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Review

Replication Features of SARS-CoV-2 and Advantages of Targeting S Protein with Aptamers to Block Viral Entry

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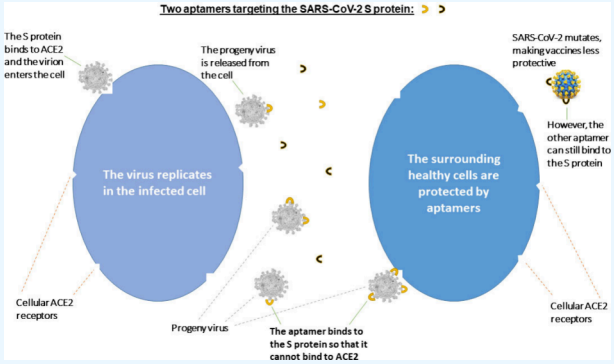
ABSTRACT: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a virus of the coronaviridae family. The virus enters the cell through binding to the corresponding receptor angiotensin-converting enzyme 2 (ACE2) on host cell membrane with the spike protein (S protein) on its envelope; thus, we can design inhibitors that bind the S protein to block the entry of the virus into cells. Aptamers are single stranded DNA or RNA molecules that can form specific three-dimensional structures and bind their target molecules with high affinity and specificity and thus are promising candidates for S protein inhibitors. This paper reviews the replication cycle and cell entry mechanisms of SARS-CoV-2 as well as the preparation principle and characteristics of aptamers, features a discussion of the advantages of using aptamers to target the S protein to prevent SARS-CoV-2 from infecting cells, and finally summarizes the research progress in S protein-blocking aptamers.



INTRODUCTION

In December 2019, a pneumonia of unknown cause emerged in Wuhan, China. In early January 2020, the pathogen of this pneumonia was identified as a novel coronavirus and was temporally named 2019-nCoV by Chinese scientists who identified it.[1] In February 2020, the International Committee on Taxonomy of Viruses (ICTV) officially named this virus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and in the same month, the World Health Organization (WHO) named this disease coronavirus disease 2019 (COVID-19).[2] The disease was transmittable between people and spread rapidly. By March 2020, it had become a global pandemic. Although by December 2022, the epidemic had been brought under control in most countries or regions of the world, it had not disappeared. According to WHO data (https://covid19.who.int/), there were still 275,553 new cases and 4,230 new deaths reported globally within the 28 days from March 3 to March 31, 2024. As of March 31, 2024, the COVID-19 epidemic had resulted in a total of 775,251,779 infections and 7,043,660 deaths, according to the WHO.

To control the spread of the disease, the first consideration is usually the use of vaccines. However, SARS-CoV-2 vaccines have not been able to effectively controlled the epidemic.[3] Furthermore, SARS-CoV-2 is prone to mutations, and variant strains can evade the immunity induced by existing vaccines.[4] Therefore, it is essential to adopt multiple intervention measures to prevent infection. This article reviews the replication cycle and cell entry mechanism of SARS-CoV-2, discusses the advantages of targeting the S protein of SARS-CoV-2 to inhibit viral infection, reviews the preparation principle and general characteristics of aptamers, and discusses the advantages of using aptamers to target the SARS-CoV-2 S protein to block viral attachment and entry into cells; it finally summarizes the research progress on aptamers that target/block the S protein to prevent SARS-CoV-2 infection.

PROPERTIES AND REPLICATION CYCLE OF SARS-COV-2

The family Coronaviridae is classified into the order Nidovirales and includes three subfamilies (Letovirinae, Orthocoronavirinae and Pitovirinae) [ICTV, Virus Taxonomy: 2023 Release----https://ictv.global/taxonomy]. The Orthocoronavirinae includes four genera: Alphacoronavirus, Betacoronavirus, Deltacoronavirus, and Gammacoronavirus, of which, the Alpha- and Beta-coronaviruses infect humans and other mammals while the Delta- and Gammacoronavirus infect birds. The Betacoronavirus can cause serious respiratory diseases in man such as severe acute respiratory syndrome (SARS, caused by SARS-CoV), middle east respiratory syndrome (MERS, caused by MERS-

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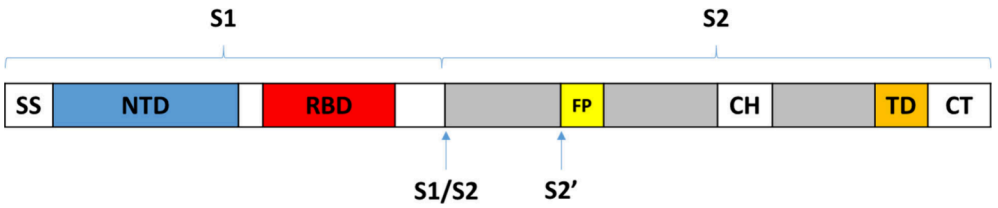

Figure 1. Peptide chain structure of SARS-CoV-2 S protein mainly showing the regions relevant to virus entry. The lengths of the regions are not drawn to scale. Abbreviations: SS, signal peptide; NTD, N-terminal domain; RBD, receptor-binding domain; S1/S2, S1/S2 protease cleavage site; S2′, S2′ protease cleavage site; FP, fusion peptide; CH, central helix; TD, transmembrane domain; CT, cytoplasmic tail.

