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An unexpected biomaterial against SARS-CoV-2: Bio-polyphosphate blocks binding of the viral spike to the cell receptor

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No other virus after the outbreak of the influenza pandemic of 1918 affected the world’s population as hard as the coronavirus SARS-CoV-2. The identification of effective agents/materials to prevent or treat COVID-19 caused by SARS-CoV-2 is an urgent global need. This review aims to survey novel strategies based on inorganic polyphosphate (polyP), a biologically formed but also synthetically available polyanionic polymeric material, which has the potential of being a potent inhibitor of the SARS-CoV-2 virus-cell-docking machinery. This virus attaches to the host cell surface receptor ACE2 with its receptor binding domain (RBD), which is present at the tips of the viral envelope spike proteins. On the surface of the RBD an unusually conserved cationic groove is exposed, which is composed of basic amino acids (Arg, Lys, and His). This pattern of cationic amino acids, the cationic groove, matches spatially with the anionic polymeric material, with polyP, allowing an electrostatic interaction. In consequence, the interaction between the RBD and ACE2 is potently blocked. PolyP is a physiological inorganic polymer, synthesized by cells and especially enriched in the blood platelets, which releases metabolically useful energy through enzymatic degradation and coupled ADP/ATP formation. In addition, this material upregulates the steady-state-expression of the mucin genes in the epithelial cells. We propose that polyP, with its two antiviral properties (blocking the binding of the virus to the cells and reinforcing the defense barrier against infiltration of the virus) has the potential to be a novel protective/therapeutic anti-COVID-19 agent.

Keywords: Inorganic polyphosphate; SARS-CoV-2; Polyanion; Spike protein; Nanoparticle

Introduction and scope of the review
Phosphorus is a central element in both materials science and life sciences. Due to its versatile structural and physicochemical properties, phosphorus-based materials, especially materials based on the salts of phosphoric acid, have drawn great attention from purely academic basic research to applied, industrial or medical directions. The phosphate anion, as a functional group in a wide variety of biomolecules, most likely developed in the photic zone of the Archean Ocean (4000–2500 million years ago). This phosphate species is not only of central importance for the origin of life, as it is part of the nucleotide building blocks of DNA and RNA, but is also an essential component of energy transfer molecules such as ATP (reviewed in: [1]). Some phosphate materials are storing metabolic energy. Pyrophosphate is the first material, which is synthesized from two phosphate units by exploiting the energy of a proton gradient coming directly or indirectly from light. Compounds with energy-rich bonds like ATP or pyrophosphate are required for the synthesis of complex biomolecules. Even though the phosphoanhydride bonds in these molecules are thermodynamically unstable, catalysts (enzymes) are needed to break these linkages under physiological conditions. Inorganic polyphosphate (polyP), an ancient and
well-conserved polymer is exceptional. It is monotonously formed from tetrahedral $\text{PO}_4$ (phosphate) structural units linked via phospho-anhydride bonds to chains of up to 1000 phosphate units; cyclic molecules also exist with smaller chain lengths. Phosphorus, as an element, has been proven to play a part as an indispensable component in energy metabolism chains, acid-base balance, and finally also in the genetic substances transfer [1,2]. Furthermore, pnictogens (like non-metal phosphorus or the metalloids arsenic and antimony) have been introduced as molecular structures with favorable applications in nanomedicine [3]. Polymeric polyanionic compounds from oxynion analogous to orthophosphate ($\text{PO}_4^{3-}$), like vanadate ($\text{VO}_4^{3-}$), sulfate ($\text{SO}_4^{2-}$) and arsenate ($\text{AsO}_4^{3-}$) are not found in nature, possibly because of their low stability compared to polyP, which only hydrolyzes very slowly at room temperature in aqueous neutral solutions [1]. PolyP is placed at the interface between inorganic chemistry and biochemistry. Since this inorganic polymer is enzymatically synthesized and hydrolyzed, it can be considered as a quasi-organic molecule, which lacks carbon (a characteristic feature of organic compounds). It behaves like a biological material, similar to bio-silica as an example for another enzymatically formed inorganic material [4], and fulfills a number of essential physiological functions and metabolic properties like other organic metabolites [1,5,6].

PolyP which is synthesized in every bacterial or eukaryotic cell, acts as a morphogenetically active material and elicits transcriptional readout of important cellular events, especially during anabolic bone mineralization in vitro and in vivo [5,7]. In addition, polyP is the only natural polymer that combines the functions of compartment formation (coacervation), signaling, and acting as a primary storage of energy in its covalent bonds. PolyP is the most ancient energy-carrying molecule [8–10] and might have functioned as the basis for proto-cell formation 3900 million years ago [11]. Due to its properties, to provide metabolic energy and simultaneously to deliver phosphate as a precursor, polyP was proven to act anabolically effective on bone and cartilage synthesis and repair (reviewed in: [1]). In addition, polyP undergoes dynamic coacervation during wound regeneration, even in animals suffering of diabetes [5]. This ability to form a coacervate also provided the basis for a successful exploitation of the material as a bioink for three-dimensional bioprinting of organ-like units [12].

PolyP is an anionic polyelectrolyte, interacting with oppositely charged ions. Polyelectrolytes can associate together through electrostatic interactions via attraction forces between two partially ionized species with opposite charges. Those electrostatic interactions, like between (poly)arginine and (poly) phosphate, can be strong and are sometimes even attributed as “covalent-like” [13]. Polyanions act as receptors for many human viruses [14]. Prominent polycations are the polyanines, which interact with negatively charged nucleic acids, proteins and phospholipids via ionic and/or hydrogen bonds [15].

In medicine, polyelectrolytes have so far mainly been used on a large scale as soft particles or as coatings for complex, oppositely charged polyelectrolytes or counterions. In contrast, most pharmaceuticals are not polymers but low-molecular-weight natural products, or derivatives of them, with arabinofuranosyladenine or arabinofuranosylcytosine as examples, which inhibit herpes simplex virus outbreak on lips or are used for a treatment of leukemia [16,17,18]. These compounds inhibit either competitively or non-competitively enzyme activities through direct or indirect binding and inhibition as ligand-like compounds at their active sites (Fig. 1(a,b)). More recently, poly-
The substrate for the enzymatic synthesis of polyP, ATP, was discovered by Lohmann [23]. The first clue that polyP is an energy-rich phosphate was provided by Ebel [24] who disclosed the structural relationship between ATP and polyP. The evidence that polyP plays significant functions in metabolism was elaborated in yeasts [25]. Especially driven by studies of Kulaev [26], Sylvia Ruth Kornberg [27] and her later husband Arthur Kornberg [28] polyP was given a prominent place in biochemistry. The synthesis of polyP was co-discovered with the one of DNA [28].
Besides of the platelets (thrombocytes), the mast cells are filled with polyP chains which are encapsulated into acidocalcisomes as well [43]. These cells have a longer half-life with ~40 d [44], while the half-life of platelets is only 8–9 d. The precursor cells of the platelets are the megakaryocytes (50–100 μm in diameter). It needs to be elaborated if in these precursor cells the polyP polymers are synthesized [45]. About 2000–5000 new platelets are formed from a megakaryocyte (Fig. 3(a)) [46]. In the platelets, two pools of polyP are present. First, a polyP fraction with a chain length of 60–90 Pi units, and second, a pool with polymers of a longer average chain length of 200–1000 Pi units [47,48]. Importantly, short-chain polyP is released in a soluble form, while the long-chain polyP molecules become encapsulated into 100 to 200 nm large particles [49]. Interestingly, only the long-chain polyP fraction binds to the platelets, while short-chain polyP does not and exists in the soluble phase [50].

