (HSase) induced reduction of the binding of SARS-CoV-2 pseudotyped virus and S-protein. This was shown for a variety of primary cells and cell lines (Bermejo-Jambrina et al., 2021; Clausen et al., 2020; Liu et al., 2021; Yue et al., 2021), including cells from human lung or other tissues, and animal cells. The inhibition of SARS-CoV-2 infection as a result of HSase treatment remains for cells with ACE2 overexpression (Clausen et al., 2020); however, increased ACE2 expression reduced the effect of HSase on the inhibition of S-protein binding (Yue et al., 2021).

Concerning SARS-CoV-2 binding to cells without ACE2 expression, studies are contradictory. Virus binding to syndecan 1 or syndecan 4 was possible (Bermejo-Jambrina et al., 2021). Syndecans are sulfated transmembrane proteins, carrying HS or CS. In contrast, blocking of ACE2 by specific antibodies, reduced the binding of virus to cells (Bermejo-Jambrina et al., 2021). Thus, the HSPG binding itself might not be strong enough to attach SARS-CoV-2 effectively to the host cells. However, the presence of degree of polymerization (dp) 20 heparin increased the amount of S-protein bound to ACE2 (Clausen et al., 2020). Thus, it can be assumed that SARS-CoV-2 binding to HS is necessary for successful ACE2-binding and infection of host cells by the virus.

In summary, the experimental studies on SARS-CoV-2-binding to host cells focused on the S-protein-binding, S-protein pseudotyped virus-binding, or full virus-binding to HS, host cells or ACE2 receptor. Most of the studies concluded a strong dependence of the SARS-CoV-2 infection on the HS-co-receptor, which specifically interacts with the S-protein. As

far as we know, no specific studies on other SARS-CoV-2 glycoproteins, e.g., from the membrane, were conducted, and no predictions on the HS-binding towards these structures was published.

2.3. Prediction of GAG-binding to SARS-CoV-2

In January 2021, a review covering the early computational studies of the SARS-CoV-2 binding to HS was published (Yu et al., 2020). Here, we extend this analysis by highlighting more recent progress in the field since, specifically focussing on the interactions among glycans and the SARS-CoV-2 S-protein as well as putative GAG-binding sites (Fig. 3). Reviewing the published articles, three main approaches were implemented to identify GAG-binding motifs on the SARS-CoV-2 S-protein: (1) the analysis of the genome for typical binding motifs, e.g., Cardin-Weintraub motifs; (2); the determination of Levenshtein distances, and (3) *in-silico* studies of HS/protein interactions. All three computational approaches come with their advantages and disadvantages (Sankaranarayanan et al., 2018). The main advantage of the identification of (1) GAG-binding motifs is the limited computational resources necessary. However, the three-dimensional structure, and, thus, accessibility of the binding site, cannot be evaluated without additional *in-silico* tools. This disadvantage is circumvented to some extent by determining (2) Levenshtein distances. These are used to categorize the similarity of amino acid sequences to other proteins known to bind GAGs, hence the folding of the putative GAG-binding sites may be predicted from known molecules. The variety of *in-silico* tools, such as homology modeling, electrostatic potential energy determination, MD, and molecular docking, gained increased attention with the improvements in computing power and software tools. They offer a toolset to screen large databases with little workforce and to understand interaction dynamics (Yu et al., 2020). Although computational approaches give new insights into molecular bindings and can accelerate structure-based drug discovery with reduced person-time by screening large databases, they face certain limitations. Both, molecular docking and molecular dynamics simulations are limited by the effectiveness of the energy functions that power them, but they provide new insights into ligand-protein interactions (Yu et al., 2020). Most studies use structures up to dp8, which is easily doable considering the computational complexity associated with calculating the free energy of binding of a ligand to a protein receptor. The length of the glycan plays an important role in binding due to bridging between basic residues. Furthermore, with increasing chain length, acidic glycans gain an increasing number of rotatable bonds that enhance their conformational flexibility. Additionally, while *N*- and *O*-glycosylation of GAG-binding proteins is often neglected, these post-translational modifications can hinder the binding to predicted sites within the protein (Paiardi et al., 2022).

2.3.1. Cardin-Weintraub motifs

In a recent work, three putative GAG-binding motifs, named 1, 2, and 3, were identified in the SARS-CoV-2 S-protein (Kim et al., 2020). The first was reported to be located in the RBD at residues 453–459 (YRLFRKS) (Kim et al., 2020) at a site not overlapping the ACE2 contacting interface of the S1 subunit (Clausen et al., 2020). The second predicted Cardin-Weintraub motif was in the proteolytic cleavage site at the S1/S2 junction (residues 681–686). This furin motif is encoded by 'BXBB' (Vankadari, 2020), and in SARS-CoV-2 the furin cleavage site is PRRAR (S. Kim et al., 2020; Tang, Bidon, et al., 2020). Here, GAG-binding was predicted to be possible in each monomer chain of the trimeric S-protein. The probability of HS-binding in this motif was supported by a reduced binding affinity of the SARS-CoV-2 S-protein after the removal of arginine (R) from the sequence (Partridge et al., 2021). The third motif encoded SKPSKRS (residues: 810–816), adjacent to the fusion peptide, and was expected to be inaccessible for GAG-binding in the trimeric S-protein as none of the basic chains was exposed in any of the S2 subunits.

2.3.2. Levenshtein distances

Using so-called Levenshtein distances, several putative protein-GAG binding were predicted. Mycroft-West et al. (2020) identified RKR 354–356, LVK 533–535, KK 557–558 and R557 as well as R346, R403, K417 and H519 in the SARS-CoV-2 S-protein. Another study proposed >60 putative GAG-binding sites across the full S-protein, each containing basic residues (Schuurs et al., 2021).

2.3.3. In-silico predictions

Docking studies with heparin oligosaccharides of varying degree of polymerization (dp)4, dp6, and dp8 (tetrasacchride through octasaccharide) predicted four binding sites, which can accommodate different fragment lengths, at residues K356, R357, R355, R466, R346, K444 and R509; in the region R457, K458 and K462; binding at R408, R403 and K417; and at LVK 533–535 and KK 557–558 (Mycroft-West et al., 2020). Additional GAG-binding options were proposed at different polar sidechains of the SARS-CoV-2 S-protein: N487, Y489, Q493, Q498, and Y505, all located in the RBD that might induce heparin binding and sterically inhibit ACE2 binding at Q498, Y489, and Y505 (Kwon et al., 2020). Similarly, binding of heparin with N448, N450, Q493, and N501

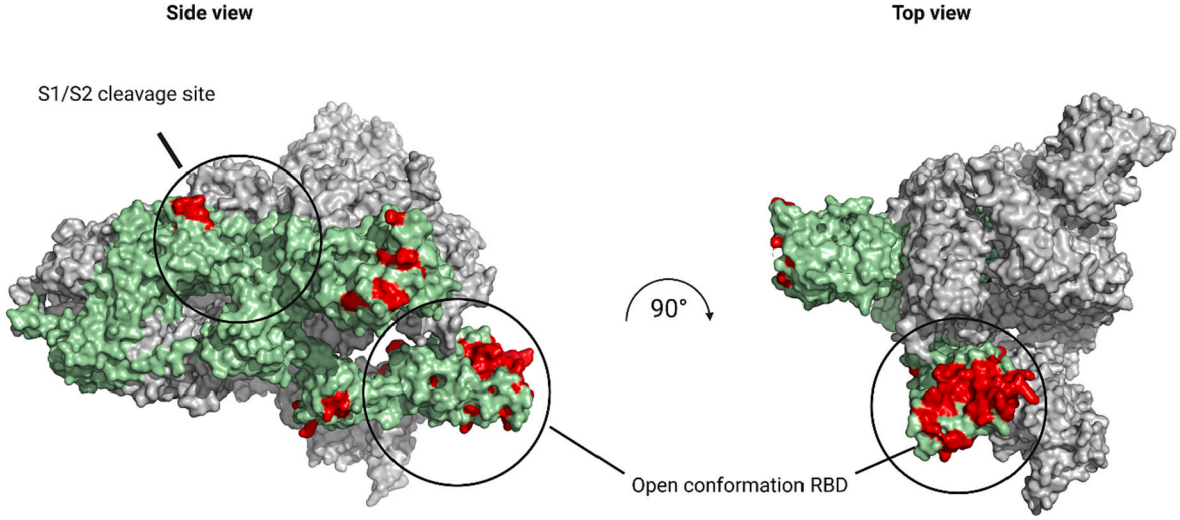

Fig. 3. Proposed binding regions of GAGs on SARS-CoV-2 S-protein wild type (wt). Trimeric S-protein (PDB ID: 6VSB) with one monomer colored in light green was displayed using *PyMOL* (Schrödinger). All putative GAG-binding sites mentioned in this chapter are marked in red. Figure prepared using biorender.com.

via a hydrogen bonding network may be possible (Kwon et al., 2020).

Docking studies suggested strong interactions of dp4 fragments with the positively charged amino acids R346, R355, K444, R466, and possibly R509 (Clausen et al., 2020). This computational study further indicated that F347, S349, N354, G447, Y449, and Y451 could take part in binding these oligosaccharides (Clausen et al., 2020). The involvement of several of these sites was confirmed by (Paiardi et al., 2022), who identified T345, R346, N354, R355, and N360 in the RBD as well as R682, R683, and R685 close to the S1/S2 junction as heparin binding sites in open and closed conformation. The binding of glycans to residue R403, identified in studies of the Levenshtein distance, was further confirmed together with the importance of residue N501 (Dwivedi et al., 2021).

The identification of a GAG-binding site in the S1/S2 junction was extended by the possibility of long HS chains bridging this site with the RBD (Gupta et al., 2021; Partridge et al., 2021; Schuurs et al., 2021). A putative binding position of a full-length heparin molecule in the open and closed conformation of the S-protein as proposed by Gupta et al. (2021) is illustrated in Fig. 4. Controversial reports have been published whether the bridging effect might induce or prevent the open conformation of the RBD, and experimental data concerning this will be discussed in more detail in the next section.