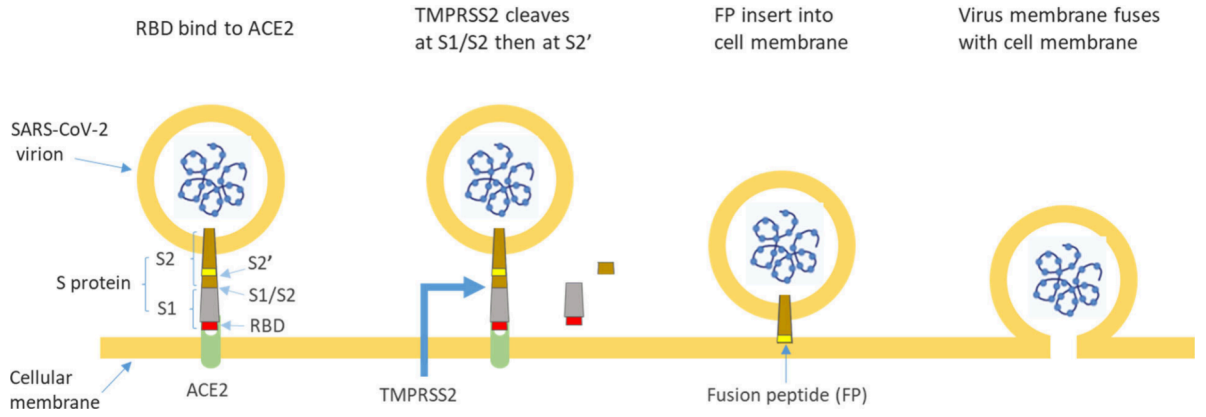

Figure 2. Schematic diagram of SARS-CoV-2 entry mechanism showing the entry process of SARS-CoV-2 mediated by the S protein and the ACE2 receptor. See Figure 1 and the text for abbreviations.

CoV), and coronavirus disease 2019 (COVID-19, caused by SARS-CoV-2).

SARS-CoV-2 belongs to the Coronaviridae family, Orthocoronavirinae subfamily and Betacoronavirus genus. Like all other members of the Orthocoronavirinae subfamily, the virions of SARS-CoV-2 are spherical in shape, 80−160 nm in diameter, and enveloped. The viral genome is a single-stranded positive-sense linear RNA (+ssRNA) approximately 30,000 bases long. The viral nucleocapsid is composed of the genomic RNA and the nucleocapsid protein (N protein), exhibiting helical symmetry. The viral envelope is embedded with three proteins: spike protein (S protein), membrane protein (M protein), and envelope protein (E protein).[5]

SARS-CoV-2 enters cells by binding its S protein to corresponding host cell receptors. After entering the cell, the + ssRNA genome employs the host cell's protein synthesis machinery to synthesize viral RNA-directed RNA polymerase (RdRP) and other nonstructural proteins. RdRP and several other nonstructural proteins form a replication and transcription complex on the endoplasmic reticulum membrane. Within this complex, a full-length -RNA strand is replicated from the genomic + RNA strand, and then the -RNA strand is used to replicate the + RNA genome of progeny viruses. Additionally, the -RNA strand is transcribed into several subgenomic mRNAs, which are subsequently translated into four progeny virus structural proteins: N protein, S protein, M protein, and E protein. The newly synthesized N protein assembles with the progeny virus + ssRNA genome to form the progeny virus nucleocapsid. The newly synthesized S, M, and E proteins are embedded in the endoplasmic reticulum membrane, and the membrane detaches to form an endoplasmic reticulum-Golgi intermediate compartment. The newly assembled nucleocapsid buds inward this compartment at the site with S, M, and E proteins, entering the lumen to form progeny SARS-CoV-2 virion. The progeny SARS-CoV-2 is released extracellularly through the host cell's exocytosis process, completing the replication cycle.[6]

S PROTEIN-MEDIATED VIRUS ENTRY MECHANISM

SARS-CoV-2 relies on its S protein to recognize receptors on the host cell surface, enabling virus attachment and entry into the cell.[7] The S protein is a homotrimeric glycoprotein with a molecular weight of 180−200 kDa, composed of three identical subunits (three identical peptide chains), and belongs to type I transmembrane proteins (i.e., each peptide chain crosses the membrane once, with the N-terminus located extracellularly). Each monomeric peptide chain is 1,273 amino acids long and is divided into S1 and S2 regions (some authors call these S1 and S2 subunits; some others call them the S1 and S2 domains), with S1 located entirely extramembranously and S2 containing extramembranous, transmembranous, and intramembranous domains (see Figure 1). The receptor-binding domain (RBD) of the S protein is located within the S1 region (223 amino acids long, amino acid 319−541).[8] The three-dimensional structure of the S protein, composed of the aforementioned three subunits, appears as an inverted long triangular pyramid when viewed from the front. The RBD is located on the top surface and can exist in either a "down" or "up" state. The "down" state cannot bind to the receptor, while the "up" state can bind to the receptor.[9]

After the RBD of the S protein recognizes and binds to its receptor angiotensin-converting enzyme 2 (ACE2) on the host cell membrane, the conformation of S1 changes. Under the action of the host's transmembrane protease/serine subfamily member 2 (TMPRSS2), the S protein undergoes cleavage at two sites: S1/S2 and S2′ (S2′ is located within S2, 130 amino acids away from S1/S2). Subsequently, the remaining peptide chain in the S2 region undergoes a conformational change with the

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fusion peptide fragment (hydrophobic amino acid sequence) at its terminus extending outward and inserting into the host cell membrane, which leads to the fusion of the viral envelope with the host cell membrane and the entry of the viral nucleocapsid into the cell[10,11] (see Figure 2).

Unlike other coronaviruses of the same genus, the S protein of SARS-CoV-2 inserts a polybasic amino acid sequence (arginine-arginine-alanine-arginine) at the S1/S2 junction.[12] This polybasic amino acid sequence allows the S protein of SARS-CoV-2 to be cleaved from the S1/S2 junction by the TMPRSS2 after binding to the receptor. In addition, the S protein is cleaved from the S1/S2 junction by furinase (a protease resident in the Golgi apparatus) at the time of synthesis in the previous cell (the cleaved S1 remains bound to S2 by noncovalent bonds).[13]

The entry of SARS-CoV-2 into cells is mainly mediated through the binding of the S protein to the ACE2 receptor as summarized above. In addition, SARS-CoV-2 can also enter cells via the phagocytosis or macropinocytosis pathway,[14,15] which also requires the participation of the S protein. After the virus is phagocytosed or macropinocytosed by the cell, the pH in the phagosome (endosome) decreases, activating cathepsins B and L on the endosomal membrane. These activated cathepsins then cleave the S protein, leading to the fusion of the viral envelope with the endosomal membrane in a manner similar to the above, and the release of the viral nucleocapsid into the cytoplasm.