Until now, a mammalian enzyme that exclusively hydrolyses the phosphoanhydride bonds of the linear polyP molecule, a polyP-specific polyphosphatase, has not been discovered. In addition to the polyP-degrading (but non-specific) ALP, the insect and vertebrate protein h-prune, a short-chain polyP exopolyphosphatase, and the diadenosine and diphosphoinositol phosphohydrolase, an endopolyphosphatase hydrolyze polyP [54,55].

### Polyphosphate conformation and configuration

The polyP polymers are flexible molecules with a bond angle of 130° at the P–O–P linkage and of 102° at the O–P–O atoms (Fig. 4(a)). The P–O bond length at the oxygen bridges of polyP is 1.62–1.66 Å [56,57]. In aqueous solution, the polyanionic polyP chain takes an extended conformation because of the Coulomb repulsion of the negatively charged Pi groups [58]. The orientation of the linked PO₄ tetrahedra adapts to either an eclipsed or a staggered conformation (Fig. 4(b and c)) [59,60]. In the Ca²⁺ salt form, the polyP chain adopts an eclipsed conformation (Fig. 4(b)), while the monovalent Na⁺ as counterion favors the staggered conformation of polyP (Fig. 4(c)) [61]. This transition might have a functional importance. This differential salt formation of polyP provides the polymer with a type of "induced fit" potential. With Na⁺ as a counterion, the P=O π bonds arranged along the polyP chain (Fig. 4(c)) have a longer spacing (~4.5 Å) compared with the exposing P–O bonds in the salt with Ca²⁺.
with \( \approx 2.5 \, \text{Å} \) (Fig. 4(b)) [62]. These values imply that for an association of polyP with peptide stretches (spacing of consecutive peptide bonds of around \( \approx 4.5 \, \text{Å} \)) a staggered conformation with Na\(^+\) will most likely match better than the eclipsed conformation.

Furthermore, mitochondria have a comparable low concentration of Ca\(^{2+}\) with \(0.2–20\, \text{mM}\) in comparison to the acidocalcisomes with \(2\, \text{M}\) [1]; the concentration of Na\(^+\) in the cytoplasm varies between 50 and 60 mM [63]. These concentration differences most likely dictate the type salt formation between the anionic polymer and the respective cations. In the mitochondria and the cytoplasm the polymer should be present in a soluble form with the counterion Na\(^+\), while in the acidocalcisomes polyP exists as Ca\(^{2+}\) salt nanoparticles [48]. In a biomimetic approach using the particles in the acidocalcisomes as a template [48], amorphous polyP nanoparticles have been prepared by our group, applying a super-stoichiometric ratio of Ca\(^{2+}\) to phosphate, in an alkaline environment at a pH of 10 [64]. The size of the mesoporous nanoparticles formed varies around 200–500 nm (Fig. 4(d-i)); they display morphogenetic activity (reviewed in: [5,65]). A similar procedure with Mg\(^{2+}\) and polyP likewise resulted in the synthesis of amorphous nanoparticles with a slightly smaller size of \(\approx 100\) nm (Fig. 4(d-ii)) [66].

**PolyP coacervation**

The process of coacervation proceeds in an aqueous phase with macromolecules, like polyP, and results first in a formation of a dense phase that is in thermodynamic equilibrium with a dilute phase. This kind of “lyophilic colloid” material forms spontaneously in water at neutral pH and constructs stable membrane-like structures. In the salt form with the cation Ca\(^{2+}\), polyP forms a coacervate phase at a close to neutral pH, which is most likely the physiological active form of the polymer [67]. Earlier studies have shown that nanoparticles tightly associate with biomolecules under formation of (hard) protein corona particles with modified and changed biomedical properties [68].

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**FIGURE 3**

Intracellular synthesis and accumulation of polyP; partially hypothetical scheme. (a) Blood platelets are the major stores of polyP. This polymer is presumably synthesized in megakaryocytes, from where the platelets originate by cytoplasmic fragmentation. (b) In mitochondria ATP is produced from reducing equivalents that accumulate during the metabolism of glucose, fatty acids, amino acids and pyruvate, like in the citric acid cycle. The reducing equivalents formed (NADH, FADH\(_2\)) drive the electron transport chain at the inner mitochondrial membrane, followed by an export of the protons into the intermembranous space. From there the protons are rechanneled via the inner mitochondrial membrane and thereby drive the ATP production by ATP synthase. Finally, ATP releases the mitochondrion via the adenine nucleotide translocase (ANT; inner mitochondrial membrane) and the voltage-dependent anion channels (VDAC; outer mitochondrial membrane) into the cytoplasm. Adjacent to the mitochondria are the acidocalcisomes. In the membranes of these organelles, the Vtc complex is located with its subunits 1–4, where, in budding yeast, polyP is polymerized and pressed into the lumen of the organelle. In vertebrates this pathway is unknown. Adapted with permission [1]. Copyright 2019 American Chemical Society.
By this, the beneficial effect of some nanoparticles become confined [69].

The polyP coacervation is prevented if, like in the experiments with Ca-polyP nanoparticles, their zeta (ζ) potential increases and in turn interferes with the fusion process [70]. An increase in the negativity of the ζ potential is favorable for implant materials [70] but not for a material with an intimate contact to the target cells in which polyP should elicit its functional activity. The ζ potential of the Ca-polyP nanoparticles is in the range of 

\[ -42.3 \text{ mV} \]

for particles with a size between 307 and 859 nm and

\[ -33.6 \text{ mV} \]

for particles of 61–198 nm. In turn, the nanoparticles are stable, also in culture medium, for about 10 h (Fig. 5(a)) [67].

However, since the ζ potential is prone to ionic polyelectrolytes or macromolecules like proteins, adsorption of these molecules to the particles reduces the ζ potential, followed by a concomitant shift of the slipping plane from the solid surface of colloidal particles [72]. The reduction of the ζ potential is paralleled with an increase of biocompatibility and a faster release of morphogenetic signals to the cellular environment [67,71]. After transfer of the Ca-polyP nanoparticles (or – even more efficient – Mg-polyP nanoparticles) into cell culture medium supplemented with fetal calf serum, the particles attract protein(s)/peptides out of the aqueous environment and integrate them into the dynamic coacervate (Fig. 5(b)) [67]. A scanning electron microscopic analysis of the transformation of nanoparticles to a coacervate is shown in Fig. 5(b-i to iv).
FIGURE 5
Transition of Ca-polyP nanoparticles to the corresponding coacervate (CoA). (a) At pH 10 Ca-polyP, or even faster Mg-polyP, nanoparticles are formed. These particles show a relatively long stability in culture medium without serum. (b) After transfer into medium and serum of pH 7, the nanoparticles undergo coacervation, under concomitant inclusion of serum peptides or other morphogenetically active supplements. After addition of peptides to the nanoparticles, with a high \( \zeta \) potential, the potential decreases and the coacervate is formed (i to iv). The coacervate phase has a lower stability.
Release of metabolic energy

