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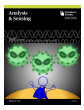
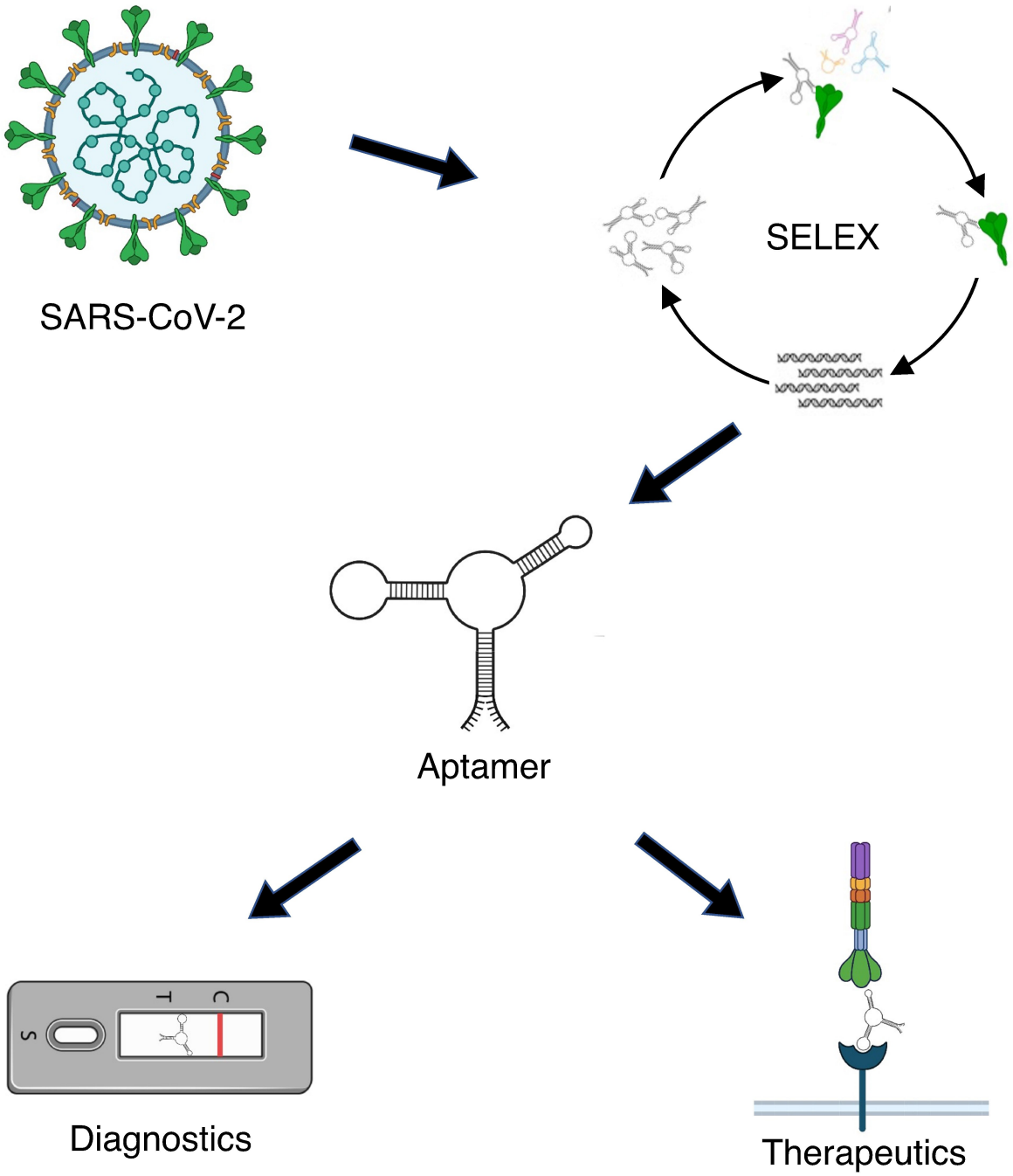


Aptamers for SARS-CoV-2: Isolation, Characterization, and Diagnostic and Therapeutic Developments

Ryan Amini[+],[a] Zijie Zhang[+],[a] Jiuxing Li[+],[a] Jimmy Gu,[a] John D. Brennan,*[b] and Yingfu Li*[a, b]



The SARS-CoV-2 virus and COVID-19 pandemic continue to demand effective diagnostic and therapeutic solutions. Finding these solutions requires highly functional molecular recognition elements. Nucleic acid aptamers represent a possible solution. Characterized by their high affinity and specificity, aptamers can be rapidly identified from random-sequence nucleic acid libraries. Over the past two years, many labs around the world have rushed to create diverse aptamers that target two important structural proteins of SARS-CoV-2: the spike (S) protein and nucleocapsid (N) protein. These have led to the identification of many aptamers that show real promise for the development of diagnostic tests and therapeutic agents for SARS-CoV-2. Herein we review all these developments, with a special focus on the development of diverse aptasensors for detecting SARS-CoV-2. These include electrochemical and optical sensors, lateral flow devices, and aptamer-linked immobilized sorbent assays.

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and its associated coronavirus disease 2019 (COVID-19), have brought an unprecedented level of disruption to society.[1] More than two years since the initial outbreak, several hundred million cases and more than 6 million deaths have been recorded. Several variants of concern (VoCs), such as the UK variant (Alpha), the South African variant (Beta), the Brazil variant (Gamma), the Indian variant (Delta), and most notably, the second South African variant (Omicron), have only increased public distress.[2] Although the introduction of vaccines has reduced COVID-19 transmissibility, hospitalizations and deaths, diagnostic and therapeutic interventions remain critical for outbreak prevention, contact tracing and treatment of severe cases.[3]

A plethora of SARS-CoV-2 diagnostic and treatment methods are available today. Each technology, however, has its limitations. For diagnostics, the reverse-transcription real-time quantitative polymerase chain reaction (RT-qPCR) is the go-to technique because of its high sensitivity and specificity.[4] However, RT-qPCR based tests not only are costly and time-consuming but also require an instrument, making them impractical for rapid, on-site testing.[5,6] Several commercially available enzyme-linked immunosorbent assays (ELISAs) and lateral flow devices (LFDs) for testing antigens from SARS-CoV-2 have been designed to provide a faster "sample-to-answer" time. However, they are considerably less sensitive than RT-qPCR assays as they require high viral loads.[7,8] In addition, such tests are highly sample dependent, and show sensitivity as low as 2.6% and 57.7% with saliva and throat samples, respectively.[9]

On the therapeutic front, only one drug, remdesivir, has been approved by the Food and Drug Administration (FDA) for COVID-19 treatment.[10] However, similar to the other drugs under emergency use authorization (EUA), remdesivir is limited in widespread use as it must be intravenously delivered in a healthcare setting.[11] Paxlovid, Pfizer's oral antiviral treatment, is a novel solution to this dilemma.[12] Yet, even with highly successful clinical trials, Paxlovid prescriptions will be complicated in the near future due to its limited supply and distribution. In addition, Paxlovid is a CYP450 inhibitor and can interfere with the metabolism of several other drugs.[13]

Constant mutations of SARS-CoV-2 present an on-going challenge to vaccines, diagnostic tests and therapeutics. Each time a new VoC is evolved, vaccines, diagnostics and therapeutics may have to be updated to remain relevant and effective. Take rapid antigen tests that detect the spike protein of SARS-CoV-2 as an example: new recognition elements may have to be derived if the existing tests can no longer recognize the spike protein of a new VoC or have a significantly reduced binding activity. Without any doubt, efforts are needed in the research community to derive more and better molecular recognition elements that can be used to develop more accurate rapid tests for ever-evolving SARS-CoV-2 variants.

Aptamers are single-stranded DNA, RNA or modified nucleic acids that have specific sequences and can create a well-defined structure to recognize a target of interest.[14–16] Aptamers are isolated from random-sequence nucleic acid libraries based on their high affinity and specificity for target recognition. The aptamer identification process is usually rapid (days to weeks). In addition, there are now well-established methods to integrate aptamers into diagnostic tests or therapeutic agents. It is worth mentioning, however, that aptamers have yet to achieve the commercial success seen with antibodies. This can be attributed to several obstacles-vulnerability to nucleases, risk of excretion by renal filtration, and sensitivity to environmental conditions (i.e., pH, temperature, etc.), just to name a few.[15] Nevertheless, aptamers still possess many desirable qualities for practical applications, and we anticipate that these aforementioned issues can be overcome with further research efforts by the functional nucleic acid community. Aptamers thus remain a worthy solution to deal with the challenges in the areas of diagnostics and therapeutics for SARS-CoV-2.

At this time, more than a dozen university labs have responded to this challenge by isolating aptamers for two antigens of SARS-CoV-2- the spike (S) protein and nucleocapsid (N) protein – and investigating these aptamers for potential diagnostic and therapeutic applications. These have led to the

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identification of several functional aptamers that show real promise as therapeutic and diagnostic agents for SARS-CoV-2. This review will provide a comprehensive summary of the findings from these studies.

2. Potential Antigens from SARS-CoV-2

Four main structural proteins define SARS-CoV-2 as a whole (Figure 1): envelope (E), membrane (M), nucleocapsid (N), and spike (S),[17,18] each of which could potentially serve as a diagnostic and therapeutic target.

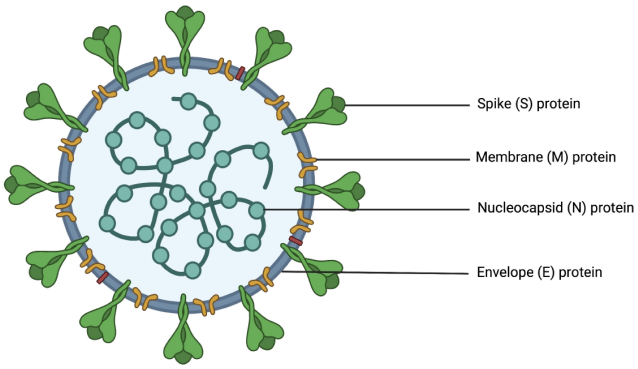
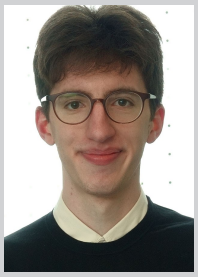


Figure 1. Illustration of SARS-CoV-2 structural proteins. Created with BioRender.com.