INHIBITING THE S PROTEIN CAN PREVENT SARS-COV-2 FROM ENTERING CELLS

As mentioned earlier, the attachment and entry of SARS-CoV-2 virions into cells are mediated by the S protein, which consists of two regions or domains, S1 and S2. S1 recognizes and binds to receptors on the cell membrane through its RBD, and under the action of host enzymes, the S protein is cleaved at the S1/S2 junction and within S2. The cleaved S2 undergoes a conformational change, exposing the fusion peptide fragment, which mediates the fusion of the viral envelope with the cell membrane or endosomal membrane, allowing the viral nucleocapsid to be released into the cell. Therefore, theoretically, designing inhibitors to bind to the S protein can inhibit S protein-mediated cellular entry, thereby preventing SARS-CoV-2 from infecting cells. Inhibitors can be designed to bind to the S protein at different or multiple sites to respectively inhibit RBD binding to ACE2, S protein cleavage, and S2 residual fragment conformational changes.[16]

A number of studies have shown that antibodies or drugs binding to the S protein can effectively inhibit SARS-CoV-2 infection of cells.[6,17,18] Wu et al.[19] isolated two monoclonal antibodies from the serum of recovered patients, both capable of blocking the binding of the S protein to cell receptors, and these antibodies bind to the S protein at different sites. Animal experiments have shown that these antibodies can reduce the viral titer in the lung tissue of infected animals. Chi et al.[20] prepared multiple humanized single-domain antibodies against RBD, which can effectively bind to RBD and block its binding to ACE2, neutralizing both SARS-CoV-2 pseudoviruses and authentic viruses. Ju et al.[21] analyzed the crystal structure of RBD antibodies bound to RBD and found that the binding of RBD antibodies to RBD inhibits the binding of RBD to ACE2 through steric hindrance, thereby preventing viral entry into cells.

ADVANTAGES OF INHIBITING S PROTEIN TO PREVENT SARS-COV-2 INFECTION OF CELLS

Inhibiting the binding of the S protein to its receptor can prevent SARS-CoV-2 infection of cells from the source. Like other viruses, the replication cycle of SARS-CoV-2 in cells includes the steps of attachment and entry, biosynthesis, and assembly and release. While various steps in the replication cycle of SARS-CoV-2 can be targeted, such as inhibiting the viral RNA-directed RNA polymerase, blocking its attachment and entry into host cells is undoubtedly the most ideal approach. This strategy prevents viral infection at the very beginning, effectively protecting cells from damage.

On the other hand, inhibiting the S protein *per se* should not have adverse effects on the body. Although we can inhibit the attachment of SARS-CoV-2 to cells by acting on the cell membrane receptor ACE2 or inhibit viral entry into cells by acting on the enzymes that cleave the S protein (i.e., TMPRSS2), inhibiting these enzymes may lead to adverse reactions in the body because both ACE2 and TMPRSS2 are normal cellular enzymes and are needed to perform their physiological functions. Therefore, a better choice would be to inhibit viral attachment and entry into cells by acting on viral components. The S protein is a viral component, and inhibiting it can prevent viral attachment and entry into cells without causing potential damage to the body.

ACE2 is a key enzyme involved in the regulation of blood pressure and other physiological processes; and so, inhibiting ACE2 may disrupt these physiological functions and lead to adverse effects on the body, particularly on cardiovascular, renal, and pulmonary functions.

ACE2 plays a crucial role in the renin-angiotensin system by converting angiotensin II (Ang II) into Ang 1−7, which has cardioprotective, vasodilatory, antigrowth, and antiproliferative effects. Inhibition of ACE2 could disrupt the balance between ACE and ACE2, leading to increased levels of Ang II. This imbalance is associated with hypertension, inflammation, oxidative stress, fibrosis, and impaired vascular function, which are major contributors to cardiovascular diseases.[22] Studies in ACE2 knockout mice have shown severe cardiac dysfunction, highlighting the importance of ACE2 in maintaining cardiac function.[23]

ACE2 is highly expressed in the kidney, particularly in the proximal tubules, endothelial and smooth muscle cells of renal blood vessels, and podocytes. Inhibition of ACE2 could exacerbate renal damage by increasing Ang II levels, which are known to mediate renal injury.[24] In diabetic mice, ACE2 deficiency has been linked to increased blood pressure, glomerular damage, and renal fibrosis.[25]

ACE2 is also expressed in the lungs, where it plays a role in protecting against respiratory infections and lung injury. Inhibition of ACE2 could compromise this protective effect, making the lungs more susceptible to damage.[24]

TMPRSS2 is a transmembrane protease that can cleave and activate multiple endogenous substrates and is involved in various cellular processes, including cell differentiation, proliferation, and apoptosis. While direct evidence of adverse effects from TMPRSS2 inhibition is limited, it is plausible that inhibiting a protease involved in multiple physiological processes could lead to unwanted side effects.

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ADVANTAGES OF MULTI-TARGET BINDING AGAINST THE S PROTEIN

The S protein mediates the attachment and entry of SARS-CoV-2 into cells through several steps, so the cellular entry mediated by this protein could be better inhibited by acting on multiple sites of the protein. For example, acting on RBD can inhibit the attachment of the S protein to its receptor, acting on S1/S2 can inhibit the cleavage and activation of the S protein, and acting on S2 can inhibit its conformational change, thereby preventing membrane fusion. Furthermore, if one site of the S protein fails to be effectively bound by the inhibitor, occasionally or due to certain reasons, binding at other sites can compensate for this. Therefore, multitarget binding against the S protein can ensure the reliability of the inhibition effect.

Multitarget binding inhibition of the S protein can also address the issue of viral mutations, for even if one site of the protein mutates, other sites can still be bound. It has been demonstrated that SARS-CoV-2 is prone to mutations, especially in certain regions of the S protein.