Which of the two main GAG-binding regions in the RBD and the S1/S2 region dominates binding of HS s is not yet fully understood. As explained before, the binding of HS at the S1/S2 junction appears to be required for viral attachment. This is supported by a docking study, which demonstrated that GAG-binding is largely independent from the binding in the S1 subunit, but involves regions in the S2 subunit (Partridge et al., 2021). Additionally, several publications reported that the RBD seems to accommodate shorter GAG chains, i.e., up to dp6 (Mycroft-West et al., 2020; Schuurs et al., 2021; Yang et al., 2020), which might limit the binding capacity of this region.

2.4. Proposed HS-dependant infection process of SARS-CoV-2

Based on reports on the putative GAG-binding sites and experimental studies on the HS-binding of SARS-CoV-2, we propose an HS-dependant infection process of SARS-CoV-2. In this review, we expand on the infection processes proposed in previous reports by Kim et al. (2020), Paiardi et al. (2022), and Kalra and Kandimalla (2021). The SARS-CoV-2 infection is expected initially to affect cells in the respiratory tract, especially nasal epithelium cells (Tandon et al., 2021). Presumably, cells with a high HS-concentration and/or high ACE2 expression are preferentially attacked (Yue et al., 2021). Interestingly, until now no correlation was found between the HS composition in human tissue and S-protein binding (Clausen et al., 2020) (Fig. 5, insert). However, studies on the anti-SARS-CoV-2 properties of GAGS found higher efficiencies by HS from lung compared to mucosa (Tree et al., 2021), and by human and porcine heparins compared to bat-origin (Yan et al., 2021). This suggests that the composition of HS on different tissues influences the SARS-CoV-2 binding and might therefore contribute to cell tropism.

We propose that HS-binding is essential for the initiation of SARS-CoV-2 infection (Fig. 5 panel 1). The host cell HSPGs act as virus "collector" due to their position on the cell membrane (Kalra & Kandimalla, 2021). SARS-CoV-2 presumably binds to host cell HS via the two main GAG-binding sites (motif 1 and 2) in the RBD and in the region of the S1/S2 cleavage site (Fig. 3). After attachment, the S-protein primarily binds to ACE2 through its RBM in the S1 subunit (Bermejo-Jambrina et al., 2021). The ACE2 receptor- and HS-binding can occur simultaneously. This has been demonstrated by experimental (Bermejo-Jambrina et al., 2021; Clausen et al., 2020; Liu et al., 2021) and computational means (Clausen et al., 2020; Paiardi et al., 2022) (Fig. 5 panel 2b). ACE2 receptor-binding involves N487, Y489, Q493, Y449, G446, T500, N501, G502, and K417 residues (Lan et al., 2020), while the HS-binding site in the RBM was proposed to engage the residues R346, R355, K444, R466, and probably R509 in electrostatic interactions and the residues F347, S349, N354, G447, Y449, and Y451, in coordinating the H-bond and hydrophobic interactions (Clausen et al., 2020). Other authors proposed additional binding patches in the RBD (Fig. 3). While the ACE2 receptor is the major protein-based receptor for SARS-CoV-2 binding to host cells, other receptors, such as AXL (tyrosine-protein kinase receptor), ASGR1 (asialoglycoprotein receptor 1) or CD147, have been recognized as potential alternative receptors independent of ACE2 (Peng et al., 2021). Until now, investigations have been focused on the interaction of SARS-CoV-2 with ACE2 and HS receptors. Further research should be conducted on the influence of HS on the binding of SARS-CoV-2 to other receptors.

The S1 subunit of the S-protein exists in two conformational states: a

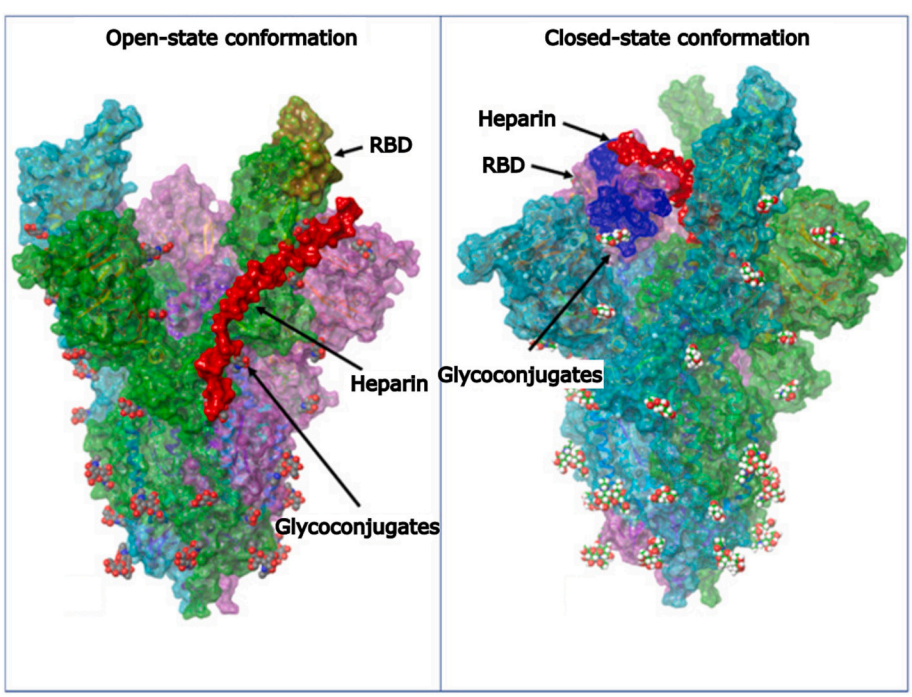

Fig. 4. Putative binding position of heparin to trimeric S-protein. The S-protein in trimeric structure, monomers in teal, green and purple, was retrieved from PDB databank (ID:7CAI). The figure illustrates the glycosylation sites as glycoconjugates of the S-protein on the open state S-protein (left panel), with the RBD (brown-green) in open conformation, and in the closed state S-protein (right panel). In the right panel, the putative GAG binding region is colored in blue. In both panels the putative binding position of full-length heparin is shown in red. The figure was reproduced with permission from (Gupta et al., 2021).

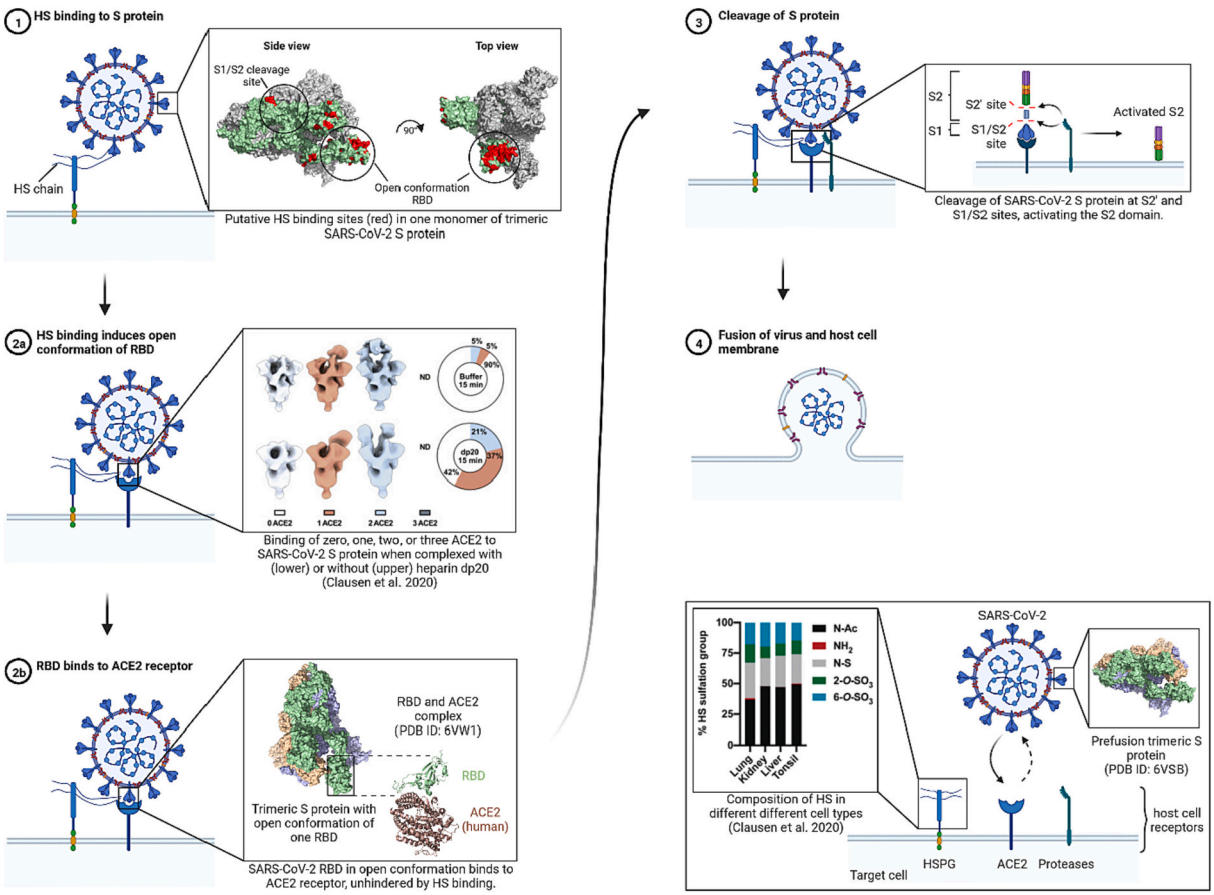

Fig. 5. Proposed mechanism of HS-dependent SARS-CoV-2 cell infection.
(1) SARS-CoV-2 trimeric S-protein binds to HSPG on host cell surface. The binding presumably is most dominant in the RBD region and close to the furin motif at the S1/S2 cleavage site. (2a) The binding of the S-protein to the HS chains induces the upfolding of the monomers of the trimeric S-protein from closed to open conformation. Only in open conformation the RBM is exposed for ACE2 binding. (2b). The complexation of RBD and ACE2 occurs in a codependent manner with the HS-binding in the RBD region. Each monomer can bind to one ACE2 receptor. (3) The S1 and S2 subunits of the S-protein are cleaved at the S1/S2 and the S2′ site, presumably S1/S2 mainly by furin and S2′ by TMPRSS2 protease receptor. The cleavage activates the S2 subunit. (4) The activated virus fuses with the host cell membrane. From here, the replication cycle inside the host cell starts. Figure prepared using biorender.com. The proteins in panel 1, 2b, and in the insert were displayed using *PyMOL* (Schrödinger). The figure displaying the HS sulfation pattern in the insert as well as the figure of the S-protein conformation (panel 2a) were reproduced with permission from Clausen et al. (2020).