2.1. Envelope (E) protein

The E protein of SARS-CoV-2 functions to protect the genetic material of the virus and plays a key role during viral morphogenesis and assembly.[19] E protein is of particular interest because of its ability to form ion channels.[20] Ion channels in coronaviruses were previously determined to play an important role in virus-host interaction, inflammasome activation, and intracellular protein trafficking.[21,22] Since all these processes are critical for viral infection, disruption of any of them could



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Jim Gu completed his B.Sc. in Biochemistry in 2008 and his M.Sc. in Biochemistry in 2011 at McMaster University in Professor Yingfu Li's laboratory, where he worked on RNA-cleaving DNAzymes, screening of bacterial toxin-antitoxin systems, and on selection of DNA aptamers for cell internalization. He continues to work on DNAzyme and aptamer selection, characterization and bioinformatics applied to a variety of applications as a Research Technician with the Li Lab.

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Dr. Yingfu Li received his B.Sc. in chemistry from Anhui University in 1983, his M.Sc. in applied chemistry from China Agriculture University in 1989, and his Ph.D. in biochemistry and chemistry from Simon Fraser University in 1997 under the guidance of Prof. Dipankar Sen. He spent the next two years at Yale University as a postdoctoral fellow with Prof. Ronald Beaker. In 1999, he joined the Department of Biochemistry and Biomedical Sciences and the Department of Chemistry and Chemical Biology at McMaster University where he is now a professor. His group studies DNAzymes, aptamers, biosensors, nanotechnology, and noncoding RNA.

prevent pathogenesis, making the E protein an interesting therapeutic target. One benefit of targeting the E protein is that its sequence identity is far more similar to SARS-CoV and MERS-CoV than the S and N proteins.[23,24] This enhances E protein as a target because of its potential to trigger cross-reactive T cells, broadening the immune response. Unfortunately, the downside of the E protein is its lack of immunogenicity,[23] which makes it a poor target for vaccine development. However, it should still represent a possible target for aptamer development.

2.2. Membrane (M) protein

The structural integrity of SARS-CoV-2 is primarily maintained by the M protein. It functions not only in the structural assembly of the virus but also in assisting intracellular homeostasis and RNA packaging.[19] Like the E protein, the M protein also enjoys the benefits of being highly conserved for its SARS-CoV and MERS-CoV counterparts.[23,24] However, also like the E protein, the M protein is poorly immunogenic for humoral responses, and thus is not a good target for vaccine creation. It has been hypothesized that this is due to its small and receding nature.[25] Consequently, both proteins have been less popular targets for COVID-19 interventions, when compared to the N and S proteins. However, it could still represent a target for aptamer identification and detection assay development.

2.3. Nucleocapsid (N) protein

The N protein of SARS-CoV-2 is the most abundant of the four structural proteins and is highly immunogenic as well.[23] The N protein is primarily responsible for assisting in arranging RNA into the viral nucleocapsid. N proteins are also highly prevalent in the support of transcription, replication, cytoskeleton reorganization, and host cell apoptosis. Needless to say, the N protein is essential for SARS-CoV-2 survival.[19] As a therapeutic target, the RNA binding domain at the core of the N protein would be expected to prevent viral transcription/replication and would thus be an effective immunological target.[19] However, therapeutic applications of the N protein have been hindered, as it has been revealed that vaccines based on the N protein unexpectedly enhanced infection rather than providing protection.[26] Nevertheless, the N protein remains a popular target for the development of SARS-CoV-2 diagnostics, and is the target detected by most of the currently available antibody-based rapid antigen tests.

2.4. Spike (S) protein

Perhaps the most defining characteristic of SARS-CoV-2 is the trimeric spike (S) glycoprotein that decorates the surface of the virus. The S protein is composed of two distinct subunits, respectively named S1 and S2.[27,28] Of particular interest is the S1 subunit, which contains the receptor-binding domain (RBD). Upon the onset of infection, the RBD of the SARS-CoV-2 S protein identifies and interacts with angiotensin-converting enzyme II (ACE2) on host cells; using a hinge-like, conformational change, the virus can mediate host entry. The S2 subunit, on the other hand, primarily serves to cause membrane fusion between the virions and host cells, which is also crucial for the perpetuation of the virus.[19,29]

Several features of the S protein establish it as an excellent target for the development of vaccines, therapeutics and diagnostics, when compared to the other structural proteins.[23,30] The S protein is highly immunogenic and its function is indispensable to the virus, as it must be present for viral entry and replication. These properties make the S protein a great target for vaccines and therapeutics. Three traits of the S protein have made the S protein the most popular choice for aptamer development. First, a large part of this protein sits on the viral surface and thus the recognition by an aptamer, as well as diagnostic tests developed with this aptamer, does not require the lysis of the virus. Second, the S protein is a trimeric protein made of three identical monomers, which offers the opportunity for engineering dimeric or trimeric aptamers to bind two or three subunits of the same S protein for enhanced affinity. Third, each viral particle carries multiple copies of the S protein on its surface, providing another mechanism for multivalent recognition by aptamers to achieve high binding affinity.

3. Aptamers and SELEX

Aptamers are short, single-stranded DNA, RNA, or modified nucleic acid molecules that are capable of binding to a target of interest with high affinity and specificity.[14–16] This section will briefly review the primary methods of generating aptamers and their key advantages over alternative biochemical moieties for diagnostic and therapeutic applications.

3.1. SELEX for Aptamer Generation

The primary and most popular method by which aptamers are created is through an *in vitro* selection technique known as SELEX (Systematic Evolution of Ligands through EXponential enrichment), which was first reported in 1990.[31,32] Conventional SELEX for isolating DNA aptamers, for example, begins with a library of 10^{14}-10^{15} random single-stranded DNA sequences, as illustrated in Figure 2 with a general scheme for selecting DNA aptamers that bind the S protein of SARS-CoV-2. Potential aptamers from a library are progressively enriched through many repetitive rounds of binding-mediated partitioning and amplification. One round may include a negative selection step to eliminate sequences binding to non-target molecules and a positive selection step to retain high-affinity sequences for the intended target. Selected sequences at the end of each cycle are amplified to create an enriched DNA pool. Iterative cycles of this procedure are performed to let the molecules compete for survival based on their affinity and/or specificity. Once the evolving pool exhibits satisfactory binding properties, sequenc-

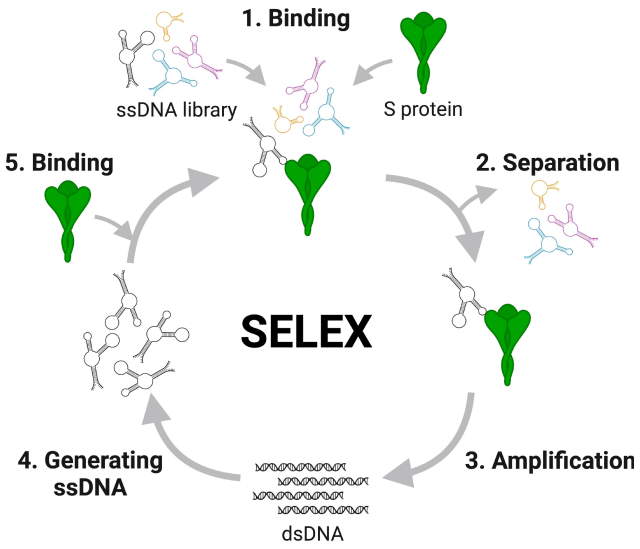

Figure 2. Schematic outline of SELEX procedure, with the SARS-CoV-2 S protein as the target for identifying DNA aptamers.

ing is used to reveal the identities of the remaining sequences in the pool.[33]

SELEX has now been used to produce aptamers for thousands of targets, including metal ions, small molecules, proteins, nucleic acids, viruses and cells.[14,15] Typically, SELEX is a rapid process that requires only days or weeks to complete, which is much faster than the discovery process for antibodies.[34,35] In addition, SELEX has expanded to include the use of chemically modified nucleotides, which can lead to better aptamers with higher stability and affinity.[36,37]

3.2. Characteristics and Advantages of Aptamers

The two defining characteristics of aptamers are their affinity (i.e., how well they bind to their designated target) and specificity (i.e., how well they distinguish the designated targets from other related and unrelated targets).[14,16] When selected properly, aptamers are capable of recognizing their intended target at extremely low concentrations while exhibiting minimal binding affinity towards other targets even at significantly higher concentrations. Aptamers can be further engineered to produce enhanced binding affinity following SELEX. For example, joining two aptamers that bind different epitopes of the same protein target can result in a dimeric aptamer with much higher binding affinity. Aptamers can also be pushed to possess high recognition specificity, often through counter-selection steps against unintended targets, which might be highly desirable for downstream applications as such aptamers can help reduce any off-target effects.[16]

Often described as "chemical antibodies", aptamers offer many advantages as components of diagnostics and therapeutics.[38,39] Firstly, aptamers are extremely small in size, which allows for minimal steric hindrance on the surface of the molecule (like a viral particle) and enables the binding of more recognition molecules.[40,41] Secondly, aptamers, particularly DNA aptamers and modified nucleic acid aptamers, are chemically and thermally more stable than proteins, allowing them to have a longer shelf life. DNA aptamers can also be manufactured in large quantities with minimal batch-to-batch variation. The chemical processes required to generate DNA allow for simple chemical modifications, all while being more cost-effective than alternatives such as antibodies. In addition, because nucleic acids are not recognized as foreign entities by the human immune system, they demonstrate low-immunogenicity and non-toxic properties.

4. Aptamers for the S Protein of SARS-CoV-2

4.1. SELEX Studies with the S Protein

Realizing the potential of aptamers as excellent molecular recognition elements that can be generated rapidly, many labs around the world have moved quickly to produce aptamers against the SARS-CoV-2 S protein. In fact, twelve SELEX studies have been conducted over a short period of time (two years) to select S protein binding aptamers (Table 1).[42–53] To the best of our knowledge, there has never been a prior case where this many SELEX experiments were performed for the same target of interest by multiple labs around the world using different nucleic acid libraries and different SELEX methods. In total, ten of these studies generated DNA aptamers, one produced RNA aptamers and the other generated aptamers using modified

Table 1. Aptamers for the S protein of SARS-CoV-2.