Several mutations in the S protein of SARS-CoV-2 including the D614G mutation, the N501Y mutation, the E484 K mutation, and the L452R mutation have been found. The D614G mutation is one of the earliest widely reported mutations in SARS-CoV-2.[26] It alters the tightness between S1 subunits, making the S protein structure looser and facilitating the production of open conformations with two or three RBDs exposed upward, thereby significantly enhancing the virus's infectivity. The N501Y mutation is located in the RBD and enhances the binding affinity of SARS-CoV-2 to ACE2, potentially increasing infectivity from an epidemiological perspective. This mutation is present in multiple variants, such as Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), and subsequent variants like 501Y.V1, 501Y.V2, and 501Y.V3.[27] The E484 K mutation is located in a highly structurally variable loop at the interface between the RBD and ACE2. It also enhances the binding of the RBD to ACE2 and is common in Beta, Gamma, and Kappa variant (B.1.617.1). Additionally, this mutation is associated with evading serum antibody recognition.[28] The L452R mutation is found in the Delta (B.1.617.2) and Kappa variant (B.1.617.1), as well as the Epsilon variant (B.1.427/429).[29,30] Pseudovirus experiments have shown that the L452R mutation can somewhat enhance viral infectivity and contribute to viral escape from neutralizing antibody recognition.

It has been found and mutated regions can no longer effectively bind to the original inhibitors.[31] Studies also showed that some SARS-CoV-2 mutants are resistant to neutralizing antibodies.[32] Thus, if we design inhibitors to bind multiple targets of the S protein, when one site of the S protein mutates, other sites can still be bound, as it is unlikely for multiple sites to be mutated simultaneously.

APTAMERS CAN BE USEFUL OR VALUABLE CANDIDATES FOR S PROTEIN INHIBITORS

Aptamers, also known as nucleic acid aptamers to distinguish them from peptide aptamers, are single-stranded DNA or RNA oligonucleotides that can form certain three-dimensional structures and can bind to target molecules with high specificity and affinity. Aptamers bind to their specific target molecules through close spatial adaptation and noncovalent interactions such as ionic bonds, hydrogen bonds, and van der Waals forces. They can identify very small differences between different target molecules, such as the presence or absence of small groups like hydroxyl groups and chiral differences. The potential targets of aptamers are wide-ranging; they can be free proteins, many small organic or inorganic molecules or even metal ions, and may be membrane-bound proteins or other membrane-bound molecules or virions.[35] With these general properties and the aditional characteristics discussed below, aptamers can be an advantageous choice of S protein inhibitors to block the entry of SARS-CoV-2 into Cells and prevent viral infection.

SELEX TECHNOLOGY FOR APTAMER SELECTION

Aptamers are usually prepared using the SELEX (Systematic Evolution of Ligands by EXponential enrichment) technique, which was developed independently by L. Gold and J. W. Szostak in 1990.[33,34] The sequences at the two terminal regions of an aptamer molecule are predesigned by the researcher and are synthesized before selection, which are essential for aptamer selection but may be deleted (or kept) afterward. The sequence in the central region is randomly synthesized during the SELEX selection process and is primarily responsible for aptamer's specific target binding.

To prepare aptamers using SELEX technology, the first task is to establish an oligonucleotide library or pool (with more than 10^{15} different DNA oligonucleotide chains). Researchers design the sequences at both ends of the oligonucleotide chain (usually 20−30 nucleotides long on each side), which must contain PCR primer sequences (for amplification of selected oligonucleotide chains during SELEX) and may also contain restriction enzyme recognition and cutting sequences (for cloning and storage of the obtained aptamers). For making RNA aptamers, a promoter sequence (for *in vitro* transcription during the SELEX selection process) must also be included. The central region of the sequence is randomly synthesized by a DNA synthesizer, with different aptamers having random sequences of varying lengths, depending on the designer and typically 25−30 nucleotides long. This length theoretically allows for the formation of 4^{25}−4^{30} (approximately 10^{15}−10^{18}) different oligonucleotide chains, among which there will certainly be chains that can bind to a specific target.[35]

The next task is to screen out the chains in the library that can bind to the specific target with high specificity and affinity through multiple cycles of selection and enrichment. The first step is to screen out oligonucleotide chains with stronger binding affinity to the target molecule from the initially established oligonucleotide library (if it is the preparation of RNA aptamers, the DNA library needs to be *in vitro* transcribed into RNA library before screening). The second step is to amplify the selected oligonucleotide chains in large quantities (for RNA aptamer preparation, the selected oligonucleotide chains need to be reverse transcribed into DNA before amplification and then *in vitro* transcribed into RNA chains before next round of screening). The remaining steps are to further screen out the chains with still stronger binding affinity from the oligonucleotide chains selected from the previous cycle and to amplify them; these steps are repeated for several times (usually 6−12 cycles are required to obtain the aptamers with satisfied binding affinity). It should be noted that as the cycles progress, the target-binding affinity and specificity of the selected chains continuously improve (i.e., enrichment takes place); that was because the fidelity of the polymerases are not perfect and new variations of the synthesized chains continuously appear. The finally obtained oligonucleotide chains that can bind to the target with high specificity and

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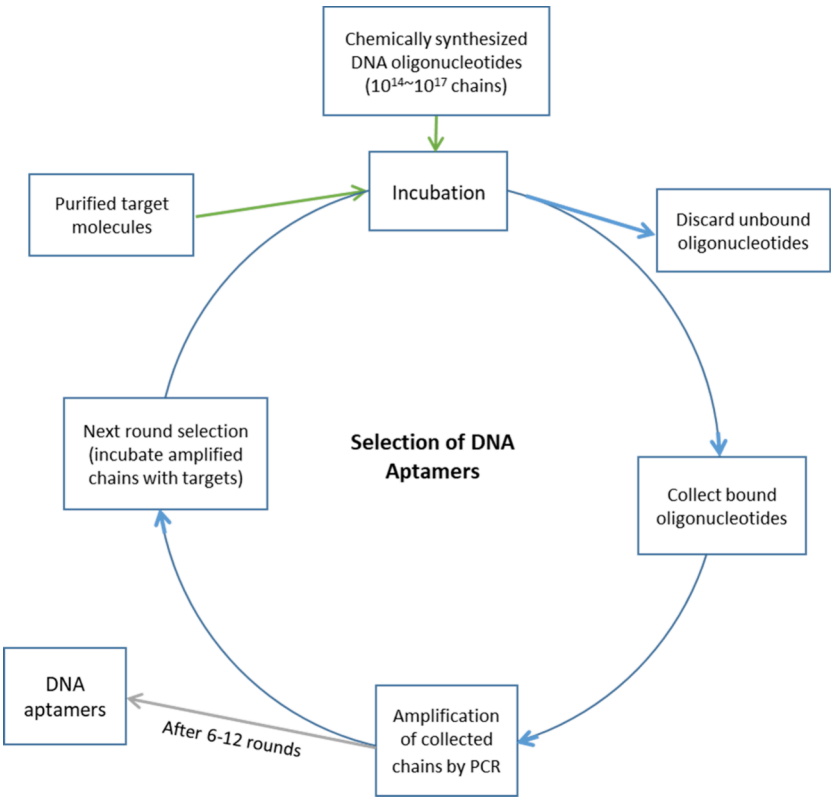

Figure 3. Schematic drawing to show the selection of DNA aptamers with SELEX technology.