closed/inactive conformation that serves as the natural metastable state, and an open/active conformation (Fig. 2), which initiates virus interaction with the host cell (Benton et al., 2020). In the closed conformation the viral RBMs are buried by the adjacent sequence (Peng et al., 2021). The open conformation of any of the trimers is the first step in S-protein binding to host cell receptors and fusion with the host cell membrane (Benton et al., 2020). Binding to the ACE2 receptor itself can promote the transition to the open conformation of the other two S1 units of the trimer, allowing for binding to one to three ACE2 molecules (Benton et al., 2020). It was proposed that the promotion of the open conformation, and thus ACE2 binding, can additionally be induced by the binding of HS to the S-protein (Clausen et al., 2020) (Fig. 5 panel 2a). This was questioned by Paiardi et al. (2022) and still remains to be evaluated by additional studies. Nevertheless, conformational changes of the SARS-CoV-2 RBD by binding to heparins have been observed (Mycroft-West et al., 2020). Binding of heparin to the S-protein lowers its unfolding temperature (Guimond et al., 2022; Schuurs et al., 2021; Tree et al., 2021) as confirmed through MD simulations combined with native mass spectrometry analysis (Yang et al., 2020). The destabilization is predicted to reduce the binding strength between ACE2 and RBD up to full binding release (Yang et al., 2020). Contrary to these result, stabilization of the ACE2-RBD-complex was observed in the presence of heparin (Paiardi et al., 2022). The impact on a structure-function relationship is not fully understood.

After successful ACE2 binding, the SARS-CoV-2 S-protein is cleaved, priming the S-protein for fusion with the host cell (Hoffmann et al., 2020; Yu et al., 2020). This occurs at two sites; the S1/S2 linkage and the S2′ site (Fig. 5 panel 3), and is mediated by different host cell proteases, e.g., furin or transmembrane protease serine protease-2 (TMPRSS-2) (Peng et al., 2021). Whether cleavage of the S-protein trimer by furin is affected by the presence of heparin is unclear. Liu et al. (2021) indicated no steric hindrance in this region of the spike, while Paiardi et al. (2022) predicted a masking of the S1/S2 junction site of all three monomers.

In conclusion, HS is categorized as a viral co-receptor and induces formation of a complex of SARS-CoV-2 S-protein and ACE-2 receptor through conformational changes. HS appears to play a major role in binding S-protein close to the furin cleavage site. Additionally, HS-binding aids in the approximation of the S-protein to the cell surface and its receptors.

2.5. Mutations in the S-protein

Mutations in the SARS-CoV-2 genome are a natural consequence of its replication. In the past two years, the number of S-protein mutations per variant has increased in the evolution of SARS-Cov-2 (Fig. 6B), with the Omicron variants posing the most dramatic increase in mutations to

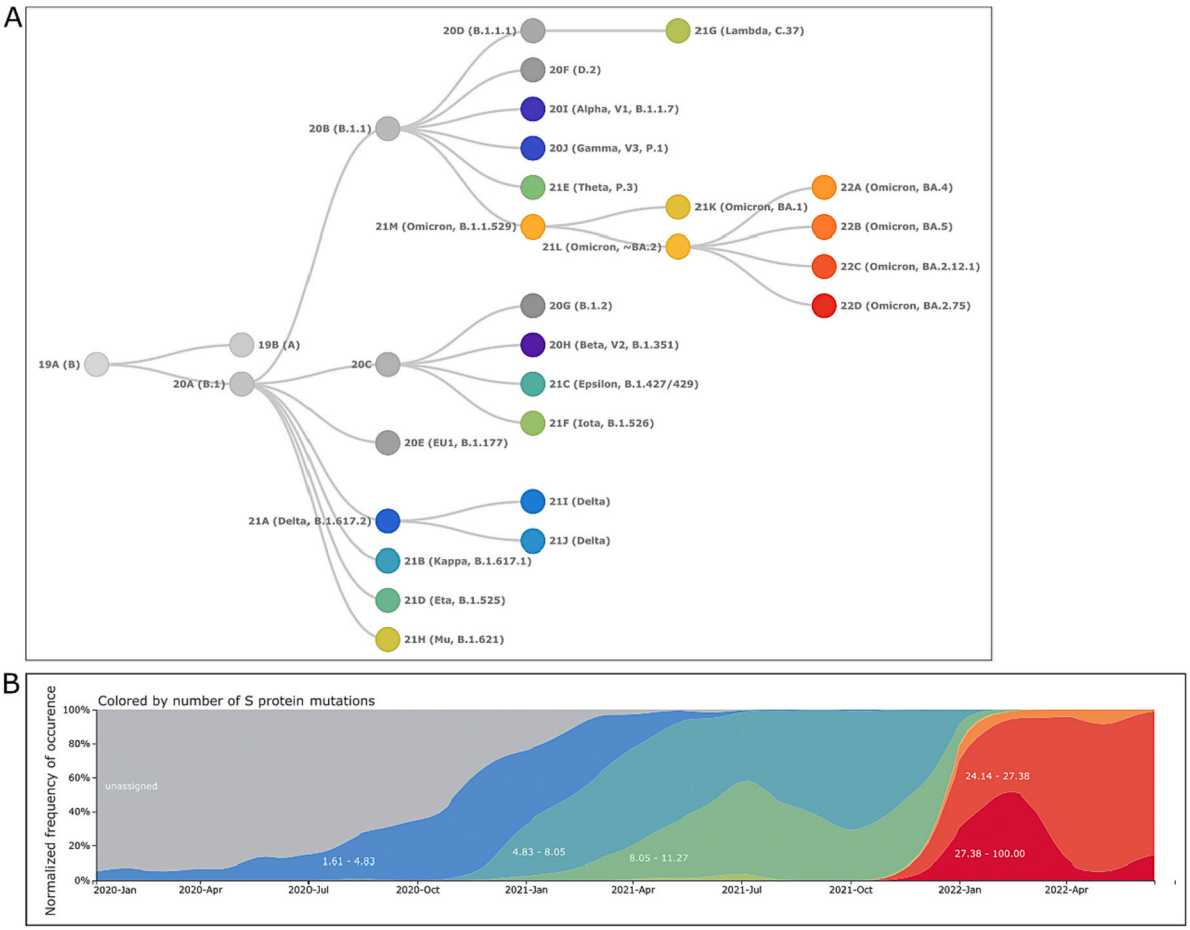

Fig. 6. Evolution of SARS-CoV-2 variants. (A) represents the phylogenetic tree of SARS-CoV-2. The image was reproduced from covariants.org/variants with credits to the nextrain.org project for the underlying data. (B) shows a timeline of mutation frequency in the S1 subunit of the S-protein, normalized to its occurrence at each date. Inside colored fields, the number of mutations in the S protein is displayed. The filtered genomes include only human origins of all clades of SARS-CoV-2. The graphic is a modified svg-screenshot from the nextrain.org webpage.

date (Fig. 7B). Interestingly, the most infectious VOCs until now, the Delta variant and the Omicron variants, evolved from two different evolutionary branches (Fig. 6B), emphasizing the need for constant surveillance and analysis of all SARS-CoV-2 variants. These variations in the S-protein have raised concern for limited effectiveness of vaccines and antivirals as seen with SARS-CoV-2 A.30 Omicron variant (Arora et al., 2021; Das, Chakraborty, Bayry, & Mukherjee, 2021; Lusvarghi et al., 2022). Thus, advancing our understanding of the conformational changes that the S-protein undergoes along with the molecular interactions with host cells are critical. The position of the mutations determines the fitness of the virus. None of the variants of concern (VOCs) show mutations in the predicted *N*-glycosylation sites (Fig. 7B). Alterations in the S-protein are most frequently found in the S1 subunit, among others in the RBM region and close to the S1/S2 region (Fig. 7A). Despite these mutations being distributed broadly across the RBD (Fig. 8A) and the S-protein in general, they do not have a pronounced impact on the folded structure of the protein. A structural alignment of the RBD regions of the wild type (wt), Delta, Omicron BA.1 and BA.2 revealed a high level of similarity between these regions (Fig. 8B).