Within the polyP polymer chain the P_i units are linked via energy-rich acid anhydride bonds to non-branched chains \[1,8,73\]. In the biotic environment two enzymes are present that process polyP and its reaction components. At first, polyP is hydrolyzed from the ends of the molecule under release of P_i and polyP_{n-1} by ALP (Fig. 6(a and c)) \[74,75\]. The degradation follows a processive mechanism (the enzyme remains attached to the polyP substrate until complete degradation before it dissociates from the substrate). Because of the higher affinity of ALP to the longer polyP molecules (lower \(K_m\) value) those chains are degraded first before hydrolysis of the shorter molecules can start \[74\]. The ALPs are promiscuous enzymes \[76\] since besides of polyP, they hydrolyze ATP, ADP, AMP, PP_i, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, \(\beta\)-glycerophosphate and others, at a pH between pH 7.5 and pH 9.4. During the ALP driven degradation, energy \((\Delta G^0)\) is released that can be at least partially transformed to biochemically utilisable metabolic energy \[77\]. Experimental data show that this energy is used for the formation of an energy-rich phosphoanhydride bond in ADP, via phosphoryl transfer to AMP (Fig. 6(b)) \[78–80\]. During this reaction the P_i units released from polyP are linked in the transition state, as metaphosphate, to an intermediate compound comprising an acid polarized carbon atom, similar to the one which exists in the mesomeric guanidino group in the protonated Arg (Fig. 6(b and d)) \[1,81,82\]. In a subsequent step, the intermediate P_i/metaphosphate unit is transferred to AMP under the formation of ADP. In continuation, ADP is then up-phosphorylated to ATP under the consumption of two moles ADP and the release of one mole AMP via the adenylylate kinase (ADK), likewise a ubiquitously distributed extracellularly localized (cell membrane bound) enzyme (Fig. 6(b)) \[83\].

The generation of ATP, after exposure of polyP to cells, is substantiated by cell culture studies \[79,80\]. Addition of the ALP inhibitor levamisole to the system blocks the synthesis of ATP from AMP \[84\]. Additional exposure with Ap5A, a dinucleoside polyphosphate which is an inhibitor of ADK, abolishes the ATP generation, also in the extracellular space \[80,83\]. Therefore,

**FIGURE 6**

PolyP as ATP generator and phosphate donor in the extracellular space. (a) The physiological polymer polyP is hydrolysed by the ALP under liberation of orthophosphate (P_i). The polyacrylamide-urea gel shows that during the 60 min incubation period with 5 \(\mu\)g/mL of commercially available ALP (calf intestine, \(\geq1500\) units/mg) the size of the Na-polyP_{40} chain (polyP with an average chain length of 40 P_i units) decreases close to the oligomer marker with 3 P_i units. Staining with toluidine blue. (b) Storage of liberated Gibbs free energy \((\Delta G^0)\) liberated during cleavage of the energy-rich phosphoanhydride bonds, which occurs stepwise by formation of an energy-rich intermediate (Im). This intermediate contains a metaphosphate species, formed during the reaction and bound to a positive, acid polarized carbon atom in a mesomeric stabilized guanidinium group of the enzyme. By this, a gradual transfer of the Gibbs free energy to AMP, under formation of ADP, takes place. Subsequently, the enzyme ADK catalyses the synthesis of ATP from ADP under liberation of AMP. Finally, ATP is channeled into the organismic metabolism, used, e.g., for substrate (S) phosphorylation reactions. (c) The hydrolysis of polyP by ALP starts at the ends of the polymer. The activated P_i intermediate (metaphosphate) formed during cleavage of the phosphoanhydride bond is transferred (d) to the guanidinium side chain (Arg-P_i interaction), or it can associate and then bind to Arg under formation of a phosphoramidyl group (Arg-P linkage). In this way, the derivatized guanidinium causes a stabilization and orientation of the Arg residues on the surface of the receptor binding domain (RBD) of the SARS-CoV-2 spike (S) protein. Adapted with permission \[1\]. Copyright 2019 American Chemical Society.
we propose a coupled ALP-ADK reaction as the metabolic chain leading to the generation of extracellular ATP from polyP.

**Binding of SARS-CoV-2 to the cellular host**

The viruses have developed intricate systems to enter the host cell and to usurp the host cellular replication machinery. As obligate intracellular parasites, all these processes are prerequisites for them to propagate and to produce progeny viruses. If the virus succeeds in penetrating the host cell, serious diseases, like in COVID-19 (coronavirus disease 2019), or chronic diseases, such as AIDS or viral hepatitis, can emerge. An inhibition of binding of SARS-CoV-2 to the respective host cells is a key for a prevention of viral infection. In the following it is outlined that polyP is an effective biomaterial which interacts with the viral binding protein and by this blocks its attachment to the cell surface.

**The virus and the disease**

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the etiologic agent of COVID-19 [85]. Already before the outbreak in Wuhan, China, and the subsequent spreading as a pandemic, COVID-19-related infectious diseases emerged with the examples of the severe acute respiratory syndrome (SARS), first emerged in 2002 in Guangdong, China, and the Middle East respiratory syndrome (MERS) in 2012 in Jeddah, Saudi Arabia. These viruses are grouped together to the Coronaviridae, a family of enveloped, positive-strand RNA viruses, which are infectious for amphibians, birds, and mammals [86]. The Coronaviridae store their genetic information in as much as 25–32 kb [kilobases] of RNA and therefore represent the viruses with the largest known RNA. In comparison, the RNA virus HIV-1 has a small genome size with less than 10 kb [87,88]. Both viruses even amplify their genetic information by transcription of more than one open reading frame of the primary RNA information carrier [89,90]. Coronaviridae have a globular morphology with a diameter of 118–136 nm and 9–12 nm large surface projections formed by their spike (S) glycoproteins that extend from the virus envelope [91]. Members of the Coronaviridae with the subfamily of Coronavirinae are widespread in animals and cause only mild respiratory or enteric infections, with the exception of SARS-CoV-2.

SARS-CoV-2 is transmitted via an airborne route. In consequence, social distancing and mandated face covering are strongly advised as crucial and effective protections to fight the pandemic [92]. This precaution will control and reduce nebulization of virus-containing particles as a result of coughing/sneezing, and less frequently by normal breathing, talking and singing of infected individuals. The vehicle of virus transmission are aerosol droplets with a diameter of ~1–5 μm. They will be exhaled via the nose and mouth and enter the lung after passing the trachea. In the respiratory tract, they come into contact with the mucus and eventually penetrate the periciliary layer covering the airway epithelia and ultimately initiate infection of the epithelium with its goblet cells and ciliated cells [93]. Patients may develop pneumonia and severe dyspnea that require intensive care. Accordingly, an acute respiratory syndrome is considered as the hallmark of severe COVID-19 even though accumulating evidence suggests that SARS-CoV-2 also attacks other organs and various body systems [94]. The polymer polyP, which is discussed later, is expected to prevent attachment in the nasopharyngeal and oropharyngeal airways system. It has been described that thrombotic processes, occurring during the progression of COVID-19, cause coagulopathies, unexpected clotting occurring in veins. Frequently, the clinical picture of COVID-19 is linked with an abnormal low level of blood platelets, with thrombocytopenia [95]. A low platelet count parallels with an increased mortality of patients [96]. This syndrome is characterized by a decrease of cytokines/chemokines and also of polyP, which is primarily stored in the platelets [29]. A low level of polyP is also found in delta-storage-pool-disease, a thrombocytopenia, which is characterized not only by a deficiency in the level of polyP but also a pathological modulation of the processes of coagulation and fibrinolysis and a diathesis for bleeding [97].