	Identifier	Random nt	Best aptamer	K_d (nM)	SELEX method	Ref.
1	Song-2020	40 (DNA)	CoV2-RBD-1	3.1	Beads-based	[42]
2	Li-2021	40 (DNA)	MSA1	1.8	Bead- and EMSA-based	[43]
3	Liu-2021	40 (DNA)	Aptamer-1	6.1	Beads-based	[44]
4	Schmitz-2021	40 (DNA)	SP5	9.2	Beads-based	[45]
5	Sun-2021	40 (DNA)	CoV2-6C3	44.8	Beads-based	[46]
6	Gupta-2021	41-44 (DNA)	S14	21.8	Beads-based	[47]
7	Yang-2021	40 (DNA)	nCoVS1-Apt1	0.33	Capillary electrophoresis-based	[48]
8	Peinetti-2021	45 (DNA)	SARS2-AR10	79	Filtration-based	[49]
9	Kacherovsky-2021	40 (DNA)	SNAP1	39.3	Beads-based	[50]
10	Valero-2021	36 (RNA)	RBD-PB6	18	Beads-based	[51]
11	Saify Nabiabad-2022	22 (DNA)	Apt1	290	Filtration-based	[52]
12	Ferreira-Bravo-2021	40 (XNA)	FANA–R8-9	1.4	Beads-based	[53]

nucleic acids (MNA) incorporating 2′-fluoro-arabino nucleic acids (FANAs) to select for nuclease-resistant aptamers.[53]

The first SELEX study was conducted by Song *et al.* in China in 2020 where the receptor-binding domain (RBD) of the S protein from the wildtype SARS-CoV-2 was used as the target.[42] RBD was also used as the target for aptamer selection in four other studies.[44,51–53] The S1 subdomain and full trimeric S proteins have also been used as targets for SELEX, as reported by three and two groups, respectively.[43,45,47,48,50] Finally, work described by Peinetti *et al.* used pseudotyped viruses that express the S protein as the target for binding.

In terms of the size of the random region in the library sequences, which is responsible for generating sequence diversity, 11 out of 12 studies used a random region of ~40 nucleotides (36–45 nucleotides). We note that a ~40-nucleotide random region has been widely used in previous SELEX studies,[15] and thus it is not surprising that most of the S protein aptamer selections adopted this size. The study by Saify Nabiabad used a random domain of only 22 nucleotides.[52] Although this size of random domain is less common, small-sized aptamers do exist. For example, the well-known thrombin binding DNA aptamer contains only 15 nucleotides.[54]

A very important step in a SELEX experiment is separating free aptamers from aptamer-target complexes, which can be achieved by several different methods (Table 1). Among the 12 SELEX efforts, 9 used bead-based separation where the protein target was immobilized onto beads, most probably due to the fact this method is easy to implement. Two studies used filtration-based methods, where a nitrocellulose membrane is instead used to immobilize the protein target. Finally, one study used capillary electrophoresis to achieve DNA-protein complex separation.[48] Compared to bead-based methods, filtration- and capillary electrophoresis-based methods avoid the immobilization of target proteins and can allow the selection of candidate aptamers that bind different epitopes of the protein targets.

Implementation of additional strategies has also been reported to achieve different goals. For example, Schmitz *et al.* used a robotic-assisted selection procedure that was capable of performing 12 consecutive, automated selection cycles, without manual interference.[55] Song *et al.* included ACE2 competition within their selection process (Figure 3) where ACE2 was incubated with bead-bound aptamers in order to select aptamers that compete with ACE2 for S protein binding so that the aptamers could be further developed as potential therapeutic agents to block viral binding to ACE2.[42] Our McMaster team combined two complex separation strategies in tandem to achieve aptamer selection: bead-based selection was used for the first 3 rounds and gel-based selection for the next 10 rounds.[43] Using multiple partitioning methods for the isolation of functional nucleic acids, which had been implemented in several previous reports,[56–58] can help eliminate selfish DNA sequences that can survive the use of a single physical separation method.[59] Finally, Peinetti *et al.* used "active" pseudotyped viruses as the positive selection target and "deactivated" pseudotyped viruses as the counter selection target with the goal of isolating aptamers that bind only to actively transmitting viruses.[49]

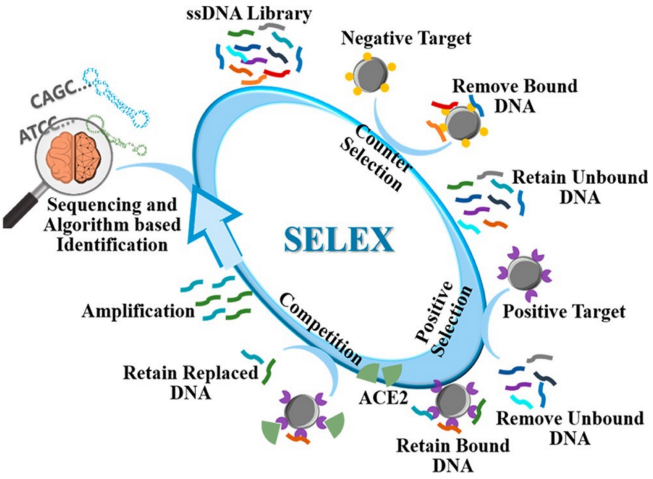

Figure 3. Summary of *in vitro* selection conducted by the Yang Lab to obtain high-affinity SARS-CoV-2 receptor-binding domain (RBD) aptamers.[42] Reprinted with permission from Ref. [42]. Copyright (2020) American Chemical Society.

Most of the SELEX studies used completely random libraries. However, our group took a different approach: the two flanking constant regions were designed with specific sequences for the creation of a pairing element that places the random-sequence domain into a hairpin structure (Figure 4A), which is often found in many published aptamers. In this way, we significantly increased the potential of finding more aptamers from the structured library. Indeed, after 13 rounds of selection, we obtained several aptamers that exhibited excellent affinity. Another advantage of such a library design is rapid identification of minimized sequences and secondary structures of these aptamers. For example, we were able to quickly identify the shortest sequences as well as the secondary structures of two top ranking aptamers from our SELEX experiment (Figure 4B).[43]

The affinity of an aptamer for a target of interest can be judged by its K_d value (i.e., dissociation constant), which can be

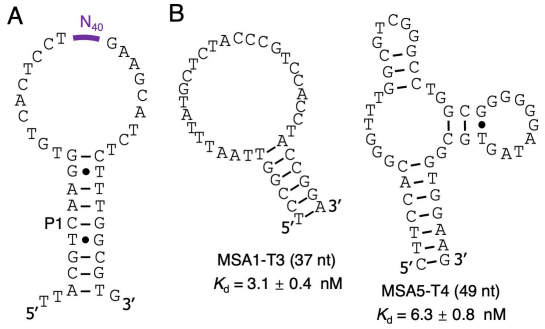

Figure 4. (A) The pre-engineered secondary structure of the DNA library used for the SELEX experiment by Li *et al.* (B) The hairpin structures of the minimized mutants of two top ranked DNA aptamers MSA1 and MSA5. Adapted with permission from Ref. [43]. Copyright (2021) Oxford University Press.

determined by a variety of methods, such as flow cytometry,[60] biolayer interferometry (BLI),[61] surface plasmon resonance (SPR),[62] dot-blot assays,[63] and capillary zone electrophoresis (CZE).[64] The best aptamer from each of the 12 SELEX studies was provided in Table 1, along with its K_d value. Interestingly, the K_d values of the 12 aptamers span almost 3 orders of magnitude, ranging from 0.33 nM to 290 nM, though most of the aptamers exhibited K_d values between 1–20 nM. However, due to the differences in measurement methods and the use of different targets (S1 protein, trimeric spike protein or pseudoviruses), the reported K_d values may not accurately reflect their true affinity ranking. As such, the reported K_d value for an aptamer to a target does not necessarily translate to its ability to function *in vivo*, necessitating the requisite for standardized assay conditions.[65] A head-to-head comparison of these aptamers using the same target and same experimental methods would be needed to allow proper affinity ranking.

4.2. Aptamer Sequence Comparison

The 12 SELEX experiments targeting the S protein of SARS-CoV-2 have generated many aptamers in addition to those listed in Table 1. Table S1 lists 43 published aptamer sequences for which a K_d value has been reported. With such an abundance of SARS-CoV-2 S protein aptamers, an interesting question is whether all these sequences are distinct. To answer this question, we have performed a BLAST multiple sequence alignment of the original random-sequence regions from the best-performing DNA aptamers listed in Table S1, and the results are provided in Figure S1. A query list of random sequence domains, where identifiable, were aligned against a database of the same list of sequences using the nblast program from the BLAST package.[66] Where alignments were detected between two sequences, the smallest E-value (most significant alignment) was extracted and plotted on a heatmap (Figure S1). The most significant alignments generally appeared between aptamers identified from the same selection library, for example within the MSA family (family refers to the alignment between each sequence having an E-value lower than 10^{-3}) aptamers and FANA family aptamers, respectively. The most significant alignment between aptamers originating from separate selections was observed between CoV2-RBD-1 and Apt1. Several lower significance alignments (E-value $> 10^{-3}$) were also observed, many of these alignments appeared between G-rich regions of aptamers and may represent a shared preference for G-quadruplex structural elements. The diversity of aptamers identified may be due to several factors, such as a variety of spike target constructs (S1, RBD or trimeric), library designs and selection methods.

There are many G-rich sequences in the selected S protein binding DNA aptamers. However, since G-rich elements are commonly found in a variety of DNA aptamers,[67] the presence of G-rich elements does not necessarily suggest that they belong to the same aptamer class. To evaluate the G-quadruplex forming ability of the SARS-CoV-2 S protein aptamers, the sequences listed in Table S1 were run through PQS Finder, a G-quadruplex prediction package, which identified 26 sequences that have the potential to form a G-quadruplex structure (Table S2). Notable aptamers with high quadruplex prediction include MSA2, MSA5, MSA7, MSA8, S1 and S14, all of which register a score of >60, suggesting that these sequences have the potential to form a G-quadruplex structure. This is a point that is worth future investigation. Once again, we note that G-rich elements and G-quadruplex structures are commonly found in many published DNA aptamers that bind other targets,[67] and thus the G-rich nature of these sequences cannot be simply used to suggest that they belong to the same aptamer family.