Given the limited structural variation in the RBD and full-length spike proteins between individual viral strains, research has focused on alternative ways of explaining the disparity in their binding interactions. One area where substantial progress has been made is in the analysis of the chemical signature of the RBD and full-length spike proteins. It is known that among coronaviruses, the RBD is poorly conserved (Tang, Bidon, et al., 2020), while other functional regions, e.g., the fusion domain, are relatively invariable (Hoffmann et al., 2020; Schuurs et al., 2021). Previously, studies of conserved regions among different coronaviruses were undertaken, e.g., in the RBD region (Schuurs et al., 2021) and the cleavage sites (Hoffmann et al., 2020). Both studies identified a high level of conservation of basic residues. Interestingly, the polybasic furin cleavage site is unique among these viruses and its origin is still unknown (Temmam et al., 2022). Concerning the different SARS-CoV-2 variants, observed mutations are more often adding basic residues than removing them (Fig. 7B). In the case of the RBD (Fig. 8C) it is possible to observe a clear mutational change in the electrostatic signature of this region across individual viral strains, which leads to a change in overall isoelectric point of the full S-protein by nearly one pH unit (Table 1). The pH shift towards a more basic isoelectric point of the SARS-CoV-2 S-protein corresponds to an increase in transmissibility from the wt to the Omicron variants. Interestingly, the Beta variant and the Delta variant evolved from a different phylogenetical branch than the Alpha, Beta and Omicron variants (Fig. 6). Nevertheless, an increase of the isoelectric point was observed for all variants, which was comparable for the Beta and Delta variants as well as for the Alpha and Gamma variants. Thus, we propose that this chemical change is beneficial for virus transmissibility and infection. Additionally, it might be a major driver of why individual viral strains display diverse infectivity profiles and why the efficacy of vaccine and therapeutic candidates have been only partially effective to date.

2.6. Impact of S-protein mutations on HS-binding

A growing number of studies are investigating the impact of SARS-

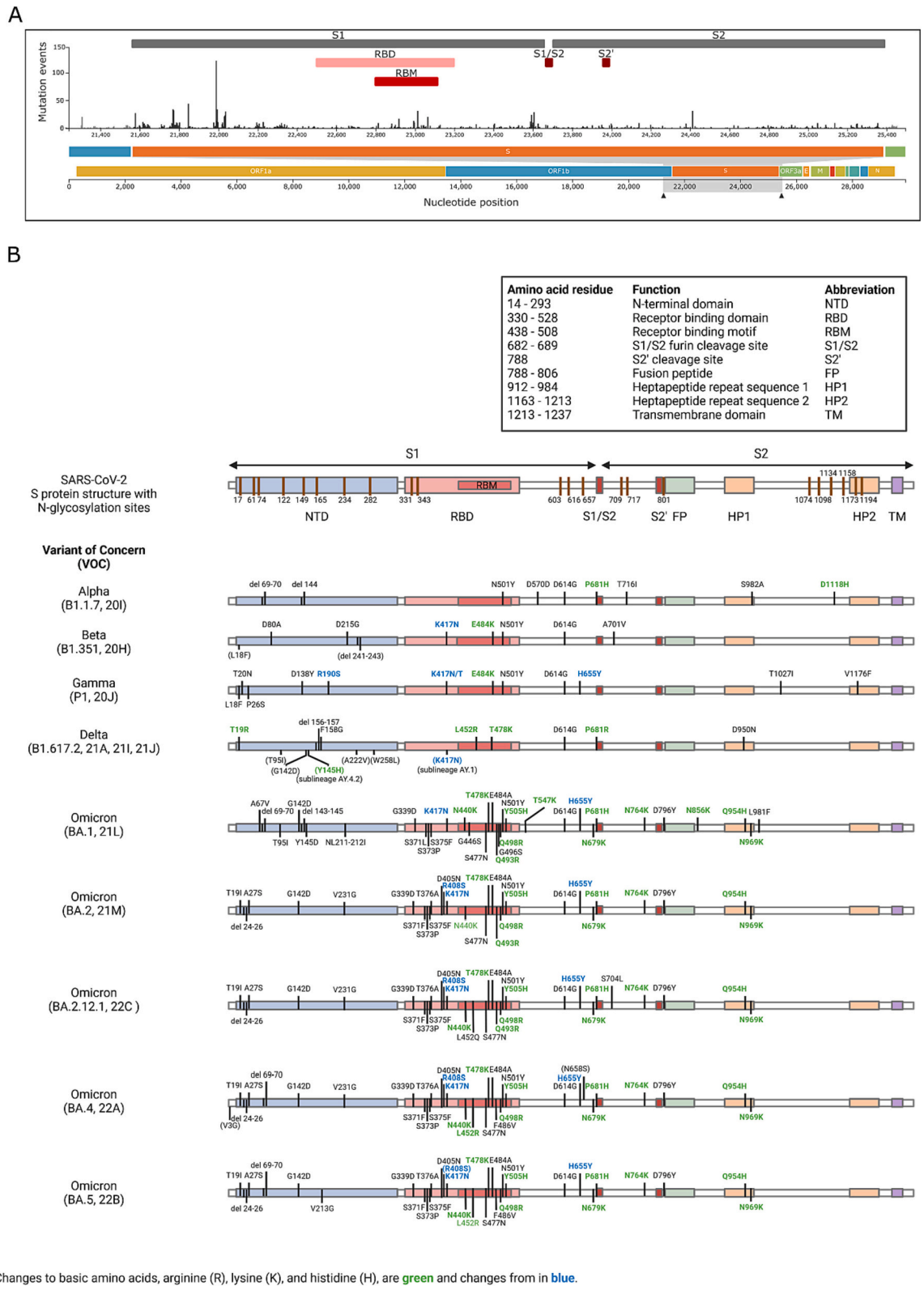

Fig. 7. Mutations in the SARS-CoV-2 S-protein. (A) specifies the mutation events per position in the nucleotide sequence, focused on the S-protein. Both images are modified *svg*-screenshots from the open-source nextrain.org project. The filtered genomes include only human origins of all clades of SARS-CoV-2 from January 2020 until June 18, 2022. (B) Mutations in the S-protein of currently known variants of concern (VOC) are presented in comparison. Changes to basic residues (arginine (R), lysine (K) or histidine (H)) are marked in green, changes away in blue. Positions of *N*-linked glycosylation sites are indicated in orange in the upper sequence of the SARS-CoV-2 wt (Schuurs et al., 2021). The image was adapted from viralzone.expasy.org/9556 and extended with information from the open-source nextstrain.com project and (Gobeil et al., 2021; Huang et al., 2020; Peng et al., 2021). Fig. B prepared using biorender.com.

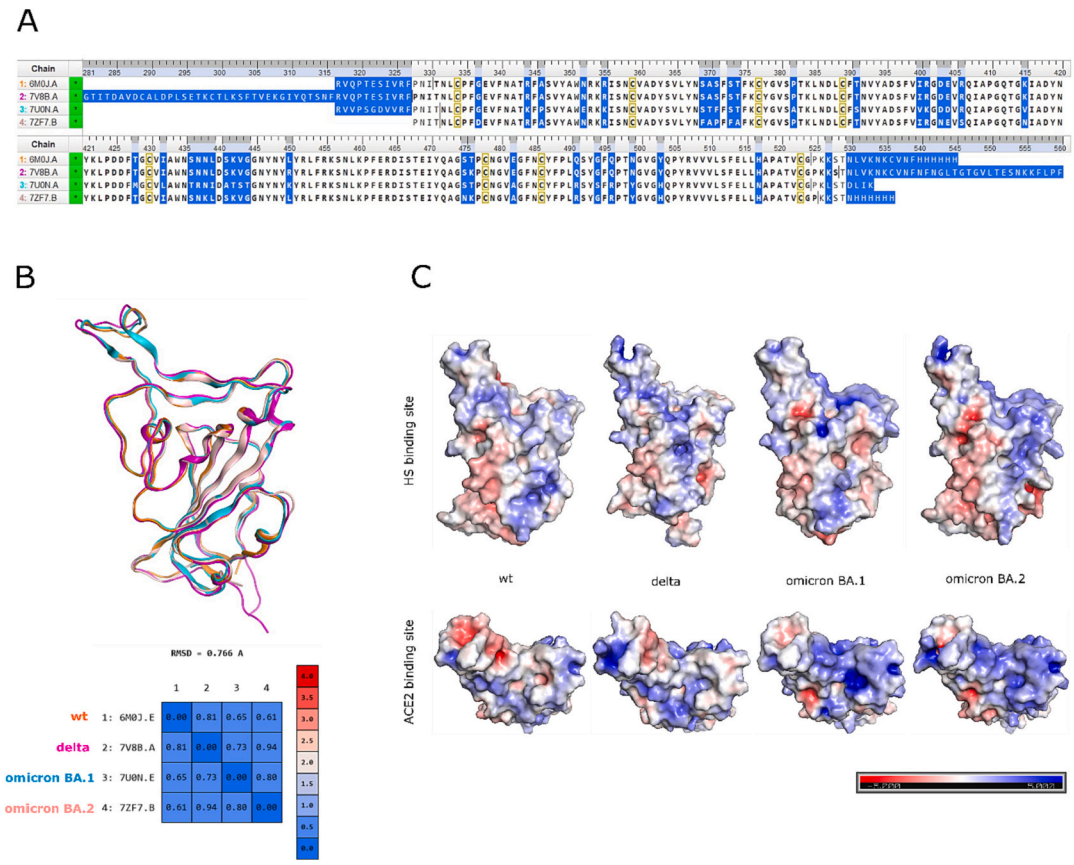

Fig. 8. Structural impact of mutations on the RBD of SARS-CoV-2. A comparative analysis of the RBDs of four SARS-CoV-2 variants, i.e., wt (wt, PDB ID: 6MOJ), Delta (PDB ID 7V8B), Omicron BA.1 (PDB ID 7U0N), and BA.2 (PDB ID 7ZF7) was performed using the Molecular Operating Environment (*MOE*) 2022 software suite (Chemical Computing Group ULC, 2022). A blosum62 substitution matrix along with default alignment parameters were used to generate a multiple sequence alignment (MSA) (A) of the four RBDs being compared. This MSA highlights the regions of sequence divergence (amino acids colored in blue). A comparison of the structural impact of these mutations can be observed by superimposing the RBDs onto each other and identifying regions of structural variation. This approach uses the Mean Square Distance (MSD) deviation of corresponding C-Alpha atoms to optimize the atomic coordinate superimposition. This approach revealed a high level of structural similarity (all structures < 1 Å) between the four RBDs (B). The impact of observed mutations on the distribution of positively charged residues on the surface of the RBDs for receptor and ligand interactions can be determined by calculating an electrostatic map of each RBD (C). The electrostatic maps shown here were generated using the Adaptive Poisson-Boltzmann Solver (APBS Electrostatics (Baker, Sept, Joseph, Holst, & McCammon, 2001)) plugin in *PyMOL* (Schrödinger). The position of the ACE2 binding site on the RBD protein was derived from the wt structure (PDB ID 6M0J) and the putative HS binding site from previous research (Clausen et al., 2020). The electrostatic maps of each RBD were displayed using *PyMOL* (Schrödinger) to highlight the variability in the density of positive charge at the heparin binding face (upper panel) and ACE2 binding site (lower panel). Blue corresponds to positively charged and red to negatively charged clusters on the protein.