**Virus attachment**

In this review, we focus on the initial phase of the viral replication cycle because of the following rationale. It appears to be evident that an initial inhibition of the attachment of the virus to the corresponding cellular receptors, e.g. by physiological binding molecules/materials like polyanionic polymers, or by components of the immune system, will neutralize the infectious agent [98,99]. Components of both the innate immune system (e.g., complement system) and the adaptive immune response (antibodies) appear to be promising candidates. Here we highlight that the polyanionic polymer polyP masks the most distal part of the viral spike protein with its cationic domain and blocks the interaction between the viral spike protein and the host cell receptor.

Like with any virus the SARS-CoV-2 life cycle is divided into three stages; entry (attachment, fusion, uncoating), genome replication (replication and protein translation), and exit (assembly, maturation, and release). This virus utilizes two kinds of molecules on the cell surface to attach and bind to the host cell. At first, the prominent protein at the outer surface of the lipid envelope that surrounds the virus particle, the spike S-protein, attaches to the corresponding host cell receptor, the angiotensin-converting enzyme 2 (ACE2) [100]. This initial interaction of SARS-CoV-2 with the cell is flanked or controlled by additional perhaps less specific cell surface molecules, like glycosaminoglycans–heparins that interact with the two primary components (S-protein and ACE2) and might link them together [101].

Subsequently, more downstream after binding of the viral S-protein with its exposed receptor binding domain (RBD) to ACE2, enzymes are involved that process the S-protein and, by this, prepare the virus for the fusion process with the host cell. These cellular enzymes are furin, transmembrane protein serine protease 2 (TMPRSS2), and cathepsin L; then, later, further enzymes act, which complete the viral replication cycle, the RNA-dependent RNA polymerase, helicase, exonuclease, papain-like protease, and 3C-like protease [102]. Inhibitors of these enzymes will block the viral reproduction cycle directly [103–106].
The SARS-CoV-2 spike S-protein is a glycoprotein composed of three monomeric subunits with a size of 180–200 kDa each. They comprise a large extracellular N-terminal segment, a transmembrane domain, and a shorter intracellular C-terminal segment [107,108]. Each monomer of this homotrimer consists of two subunits, the S1 subunit which carries the RBD responsible for the cell attachment [109,110]. The S2 subunit which contains the fusion peptide and the two HR [hydrophobic repeat regions] domains (HR1 and HR2) mediates the fusion of the viral envelope with the host cell membrane [108,111–113]. The polysaccharide side chains of the S-protein facilitate the passage through the mucus on the surface of the epithelia (Fig. 7 (a)). Prior to binding to the cellular receptor ACE2, the RBD has to undergo a conformational change from a closed, “down” state to an opened, “up” conformation which is needed for receptor binding (Fig. 7(b)). The cellular receptor used by SARS-CoV-2, the ACE2 protein, is a zinc-containing metalloenzyme (a carboxypeptidase) that converts angiotensin I to angiotensin 1–9, and angiotensin II to angiotensin 1–7. In contrast to its homolog ACE, which forms angiotensin II [114], ACE2 is a dimeric protein, which is primarily expressed in cells of the respiratory tract, in particular of the alveolar epithelial type II. In addition, it is present on cells of the vascular endothelium of lung, heart, intestine, and kidney [115,116]. The binding of the viral S-protein to ACE2 occurs via the RBD domain of the S1 subunit which binds to the host cell as a trimer [117]; (Fig. 7(c)). The dissociation constant of the SARS-CoV-2 S – ACE2 interaction is high and measures $K_D = 14.7\text{-nM}$ [117]. This docking reaction is paralleled by a conformational change of the S1 subunit. Only, in the opened, “up” conformation the RBD is accessible for the receptor, while in the closed, “down” state this domain is unable to efficiently bind to the receptor protein (Fig. 7(b and c)) [108,117]. The subsequent step, membrane fusion/virus entry, requires a proteolytic cleavage of the S-protein by host cell proteases into the S1 and S2 subunits (Fig. 7(d)) [118,119]. Two cleavage sites exist, the S1/S2 site, at the boundary of the S1 and S2 subunits, and the S2’ site, close

**FIGURE 7**

Initial steps of the SARS-CoV-2 replication cycle. (a) Passage of the virion across the mucus barrier. (b) Sensing of the viral envelope with its homotrimeric spike S-proteins, consisting of the S1-subunit with the receptor binding domain (RBD), and the S2-subunit, for a suitable surface receptor, for ACE2. (c) Binding of the RBD of S1 to the dimeric host cell ACE2 receptor. (d) Enzymatic/proteolytic cleavage of S-protein at the S1/S2 site. (e) Post-cleavage phase and prehairpin formation. (f) Fusion of the virus envelope with the host cell membrane. Further details are given in the text.
to the fusion peptide [120–122]. The major cleavage site is the S1/S2 site, which is characterized by the presence of a cluster of basic (Arg) aa residues [108,117]. Several host cell proteases catalyze the proteolytic activation/cleavage of the S-protein at the S1/S2 site. The main proteases involved are the serine protease, TMPRSS2, located in the host cell membrane, as well as the serine proteases furin and cathepsin L [123–125]. The plasma membrane-bound TMPRSS2 and furin are highly expressed in lung epithelial cells [126]. Proteolytic activation of the S-protein by TMPRSS2, after SARS-CoV-2 binding to ACE2, allows the virus to fuse directly with the host cell membrane (Fig. 7(e and f)). During this process, a trimeric hairpin structure is formed by the two heptad repeats HR1 and HR2. After this step, viral RNA is channeled into the host cell [124].

During the initial interaction of the viral RBD with the cellular ACE2, the virus particles have to cross not only the mucus protection layer on the outer surface of the epithelial layer but also to penetrate the bulky heparan sulfate (Fig. 8(a)). Both systems form a strong barrier between the cells and the outer respiratory system, which is in contact with the respiratory air. The glycosaminoglycan polymeric chains control the association of SARS-CoV-2 and the subsequent internalization of the virus [127]. This association has been localized at the glycosaminoglycan binding motif at the S1/S2 proteolytic cleavage site [127]. While the anionic and cell membrane associated heparan sulfate molecules link the virus to the cell surface and modulate infection (Fig. 8(c)) the anionic polyP, as a soluble, not membrane-associated polymer, blocks the binding of the viral spike protein to the cellular receptor (Fig. 8(b)). Until now it cannot predicted if, besides of the association step of the viral RBD to the cellular ACE2, polyP also inhibits the endocytosis process. Data are lacking for soluble polyP and polyP nanoparticles, but the potential seems to be there [128].

### The RBD of SARS-CoV-2 and its cationic groove

The RBD at the S-protein is the initial sensor of the virus, screening for suitable receptors at the host cell membrane, a common

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**FIGURE 8**

The hurdle-rich migration of the SARS-CoV-2 to the host cell. (a) During the travel of the virus particle with its RBD at the S-protein to the cellular ACE2 receptor two functional layers have to be crossed: the mucus protection layer and the heparan sulfate shield. The virus particles (diameter 80–140 nm) approach the target cells via the airways and there through a mucus layer as well as the cell membrane-associated heparan sulfate shield. The bulky mucus layer, measuring ~5 μm, covers the epithelial cells. In addition to mucin, the cell surface is decorated with up to 170 nm long heparan sulfate macromolecules. (b) In contrast to the heparan sulfate molecules, the polyP chains are freely present outside of the epithelial cells and block virus binding to the cells. (c) The heparan sulfate polymers interact both with the SARS-CoV-2 spike protein and the cell surface and modulate infection.
feature for all members of the Coronaviridae [119,129,130]. In SARS-CoV-2 the RBD sequence has a length of 218 aa, a theoretical molecular weight of 24,507 Da and an isoelectric point of 8.9.