4.3. Secondary Structures

Nucleic acid aptamers have been observed to possess numerous secondary structures, such as hairpin loops, internal loops, multi-branched loops, single-stranded elements, bulges, etc.[68] These secondary structures are known to play a key role in directing the binding interactions between an aptamer and its protein target.[68]

To determine which structural elements are more essential than others, researchers commonly conduct aptamer truncation strategies. Aptamer truncations involve the removal of non-essential nucleotides, which subsequently decreases production costs for future applications and improves aptamer-target accessibility. Several truncated aptamers have been reported, which are listed in Table S3. These shortened sequences have been fully tested and show binding affinities similar to their parent sequences (Table S3).

The proposed secondary structures of 28 aptamers are provided in Figures S2, S3 and S4, some of which were provided in the original publications and the remainder created by us using the Mfold program. In one example, the secondary structures of 3 aptamers from our studies – MSA1, MSA3 and MSA5 – have been described (Figure S3, panels A, B and C), and their activities have been confirmed with a series of truncated mutants.[43] In another example, *Kacherovsky et al.* have also reported the secondary structure of their best performing aptamer SNAP1 (Figure S3, panel D).[50] An initial 20 nucleotide truncation, and a longer 50 nucleotide truncation, of SNAP1 revealed that the multi-branched loop and the three hairpin loops were necessary for binding, whereas the stem and internal loops could be completely removed. Once again, this is consistent with the idea that hairpin loops are critical for target binding, and that the best, high-affinity DNA aptamers adopt hairpin structures.[69–71]

4.4. Dimeric and Multimeric Aptamers

High-affinity aptamers can be derived by engineering dimeric or multimeric aptamers through the joining of two or more monomeric aptamers.[72–76] For example, the Mayer group has combined two thrombin aptamers into a dimeric aptamer, which improved affinity by ~30-fold.[74] The Soh group has also

created an impressive bivalent aptamer using a selection approach.[72] Given the fact that each SARS-CoV-2 virus expresses ~30 spike proteins on its surface and that the spike proteins are homotrimers,[77] dimeric or multimeric aptamers can provide multivalent recognition towards spike proteins for enhanced affinity. Herein we summarize the reported dimeric and multimeric aptamers for spike proteins of SARS-CoV-2 and compare their affinities in Table 2 (the sequences of these aptamers are provided in Table S4).

Yang's group reported the first dimeric DNA aptamer, cb-CoV2-6C3, which was derived by ligating two monomeric aptamers selected using RBD as the target.[46] A circular dimeric aptamer was engineered to improve both stability and affinity,

Table 2. Dimeric and multimeric aptamers for SARS-CoV-2 S protein.

	Identifier	Dimer/ Multimer	Best aptamer	Affinity for protein (pM)	Affinity for pseudovirus	Ref.
1	Sun-2021	Dimer	cb-CoV2-6C3	130 (RBD)	0.42 nM (IC_{50})	[46]
2	Valero-2021	Dimer	RBD-PB6-Ta	72 (RBD)	387 nM (IC_{50})	[51]
		Trimer	RBD-PB6-Ta	39 (RBD)	46 nM (IC_{50})	[51]
3	Zhang-2021	Dimer	DSA1N5	120 (Spike)	2.1 pM (K_d)	[78]
4	Sun-2021	Multimer	SNAP	3.9 (RBD)	0.1428 pM (IC_{50})	[79]

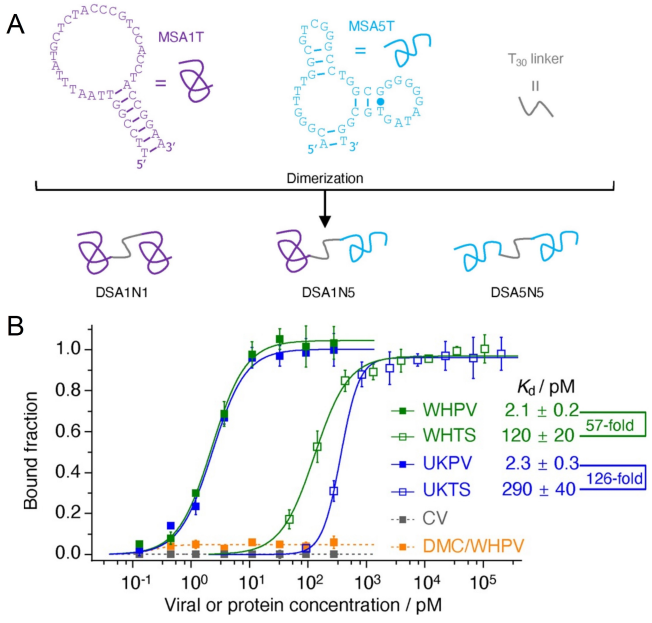

Figure 5. (A) Schematic of engineering dimeric aptamers by ligation of two monomeric aptamers with a polythymidine linker. (B) Determination of binding affinity of heterodimeric aptamer DSA1N5 binding for the spike proteins and pseudotyped viruses of the original SARS-CoV-2 and alpha variant. WHPV and UKPV: lentiviruses pseudotyped with the spike protein of the wildtype SARS-CoV-2 and the alpha variant. WHTS and UKTS: trimeric spike protein of the wildtype SARS-CoV-2 and the alpha variant. CV: control lentivirus. DMC: inactive mutant dimeric aptamer control. Adapted with permission from Ref. [78]. Copyright (2021) Wiley-VCH.

and was utilized to block virus infections by inhibiting the interactions between the RBD and human ACE2 receptors. cb-CoV2-6C3 exhibits a high affinity (K_d = 0.13 nM) for the RBD and an IC_{50} of 0.42 nM for inhibiting SARS-CoV-2 infections (Table 2).

Valero *et al.* engineered dimeric and trimeric RNA aptamers by ligating an RNA homo-monomeric aptamer with a poly-adenosine linker.[51] Although these aptamers were demonstrated to be effective in neutralization of SARS-CoV-2 infections, the affinities for viruses were still in the nanomolar range (Table 2).

We recently constructed a series of dimeric aptamers by linking two monomeric aptamers with a polythymidine linker. These include two homodimers DSA1N1 and DSA5N5 as well as a heterodimer DSA1N5 (Figure 5A).[78] We found that the heterodimeric aptamer DSA1N5 had the highest affinity among reported dimeric or trimeric aptamers for recognizing pseudo viruses of both the original SARS-CoV-2 and the Alpha variant with K_d values of 2.1 pM and 2.3 pM, respectively (Table 2 and Figure 5B). The affinities for viral recognition were more than 57 and 126-fold better than for their spike proteins (K_d of 120 pM and 290 pM, respectively; Figure 5B), highlighting the advantage of engineering bivalent aptamers for viral particle recognition.

In addition to dimeric and trimeric aptamers, the Yang and Tan groups recently developed a spherical multivalent aptamer-integrated nanoparticle (SNAP) by coupling multiple monomeric DNA aptamers onto gold nanoparticles for blocking SARS-CoV-2 infections.[79] SNAP exhibited high affinity binding to the RBD with a K_d of 3.9 pM and potent neutralization against authentic SARS-CoV-2 with an IC_{50} of 142.8 fM (Table 2), which were about 2 to 3 orders of magnitude stronger than those reported for other neutralizing aptamers and antibodies, providing an effective strategy for constructing multivalent aptamer systems for potential therapeutic applications.

4.5. Aptamer Binding to Variants of Concern

Since the emergence of wild-type SARS-CoV-2, the virus has been undergoing constant mutations, causing the development of many variants of concern (VoCs). These variants are characterized by multiple amino acid alterations in the S protein, specifically in the RBD. The Delta variant, for example, was observed to have 11 key mutations in the S protein.[80] In response to the changing immune profile of humans, it is understood that the virus develops these mutations to enhance S protein-ACE2 interactions.[81] Neutralizing antibodies present in those vaccinated against SARS-CoV-2 primarily target the RBD. With changes to the S protein, new variants have been proven to increase infection, improve transmissibility and evade humoral immunity altogether.[82] To make matters worse, at the time of writing, a novel, second variant from South Africa has most recently emerged.[83] This "Omicron" strain has substantially increased transmissibility and partially evades vaccines, and has produced a large global wave of infection which started in late 2021.[84,85] At the time of writing, a relative of the

main Omicron variant, known as BA.2, is displacing its original sibling BA.1 and rapidly spreading around the world due to its increased transmissibility over BA.1.[86] Considering that several of the previously mentioned aptamers bind to the RBD, or have been targeted to the RBD, it would follow that affinity would decrease in the recognition of these new spike variants. Taken altogether, these variants are complicating the epidemiological strategy of the COVID-19 pandemic and necessitate additional diagnostic and therapeutic solutions.

To address this issue, we recently made an effort to select for universal DNA aptamers that recognize several VoCs.[87] Our previous DNA aptamers, mainly MSA1 and MSA5, were targeted specifically to the S1 subunit of the wildtype spike protein. However, it was realized that the epitopes recognized by these two aptamers were sensitive to the changes caused by the mutations to the S protein. To reselect aptamers that can function as universal affinity agents, we conducted five parallel, one-round SELEX experiments with our previously established Round 13 aptamer pool and five VoC spike proteins (Figure 6A). One particular aptamer, named MSA52 (Figure 6B), displayed a significant increase in the pools selected using the variant spike proteins. Upon binding analysis, we found that this aptamer indeed displayed high affinity for pseudotyped lentiviruses expressing each variant protein (Figure 6C). Furthermore, to test its universality, MSA52 was also assessed with the Kappa, Delta and Omicron variants, which were not yet available at the time of the original reselection experiment. The binding assays demonstrated that MSA52 could also recognize these variants, thus confirming MSA52 as a "universal" aptamer for SARS-CoV-2 S protein. Perhaps what is most impressive is that the entire discovery process took place in less than a week, which is a testament to the rapidity of the SELEX process.