Table 1
Isoelectric points of SARS-CoV-2 S-proteins of different VOCs. Calculated with the indicated amino acid sequences at web.expasy.org/compute_pi/.

SARS-CoV-2 variant	GenBank code	Isoelectric point
wt	YP_009724390.1	6.24
Alpha	QWE88920.1	6.35
Beta	QRN78347.1	6.64
Gamma	QVE55289.1	6.39
Delta	QWK65230.1	6.78
Omicron BA.1	UFO69279.1	7.14
Omicron BA.2	UJE45220.1	7.16
Omicron BA.3 (BA2.12.1)	UPP11028.1	7.16
Omicron BA.4	UPP14409.1	7.14
Omicron BA.5	UOZ45804.1	7.14

CoV-2 mutations on viral fitness (see Guruprasad, 2021; Guruprasad, 2022; Harvey et al., 2021; Hirabara et al., 2021; Liu, Wei, Kappler, Marrack, & Zhang, 2022; Takeda, 2022 for recent articles and reviews). Here, we focus on the influence of mutations in the HS-binding sites and HS-induced changes in viral fitness.

Facilitated virus transmission was reported to evolve through increased occurrence of an open/active conformation of the S-protein, which is required for the binding of the virus to the ACE2 receptor (Benton et al., 2020), elevated stability of the S-protein bound to the cell receptors, and by tighter receptor binding (Wrobel et al., 2022). Additionally, the cleavage of the S1/S2 subunit was shown to be critically important for membrane fusion of the virus (Takeda, 2022). As indicated in the previous chapter, mutations in SARS-CoV-2 S-protein are clustered in the C-terminal domain of the S1 subunit, the RBM, and close to the S1/S2 furin cleavage site. Putative HS-binding sites were reported in the latter two regions. The HS-binding site located in the RBM, has become more cationic in the Omicron variants (Fig. 8C) to the SARS-CoV-2 wt. This and the overall more basic S-protein (Table 1) is expected to facilitate HS-binding on the S-protein. We expect this to increase the virulence of SARS-CoV-2. A docking study of the binding of the full heparin molecule identified a high level of residue conservation in the predicted binding regions on the S-protein with nearly no change in binding affinity among the different variants (Gupta et al., 2021). Concerning the size of the heparin molecule and therefore the number of interaction sites, single mutations are expected to have only a minor influence on the binding (Gupta et al., 2021; Paiardi et al., 2022).

Experimental data showed that the N501Y mutation in the Alpha,

Beta, Gamma, and all Omicron variants reduced the affinity for heparin binding (Dwivedi et al., 2021; Song et al., 2021; Zhang et al., 2022), while the L452R mutation (Song et al., 2021; Zhang et al., 2022), present in Delta and Omicron BA.2 and BA.5, and the D614G mutation (Yue et al., 2021), increased the binding affinity. A study of the involvement of the S247R mutation in the lambda variant in HS-binding showed that long-chained GAG molecules may use this mutation to bridge the S1/S2 junction with two other basic amino acids, H245 and R246, thereby improving GAG-binding (Schuurs et al., 2021). These mutations seem to affect the binding pattern to different GAGs, e.g., binding of sulfated galactan is stronger for the N501Y mutation than heparin (Kim et al., 2022). Thus, further research is necessary to understand the structure activity relationship of GAGs and SARS-CoV-2. For this purpose, the analysis of conserved HS-binding domains in the SARS-CoV-2 S-protein might improve the understanding of virus binding to the cell surface across different coronavirus strains and variants.

Among others, HS interaction with SARS-CoV-2 involves two motifs in the wild-type Wuhan strain, at residues 453–459 (YRLFRKS) and at residues 681–686 (PRRARS) (Kim et al., 2020), Compared to the SARS-CoV-2 strain Wuhan-Hu-1/2019 (accessed from the NCBI *SARS-CoV-2 Variants Overview* (ncbi.nlm.nih.gov/activ) and the nexstrain.org project (nextstrain.org/nextrain.org), last accessed April 15, 2022), the highest rate of alterations in these motifs was found at residue P681, located at the S1/S2 furin cleavage site. This aligns with the observations made by several authors of a maximum mutation density in proximity of this protease cleavage site (residues 675–692) (Guruprasad, 2021; Takeda, 2022). In detail, the proline (P) in residue 681 was substituted by an arginine (R) in the variants Alpha, Delta, and some early variants as well as a histidine (H) in Alpha, Gamma, Delta, Omicron, and early variants. The basic P681H mutation elevates the resistance against disruption of the S-protein after binding to the ACE2 receptor and induces near-complete cleavage of the subunits (Takeda, 2022; Wrobel et al., 2022).

Within the PRRARS GAG-binding region a partial deletion (residues 681–685), was sequenced in samples of the Delta variant. As described above, this mutation directly increased the cleavability at the S1/S2 site, resulting in enhanced membrane fusion of the SARS-CoV-2 virus (Takeda, 2022). Concerning the putative HS-binding YRLFRKS (Kim et al., 2020), studies on the Y453F mutation showed increased ACE2 receptor-binding (Starr et al., 2022) (jbloomlab.github.io/SARS-CoV-2-RBD_DMS/, last accessed April 15, 2022).

Three of the residues predicted for GAG-binding by Kwon et al. (2020) were mutated in the Omicron variants BA.1 and BA.2; namely, Q493R, Q498R, and Y505H (covariants.org/shared-mutations, last accessed April 15, 2022). Omicron BA.4 and BA.5 only share the latter two mutation sites (Fig. 1). In all three cases, uncharged residues are substituted by basic residues, presumably increasing GAG-binding affinities.

Dwivedi et al. (2021) performed docking studies with dp2 of four holothurian sulfated glycans derived from sea cucumber and predicted similar binding behavior at residues R403, Q498 and N/Y501. However, the same studies using heparin dp2 showed contradictory results. Confirming the experimental data, the N501Y mutation was predicted to have a negative impact on heparin binding as it lacks a key GlcNS(6S)–arginine interactions (Dwivedi et al., 2021). Interestingly, the N501Y mutation occurs in myriad VOCs, including Alpha, Beta, Gamma, and Omicron (covariants.org/shared-mutations, last accessed April 15, 2022). However, several reports identified increased binding to the ACE2 receptor (Starr et al., 2022) as well as higher likelihood of the S1 subunit to exist in its open conformation (Teruel, Mailhot, & Najmanovich, 2021) in the presence of the N501Y mutation. One explanation for the tighter binding of N501Y mutant S-protein to ACE2 might be the reported change in heparin binding sites. N501 is part of six amino acid residues that confer a tight interaction of the S1 subunit with the RBD (Sanches et al., 2021). Dwivedi et al. (2021) modeled an interaction of the sites R403, Y453, Q498, and N501 with heparin dp2 for the wild-type, and R403, D405, R408, Q409 and K417 sites for the N501Y variant. Comparing these sites with the predicted contact sites of ACE2 with the S-protein, the binding of heparin dp2 with the N501Y mutant is transferred from the center of ACE2 binding in the wild-type N501 to its edge, only interfering with K417 of the ACE2 contacting residues. Concerning residue 417, apart from the Alpha variant, all N501Y mutants additionally share the K417T/N mutation. This K417 mutation induces a more open spike trimer (Wrobel et al., 2022). Until now, the impact of this mutation on glycan binding has not been determined. The presence of the N501Y mutation in the RBD might also increase ACE2-binding affinity in conjunction with the D614G substitution (Wrobel et al., 2022). The D614G mutation elevates the tendency of the S-protein trimer to transform into the open/active conformation (Wrobel et al., 2022).

In conclusion, these findings highlight the impact of mutations in the whole S1 subunit for interactions in other regions of the protein.

2.7. Anti-SARS-CoV-2 activities of GAGs

GAGs exhibit strong antiviral activity against many viruses. This is proposed to rely on GAG-binding to the HS receptors of viruses, inducing steric hindrance and hereby inhibition of infection (Cagno et al., 2019). Prominent examples are herpes simplex virus, dengue virus, human immunodeficiency virus, yellow fever virus, zika virus and many more (Bauer et al., 2021; Cagno et al., 2019; Kamhi et al., 2013). The mechanism of inhibition of SARS-CoV-2 infection appears to rely on three different binding positions of GAGs on the S-protein (Paiardi et al., 2022): (1) The binding of GAGs to the S-protein can induce a direct competition of attachment to the HSPGs by binding to a GAG-binding site (Fig. 3), (2) GAGs can allosterically hinder S-protein binding to host cell receptors by blocking the RBD to change from the closed to the open conformation, and (3) GAGs can prevent the furin-mediated spike cleavage by physically associating with the S1/S2 site. Additionally, ACE2 receptor has a GAG-binding site itself, which upon GAG-binding might serve to sterically inhibit interaction with the S-protein (Salih et al., 2021). Presumably, a combination of the different mechanisms engenders the inhibitory effects of heparin in SARS-CoV-2 infection. Exemplary for this is the observation of only a modest inhibition of binding of S-protein to the ACE2 receptor in the presence of heparin (Liu et al., 2021), while other authors observed substantial heparin-mediated reduction in the binding of the full SARS-CoV-2 to ACE2 receptor and inhibited infection (Gupta et al., 2021; Kwon et al., 2020; Mycroft-West et al., 2020; Tree et al., 2021; Zhang et al., 2020). We conclude that the inhibition of ACE2 receptor binding itself does not play the dominant role in preventing an infection process, however, other binding regions like the S1/S2 furin cleavage site are blocked and hinder viral entry. Hence, the experimental design of inhibition studies and the targeted receptors and binding sites should be considered. Next to the inhibition of binding and infection of SARS-CoV-2, GAGs might be applied for their antiviral activity by inhibiting the activity of enzymes, e.g., the main protease M^{Pro} needed for viral replication in the host cells (Li, Zhang, Pang, & Li, 2022).