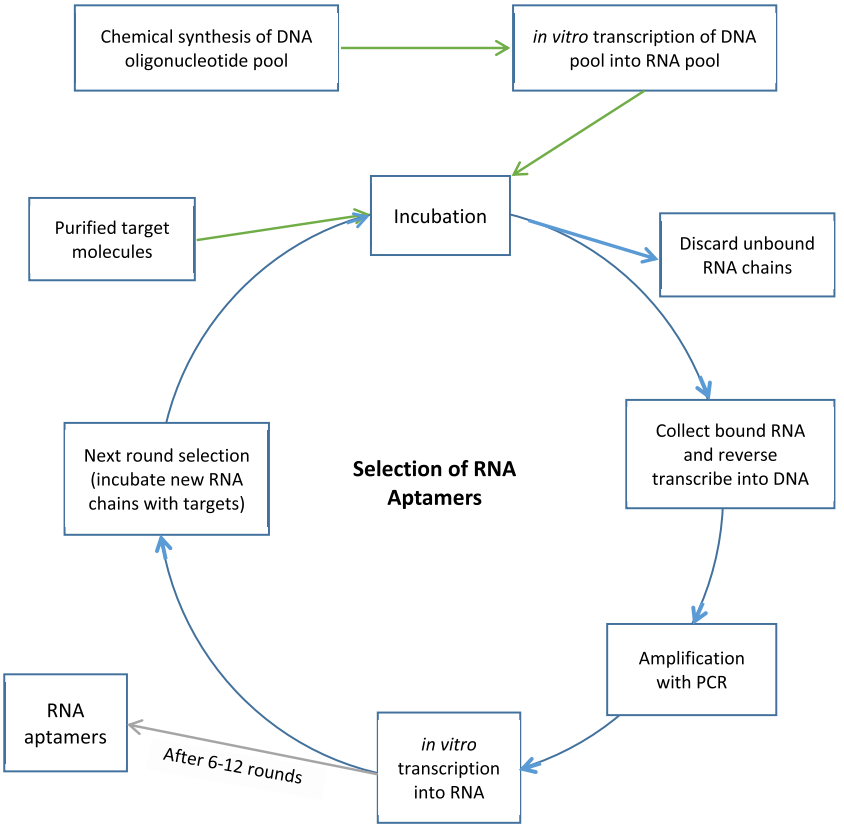

Figure 4. Schematic drawing to show the selection of RNA aptamers with SELEX technology.

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affinity are the nucleic acid aptamers for that target.[35] (See Figure 3 for DNA aptamer preparation and Figure 4 for RNA aptamer preparation)

ADVANTAGEOUS CHARACTERISTICS OF APTAMERS

Essential advantageous characteristics of aptamers:[35−37] 1) Compared to monoclonal antibodies, the production of aptamers is significantly faster and cheaper. 2) Unlike monoclonal antibodies, aptamers show no batch-to-batch variations and have good reproducibility. 3) Aptamers are easy to modify and their original binding properties retain after the modifications; this is especially important for RNA aptamers which are susceptible to nuclease degradation and often require modification to improve stability. 4) Aptamers have good tolerance and can withstand high temperatures up to 80 °C (at this temperature, antibodies usually denature). Even if they are denatured, they can easily renature and restore their original spatial structure, which allows for long-term storage.

Advantages of aptamers as medications: Both aptamers and monoclonal antibodies can bind to target molecules with high specificity and affinity, but aptamers have some advantages in clinical treatment.[35,38] 1) The biggest advantage of aptamers for therapeutic use over monoclonal antibodies is their lack of immunogenicity. They do not elicit immune rejection in patients, allowing for repeated dosing. Experiments in monkeys showed only mild immune reactions at doses 1,000 times the therapeutic dose.[39] 2) Aptamers are smaller in molecular weight than antibodies, making it easier for them to penetrate tissues and reach the site of action when used therapeutically.[36]

ADVANTAGES OF USING APTAMERS TO INHIBIT SARS-COV-2 S PROTEIN

Both aptamers and antibodies can bind to various types of target molecules. For protein targets, aptamers mainly bind through electrostatic interactions (salt bridges or ionic bonds), hydrogen bonds, and van der Waals forces in order, while antibodies bind through hydrophobic interactions, electrostatic interactions, hydrogen bonds, and van der Waals forces in sequence. Since aptamers bind to protein targets primarily through electrostatic interactions and the bond energy of electrostatic interactions is significantly greater than that of hydrophobic interactions, hydrogen bonds, and van der Waals forces, aptamers have stronger binding affinity than antibodies to the targets that are rich in positive charges.

The polybasic amino acid sequence (RRAR) at the S1/S2 cleavage site of the S protein carries multiple positive charges, which can form strong electrostatic interactions with the multiple negative charges carried by aptamers (the phosphate group of each nucleotide carries a negative charge). Particularly, as has been mentioned earlier, the S1/S2 cleavage site is crucial for S protein cleavage, activation, and the ensuing membrane fusion, and therefore the binding of aptamers to this site can more effectively inhibit S protein-mediated cell entry.[40]

As discussed in a previous section, multitarget binding inhibition of the S protein is advantageous. Aptamers are generally easier to achieve multitarget binding than antibodies. The monomeric immunoglobulins IgG and IgA1 are about the same size range as the S protein (the molecular weight of IgG being ∼150 kDa and that of IgA1 being ∼160 kDa) while the dimeric IgA2 and the pentameric IgM molecules are larger than the S protein, so when antibodies bind to the S protein, usually one molecule is bound. However, aptamers are much smaller than antibodies (approximately one tenth in size of antibodies and less than one tenth of the S protein, depending on the size of a specific aptamer) and thus multiple site binding to the S protein is possible. Besides, the S protein is coated with polysaccharide molecules which may cause antibody binding difficult.[41,42] Nonetheless, the small size of aptamers facilitate the binding or multitarget binding.