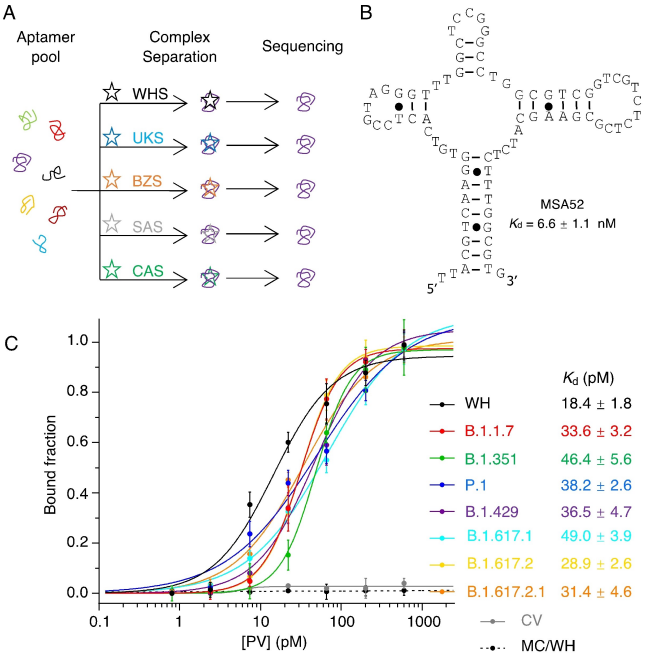

Figure 6. A universal aptamer for the S protein. (A) Pictorial representation of the reselection process for the discovery of universal aptamers. (B) Secondary structure MSA52. (C) Binding between MSA52 and pseudotyped lentiviruses expressing the spike of the wildtype (WH) and 7 variants of SARS-CoV-2. CV: control lentivirus; MC: inactive mutant of MSA52 as the control sequence. Adapted with permission from Ref. [87]. Copyright (2022) Wiley-VCH.

5. Aptamers for the N Protein of SARS-CoV-2

Although far less common than S protein aptamers, aptamers for the N protein of SARS-CoV-2 have also been discovered. At present, three SELEX studies have been reported (Table 3 and Table S5).[88–90]

Reuse of existing N-protein binding aptamers created for an earlier coronavirus represents an option for quickly identifying aptamers for SARS-CoV-2. A SELEX study, conducted in 2011 by Cho *et al.*, targeted the N protein of the initial severe acute respiratory syndrome coronavirus (SARS-CoV). This study used a DNA library with a 45-nt random domain and a bead-based SELEX strategy, which led to the isolation of several high-affinity DNA aptamers. In 2020, Chen *et al.* examined some of these aptamers for binding to the N protein of SARS-CoV-2.[91] They found an aptamer named N aptamer 1 that bound with the N protein of SARS-CoV-2 with a K_d of 4.92 nM.[91] This is not particularly surprising, considering the N protein of SARS-CoV-2 shares 91% sequence homology with the N protein of SARS-CoV.[92,93] The authors also developed a detection assay using these aptamers, which will be discussed in Section 6.[91]

Zhang *et al* published a study in 2020 where a DNA library containing the 36-nt random domain and a bead-based SELEX strategy was used to search for aptamers that bind the N protein of SARS-CoV-2.[89] Four aptamers were discovered, with the best aptamer exhibiting a K_d of 0.49 nM. Interestingly, upon analysis, it was found that these aptamers interacted with the N protein in a sandwich style. Sandwich-type interactions occur when a target antigen is bound to two recognition elements, in this case, two aptamers. Given biological samples may contain molecules that potentially interfere with aptamer-target binding, sandwich-type interactions are particularly attractive in terms of potentially mitigating this interference. Two other labs have now utilized the aptamers from this work to create diagnostic tests for COVID-19 (as discussed in Section 6).[94,95]

Kang *et al.* have also developed several N protein specific aptamers in a recent study using a DNA library containing a 53-nt random region.[90] These aptamers were isolated using a

Table 3. DNA aptamers for the N protein of SARS-CoV-2.

Study #	Identifier	Random domain (nt)	Name of best aptamer(s)	K_d (nM)	SELEX method	Ref.
1	Cho-2011	45	N aptamer 1	4.92	Beads-based	[88]
2	Zhang-2020	36	A48	0.49	Magnetic beads-based	[89]
3	Kang-2021	53	SARS-CoV-2_apt2	0.57	Microtiter plate-based	[90]

previously developed SELEX method called H-sandwich SELEX.[96] The technique involves first immobilizing antibodies for the N protein onto a microtiter plate so that the N protein can be captured onto the plate for aptamer selection. The resultant aptamers are programmed to exhibit sandwich forming capabilities with their target and the antibody. The best aptamer from this work is named SARS-CoV-2_apt2 with a K_d of 0.57 nM (Table 3).

6. Detection of SARS-CoV-2 with Aptamers

Aptamer-based biosensors, or "aptasensors", have been an intensive area of research since the early 1990s, and have been used for detection of diverse targets using various signal transduction mechanisms.[97,98] With the development of many aptamers for both the S and N proteins of SARS-CoV-2, many research groups around the world have made efforts towards developing aptasensors for detecting SARS-CoV-2,[99] though at present, none of the aptamers have been used in commercial tests. In this section, we will discuss recent advancements in SARS-CoV-2 aptasensors in the following categories: electrochemical sensors, optical sensors, rapid lateral flow assays (LFAs), and aptamer-linked immobilized sorbent assays (ALISAs). A summary of the performance of these aptasensors, including the limit of detection (LOD) values and targets, is provided in the Table S6.

6.1. Electrochemical sensors

Electrochemical aptasensors involve the surface-immobilization of selected aptamers to capture the target biomolecule. Detection occurs through one of two mechanisms, either by a potentiometric technique or an amperometric method.[98,100] Common techniques include square wave voltammetry (SWV), differential pulse voltammetry (DPV), and electrochemical impedance spectroscopy (EIS).[98] Electrochemical detection methods are distinguished by several advantages.[101] Both the electrodes and the potentiometers can be easily miniaturized and assays can be automated to provide high detection speeds and low costs. Furthermore, electrochemical sensors can often be integrated with a smartphone to allow simple signal processing and readouts using an associated app.[78] Given these advantages, many aptamer-based electrochemical sensors have been developed for the detection of diverse human diseases.[16,102–105]

There are several recent examples of aptamer-based electrochemical sensors for SARS-CoV-2. For example, Idili *et al.* developed an electrochemical sensor by simply immobilizing an S protein-specific DNA aptamer on electrodes for quantitative detection of SARS-CoV-2 (Figure 7).[106] The platform utilized conformational changes of the immobilized aptamers upon binding with the S protein to bring a redox probe into close proximity to the electrode surface, producing an increase in current. This sensor was easy to manufacture, rapid (within minutes), and could conduct direct testing in clinical samples

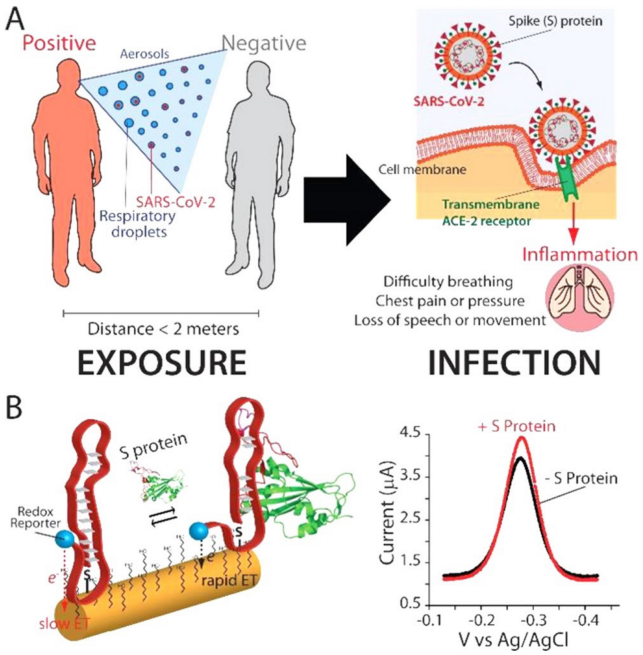

Figure 7. Electrochemical aptamer-based (EAB) sensors exploit the binding-induced conformational change of a covalently attached, redox reporter-modified aptamer to generate an easily measurable electrochemical signal. Reprinted with permission from Ref. [106]. Copyright (2021) American Chemical Society.

(serum and saliva). However, this direct immobilization strategy generated a high background signal in the absence of targets, leading to low sensitivity and a relatively high false-positive rate.

Electrochemical impedance spectroscopy (EIS) is one of the most sensitive electrochemical techniques and can deliver measurable signal changes resulting from small fluctuations in biomarker concentration.[107] This method has been used to develop several electrochemical sensors for the detection of SARS-CoV-2.[108–110] Our groups recently developed a "Cov-eChip" sensing platform by immobilizing the dimeric DSA1N5 aptamer on gold electrodes for highly sensitive detection of SARS-CoV-2 in unprocessed saliva samples (Figure 8).[78] Binding of either S protein, pseudovirus or SARS-CoV-2 lead to an increase in electrochemical impedance that could be read using a smartphone, providing a detection limit of 1000 viral particles per mL of saliva in 10 min for wildtype SARS-CoV-2, with similar detection levels for both the Alpha and Delta variants. Evaluation of 36 positive and 37 negative saliva samples produced a clinical sensitivity of 80.5% and specificity of 100%, and the sensor could detect the wildtype virus as well as the Alpha and Delta variants in the patient samples. The Cov-eChip sensor promises a rapid, single-step detection method that can be conveniently paired with simple, handheld instrumentation.

To achieve a highly sensitive aptamer-based sensing platform, Zakashansky *et al.* used stretchable polymers based on Shrinky-Dink© plastics to create a wrinkled polymer that was then coated with a gold film to generate high surface area electrodes for aptamer coupling.[107] Using a portable potentiostat connected to a smartphone, an SWV method was shown to

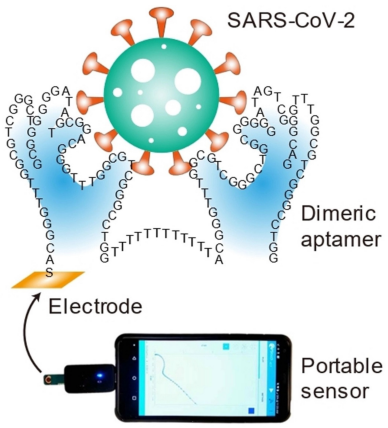

Figure 8. Illustrated representation of the "Cov-eChip", a rapid and simple electrochemical sensor for SARS-CoV-2 diagnosis.[78] The sensor is anchored by a dimeric aptamer capable of selectively binding to the S protein and its variants. Reprinted with permission from Ref. [78]. Copyright (2021) Wiley-VCH.

have very high sensitivity, being able to detect down to 1 ag mL^{-1} of S protein spiked into saliva, though there was no validation step using clinical samples.