Two types of key experiments were conducted to evaluate GAG-mediated inhibition of SARS-CoV-2 infection. In one experimental approach protein-GAG-interactions are studied by using surface plasmon resonance spectrometry (SPR) or structurally defined oligosaccharide sequences in a microarray high throughput format (Hao et al., 2021; Liu et al., 2021; Watanabe et al., 2021; Yu et al., 2020). Both strategies take advantage of competitive binding of SARS-CoV-2 or the S-protein to heparins in the presence of other GAGs, which allows for rapid screening studies. Heparin is often chosen for these studies due to its structural similarity to the chains of HSPGs. A second experimental approach involves inhibition of binding of SARS-CoV-2 or its substructures to cells or receptors, such as ACE2. Here, both the binding of GAGs to the virus and the subsequent binding of the complex to cell receptors are examined. Thus, the impact of steric hindrance and inhibition of the natural infection process can be observed more closely. An

extensive list of tested GAGs with potential SARS-CoV-2 inhibition is summarized in Table 2. Differences between the reported studies exist, which is not surprising as deviations are expected due to varying protein and glycan structures. In any event, there are general trends that have been observed regarding GAG-SARS-CoV-2 interactions as follows:

- SARS-CoV-2 binding to **GAGs** is the strongest for HS, including heparin, and can become stronger when complexed with metal ions. For example, Mg^{2+}-complexed heparin had increased anti-SARS-CoV-2 activity compared to native heparin (Mese et al., 2021). Furthermore, several GAGs like fucans and galactans showed high affinity binding to SARS-CoV-2 (Tandon et al., 2021). On the other hand, ACE2 receptor-binding to S-protein is not inhibited by sulfated galactofucan and glucuronomannan (Jin et al., 2020), and kappa-carrageenan and lambda-carrageenan were less effective than iota-carrageenan (Morokutti-Kurz et al., 2021). A low binding affinity of the virus was observed towards CS (Dwivedi et al., 2021; Song et al., 2020; Tandon et al., 2021; Watanabe et al., 2021), DS (Dwivedi et al., 2021), or HA (Liu et al., 2021). CS, however, might act as alternative binding partner for the S2 subunit throughout infection (Watanabe et al., 2021). Further details are summarized in Table 2.
- The **monosaccharide** composition itself seems to have a small influence (Hao et al., 2021) but certain structures, e.g., difucosyl branches with the α-Fuc2,4*S* unit at the terminal monosaccharide (Dwivedi et al., 2021), are preferred binding partners. Furthermore, combined with their sulfation pattern, the IdoA2*S* and IdoA2*S*-GlcN*S*6*S* repeating units were found to increase HS-binding to SARS-CoV-2 S-protein (Hao et al., 2021; Liu et al., 2021). Thus, the fine structural composition and charge-distribution seems essential.
- The **chain length** of HS is critical, i.e., stronger binding is obtained with longer chains or higher molecular weights (Jin et al., 2020; Liu et al., 2022; Paiardi et al., 2022; Schuurs et al., 2021; Yan et al., 2021). The use of low molecular weight heparin (LMWH) was less effective than unfractionated heparin (UFH) (Mycroft-West et al., 2020; Tree et al., 2021). Short chains, e.g., up to nine monomers (Hao et al., 2021) or dp18 (Kim et al., 2017; Tandon et al., 2021), showed little inhibition. Thus, steric hindrance might be necessary to prevent infection by GAG inhibition. Another explanation for the importance of the chain length is that longer chains can interact with multiple putative GAG-binding sites on the SARS-CoV-2 S-protein (Paiardi et al., 2022). To this end, Clausen et al. (2020) published evidence that long-chained heparin can bind open and closed S-protein conformations, bridging the GAG-binding sites. This was supported by the previously mentioned theory that full-length heparin can hinder the S-protein from opening into the active conformation by locking it in the closed conformation (Gupta et al., 2021; Paiardi et al., 2022). The need for longer chain GAGs may also originate from glycans on the S-protein, which shield the viral surface from GAG-binding, and longer chains may overcome this shielding (Schuurs et al., 2021).
- The influence of **sulfation** pattern is quite complex. Generally, higher sulfation levels increase binding affinity (Hao et al., 2021; Jin et al., 2020; Mycroft-West et al., 2020; Paiardi et al., 2022; Tandon et al., 2021; Watanabe et al., 2021; Yue et al., 2021; Zhang et al., 2022). This suggests the importance of electrostatic interactions between the S-protein and cellular HSPGs. Certain sulfation sites were designated a prerequisite for HS-binding to the S-protein in most studies. Those are 2-*O*-sulfation (Kim et al., 2020; Liu et al., 2021; Mycroft-West et al., 2020; Watanabe et al., 2021), 6-*O*-sulfation (Dwivedi et al., 2021; Hao et al., 2021; Kim et al., 2020; Liu et al., 2021; Mycroft-West et al., 2020; Watanabe et al., 2021), and *N*-sulfation (Kim et al., 2020; Tandon et al., 2021; Watanabe et al., 2021). Exceptions from this are that the inactivation of 6-*O*-sulfotransferase had only a mild effect on the inhibition of infection by pseudotyped virus (Clausen et al., 2020; Tandon et al., 2021), but a significant reduction on S-protein binding (Clausen et al., 2020). Additionally, a study by Yue et al. (2021) postulated that 2-*S*- and 6-*S*-sulfation is not a necessity for heparin to inhibit S-protein binding to the ACE2 receptor. The presence of 3-*O*-sulfation, the molecular structure that gives heparin its antithrombotic activity, improves S-protein binding (Liu et al., 2021), albeit it is not mandatory for SARS-CoV-2 inhibition (Clausen et al., 2020; Yue et al., 2021). The increased electrostatic interaction of highly negatively charged HS with positive amino acid residues on the S-protein and the polysaccharide conformation itself might be decisive as high degrees of sulfation enhance the rigidity of polysaccharides.
- The **sialylation** pattern has not been extensively studied. However, sialic acid in the form of *N*-acetylneuraminic acid did not show substantial binding to the SARS-CoV-2 S-protein (Hao et al., 2021).

These general observations do not apply for all tested GAGs. Several compounds showed equally high binding affinities with SARS-CoV-2 as heparin, albeit they do not share monosaccharide composition, glycosidic linkage sites or stereochemistry, nor sites of sulfation (Tandon et al., 2021). However, other GAGs, e.g., keratan sulfate (Kwon et al., 2020), synthetic pentsaccharides heparinoid, frondaparinux (Partridge et al., 2021), galactose-4-sulfate (Morokutti-Kurz et al., 2021), sulfated lactobionic acid, Sulodexide, Defibrotides, and 4-*t*-butylcalix arene-*p*-sulfonic acids (Zhang et al., 2022), did not exert any inhibitory activity. These observations highlight the difficulty in deciphering the structure-affinity relationships and reenforce the need for further investigation.

3. Therapeutic application of GAGs in COVID-19 treatment

GAGs may be employed in the treatment of diseases and ameliorating their symptoms based on the anti-inflammatory, anticoagulant and mucolytic properties (van Haren et al., 2020). As an example, severe COVID-19 which may result in thrombosis, coagulopathy, hyper-inflammation, and alveolar damage may be treated with heparin (Goligher et al., 2021; Gozzo, Viale, Longo, Vitale, & Drago, 2020; Thachil, 2020; van Haren et al., 2020). Additionally, GAGs can serve as chemopreventatives against HS-binding pathogens, such as SARS-CoV-2. In the following sections, an overview of the use of GAGs as therapeutic anti-inflammatory, anti-coagulative, and anti-viral agent is given. Additionally, the challenges in applying GAGs as therapeutics are discussed.

3.1. Anti-inflammatory effects

The S-protein acts as a pathogen-associated molecular pattern (PAMP) to directly cause neuroinflammation (Frank et al., 2022), and the S1 and S2 subunits of the virus induce cytokine activity (Khan et al., 2021). The S1/S2 furin cleavage site was identified as a superantigen motif, causing hyperinflammation (Cheng et al., 2020). The S1/S2 region also has been identified as an HS-binding motif; thus, heparin may mask the site and interfere with the inflammatory process. Additionally, heparin and its derivatives can palliate inflammation by reducing the expression of pro-inflammatory mediators, e.g., cytokines, and by inhibiting inflammatory cell recruitment due to limited heparanase activity (van Haren et al., 2020). For example, by reducing interleukin 6 (IL-6) levels and increasing the concentration of lymphocytes, LMWH exerted anti-inflammatory effects in severely-ill COVID-19 patients (Shi et al., 2020). This reduced the incidence of “cytokine storms”, which arise as a result of hyper-inflammation and can lead to patient death (Shi et al., 2020). Additionally, in COVID-19 patients, UFH has been shown to be more effective in reducing inflammatory processes than LMWHs (van Haren et al., 2020). For further reading, we recommend the following literature sources (Braz-de-Melo et al., 2021; Hippensteel et al., 2020).

Table 2
List of GAGs tested for binding to SARS-CoV-2 wild-type.
SPR competition assays are always with heparin chips if not stated otherwise.