Finally, aptamers do not exhibit antibody-dependent enhancement (ADE) of infection. ADE refers to the phenomenon where antibodies (typically non-neutralizing antibodies) bind to certain viruses and, instead of inhibiting viral infection, facilitate viral entry into cells.[43] This mechanism involves the Fab segment of the antibody binding to the virus and the Fc segment binding to the cell; these bindings mediate viral entry. ADE has been observed in multiple viruses, including SARS-CoV and MERS-CoV. Preliminary studies on whether SARS-CoV-2 exhibits ADE have shown that antibodies targeting certain epitopes of the SARS-CoV-2 RBD, despite having neutralizing effects, can also mediate ADE.[44] Typical aptamers certainly do not cause ADE because they do not have the structures that binds to both the virus and the cell simultaneously.

EXPERIMENTAL AND CLINICAL EVIDENCE FOR APTAMER'S BINDING AND BLOCKADE EFFECTS

Numerous studies have shown that aptamers can specifically bind their targets and inhibit the function of the target molecules or block their interaction with ligands,[45−50] such as inhibiting or blocking the binding of viruses, including SARS-CoV-2, to their receptors.[51,52] For instance, aptamers targeting PD-1 and PD-L1 can inhibit the interaction between PD-1 and PD-L1.[53] Aptamers targeting the envelope protein of hepatitis C virus (HCV) can inhibit HCV binding to its receptor and entry into cells.[54,55]

Aptamers have already been used as drugs in disease treatment. The first aptamer drug approved by the US FDA for therapeutic use is Pegaptanib sodium injection, an RNA aptamer marketed under the name Macugen.[56,57] This aptamer binds to vascular endothelial growth factor and inhibits its function and is primarily used for the treatment of age-related macular degeneration (AMD). Izervay (Avacincaptad Pegol; formerly Zimura) is another aptamer (also a RNA aptamer) approved by the FDA for therapeutic use. Izervay was approved in 2023 for the treatment of geographic atrophy, a severe form of (secondary to) AMD. Izervay is a complement C5 inhibitor that inhibits the activation of the downstream common complement pathway.[58,59] In addition, there have also been several reports on aptamers in Phase I or Phase II clinical trials.[60−62]

RESEARCH PROGRESS ON APTAMERS TARGETING THE S PROTEIN OF SARS-COV-2

In 2020, Song et al.[52] first developed two DNA aptamers targeting the SARS-CoV-2 S protein with K_d values of 5.8 nM and 19.9 nM respectively; the aptamers could bind several amino acid residues in the RBD of the S protein that are key to ACE2 binding; simulation models and competitive experiments suggested that the two aptamers may share some binding sites with the RBD to ACE2. In 2021, Liu et al. developed a DNA aptamer against the RBD of the S protein that had a $K_d \approx 7$ nM and a IC50 ≈ 5 nM.[63] Sun et al. in 2021 developed a longer DNA aptamer targeting the RBD of the S protein at first; they then

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Table 1. Aptamers Targeting the S Protein of SARS-CoV-2 to Block Viral Entry into Cells

Aptamer name	DNA/RNA	Length	K_d	Target	IC50	Special properties	Ref.
CoV2-RBD-1C	DNA	51 bases	5.8 nM	several RBD amino acids key to ACE2 binding	-		52
CoV2-RBD-4C		67 bases	19.9 nM		-		
No name	DNA	40 bases	7 nM	RBD of S protein	5 nM		63
CoV2-6C3	DNA	46 bases	44.78 nM	RBD of S protein	446.68 nM		64
cb-CoV2-6C3			0.13 nM		0.42 nM	a circular bivalent aptamer	
nCoV-S1-Apt1	DNA	40 bases	0.327 ± 0.016 nM	RBD of S protein S1 region	80.12 nM		65
nCoV-S1-Apt2			0.313 ± 0.078 nM		-		
nCoV-S1-Apt3			0.118 ± 0.033 nM		-		
nCoV-S1-Apt4			28.422 ± 3.666 nM		-		
nCoV-S1-Apt5			18.829 ± 3.806 nM		-		
nCoV-S1-Apt6			85.61 ± 14.219 nM		-		
MSA52	DNA	40 bases	2−10 nM	S protein S1 region	-	recognize 7 S protein variants: α, β, γ, ε, κ, σ, and o	66, 67
TMSA52	DNA		8.8−23.7 pM	three subunits of the S protein	-	a branched homotrimeric aptamer, matching the homotrimeric shape of the S protein	68
S2A2C1	DNA	-	35 ± 4.3 nM?	S2 region of the S protein, not RBD	-		70
S1B6C3-A5-S2A2C1	DNA	-	35.8 ± 4.2 nM	RBD and S2 of S protein	-	a fusion aptamer	
AYA2012004_L	DNA	86	-	trimer S protein of SARS-CoV-2	-	mainly an *in vivo* study	71
RBD/S-A1	DNA	-	1.74 ± 0.2 nM	RBD of S protein	-		72
circSApt-NASO	DNA	-	-	S protein of SARS-CoV-2	-	a dual-function circular aptamer -ASO chimera	73
SOMAmers	DNA	-	Many <10 nM; some <1 nM	S protein S1 and S2 regions and RBD	Maximum 125 pM; mostly 0.09−20 nM	modified DNA-bases (dU/dC)	74
K1	DNA	77	26 ± 6 to 66 ± 4 pM	S protein of SARS-CoV-2		Similar binding affinities to wild-type and α, β, and o strains	75
M40	DNA	51	155 ± 26 to 280 ± 63 pM				
AM032-0	DNA	80	3−10.5 nM	RBD of S protein	IC50 = 188 nM against the pseudovirus with D614G spike, = 197 nM against the pseudovirus with the delta spike		76
AM047-0	DNA	52	0.04−0.4 nM	RBD of S protein	IC50 = 134 nM against the pseudovirus with D614G spike, = 141 nM against the pseudovirus with the delta spike		
AM-B1 (a bivalent aptamer)	DNA		-	RBD of S protein	47.0 nM	5′ end of AM032−4 connected with 3′ end of AM047−6 using a linker	
AM-B2 (a bivalent aptamer)	DNA		-	RBD of S protein (a distinct binding site from AM-B1)	159 nM	5′ end of AM047 connected with 3′ end of AM032−4 using a linker	
R	DNA	78	125.26 nM	RBD of S protein	-	With primer regions	77
J	DNA	40	-	RBD of S protein	-	without primer regions	
FANA aptamers		∼79 bases, with a 40-base central region	10−20 nM	RBD (R319-F541) and S1 (V16-R685)		2′-fluoro-arabino nucleic acid (FANA) modification	78
SPA1- M13	RNA/DNA hybrid	26	2.8 nM toward RBD; 0.39 nM toward S protein trimer	RBD and S protein trimer	-		80
AM032-4	DNA	44	-	RBD of SARS-CoV-2 variants o and JN.1	-		82
MEZ	DNA	31	6.5 nM	RBD of SARS-CoV-2 Wuhan-Hu-1 strain (the same binding site as ACE2)	-	does not form complexes with the RBD of β, δ, and o strains	83