The Lu group reported an aptamer integrated nanopore electrochemical sensor that was able to detect and differentiate infectious SARS-CoV-2 from non-infectious (inactivated) viruses.[49] The DNA aptamer specific to the infectious SARS-CoV-2 was selected and incorporated into a solid-state nanopore, where the tested viral particles were strongly confined in the nanopores resulting in blockage of the through-pore ion current, producing excellent sensitivity, and allowing detection down to 10^4 copies mL^{-1} of SARS-CoV-2 in different types of water, saliva, and serum samples without any sample pre-treatment.

Another example was reported by Tian *et al.* This group had taken the N-protein aptamers previously reported by the Luo Lab and constructed their own sandwich-type electrochemical sensor. In this case, two thiolated aptamers were first immobilized on a modified Au electrode to allow the sandwich structure to form upon binding of the N protein. Then, specific nanomaterial composites comprised of Au@Pt/MIL-53 (Al) (a metal organic framework material) decorated with both HRP and hemin/G-quadruplex DNAzymes were added as signaling nanoprobes. The nanoprobes could co-catalyze the oxidation of hydroquinone in the presence of hydrogen peroxide, producing an electrochemical current that was measured by DPV.[111] This method was able to detect 8.33 pg mL^{-1} of N protein, though the sensor was not evaluated with clinical samples.

Another example involved the development of photo-electrochemical (PEC) aptasensors, which used a photosensitive electrode material (a graphitic carbon nitride-cadmium sulfide quantum dot nanocomposite) to allow light-generated electrochemical currents for detection of SARS-CoV-2 S protein using the RBD-1 C aptamer.[112] The aptamer was immobilized onto a chitosan-coated PEC electrode, was able to detect N protein with a detection limit of 120 pM, and was capable of measurements in spiked human saliva samples. However, this sensor system was complicated and difficult to operate, and has not been demonstrated for the detection of viruses.

To generate a simplified electrochemical sensor system, Singh *et al.* developed an aptamer-based assay for SARS-CoV-2 that could use a personal glucose meter (PGM) to generate an electrochemical readout. This avoided the need to produce aptamer-modified electrodes, and offered a low-cost (~$3/test), easy-to-use, highly scalable testing platform for rapid screening of SARS-CoV-2 (Figure 9).[113] In this assay, DNA aptamers for either the N or S protein of SARS-CoV-2 were pre-hybridized with antisense sequences and conjugated on magnetic beads. Binding of the protein caused a structure switch to release an invertase-modified DNA strand, followed by removal of MBs with unreacted aptamer-invertase complexes, leaving only free invertase. Sucrose was then added to the invertase solution, which caused hydrolysis of sucrose to produce glucose. The PGM then measured the glucose concentration to produce a current that could be related back to the concentration of viral protein. The assay produced LODs of 1.50 pM and 1.31 pM for the N and S proteins in buffer, and 5.27 pM and 6.31 pM for N and S proteins in saliva. Validation using patient saliva samples produced sensitivity and specificity values of 100%. While impressive, the assay required both a separation step and a need to add sucrose, which makes it incompatible with rapid testing.

6.2. Optical sensors

Optical aptasensors involve changes in optical signals generated once the target-specific aptamer recognizes and binds the molecule of interest. Optical aptasensors boast high sensitivity, simplicity and rapid response times. To date, their practical application has been demonstrated for the detection of various targets, including small molecules, proteins, bacterial cells and viruses.[114] Optical detection techniques include absorbance, fluorescence, chemiluminescence, surface plasmon resonance (SPR), surface-enhanced Raman scattering (SERS), and interferometry.[98] Key examples for the detection of SARS-CoV-2 are presented below.

An early example of an optical aptasensor for SARS-CoV-2 was reported by Liu *et al.*, who used the N protein aptamers selected by Zhang *et al.* to develop a novel "aptamer-assisted proximity ligation assay" (Apt-PLA; Figure 10).[94] In this case, two aptamer probes bind to the N protein in a sandwich configuration, leading to proximity ligation of the extended DNA regions in each aptamer, allowing ligation-dependent recognition via qPCR. Quantitation of the N protein, which is inversely related to the threshold cycle (Ct) value of the qPCR assay, could be analyzed using the fluorescent dye TB green. This sensing platform reached a limit of detection (LOD) of 37.5 pg mL^{-1} of N proteins. However, this method required both sample pre-treatment and expensive instrumentation and reagents, and had a relatively long testing time (~2 hours), limiting the potential for rapid testing.

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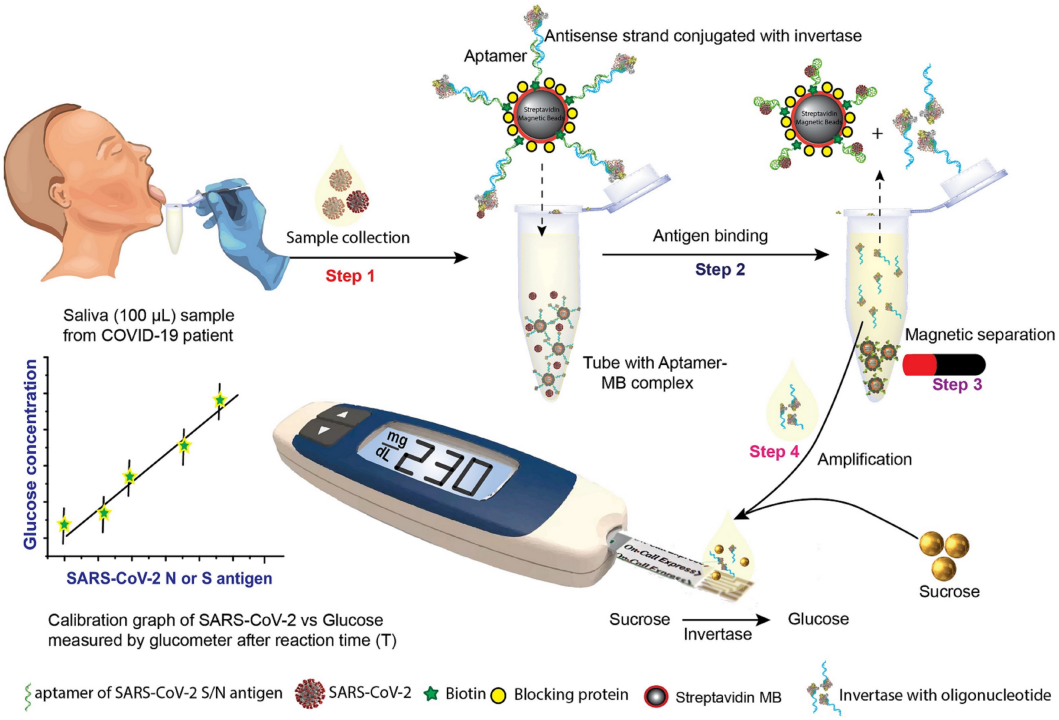

Figure 9. Overview of the point-of-care SARS-CoV-2 salivary antigen testing with an off-the-shelf glucometer. The glucose concentration is correlated with the SARS-CoV-2 N or S protein concentration. Reprinted with permission from Ref. [113]. Copyright (2021) Elsevier.

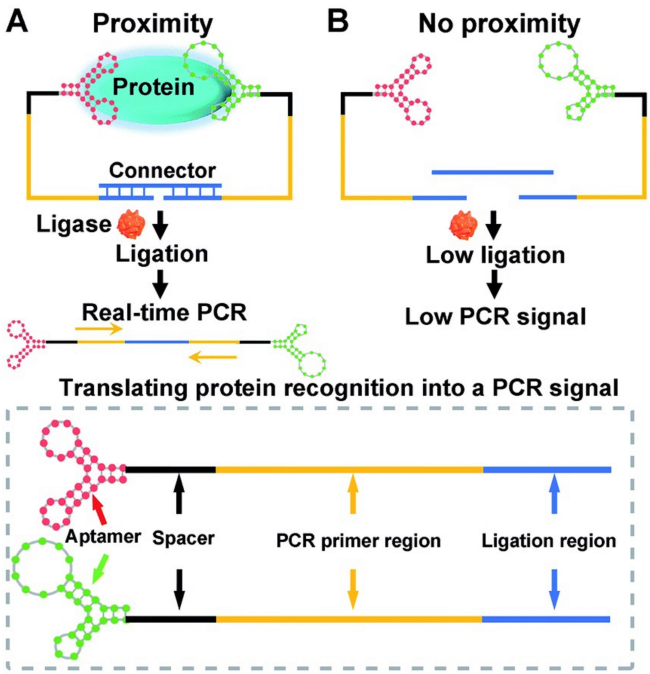

Figure 10. Demonstration of the Apt-PLA system for the SARS-CoV-2 N protein and COVID-19.[94] (A) Ligation-dependent qPCR occurs when the aptamer probes bind to the common protein target. (B) The absence of the N-protein target results in minimal ligation and thus a low PCR signal. Reprinted with permission from Ref. [94]. Copyright (2020) Royal Society of Chemistry.

Two additional optical aptasensors were developed based on either SPR or SERS techniques. In the first case, Huang *et al.* developed a sensitive aptamer integrated SERS sensor that was able to directly detect the RBD protein in urine and blood with a limit of detection down to 1.25 ng mL^{-1} in 15 min.[115] In the other example, Lewis *et al.* reported an aptamer functionalized localized SPR strategy to detect the S1 protein of SARS-CoV-2 in saliva and serum samples with a LOD of 0.26 nM.[116] Although these approaches are relatively rapid and are amenable to measurements in biological matrixes, both sensing platforms require extensive equipment for signal generation, and are difficult to implement for large-scale screening of SARS-CoV-2.[115–118]

Recently, the Tan group reported a laser assisted one-step thermophoretic assay using a fluorescently labelled DNA aptamer for the S-protein. In the absence of pseudovirus particles, the laser generates a thermal gradient that results in rapid mobility of the free aptamer within a polyethylene glycol (PEG) solution. Increased concentrations of pseudovirus led to the formation of aptamer-pseudovirus complexes, which showed reduced mobility owing to their larger size. For quantitative detection of SARS-CoV-2, fluorescence imaging was used to determine the accumulation of viral particles in laser-induced gradients of temperature and PEG concentration, which dramatically enhanced the assay sensitivity, producing a limit of detection of 170 copies per µL (Figure 11). The assay provided 100 % accuracy for testing oropharyngeal swab samples using an assay time of 15 minutes and no sample pre-treatment.[119] However, the method was again dependent on expensive instrumentation, making it difficult to implement for large scale rapid testing.