GAG	Method	Efficiency	SARS-CoV-2 variant	Reference
Heparin/UFH (not specified)	Infection/transduction with pseudotyped virus	$EC_{50} = 2.11$ μg mL^{-1}	wt	(Kim et al., 2022)
		$IC_{50} = 5990$ μg mL^{-1} (0.4 nM)	wt	(Tandon et al., 2021)
		60 % inhibition at 1000 nM	wt	(Zhang et al., 2020)
		78 % inhibition at 50×10^6 μg mL^{-1}	wt	(Dwivedi et al., 2021)
		$IC_{50} = 249{,}700$ μg mL^{-1}		
	Infection with virus	$IC_{50} = 125$ μg mL^{-1}	wt (BetaCoV/Netherlands/01/NL/2020)	(Conzelmann et al., 2020)
		>60 % inhibition at 125–250 μg mL^{-1}		
		$IC_{50} = 12.3$ nM	wt (SARS-COV-2/MT020880.1)	(Gupta et al., 2021)
		n.s.	wt (USA-WA1/2020)	(Clausen et al., 2020)
	Competitive binding of S-protein to cells	$IC_{50} = 0.033$ U mL^{-1}	wt	(Partridge et al., 2021)
		Inhibition at 10 μg mL^{-1}	wt	(Liu et al., 2021)
	SPR competition with S-protein	$IC_{50} = 76.7$ nM	N501Y mutant	(Dwivedi et al., 2021)
		n.s.	wt	(Kwon et al., 2020)
Heparin/UFH (porcine intestinal)	Infection/transduction with pseudotyped virus	72 % inhibition at 2000 nM	wt	(Yue et al., 2021)
		n.s.	wt	(Kwon et al., 2020)
	SPR competition with pseudotyped virus	$IC_{50} = 18.2$ μg mL^{-1}	wt	(Yan et al., 2021)
		n.s.	wt	(Song et al., 2021; Zhang et al., 2022)
	SPR competition with S-protein	$IC_{50} = 0.750$ μg mL^{-1}	wt	(Song et al., 2021)
		$IC_{50} = 56$ nM	wt	(Kim et al., 2020; Zhang et al., 2022)
		n.s.	wt	(Kwon et al., 2020)
Heparin/UFH (porcine mucosal)	Infection with virus	n.s.	wt (Italy/UniSR1/2020)	(Mycroft-West et al., 2020)
	Competitive binding of S-protein to cells	52 % inhibition at 100 μg mL^{-1}	wt	(Guimond et al., 2022)
	SPR competition studies with RBD	$K_i = 1700$ μg mL^{-1}	wt	(Mycroft-West et al., 2020)
Heparin/UFH (mucosa and lung of porcine and bovine origin)	Infection with virus	$IC_{50} = 25$–41 μg mL^{-1}	wt SARS-CoV-2 (Australia/VIC01/2020)	(Tree et al., 2021)
LMWH (enoxaparin)	Infection/transduction with pseudotyped virus	$IC_{50} = 1080$ μg mL^{-1}	wt	(Tandon et al., 2021)
		$IC_{50} = 7810$ μg mL^{-1}	wt SARS-CoV-2 (Australia/VIC01/2020)	(Tree et al., 2021)
		n.s.	wt and (hCoV-19/Italy)	(Bermejo-Jambrina et al., 2021)
	Infection with virus	n.s.	wt and (hCoV-19/Italy)	(Bermejo-Jambrina et al., 2021)
	Competitive binding of S-protein to cells	$IC_{50} = 0.072$ U mL^{-1}	wt	(Partridge et al., 2021)
	SPR competition studies with RBD	Approx. $K_i = 50{,}000$ μg mL^{-1}	wt	(Mycroft-West et al., 2020)
LMWH (tinzaparin)	Infection/transduction with pseudotyped virus	$IC_{50} = 3721$ μg mL^{-1}	wt SARS-CoV-2 (Australia/VIC01/2020)	(Tree et al., 2021)
		n.s.	wt and (hCoV-19/Italy)	(Bermejo-Jambrina et al., 2021)
	Infection with virus	n.s.	wt and (hCoV-19/Italy)	(Bermejo-Jambrina et al., 2021)
LMWH (dalteparin)	Infection/transduction with pseudotyped virus	n.s.	wt and (hCoV-19/Italy)	(Bermejo-Jambrina et al., 2021)
	Infection with virus	$IC_{50} = 3428$ μg mL^{-1}	wt SARS-CoV-2 (Australia/VIC01/2020)	(Tree et al., 2021)
		n.s.	wt and (hCoV-19/Italy)	(Bermejo-Jambrina et al., 2021)
	Competitive binding of S-protein to cells	$IC_{50} = 0.558$ U mL^{-1}	wt	(Partridge et al., 2021)
LMWH (nadroparin)	Infection/transduction with pseudotyped virus	n.s.	wt and (hCoV-19/Italy)	(Bermejo-Jambrina et al., 2021)
TrisS HS	Infection/transduction with pseudotyped virus	n.s.	wt	(Kwon et al., 2020)
	SPR competition with S-protein	$IC_{50} = 120$ nM	wt	(Kim et al., 2020)
		n.s.	wt	(Kwon et al., 2020)
NACH	Infection/transduction with pseudotyped virus	n.s.	wt	(Kwon et al., 2020)
	Competitive binding of S-protein to cells	Inhibition at 10 μg mL^{-1}	wt	(Liu et al., 2021)
	SPR competition with S-protein	$IC_{50} = 26{,}000$ nM	wt	(Kim et al., 2020)
Pentosan polysulfate		n.s.	wt	(Zhang et al., 2022)

(*continued on next page*)

Table 2 (*continued*)

GAG	Method	Efficiency	SARS-CoV-2 variant	Reference
	SPR competition with pseudotyped virus			
Pentosan polysulfate	SPR competition with S-protein	IC_{50} = 35 nM	wt	(Zhang et al., 2022)
Pixatimod	Infection with virus	EC_{50} = 2.7–13.2 μg mL^{-1}	wt (strain VIC01, DE-Gbg20, QLD02, QLD935)	(Guimond et al., 2022)
	Competitive binding of S-protein to cells	78 % inhibition at 100 μg mL^{-1}	wt	(Guimond et al., 2022)
Mucopolysaccharide polysulfate	SPR competition with pseudotyped virus	n.s.	wt	(Zhang et al., 2022)
Mucopolysaccharide polysulfate	SPR competition with S-protein	IC_{50} = 9 nM	wt	(Zhang et al., 2022)
Sulfated polysaccharide mix from sea cucumber	Infection/transduction with pseudotyped virus	Inhibition at 100 μg mL^{-1}[a]	wt	(Song et al., 2020)
	Infection with virus	IC_{50} = 9.1 μg mL^{-1}[a]	wt	(Song et al., 2020)
Fucoidan RPI-27	Infection/transduction with pseudotyped virus	EC_{50} = 8.3 ± 4.6 μg mL^{-1} (83 nM)[b]	wt	(Kwon et al., 2020)
	SPR competition with S-protein	n.s.	wt	(Kwon et al., 2020)
Fucoidan	Infection/transduction with pseudotyped virus	50 % inhibition at 100 μg mL^{-1}[c]	wt	(Morokutti-Kurz et al., 2021)
Fucoidan	Infection with virus	IC_{50} = 15.6 μg mL^{-1}[d]	wt	(Song et al., 2020)
Sulfated fucan[e]	Infection/transduction with pseudotyped virus	IC_{50} = 33,200 μg mL^{-1}[e]	wt	(Tandon et al., 2021)
Sulfated galactofucan	SPR competition with pseudotyped virus	K_i = 165 nM[b]	wt	(Jin et al., 2020)
Sulfated galactan	Infection/transduction with pseudotyped virus	IC_{50} = 54,000 μg mL^{-1}[f]	wt	(Tandon et al., 2021)
	Infection/transduction with pseudotyped virus	EC_{50} = 2.00 μg mL^{-1}[f] Low MW fractions without coagulation activity: EC_{50} = 3.55–6.59 μg mL^{-1}[f]	wt	(Kim et al., 2022)
Holothurian sulfated glycans	Infection/transduction with pseudotyped virus	IC_{50} = 19,600–27,900 μg mL^{-1}[g]	wt	(Dwivedi et al., 2021)
	SPR competition with S-protein	IC_{50} = 1.9–12.3 nM[g]	N501Y mutant	(Dwivedi et al., 2021)
Iota-carrageenan	Infection/transduction with pseudotyped virus	IC_{50} = 2.6 μg mL^{-1}[h] 80 % inhibition at 10 μg mL^{-1}[h]	wt	(Morokutti-Kurz et al., 2021)
	Infection with virus	IC_{50} < 125 μg mL^{-1}	wt	(Song et al., 2020)
	Infection with virus	Near complete inhibition at 3750 nM[h]	wt (PR-1)	(Morokutti-Kurz et al., 2021)
Iota-carrageenan with xylitol	Infection with virus	IC_{50} < 6.0 μg mL^{-1}[h]	wt (USA-WA1/2020)	(Bansal et al., 2021)
Kappa-carrageenan	Infection/transduction with pseudotyped virus	80 % inhibition at 100 μg mL^{-1}	wt	(Morokutti-Kurz et al., 2021)
Lambda-carrageenan	Infection/transduction with pseudotyped virus	80 % inhibition at 100 μg mL^{-1}	wt	(Morokutti-Kurz et al., 2021)
	Infection with virus	EC_{50} = 0.9 ± 1.1 μg mL^{-1}[i]	wt (BetaCoV/Korea/-KCDC03/2020)	(Jang et al., 2021)
Rhamnan sulfate	Infection/transduction with pseudotyped virus	IC_{50} = 2.39 μg mL^{-1}[j] >80 % inhibition at 1 μg mL^{-1}[j]	wt	(Song et al., 2021)
	SPR competition with S-protein	IC_{50} = 1.6 μg mL^{-1}[j]	wt	(Song et al., 2021)
Glucuronomannan	SPR competition with pseudotyped virus	K_i = 165 nM[b]	wt	(Jin et al., 2020)

NACH, non-anticoagulant low molecular weight heparin.
n.s., not specified efficiency.
TriS, non-anticoagulant trisulfated heparin.
[a] From *Stichopus japonicus*.
[b] From *Saccharina japonica*.
[c] From *Undaria pinnatifida* and *Fucus vesiculosus*.
[d] From *Padina boryana*.
[e] From *Lytechinus variegatus*.
[f] From *Botryocladia occidentalis*.
[g] From *Botryocladia occidentalis*, *Lytechinus variegatus*, and *Isostichopus badionotus*.
[h] From *Euchema spinosum*.
[i] From *Chondrus crispus*.
[j] From *Monostroma nitidium*.