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ACS Omega 2025, 10, 15840−15851

Table 1. continued

Aptamer name	DNA/RNA	Length	K_d	Target	IC50	Special properties	Ref.
MBASSA1	DNA	40-base central sequence	picomolar range	S protein of *o* strain	-	acquired 22 mutations within its 40-nt central sequence	84
S901 and S702	Commercial products, may have competing financial interest			S protein S1 region	-	aptamer-Hytac chimeras that can trigger S1 degradation	85

truncated the longer aptamer to a 46-nt aptamer; the K_d value (44.78 ± 9.97 nM) of the truncated aptamer was about half that of the full length aptamer; the authors further reconstructed the truncated aptamer into a circular bivalent aptamer with still improved K_d, IC50, and stability.[64] Also in 2021, Yang et al.[65] reported the development of several DNA aptamers with high neutralizing activity that can block the RBD of the S protein region 1 from binding to its receptor. In 2022, Zhang et al.[66] and Li et al.[67] reported the development of a DNA aptamer that can recognize both the wild-type SARS-CoV-2 and its variant S proteins, showing universal high affinity for the S protein of the wild-type and seven variants (α, β, γ, ε, κ, σ, and o) with K_d values ranging from 2 to 10 nM. The aptamer developed by Li and Zhang as mentioned above was monomeric, possessing a universal affinity for SARS-CoV-2 spike protein variants; the limitation of this aptamer was that it could bind only one of the three subunits of the SARS-CoV-2 spike protein. The same group latter constructed a branched homotrimeric aptamer (three aptamers tethered to a central linker) with 3-fold rotational symmetry; this aptamer could align with and bind to the homotrimeric subunits of the S protein of several SARS-CoV-2 variants with an improved affinity at picomolar level.[68] In 2022, Silwal et al.[69] developed a universal aptamer against the S proteins of diverse SARS-CoV-2 variants via SELEX toward the wild-type S protein and named it A1C1. A1C1 bound to the wild-type S protein of SARS-CoV-2 or variants such as σ and o with low nanomolar affinities and inhibited the interaction between ACE2 and various SARS-CoV-2 S proteins by 85−89%. This group in the same year[70] reported that they obtained a DNA aptamer that bind to the S2 region of the SARS-CoV-2 S protein and could efficiently block the S protein/ACE2 interaction, suggesting an RBD-independent inhibition approach (the RBD is located in the S1 regions of the S protein). The authors next conjugated the S2-binding aptamer with a S1/RBD-binding aptamer and found it exhibiting high-affinity of binding to the S protein of the wild-type and the variants σ and o, inhibiting their binding to the ACE2 receptor. Ayass et al.[71] in 2022 performed an *in vivo* research, in which they administered their aptamer to the lungs of mice by intubation, along with an *in vitro* research, in which they tested the efficacy and safety of their aptamer; the results showed that their aptamer was safe, effective, and could neutralize the uptake of pseudovirus-like particles by lung cells when administered locally. In the same year, Chen et al.[72] developed a DNA aptamer that recognized the RBD of the SARS-CoV-2 S protein with a K_d of 1.74 ± 0.2 nM.

In 2023, Yang et al.[73] developed a dual-function circular aptamer-ASO chimera that demonstrated high serum stability through artificial cyclization; the binding of the circular aptamer to spike protein enabled the chimera to be efficiently delivered to the host cells expressing ACE2, which achieved efficient inhibition of SARS-CoV-2 replication. Gelinas et al.[74] in 2023 reported base modification of DNA aptamers targeting the S1 and S2 regions and the RBD of the S protein; these modified DNA aptamers exhibited high degree of nuclease resistance. In the same year, Le et al.[75] screened broadly reactive aptamers that bind to the S protein of wild-type SARS-CoV-2 and all its variants with extremely high affinity, with K_d values in the picomolar range. To understand aptamer's mechanism in inhibiting viral infection, Rahman et al.[76] determined the three-dimensional structures of aptamer-RBD complexes using cryogenic electron microscopy in 2023; they combined aptamers and developed bivalent aptamers that showed a