Conservation of the RBD from SARS-CoV-2
The initial interaction of SARS-CoV-2 with the host cells occurs via the RBD and ACE2. This interaction is specific, since the sequence of the RBD with its characteristic pattern of basic aa, the cationic groove, is exclusively present in SARS-CoV-2. Data bank analysis with the deposited sequences revealed an expect value of $2e^{-166}$ [131]. The lower this value, i.e. the closer to zero, the more significant is the result, meaning it is unlikely that this pattern is found again in the data bank [119].

Furthermore, it is notable that none of the Arg residues arranged at the cationic groove of the SARS-CoV-2 RBD has been changed if compared with other strains of SARS-CoV-2, even to mutated SARS-CoV-2 like the one with the accession number 6XDG_E, expect value $4e^{-160}$. This finding implies that the evolutionary adaptation of the RBD is close to optimal for the present-day environment of the SARS-CoV-2 within the global human population [132].

The polycationic groove on the RBD surface
One prominent aa pattern exists on the surface of the RBD. This domain comprises a continuous stretch of the basic aa Arg and Lys [109,119]. In more details, the RBD has a surplus of basic aa (with Arg + Lys + His: 11 + 10 + 1) over the acidic aa (Asp + Glu: 15). In consequence, the calculated theoretical isoelectric point (pI) of this domain adjusts to 8.9, reflecting the abundance of basic aa over acidic aa. The pI’s of the basic aa Arg (pI of 10.8), Lys (9.5) and His (7.5) indicate that below these pH values the aa are mainly positively charged. Therefore, polyP as a polyanionic inorganic material [131] and the organic polyanionic biopolymer heparin [133], with their polyanionic surfaces, have been proposed to interact, via electrostatic interactions, with the RBD of the S-protein of coronaviruses in general [134], and of SARS-CoV-2 in particular [131,133].

On the surface of the COVID-19 RBD six basic aa (four Arg and two Lys units) are clustered together, forming a continuous trail (cationic groove) (Fig. 9a and b). Interestingly two of the Arg residues Arg 457 and Arg 466 are spatially connected with Asp 467 and Glu 465 (anionic pair), two aa that are known to build up a strong intramolecular proton transfer system [135,136]. Such reactive centers, which are formed by two cationic aa, Arg, that are traversed by the two anionic aa, Asp and Glu, facilitate a covalent-like reaction with addition to the Arg guanidinium group [137].

Using the algorithm of Kyte and Doolittle [138] a continuous stretch of aa with a hydropathy index of $-4.5$ exists, reflecting the clusters of Arg (index $-4.5$) and Lys (index $-3.9$). This path matches with the cationic groove on the RBD surface (Fig. 9c). In parallel, the electrostatic potential has been calculated by applying Poisson-Boltzmann equation [139]. The prediction revealed that the continuous Arg-Lys-rich patch (cationic groove) coincides with the positively charged areas while the

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**FIGURE 9**
Surface mapping of the S-protein RBD from SARS-CoV-2, adopted from PDBID:6M0J. In this view a polyP chain, with a length of 12 phosphate units, was used as a polyanionic model for molecular docking. (a) On the surface of the RBD space-filling model, the aa residues Arg (R) and Lys (K) are marked with their respective sequence positions. They form the cationic groove (colored in green). The crossing anionic pair of Asp (D) and Glu (E) is shown in purple, and the crossing area is circled (yellow). (b) Alignment of the polyP chain (red and purple) with 12 Pi units (P-1 to P-12) on the surface of the RBD (transparent), following the cationic groove (green). The crossing area with Asp (D) and Glu (E) is circled (yellow). The interatomic distances of the Pi units to selected Arg residues are given for R457 and P-1, R466 and P-5, R355 and P-7, as well as for R357 and P-10. (c) Hydrophobicity distribution pattern of the surface of the RBD is highlighted between the indices $-4.5$ (hydrophilic; blue) to $+4.5$ (hydrophobic; red). (d) Electrostatic potential on the RBD within $-10$ (red) to $+10$ (blue). (e) Surface topology of the RBD in the newly emerged mutant SARS-CoV-2 B.1.1.7. The spatial aa arrangement of the cationic groove is not changed. The location of the mutation asparagine (N) by tyrosine (Y) is circled in black. Adapted with permission [156]. Copyright 2020 The Royal Society of Chemistry.
crossing negatively charged stretch matches with the transvers-
ing Asp/Glu aa pair, the anionic pair (fig. 9(d)). This cationic
Arg-rich cluster of the RBD matches intriguingly perfect with
the spacing of the anionic phosphate units within the polyP
chain (fig. 9(b)). In contrast, in other organic polyanionic mole-
cules, like in heparan sulfate, non-charged building blocks inter-
rupt the charged units, the monosaccharides.

**Conservation of the cationic groove in the mutant SARS-CoV-2**
**B.1.1.7**

In September 2020 [140] a new mutant of SARS-CoV-2, termed
lineage B.1.1.7 emerged in the United Kingdom, in Kent and in
Greater London. This mutant accumulated 17 mutations in its
genome, eight of them are located in the gene that encodes the
S-protein on the virus surface. This mutational change came
not unexpected, since this virus is not equipped with a nucleic
acid repair system. In turn, a median mutation rate of
$1.12 \times 10^{-3}$ mutations per site per year has been calculated for
the SARS-CoV-2 [141]. However, this mutant turned out to be
more virulent, with a higher disease propensity. Among the
evolved mutations in the lineage B.1.1.7, one of them, the
N501Y mutation, was predicted to have a higher affinity to
human ACE2 protein than the earlier strain [142]. This mutation
took place at position 501 with asparagine (N) by tyrosine (Y)
(fig. 9(e)) [140]. While mutations in SARS-CoV-2 are usually
attributed to the zoonotic nature of this virus and facilitate a
jumping between vertebrate species, the B.1.1.7 lineage emerged
in patients and has been implicated with a higher affinity of the
RBD to ACE2, and, in parallel, with a more severe disease progres-
sion [143,144]. The modeling experiment suggests that the new
mutant N501Y should not affect the topology of the cationic
groove, since the location of the tyrosine is at the interface
between the RBD and ACE2 (fig. 9(e)).

**Inhibitory effect of polyP on the interaction of the**
**viral RBD with cellular ACE2**

It is conceivable that for a selective inhibition of the interaction
between a ligand (like the RBD of the viral S-protein) and the cor-
responding receptor, e.g. with ACE2, a unique sequence or pat-
tern must be present on the RBD. As outlined, such as sequence was identified with the cationic groove on the SARS-
CoV-2 RBD surface (fig. 9(a)).

**Alignment of polyP on the RBD**

The mentioned hydrophilicity/hydrophobicity analyses of the
surface of RBD, together with the electric charge distribution pat-
tern and the presence of aa with negative hydropathy indices
suggested that the highly charged polyanionic polymer polyP
with its high charge distribution shell could tightly interact with
the RBD. In the first studies on the effect of polyP on the binding
affinity between the viral RBD and cellular ACE2 receptor a poly-
mer with a chain length of ~40 P_i units has been tested [131]. For
the docking experiments polyP with 12-15 P_i units is used since
this length can span the entire RBD.