3.2. Anti-coagulative effects

Systemic hypercoagulability, especially venous thromboembolism (VTE), is a high risk side-effect of COVID-19 (Cate, 2021). Interestingly SARS-CoV-2 is unique in that the S-protein, responsible for viral attachment, acts as a strong coagulant in affected patients (Gozzo et al., 2020). This is presumably caused by the binding of SARS-CoV-2 to HS, inhibiting endogenous heparin in the body, particularly in the lung, and leading to increased risk of blood clots (Zheng et al., 2021). The reports on the effect of prophylactic treatment with heparins to prevent coagulopathy are contradictory. On the one hand beneficial outcomes after treatment of critically-ill COVID-19 patients with UFH or LMWH were

observed (Spyropoulos et al., 2021; Tang et al., 2020). On the other hand no improvement with this therapy was reported (Goligher et al., 2021). It is suspected that non for noncritically ill patients benefit more from the heparin treatment (Lawler et al., 2021). Thus, the National Institutes of Health (NIH) recommends in a statement paper the use of anticoagulants as antithrombotic therapy in hospitalized COVID-19 patients only if thromboembolic disease is diagnosed or respective pre-indications exist (National Institutes of Health, 2022). Considering the versatile modes of action of heparin in COVID-19 treatment, we recommend the consultation of the following literature body for deeper insights (Braz-de-Melo et al., 2021; Hippensteel et al., 2020).

3.3. Anti-viral effects

The antiviral activity of GAGs against SARS-CoV-2 has been extensively demonstrated. However, GAGs must be administered prophylactically or in the very early stages of a SARS-CoV-2 infection to prevent viral infection. Prophylactic treatment with heparin poses the challenge of risk assessment concerning a severe COVID-19 course and unpredictable side effects, e.g., severe bleeding. Thus, non-systemic and non-parenteral approaches might offer advances for therapy. Considering that the main route of entry for SARS-CoV-2 is the respiratory tract, the inhalation of the drug is a possible site-specific route of administration (Conzelmann et al., 2020; Erelel et al., 2021; van Haren et al., 2020). Two carrageenan nasal spray, Boots Dual Defence (Salih et al., 2021) and Coldamaris (Erelel et al., 2021; Salih et al., 2021) have shown activity against coronaviruses. Another product, using UFH from porcine mucosa (van Haren et al., 2020), is expected to convey inhibitory effects on SARS-CoV-2 infection in its applied concentration range (Tree et al., 2021).

3.4. Clinical trials

At the time of this review, 112 clinical trials for the use of heparins in COVID-19 patients were registered with the FDA, and 41 of those were completed (retrieved from clinicaltrials.gov). The heparins most often mentioned are UFH or different LMWH, e.g., dalteparin and enoxaparin (Table 3). In addition, sulfated polysaccharides such as carrageenan are applied.

Summarizing the studies on the benefits of heparins for COVID-19 patients, the results are contradictory (Chakraborty, Sharma, Bhattacharya, Agoramoorthy, & Lee, 2021): Reports on the improvement of clinical outcomes (Giossi et al., 2021; Paranjpe et al., 2020; Sholzberg et al., 2021; Tang, Bai, et al., 2020) are described as well as no effect (Goligher et al., 2021), or even worse outcomes due to side-effects such as major bleeding (Cate, 2021). Such differences might originate from the severity of the disease (Cate, 2021; Sholzberg et al., 2021; van Haren et al., 2020), e.g., the thrombotic and inflammatory damage in critically ill patients is too severe to be influenced by heparins (Cate, 2021). Additionally, the dose of the heparins plays a major role and is still an open question (Giossi et al., 2021; National Institutes of Health, 2022).

Table 3
List of GAGs being tested in clinical trials against SARS-CoV-2 infections. The information was retrieved from clinicaltrials.gov with the respective GAG name and "SARS-COV-2" as search items using all substances summarized in Table 2.

GAG		# of clinical trials	Comments
UFH		114	Mostly used as thromboprophylaxis
LMWH	Dalteparin	76	Mostly used as thromboprophylaxis
	Enoxaparin	57	In some trials as control
	Tinzaparin	65	
	Nadroparin	1	
Carrageenan		7	Mostly used to prevent infection or to reduce virus load
Mucopolysaccharide		3	

In conclusion, the administration of heparins in SARS-CoV-2 infected patients is still a case-to-case decision depending on the severity of the illness and the accompanied symptoms.

3.5. Challenges of GAG-based therapeutics

The described array of effects from therapeutic GAG application indicates the potential against SARS-CoV-2 infection and for COVID-19 treatment. GAGs like heparin are unique in their ability to inhibit virus infection and at the same time, answer to the body's immune response by suppressing coagulation and inflammation. However, these effects can be challenging to control, especially in severely ill patients, as described in the previous section. Additionally, accompanying heparin implementation, the risk of significant bleeding and immune-mediated heparin-induced thrombocytopenia needs careful evaluation (Hippensteel et al., 2020). Unfortunately, the risk factors for complications with heparin treatment correlate with the patients, which are most commonly severely ill with COVID-19 (Hippensteel et al., 2020). Thus, health agencies, e.g., the US National Institute of Health, propose treatment options depending on several factors to reduce the risk of side effects such as major bleeding (National Institutes of Health, 2022). The latter publication offers a comprehensive review of studies conducted within the field of UFH and LMWH application to treat COVID-19 and as prophylaxis. Here, another review should be recommended, which extensively summarized immunotherapeutic treatment options for COVID-19 (van de Veerdonk et al., 2022).

Some natural GAGs lack the heparin-specific anti-coagulant and anti-inflammatory character. Thus, their application, e.g., as site specific protective barrier, is more likely. Here, they are not systemic, and thus offer safe application. Overall, the application of GAGs is widely known and a common approach to treat different diseases. Thus, making it easy to be quickly repurposed for other indications (Hippensteel et al., 2020). The pharmaceuticals are cheap, e.g., compared to antibodies.

Other anti-SARS-CoV-2 strategies, such as inhibition of binding to the ACE-2 receptor, e.g., with alunacedase alfa, or the TMPRSS2 receptor, e.g., with camostat, nafamostat or upamostat, are being tested, but their side-effects by inhibiting the essential receptors should be carefully considered. These and other strategies are extensively discussed in the reviews by Chitsike and Duerksen-Hughes (2021) and W. Yan, Zheng, Zeng, He, and Cheng (2022).

4. Perspectives for future GAG-based therapeutics against SARS-CoV-2 infection

While many things are unknown about the future of SARS-CoV-2, it is certain that the virus, in general, and the spike protein specifically will continue to evolve, thereby requiring more treatment and prevention options apart from adapted vaccines. Considering the mutations of the virus over the past two years towards a more basic surface charge of the S-protein, we expect the binding to cell HS to increase in affinity with future variants. With this prospect, it might be possible to get first insight into the binding affinity of SARS-CoV-2 to the HSPGs of the host cells and thus the chance of infection only based on the amino acid sequence. Additionally, the interference with SARS-CoV-2-attachment to host cell receptors using GAGs can be predicted with this information and computational approaches. Nevertheless, a hybrid approach incorporating, both in silico and experimental methods, is needed to evaluate the efficacy of promising candidates. Individual methods by themselves demonstrate limited accuracy for identifying viable drug candidates (Llanos et al., 2021; Muratov et al., 2021). Such studies have been undertaken only by few authors for COVID-19 drugs (Kim et al., 2022; Yang et al., 2020).

With regards to the application of different GAGs for anti-SARS-CoV-2 treatment, traditional heparin shows the best results in preventing binding of SARS-CoV-2 to host cells, however, low molecular weight heparin and analogs were promising with fewer side effects, i.e.,

systemic bleeding (Thachil, 2020; Tree et al., 2021). For example, enoxaparin (Tandon et al., 2021), the non-carbohydrate heparin analog, suramin (Salih et al., 2021), and pixatimod (Guimond et al., 2022) have shown protective activity against SARS-CoV-2 in cell culture without cytotoxicity (see Table 2). These molecules could provide possible prophylactic options against infection or to reduce virus load. Certainly, further FDA-approved GAGs could be repurposed, including pentosan polysulfate or mucopolysaccharide polysulfate, which induced higher inhibitory properties than heparin (Zhang et al., 2022). In addition, naturally occurring GAGs without cytotoxicities and anticoagulating activity might serve as promising pharmaceuticals for the inhibition of SARS-CoV-2 infection (Dwivedi et al., 2021; Jin et al., 2020; Song et al., 2021). They are often widely available, cheap, and biocompatible compounds.

Finally, next to the choice of the antiviral agent, the route of application may be decisive. Site specific treatment via inhalation of nasal sprays might prevent virus infection without systemic application of GAGs (Conzelmann et al., 2020; Erelel et al., 2021; Tandon et al., 2021; van Haren et al., 2020). The inhalation approach circumvents heparins entering the coagulation system due to its poor serum bioavailability (Tandon et al., 2021; Tree et al., 2021). An example is carrageenan from algae, which is already widely used as nasal sprays and can offer at-site protection against infection, e.g., for health care personnel.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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No data was used for the research described in the article.

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