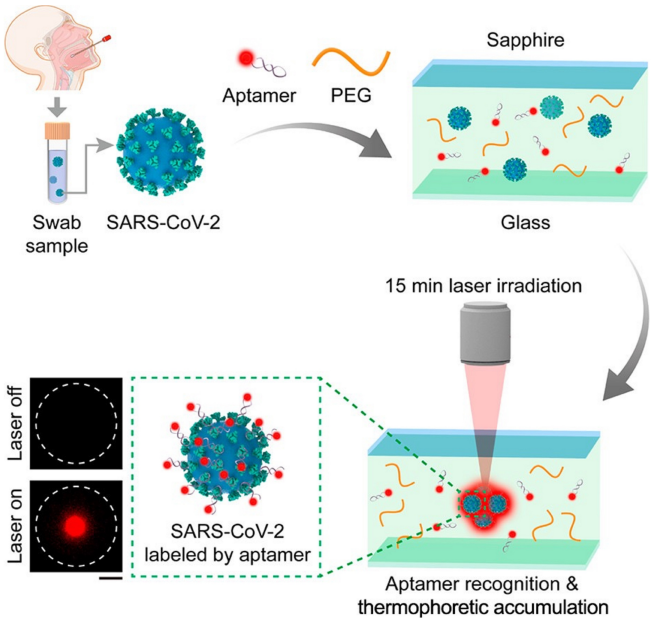

Figure 11. Schematic of the one-step aptamer-based thermophoretic fluorescence assay for rapid detection of SARS-CoV-2 viral particles. Reprinted with permission from Ref. [119]. Copyright (2021) American Chemical Society.

A recent assay based on aptamer-coated photonic crystals has been used for interferometric imaging of the scattering of bound pseudotyped SARS-CoV-2, showing single virion resolution and a detection limit of 10^3 copies of virions per mL with no need for amplification.[120] The assay did not require labelling of any species, but was relatively slow and labour intensive, making it difficult to implement for rapid, large-scale testing of infections.

6.3. Lateral flow assays (LFAs)

LFAs are a widely used paper-based rapid testing platform that can provide inexpensive, equipment-free point-of-care detection of analytes.[121] LFAs typically consist of four components that are assembled on a backing card: a sample pad for sample loading, a conjugate pad containing detection moieties, a nitrocellulose membrane containing test and control lines with immobilized capture agents, and an absorbent pad. Target molecules bind to the detection moiety, typically an antibody or aptamer labelled with a gold nanoparticle, and are then captured by the immobilized capture agent (a second antibody or aptamer) to produce a colored test line. The system is validated by the presence of the control line. While currently available commercial devices are mainly based on antibodies, many aptamer-based LFAs have been reported, particularly for small molecules, including those for dopamine detection in urine and cortisol detection in sweat.[122,123]

At this time there are only a few reports of aptamer-based LFAs for detection of SARS-CoV-2. The Pun group developed an LFA that used the SNAP1.50 DNA aptamer (a truncated version of SNAP1) as a capture agent to bind the N-terminal domain of the S protein of SARS-CoV-2, and an AuNP-modified antibody as the detection agent (Figure 12, A and C).[50] The LFA was able to detect as low as 250 pM of S protein and 10^7 copies mL^{-1} of pseudotyped lentiviruses of SARS-CoV-2 in 10–30 min (Figure 12C), though this was significantly poorer than the LOD of 10 pM for S protein and 10^5 copies mL^{-1} of pseudotyped lentiviruses obtained using a luminescence-based ELISA (Figure 12, B and D). Given that the concentration of SARS-CoV-2 detected in saliva and nasopharyngeal samples of COVID-19 positive patients by RT-qPCR in the first two weeks after onset of symptoms ranged from 10^4 to 10^{10} copies mL^{-1},[124] the LFA is likely to only be able to show positive results for patients with high viral loads.

The Luo Lab developed an alternative aptamer-based LFA using their sandwich-type N protein aptamers.[89] The conjugate pad of the LFA contained biotinylated versions of the Apt48 and Apt58 aptamers bound to streptavidin to form a bidentate binding structure. The streptavidin was also conjugated to a gold nanoparticle for signal generation. The test line was coated with an anti-N antibody, and detection was based on the N proteins in saliva forming a sandwich complex with the anti-N antibodies and the anti-N aptamers. This LFA was ultimately able to detect down to 1 ng mL^{-1} of N proteins from samples within 15 minutes. However, the LFA was not evaluated in saliva or NPS samples and no clinical validation studies with patient samples were reported.

6.4. Aptamer-linked immobilized sorbent assays (ALISAs)

Aptamer-linked immobilized sorbent assays apply the same principles as an ELISA (enzyme-linked immunosorbent assay) but involve the substitution of the antibody with a high-affinity DNA aptamer.[125] Such assays possess advantages such as high sensitivity, ease of operation and high throughput.[126] There are now several reports of ALISAs for the detection of SARS-CoV-2.[43,50,113,127,128] Svobodova *et al.* reported an ALISA using aptamer pairs to form a sandwich complex with the S-protein. A total of 8 aptamer pairs were screened for affinity and specificity and the Apt1T/Apt5B pair was selected for ALISA development. Thiolated Apt1T was bound to a gold surface as the capture aptamer, and biotinylated Apt5B was used for detection. Upon the formation of the sandwich complex, poly-HRP streptavidin was added to allow color generation based on a peroxide/TMB reaction.[127] The assay was able to detect down to 190 pM of S protein of SARS-CoV-2 in viral transport media, but only produced a sensitivity of 80% and specificity of 40% for clinical NPS specimens.

We recently selected diverse high-affinity DNA aptamers for the spike protein of SARS-CoV-2 and used one aptamer named MSA1 for ALISA.[43] We designed an ALISA sandwich assay using biotinylated MSA1 as both the recognition element (bound to streptavidin-coated microwells) and detection element, which could bind to HRP-conjugated streptavidin to allow colorimetric detection using the peroxide/TMB system described above. This assay produced a detection limit of 400 fM of pseudotyped lentivirus of SARS-CoV-2 in 50% saliva.

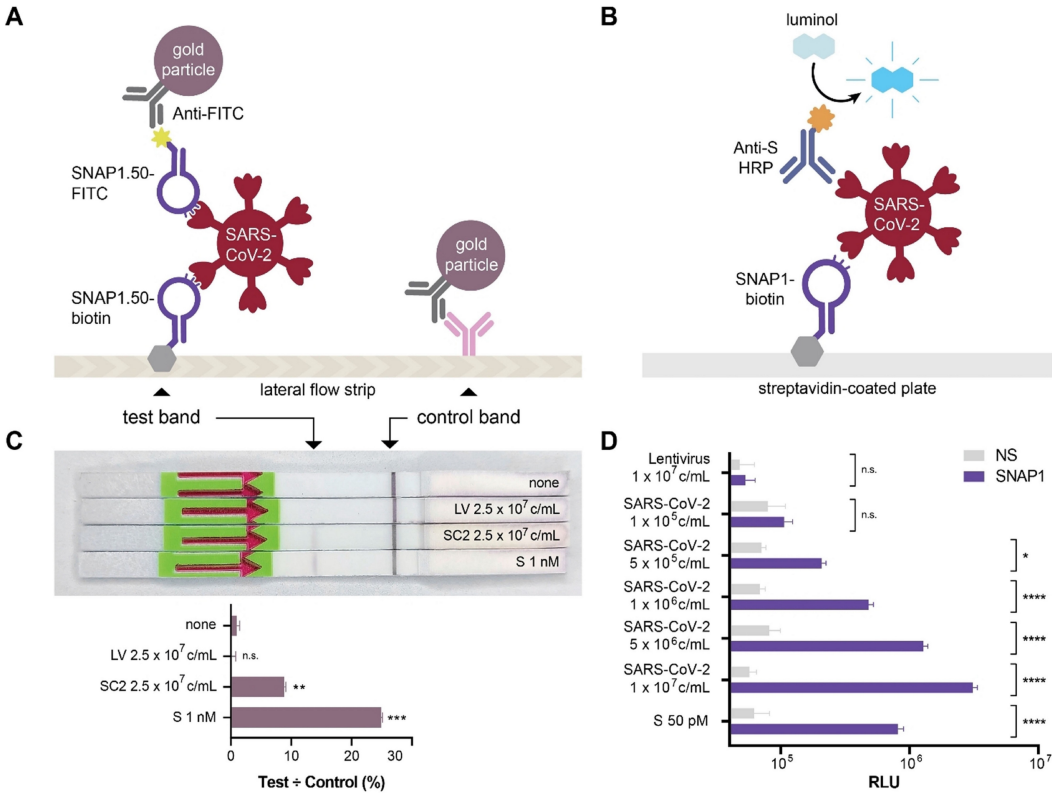

Figure 12. Aptamer-based detection of UV-inactivated SARS-CoV-2 virus. (A) Schematic of HybriDetect LFA. (B) Schematic of aptamer-antibody sandwich ELISA. (C) HybriDetect LFA strips were dipped in solutions of S protein (S), control lentivirus (LV), or UV-inactivated SARS-CoV-2 virus (SC2) incubated with SNAP1.50. (D) ELISA using NS-biotin or SNAP1-biotin as capture agents to detect UV-inactivated SARS-CoV-2 using anti-S HRP antibody for detection. The bar graph shows the mean and standard deviation of three replicates. Reprinted with permission from Ref. [50]. Copyright (2021) Wiley-VCH.