PolyP provides the steric prerequisites to tightly interact with
the RBD due to the rotational flexibility of the polymer and its
ability to bend at the anhydride linkages with 130° and 102°
[1]. Longer chains or overhangs of the polymer, after binding
to the RBD and to the RBD:ACE2 complex, could even bind to
the ACE2 receptor as well (fig. 10). Interestingly, at the inter-
face/docking site between RBD and ACE2 a likewise cluster of
the basic aa Arg (R393), Lys (K31 and K68) and His (H34) is present
which would allow longer polyP chains, after binding to the
RBD, to turn back and also to bind at this interface. Such bending
would be facilitated by a partial interaction of the polyP chain
with cations. At a higher resolution polyP binds to the RBD at
the Arg/Lys (R/K) residues at position 457, 462, 466, 355 and
357 (fig. 9(b) and fig. 10). There, the association of the polyP
with the RBD surface is tight, at the binding sites with a distance
of ~3 Å. This number matches with the published distances
between Arg and phosphate [145,146]. The attachment sites
within the polyP chain to the RBD have a spacing of 5 P_i units
at one end (between Arg residues R457 and R466) and 3 P_i units
in the middle and again 5 P_i units at the terminus (Arg residues
R355 and R357). This arrangement suggests an odd number of
the RBD-binding P_i units within the polyP, suggesting a stag-

![FIGURE 10](image)

Diagrammatic sketch of the interaction of polyP with the cellular ACE2 and the viral RBD. The docking interface of RBD and ACE2 is depicted. The basic aa of ACE2 at the RBD:ACE2 interface that potentially interact with polyP are labeled with position numbers and marked in green, like the basic aa on the RBD. Adapted with permission [131]. Copyright 2020 Elsevier.
gered conformation of the polymer between these attachment points and excluding Ca$^{2+}$ as binding counterions, at least at the binding area. These experimental data also imply that polyP molecules of different chain lengths interfere with the interaction of the virus with the cells surface and modulate the infection process.

**Modifications of Arg at RBD**

The Arg residues at the RBD have been modified in two directions, first by covalent addition of a cyclohexanedione ring, and second by P$_i$ produced in vitro from polyP. Firstly, the RBD has been incubated with 1,2-cyclohexanedione (CHD) in a sodium borate buffer (pH 8–9) to derivatize the guanidinium group under formation of N$^7$N$^8$(1,2-dihydroxycyclohex-1,2-ylene)-l-arginine [DHCH-Arg] [147,148]. Prior to this modification the charged guanidinium group has a planar structure and is largely unreactive due to the resonance stability in this group. After derivatization the Arg moieties, DHCH-Arg, become strong electrophiles [149] and can react with its vacant orbitals to electron-rich centers (Fig. 11(a)). Especially if Arg is localized adjacent to an acidic aa, like with the two Arg residues 457 and 466 which are spatially associated with Asp$_{467}$ and Glu$_{465}$, a site of strong intramolecular proton transfer is formed [135] allowing a strong “covalent”-like binding to phosphate. Arg and especially exposed derivatized Arg moieties can even passively penetrate across cellular membranes [150]. After modification, the DHCH-Arg units comprise with their DHCH groups at physiological pH a strong electrostatic potential. In turn, the amino acid can invade into the first dipole water layer that forms a bulky, 1–1.5 nm thick, hydration shell [151]. In addition, water mole-

**FIGURE 11**

*Increased electrostatic interaction potential of Arg after its modification. (a) The Arg unit on the surface of the RBD undergoes covalent modification with 1,2-cyclohexanedione (CHD) in a borate buffer, forming DHCH-arginine which has a negative charge. After this, the modified Arg units can interact with polyP via divalent cations. (b) Similarly, if polyP is enzymatically cleaved by ALP, releasing a P$_i$ unit and a proton (or an intermediate metaphosphate species), a likewise modification of the guanidinium group of Arg proceeds transitionally. An overall negative charge is built at the guanidinium group. (c) Penetration of Arg into the hydration (water dipole) layer at the protein surface. Molecular models of arginine (middle), DHCH-arginine (left) and phospho-arginine (right); the guanidinium group at Arg is marked. Both the modification of the guanidinium terminus of Arg with CHD and with a phosphoryl group increases the reactivity of Arg. The modified aa penetrates with $\sim$2 Å through the 10–15 Å thick hydration layer, formed around Arg on the protein surface.*
cules surround the modified Arg and build a gradient of dynamic dipoles providing the aa with an increased ligand reactivity [152]. Additionally, DHCH-Arg is bending the guanidinium group out of the overall surface plane. Therefore, Arg by itself and especially the modified DHCH-Arg is penetrating further into the hydration layer (Fig. 11(c)).

A second, physiological derivatization of Arg proceeds with P_i groups along the pathway seen in cells (Fig. 11(b)); there a stable linkage of the P_i to the carbon atom of the guanidinium group occurs. In intact cells a physiological synthesis of phosphoarginine, exposing a phosphoramidyl amino acid residue (N^+)-position) is known [153,154]. Projecting this finding to the situation with the RBD, especially in the physiological environment, the added polyP will be enzymatically hydrolyzed by the ALP under the release of P_i. This P_i unit will at least transitionally bind to the polarized carbon atom in the Arg guanidinium group (Fig. 11(b)). Also this change at Arg will not only increase the electrostatic potency of the aa but also generate an Arg derivative that protrudes into the hydration shell surrounding the protein, here the RBD (Fig. 11(c)).

PolyP inhibits binding of the viral RBD to the cellular ACE2

Binding assay for the polyanionic polyP to the cationic groove

The mentioned modeling studies showed that on the surface of the RBD a cationic groove, formed of the basic aa Arg and Lys, as well as His, exists that could interact with polyanionic polymers. In order to test the efficacy of potential inhibitors of the RBD:ACE2 binding, an assay system based on recombinant proteins has been applied [131,155]. The recombinant ACE2 was attached to a well plate and the recombinant RBD of the S-protein (labeled with biotin) was used as the interacting ligand. The binding strength between the RBD to the ACE2 is sensitively measured after incubation with labeled streptavidin in a chemiluminescence assay (Fig. 12(a)) [131,156].

Inhibition of the RBD:ACE2 binding by polyP

Incubation of the RBD with polyP causes a reduction of the binding propensity to the ACE2 protein. Under controlled conditions with non-modified polyP a significant inhibition, a reduction of binding to 72%, is measured with 1 μg mL⁻¹ [131]. The inhibition increases, reflected by a decrease of binding between RBD and ACE2, to 28% at the high concentration of 100 μg mL⁻¹ of polyP (Fig. 12(b)). While this series of experiments was performed with a polyP sample of 40 P_i units (Na-polyP_{40}), a smaller polymer with a chain length of only 3 P_i residues (Na-polyP_3) was selected in comparison. Surprisingly, a likewise strong inhibition was measured Fig. 12(b). Two conclusions can be drawn; either the strength of inhibition is saturable with Na-polyP_{40} due to a steric hindrance of Na-polyP_{40} at the Arg residues, or the short phosphate oligomers act inhibitory but cover only a portion of the cationic groove. To proof this proposition the Arg residues at the RBD were chemically modified [156].