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ACS Omega 2025, 10, 15840−15851

stronger inhibitory effect on virus infection; they found that one aptamer blocked ACE2-binding at one site of RBD while the other bound to a distinct site of the RBD and allosterically inhibited ACE2 binding. Using a pseudotyped viral entry assay, Halder et al.[77] shown that their aptamer specifically inhibited the entry of a SARS-CoV-2 pseudotyped virus in HEK293T-ACE2 cells but did not inhibit the entry of a vesicular stomatitis virus glycoprotein pseudotyped virus, indicating the specificity of the aptamer; the antiviral potential of the aptamer was tested and showed 95.4% inhibition against the SARS-CoV-2 virus. DeStefano group in 2021[78] generated aptamers to the RBD with 2′-fluoro-arabinonucleic acid modification; the ∼79 nucleotide aptamers bound RBD Arg319-Phe541 and S1 domain Val16-Arg685 of the S protein with equilibrium dissociation constants of 10−20 nM; the binding half-life for the RBD, S1 domain, and the full trimeric S protein was 53 ± 18, 76 ± 5, and 127 ± 7 min, respectively; these aptamers inhibited the binding of the RBD to ACE2 in an ELISA assay with high specificity---- the binding affinity was about 10-fold lower to the related S1 domain of the SARS virus. In 2023, the same group[79] further assessed the entry inhibiting effect of their aptamers *in vivo*; using an in vitro model of human airway epithelium and the SARS-CoV-2 virus, they showed that their aptamers significantly reduced viral infection whether added at the time of inoculation or several hours later.

Adachi et al.[80] in 2024 optimized aptamer length and chemistry with a deep-learning-based algorithm, RaptGen; they conducted a primer-less SELEX against the RBD of the SARS-CoV-2 spike protein with an RNA/DNA hybrid library, and based on the result of RaptGen analysis, a short truncation aptamer of 26 nucleotides was obtained and was further optimized by chemical modification; the resulting aptamer not only bound to the RBD of wildtype SARS-CoV-2 but also to its variants. In 2024, Kim et al.[81] reported that their DNA aptamers not only bind to the SARS-CoV-2 S protein and inhibit or block its binding to ACE2 but also inhibit pro-inflammatory cytokines induced by the S protein in HEK293 cells overexpressing ACE2. Ding et al.[82] utilized steered molecular dynamics simulations to elucidate the binding mechanisms of a DNA aptamer to the RBD of the SARS-CoV-2 variants Omicron and JN.1; the simulations revealed detailed molecular interaction of the aptamer and RBD, demonstrating aptamer's potential to sustain effective binding with rapid viral mutation. Khrenova et al.[83] identified an aptamer that is highly specific to the RBD of the spike protein of the SARS-CoV-2 strain Wuhan-Hu-1; the aptamer was highly selective for the RBD of the Wuhan-Hu-1 strain rather than the RBD of β, σ and o strains; the aptamer occupied the same binding site of the RBD that binds to ACE2. To fight SARS-CoV-2 mutations, Wang et al.[84] mutated an existing DNA aptamer that was originally selected for the S protein of wildtype SARS-CoV-2; the new aptamer had acquired 22 mutations within its central region of 40-nucleotides and the binding affinity to the S proteins of various o subvariants was improved 100-fold greater than the parental aptamer (from nanomolar to picomolar range). Fàbrega et al.[85] developed aptamer-hydrophobic tag chimeras (the hydrophobic tag can trigger the degradation of targets recruited in the context of a proteolytic chimera) for targeted degradation of SARS-CoV-2 S protein S1 region; the results placed aptamer-hydrophobic tag chimeras as promising tools for counteracting coronavirus infection.

Multimeric aptamer strategies provide an added benefit of enhanced binding avidity, however, the characterization of different aptamer-target pairings adds more steps to an already lengthy procedure. Moreover, the multimeric aptamers in most cases are constructed by joining pre-existing monomeric aptamers derived from in vitro selection, which adds still more steps to aptamer production. Amini et al. in Dec 2024 adopted an aptamer engineering strategy that directly selects for multimeric aptamers. They reported an in vitro selection strategy using a prestructured DNA library that forms dimeric aptamers rather than using a library containing a single random region; the library contained two random regions separated by a flexible poly thymidine linker. After 16 rounds of selection against the SARS-CoV-2 S protein, the top aptamers displayed excellent binding affinity with K_d values reach as low as 150 pM. They further found that each of the random regions functions as a distinct binding moiety and works together to achieve the high binding affinity.[86] See Table 1 for aptamers that have been developed to target the S protein to prevent SARS-CoV-2 infection.

CLOSING REMARKS

SARS-CoV-2 virions adsorb to and enter into cells through the mediation of the viral S protein, therefore inhibiting the S protein can in principle prevent viral infection. Both antibodies and aptamers are capable of binding to the S protein to inhibit its function; however, since there has already been a considerable amount of reviews on antibodies,[87−100] this article, after introducing the replication cycle and entry mechanism of SARS-CoV-2 and the advantages of targeting the S protein to constrain viral infection, mainly discussed the technology to produce aptamers, the advantageous properties of aptamers as well as the advantages of using S protein-targeting aptamers as inhibitors to prevent SARS-CoV-2 infection of cells, and finally the research progress on SARS-CoV-2 S protein-binding/blocking aptamers.

Although aptamers have promises in the prevention of SARS-CoV-2 infection as well as the treatment of some diseases such as cancers and some rare diseases, challenges exist. As of June 2022, a total of 162 antibody drugs have been approved by at least one regulatory agency in the world,[101] however until August 2023, only two aptamer drugs have been approved. This is an indirect indication that the translation from laboratory research to clinical application regarding aptamers is by no means an easy task. Concerted efforts and significant investments are required to address challenges such as drug delivery (pertaining to the S protein-blocking aptamers, effective delivery of the aptamers to the site of viral infection, in particular the respiratory tract membrane, is a serious challenge), adverse reactions and off-target effects, and insufficient clinical data about the safety and efficacy of aptamers in humans, thereby expediting the advancement of aptamer-based therapeutics. Anyhow, the approval of the second aptamer drug in Aug 2023 has given more hopes to and ignited more interest in the research community and the industry as well.

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Author Contributions

#Jun Zheng and Qiuxia Pang share the first authorship. Zhaoying Fu conceptualized, wrote and revised the text. Jun Zheng and Qiuxia Pang were responsible for data collection and visualization. Jun Zheng provided financial support. All authors have read and approved the final manuscript for publication.

Notes

The authors declare no competing financial interest.

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