7. Inhibition of Viral Infection with Aptamers

In addition to their use for diagnostic testing, aptamers have also shown promise as therapeutic agents. Considerable efforts have been made to use aptamers to target various diseases and disorders, and several aptamers have successfully entered clinical trials for the treatment of macular degeneration, cancer, diabetes, ocular disorders, autoimmune and cardiovascular diseases.[43,50,113,127,129] For SARS-CoV-2 and COVID-19, there are two potential therapeutic strategies. The first strategy is to select anti-RBD aptamers to prevent the S protein-ACE2 interaction, and thus disrupt virus entry altogether (Figure 13). This was the key motivation for several studies.[42,44–46] The first group to pursue this approach for SARS-CoV-2 was Sun *et al.*, who identified a DNA aptamer named CoV2-6, which was further shortened and then engineered into a circular bivalent aptamer (cb-CoV2-6C3; Table S4).[46] The bivalent circular aptamer showed excellent stability in serum (stable in serum for 12 h) and improved binding affinity for the RBD of the spike protein (K_d = 0.13 nM). More importantly, it could inhibit infection by authentic SARS-CoV-2 virus against ACE2-expressing 293T cells (IC_{50} = 0.42 nM). Independently, Zu *et al.* demonstrated that Aptamer-1 and Aptamer-2 were able to neutralize S protein-expressing pseudotyped virus and prevent host cell infection,[44] while Huang *et al.* showed nCoVS1-Apt1 (K_d = 0.33 nM) was also able to block pseudotyped virus infection against human cells.[48]

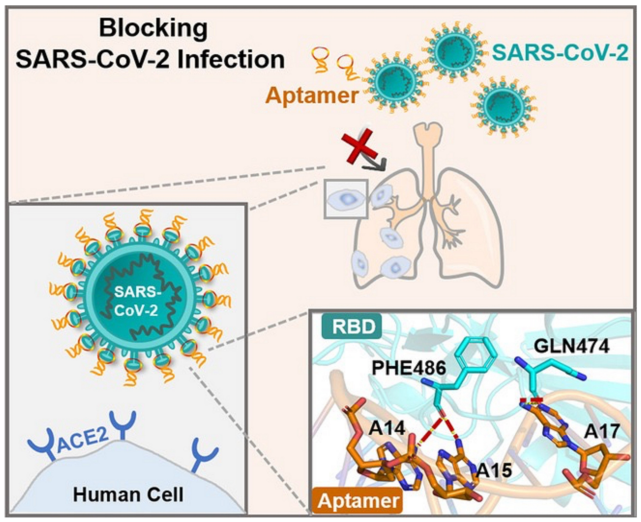

Figure 13. Pictorial demonstration of aptamer inhibition of SARS-CoV-2 infection.[46] *Sun et al.* have demonstrated an aptamer blocking strategy, whereby the oligonucleotides bind to the region of the receptor-binding domain (RBD) that directly mediates angiotensin-converting enzyme 2 (ACE2) receptor engagement. Reprinted with permission from Ref. [46]. Copyright (2021) Wiley-VCH.

With the emergence of SARS-CoV-2 mutants, it is of paramount importance to develop a therapeutic method for the prevention of mutant viral infections. Mayer identified an aptamer that specifically interacts with the S protein of SARS-CoV-2 but away from the RBD, and this aptamer does not block the RBD-ACE2 interaction.[45] Interestingly, the aptamer was still able to inhibit the infection of a pseudotyped virus against Vero E6, a mammalian cell line. The authors argue that such an approach may produce therapeutic agents that can deal with escape mutants of SARS-CoV-2.

In a follow up to their initial study, Sun *et al.* developed a spherical cocktail neutralizing aptamer-gold nanoparticle (SNAP) to block the binding between the RBD of the spike protein and ACE2 of host cells.[79] SNAP exhibited a potent neutralization effect against authentic SARS-CoV-2 (IC_{50} = 142.8 fM) due to multivalent aptamer binding to viral particles and steric hindrance of viral particles when bound with aptamer-modified gold nanoparticles. SNAP was also shown to be effective against the infection of CE2-expressing 293T cells by pseudotyped SARS-CoV-2 viruses carrying mutations observed in some VoCs, such as N501Y, D614G and K417N:E484K:N501Y. A DNA aptamer for RBD recognition has also been conjugated with gold nanostars (GNS) and the aptamer-attached GNS have been shown to effectively inhibit pseudoviral infection against ACE2 expressing HEK293T cells at a concentration of 100 ng/mL.[130]

To be utilized for clinical practice, aptamers are required to possess excellent stability in bodily fluids, and particularly against nuclease degradation. Circularization of aptamers is one option, as described above,[46] while chemical modification is another. DeStefano *et al.* selected a 2′-fluoro-arabinonucleic acid (FANA)-based aptamer (K_d = 10–20 nM) for RBD and showed the aptamer was able to inhibit the binding between RBD and ACE2.[53] Kjems *et al.* also reported a serum-stable 2′-fluoro protected RNA aptamer that binds the RBD of SARS-CoV-2.[51] They also designed a trimeric aptamer that changed the binding affinity from 18 nM to the low picomolar range. The trimeric aptamer was further shown to exhibit strong inhibition activity against the infection of pseudotyped viruses towards ACE2- and TMPRSS2-expressing Vero E6 cells (IC_{50} = 46 nM).

Xiang *et al.* have developed a strategy to modify DNA aptamers with sulfur (VI) fluoride exchange (SuFEx) chemistry so that the modified aptamers can create a covalent bond with its binding partner.[131] They demonstrated the idea with 6C3, a DNA aptamer that binds the RBD of the spike protein and SARS-CoV-2. The modified 6C3 exhibited strong inhibition of pseudotyped viruses against ACE2-expressing 293T cells, with an IC_{50} of ~30 nM, compared to the unmodified aptamer (IC_{50} > 200 nM).

Nabiabad *et al.* utilized RBD-targeting aptamers for a different purpose: delivering an RNAi drug.[52] They functionalized lipid nanoparticles (LNPs) with an RBD-binding DNA aptamer, and the LNPs encapsulated an RNAi drug.[52] It was found that Apt-LNPs-RNAi exhibited an antiviral effect that was better than Remdesivir.

The second main therapeutic strategy is to develop aptamers that target ACE2 to prevent binding of the spike protein. This approach was reported by Villa *et al.*, who used SELEX to generate anti-ACE2 aptamers.[132] This alternative method is particularly attractive because the derived aptamers can always prevent cell infection by preventing the binding of the spike protein to ACE2 without concerns for S protein mutations. In fact, given that the interaction between the RBD of S protein and ACE2 are conserved among the *Orthocoronavirinae* subfamily,[133] these aptamers could protect against both present and future coronaviruses. Villa *et al.* also claim that toxicity effects are not predicted to be an issue, since the viral site of interaction for ACE2 is distinct from the enzymatic domain, an integral component of various homeostatic functions.

Thus far, most of the therapeutic aptamer candidates have been tested in an *in vitro* manner, using neutralization assays involving pseudotyped viruses, enzyme-linked oligonucleotide assays (ELONAs), or ELISA-based assays. Absent from many of these papers, however, are *in vivo* experiments to fully validate the therapeutic values of the reported aptamers. Future follow-up studies on these aptamers will be imperative to move the aptamers forward toward therapeutic agents. In addition, the cost of aptamer therapeutics must also be considered, as aptamers are relatively large molecules, and thus their cost is certainly higher than small-molecule drugs. However, since aptamers can be chemically synthesized at a cost lower than antibodies, they still represent an attractive option as therapeutics for treating and/or preventing viral infections in a pandemic like COVID-19.

8. Conclusions and Future Perspectives

The COVID-19 pandemic has produced widespread negative impacts on social, mental, and economic well-being.[83] Despite the implementation of vaccines, cycles of restrictions and re-openings continue, leading to "COVID fatigue" and mounting frustration with public health directives. This is certainly not aided by the onslaught of incoming VoCs; the recent "Omicron" variants have produced the largest global wave thus far, including a rise in hospitalizations and ICU admissions, and have resulted in a corresponding increase in new restrictions in many countries. The recently reported BA.2 variant may well produce an additional wave of infections and further emergency orders. Many countries also continue to suffer from vaccine shortages,[134] making both improved diagnostic testing and the development of new therapeutic interventions of critical importance.

At this time many countries are also shifting away from mass testing using RT-qPCR and are aiming to implement rapid testing regimens as a means to respond to increasing case counts during major waves of infection, and also for simplified testing of incoming travelers. However, current rapid tests, primarily based on lateral flow tests using N protein-binding antibodies, show relatively poor sensitivity for emerging VoCs and thus require high viral loads to generate accurate results, making it imperative to develop improved diagnostic tests. Currently approved therapeutics for SARS-CoV-2 also have

drawbacks, and hence improved therapeutic agents are clearly needed. In this review, we have analyzed the effectiveness of aptamers as a tool for SARS-CoV-2 diagnosis and treatment. Aptamers have thus far been selected and further engineered for both the SARS-CoV-2 spike and nucleocapsid proteins, given their immunogenic properties and critical roles in mediating infectivity. These have included monomeric aptamers, multivalent aptamers, circularized aptamers and aptamers with modified nucleic acids, each of which provided improvements in affinity or stability for diagnostic or therapeutic applications.

In the area of diagnostics, a variety of sensors have been described, and several of the sensors have now been evaluated using clinical samples. Some of these sensor systems are still too complicated or require expensive instrumentation, limiting their potential for commercialization. It is likely that simple sensors based on single step electrochemical assays or LFA formats could be moved ahead toward commercialization and may compete favourably with currently available rapid tests.

Regarding therapeutic aptamers for SARS-CoV-2, most reports to date involve early-stage *in vitro* studies, which have provided strong evidence that aptamers can prevent infection based on blocking the S protein-ACE2 interaction. Both *in vivo* studies and clinical trials remain to be performed, which will certainly delay the availability of aptamer-based therapeutics. Issues related to susceptibility of aptamers to nucleases, rapid excretion by renal filtration, and reduced *in vivo* binding affinity will also need to be addressed.[129] Further research is undoubtedly required for aptamers to become a mainstay in the therapeutic and diagnostic arenas. Given their numerous upsides, we anticipate that aptamers will continue to see improvements, and will eventually prove to be an efficient tool for current and future pandemics.

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Conflict of Interest

The authors declare no conflict of interest.

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