An increase in the inhibitory potency of polyP could be achieved with the RBD which exposes the derivatized Arg amino acids. The modification of Arg was performed with CHD to obtain DHCH-Arg [156]. This change turns Arg to an aa with a strong electrostatic potential around the Arg guanidinium group and allows a stronger access of the anionic polyP to the Arg moieties at the RBD. This was confirmed experimentally by showing that the inhibitory potency of polyP in the RBD:ACE2 binding assay increases. A comparative inhibition analysis between Arg and DHCH-Arg showed that by application of Na-polyP_{40} at a concentration of 0.1 μg mL⁻¹ the binding efficiency was only reduced to 88% (non-modified Arg at the RBD), while a much stronger reduction to 23% was measured with the DHCH-Arg-modified RBD in the binding system (Fig. 12(c)). After a further increase in the concentration to 10 μg mL⁻¹ only a reduction of the binding to 60% was seen for the non-modified RBD, but a much stronger inhibition to 3% with the DHCH-Arg-modified viral binding protein [156].

For a molecular stoichiometric analysis, the strength of inhibition has been assessed on a molecular basis, correlating the concentrations of the RBD molecules in the assay (applying the DHCH-Arg-modified RBD) with Na-polyP_{40}. At a concentration of 0.05 μg mL⁻¹ of polyP a 50% inhibition was reached in the binding assay with DHCH-Arg-modified RBD. Based on the molecular weight of the RBD with ~24,500 Da, a ratio between 6.85×10⁻⁸ mmol polyP and 8.16×10⁻⁸ mmol RBD was calculated to be present in the binding assay at 50% inhibition. This calculation implies that every molecule of RBD is associated with one molecule of Na-polyP_{40}, also reflecting that the binding of polyP to the DHCH-Arg-modified RBD and the following interaction with the cellular ACE2 receptor is highly sensitive and likewise selective.

The concentration of the two fractions of polyP, existing in the circulating blood, the relatively short polymers with 60–100 phosphate units and the long chain polymers were determined in human serum/plasma. For the short chain fraction a concentration of around 10 μM (with respect to orthophosphate units; equivalent to 0.8 μg mL⁻¹) and for the long chain fraction of ~40 μM (3.2 μg mL⁻¹) was quantitated [157]. It must be stressed that at present the quantification methods are not very precise. In addition, polyP levels in blood are mostly estimates based on the soluble, short-chain polyP fraction released after platelet activation [47], neglecting the insoluble, particulate fraction of polyP on the platelet surface [48]. Nevertheless, the concentrations found to be inhibitory in the assay with the viral RBD, derivatized DHCH-Arg, reach values determined for polyP within the physiological range.

Potential anti-SARS-CoV-2 activity of organic polyanions

PolyP is an inorganic biomaterial, which reduces the interaction between the viral RBD and the corresponding cellular ACE2. Therefore, it was interesting to clarify if also organic polyanions cause this inhibitory effect, like heparin. Heparin is an established anticoagulant and inhibitor of enzymes that mediate blood clotting [158–161]. This polymer has been reported to hold anti-viral activity [162]. Recently three groups published an interaction of heparin, and related compounds like heparan sulfate, with the RBD of the SARS-CoV-2 S-protein [101,163,164]. Based on these data an application for patients has been proposed [165,166]. Applying circular dichroism spec-
mucins, as a protective barrier

In the respiratory airways system, a two-phase mucus/mucin layer covers and protects the epithelial cell layer. This layer is particularly bulky on the surface of the oropharyngeal airways epithelia. From there the viruses enter the pulmonary circulatory system. The mucus is produced in the epithelial cells as slippery aqueous secretion. This viscous colloidal fluid is supplemented with inorganic salts, antimicrobial proteins/enzymes (lysozymes, lactoferrin), immunoglobulins, and the mucins. By this, the mucus acts as a physical barrier against foreign materials, both of physical (e.g., dust particles) and (bio)chemical origin, including bacteria or viruses [169,171,172]. The mucins, the major functional component of the mucus, serve as a protective shield against virions [169,171,172].

Role of mucins as a protective barrier

In the respiratory airways system, a two-phase mucus/mucin layer covers and protects the epithelial cell layer. This layer is particularly bulky on the surface of the oropharyngeal airways epithelia. From there the viruses enter the pulmonary circulatory system. The mucociliary transport layer, exteriorly present on the epithelial cells, acts as a self-clearing and self-cleaning mechanism of the airways in the respiratory system. It directs the mucus to the pharynx from where it is either swallowed or coughed up; the clearance velocity is fast with $\sim 5.5$ mm min$^{-1}$ [167].

The virus is entering the nasal lumen [168] from where it spreads via an airborne transmission route. Then viral particles reach the surface region of the respiratory epithelia and either resist there or invade through the mucus/mucin layers the epithelial cells via the entry molecules ACE2 and TMPRSS2 [169].

In healthy individuals the mucus is an efficient protection system against an immediate infection of cells with the virus [170].

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In healthy individuals the mucus is an efficient protection system against an immediate infection of cells with the virus [170].
ponents of the mucus, are a large family of heavily glycosylated proteins, containing both N-linked and O-linked glycans.

Until now 22 mucin-type glycoproteins have been identified, which are collectively termed MUC [172]. The family of mucins are grouped into the secreted mucins, gel-forming components of viscoelastic mucous gels protecting the epithelia (like MUC5AC and MUC5B) and the membrane-bound mucins with the prevalent MUC1 [172]. The epithelium is composed of the goblet cells, which produce the mucus, and the ciliated cells, which are decorated with ~200 cilia each of 7 µm in length (Fig. 13(a)). The cells secrete the membrane-tethered mucins (MUC1, MUC4, and MUC16) deposited in the periciliary layer, while the outer layer of the mucus of the respiratory tract contains the two gel-forming mucins (MUC5AC and MUC5B) [172].

Extracellularly, the different mucins with their functional groups form a tight meshwork. The termini of the glycans are usually built by charged sialic acid units [173–175] and by this have the propensity to form salt bridges. From there the hierarchical organization to a network starts by building up Ca^{2+}-mediated links between monomeric mucins, stabilized with hydrogen bonding. Finally, disulfide bonds link the monomeric mucins to a solid scaffold into which host cells and foreign bacteria can be entrapped (Fig. 13(b)) [176]. Also additional non-mucin proteins, like cytokines can be included [177].

### Mucin protection against virus invasion

Due to their small sizes viruses can penetrate the mucus barrier and reach the epithelium. Some viruses, like the influenza viruses, are secreting their own neuraminidase, which facilitates their migration through the extracellular glycoprotein shield and through the mucus [178]. In contrast, larger virus particles like the herpes simplex virus (size of 180 nm) remain immobilized within the mucus layer. As a self-defense of the epithelial cells against influenza viruses they induce an upregulation of mucins, especially in human nasal epithelial cells [179].

Only little is known about the presence of potential antivirally acting factors/compounds in the mucus. Some intrinsic antiviral activity in mucins has been measured, which has been attributed to the adhesive and enveloping property of the heavily glycosylated proteins [180].

**Mucin does not abolish the inhibitory activity of polyP**

As outlined the polyanionic polymer, the inorganic polyP, which abolishes the binding affinity of the RBD to the ACE2 receptor, also remains active in the mucin environment [181]. Even more, the inhibition is even found at polyP concentrations which are close to those measured in the circulating blood. This finding has therapeutic relevance, since for COVID-19 patients it has frequently been shown that they display clinical symptoms of thrombocytopenia, which is characterized by abnormally low levels of platelets [96]. From this finding it can be deduced that not only the release of cytokines from platelets but also the release of polyP from them into the mucus layer is impaired. In order to elucidate if the anionic polyP interferes with the anionic mucins combinatorial experiments have been performed. Commercial mucin (from bovine submaxillary glands) was selected. Electron micrographs of the mucin added to the system show that during the incubation in the binding assay (supplemented with MgCl2) small polyP nanoparticles of a size of ~20 nm are formed in the mucin matrix (Fig. 14(a) versus (a-ii)). After addition of mucin together with polyP (10 µg mL^{-1} and 100 µg mL^{-1}) no reduction of the polyP inhibitory activity was measured in the RBD:ACE2 binding assay (Fig. 14(b)). The physiological concentration of mucin in the airway surface liquid is ~100 µg mL^{-1} [182]. These findings suggest that mucin does not interfere with the inhibitory potency of polyP in the RBD:ACE2 binding assay.
To elucidate the potential benefit of polyP as a protective material against virus infections, gene expression studies with the alveolar A549 cells, which are positive for the ACE2 receptor [183], were performed. Like determined in other cell systems also here polyP, even in the presence of mucin, elicits morphogenetic potential [5,181]. In the steady-state-expression system using alveolar A549 cells both the MUC1 gene and the MUC5AC gene became significantly upregulated (Fig. 14(c)). Additionally, it was confirmed that in this cell system ATP is produced extracellularly after exposure to polyP and mediated by the cell-bound ALP and ADK [1].

Conclusions and perspectives
As long as no efficient drugs are available for the treatment of COVID-19 patients and the immunization of the population is not sufficient, preventive protective measures are indicated. Such protective measures can be based not only on the use of nose and mouth masks in combination with a suitable lifestyle, as well as disinfectants for contaminated surfaces or skin, but also on materials that show antiviral activity against specific viral molecules or components. In this review it is highlighted that such preventive/protective tools against the initial phase of SARS-CoV-2 infection are at the horizon or beyond. The materials/polymers in focus are polyanionic inorganic polyP and perhaps also organic polysaccharides (heparin/heparan sulfate) that are directed against distinct patterns on the target virus necessary for host cell recognition and infection.

Innate immunity is the initial response of animals/humans to microbes that prevents, controls and eliminates infection of the host with bacteria, viruses and other pathogens [184]. It is an evolutionary old system and evolved together with the Porifera ~800 million years ago [185,186]. This first-line defense system is efficient and complex and provides an immediate response to microbial assaults. The innate immune response to SARS-CoV-2 has been elucidated to some extent [187]. The single-stranded RNA of this virus is interacting with extracellular and endosomal pattern recognition receptors, the Toll-like receptors (TLRs). After their activation the secretion of cytokines starts with the interferons as the most important molecules of the antiviral defense arsenal. Subsequently other cytokines, like the proinflammatory tumor necrosis factor α and interleukins −1, −6 and −18 are released that potentiate the adaptive immune response [187]. In the first phase of infection the SARS-CoV-2 S-protein with its RBD has to bind to the cells and, therefore, has to break the barrier of the protective covering mucus layer on the epithelial surfaces. At the beginning of the infection the airway epithelial cells with their mucus layer function as an innate sensor and act as a first mechanical barrier defending against the virus. The epithelial cells can suppress proximate inflammatory processes by inhibition of the pattern recognition receptors signaling chain and the secretion of inhibitory cytokines, eicosanoids, or glucocorticoids [188].

The innate immune defense system provides not merely a passive protection against the pathogen, like in a passive immunization treatment, but includes also an active response of the body through self-production of defense molecules [189]. Passive immunization using immunoglobulin therapy is indicated for individuals with B cell immunodeficiencies to support the antibody-antigen neutralization steps. This passive treatment is usually supplemented with adjuvants that are additionally used to improve the immune status of the patient and boost the host immune responses [190].

As summarized in this review polyP inhibits the RBD:ACE2 interaction even at around physiological concentrations. This passive protection is flanked by an active, a morphogenetic induction of mucin gene expression in cells exposing the ACE2

![Image](54x120 to 564x297)
FIGURE 14
Preparation of a mucin/collagen-based hydrogel (HG) to study the inhibitory potency of polyP in a bioinspired environment. (a) Morphology of (i) dried collagen/mucin (5 mg mL$^{-1}$, 100 μg mL$^{-1}$) as well as of the (ii) collagen/mucin/polyP HG (5 mg mL$^{-1}$, 100 μg mL$^{-1}$, 10 μg mL$^{-1}$); scanning electron microscopy. Nanoparticles are marked (> -). (b) Coincubation of polyP with mucin in the RBD:ACE2 binding assay. The studies were performed in parallel in the absence or presence of mucin (100 μg mL$^{-1}$) and two concentrations of polyP (10 μg mL$^{-1}$ and 100 μg mL$^{-1}$). (c) Influence of collagen alone (1 mg mL$^{-1}$), collagen (1 mg mL$^{-1}$) and mucin (100 μg mL$^{-1}$) together, and collagen/mucin with polyP (10 μg mL$^{-1}$) on the expression of the MUC1 and MUC5AC gene in the HG during a 6 d incubation period. n = 5; *p < 0.05.
The upregulation of the mucin genes is seen in A549 cells, which are related to the respiratory epithelial cells [191]. In these cells, the expression of the mucin genes is in cis, meaning at the site from where polyP is reaching the cells. More specific, polyP induces the steady-state-expression of the MUC1 gene. MUC1 contributes to the formation of the periciliary layer and contributes to the antiviral activity of the mucus. In addition, polyP induces an activation of MUC5AC. This mucin is localized on top of the periciliary layer. There, in the MUC5AC layer the virus particles become trapped into the mucus (Fig. 13(a) and Fig. 15(a)) [181]. In turn, polyP will surely contribute to restoration of the cellular integrity and function of the epithelium. When inhaled through the airways, the SARS-CoV-2 particles face the bulky mucin layer which frames the airway epithelium (Fig. 15(a) and (b)) [194]. When the polymer comes into contact with the mucus, a glyco-proteaceous fluid, polyP undergoes coacervation and embeds virus and virus-like particles [71]. In turn, polyP strongly binds to the RBD at the S-protein of SARS-CoV-2, as summarized here. Finally, polyP provides metabolic energy to the epithelial cells, amplifying their natural defense properties [195].

In conclusion, the polyanionic polyP material acts on three levels as antiviral or antiviral protective material. When inhaled through the airways, the SARS-CoV-2 particles face the bulky mucin layer which frames the airway epithelium (Fig. 15(a) and (b)) [194]. When the polymer comes into contact with the mucus, a glyco-proteaceous fluid, polyP undergoes coacervation and embeds virus and virus-like particles [71]. In turn, polyP strongly binds to the RBD at the S-protein of SARS-CoV-2, as summarized here. Finally, polyP provides metabolic energy to the epithelial cells, amplifying their natural defense properties [195].

The results highlight that the inorganic biomaterial polyP, as the only polyanionic polymer to date, is a promising candidate for the rational design and the further development of a targeted antiviral defense against SARS-CoV-2. This material is physiological and is metabolized in the body and eliminated via physiological routes.

Data availability

The authors declare no data availability.

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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