Aptamers for Viral Detection and Inhibition

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ABSTRACT: Recent times have experienced more than ever the impact of viral infections in humans. Viral infections are known to cause diseases not only in humans but also in plants and animals. Here, we have compiled the literature review of aptamers selected and used for detection and inhibition of viral infections in all three categories: humans, animals, and plants. This review gives an in-depth introduction to aptamers, different types of aptamer selection (SELEX) methodologies, the benefits of using aptamers over commonly used antibody-based strategies, and the structural and functional mechanism of aptasensors for viral detection and therapy. The review is organized based on the different characterization and read-out tools used to detect virus–aptasensor interactions with a detailed index of existing virus-targeting aptamers. Along with addressing recent developments, we also discuss a way forward with aptamers for DNA nanotechnology-based detection and treatment of viral diseases. Overall, this review will serve as a comprehensive resource for aptamer-based strategies in viral diagnostics and treatment.

KEYWORDS: viruses, sensing, inhibition, aptamers, aptasensors, DNA nanostructures

INTRODUCTION

Detection and treatment of viral infections is an ever-necessary aspect of biomedical science, with viruses such as human immunodeficiency virus-1 (HIV-1) and hepatitis C virus (HCV) killing millions of people every year.1-3 While several diagnostic methods have been developed for viral infections, global pandemics such as the current COVID-19 scenario demonstrate the need for multiple alternate strategies for rapid detection of viral infections. There is also a need for more efficient diagnostic tools with the focus on aspects such as rapid detection, accuracy, affordability, and portability of the assay so that detection strategies are useful in low-resource settings. For treating viral infections, new methods that address molecular mechanisms of viral infection could be potent in creating therapeutics for a range of viral diseases with similar infection routes. Recently, several materials have been developed toward more effective viral diagnostics and treatment.4-6 For example, graphene-based materials have been used for detection of viruses,7 and nanoporous carbon-based materials and thin-film-based coatings have been used in developing protective equipment against viruses.7 Nucleic acid engineering has also been a rapidly developing area in biosensing and drug delivery. Among these, aptamer-based methods have attracted more attention due to their applicability in a wide range of disciplines such as pathogen and toxin diagnostics,7 therapies,10,11 water quality control,12 and imaging.13

Aptamers are single-stranded oligonucleotide (ssDNA or ssRNA) ligands comprising 10 to 100 nucleotides that can exhibit high affinity and specificity to their selected target. Originally discovered by Gold and Szostak,14,15 they have now been developed by several research groups and are considered as the nucleic acid equivalent of the antibody (Ab). Their low immunogenicity, small size, batch to batch reproducibility, ability to be chemically modified, and low cost of production have positioned aptamers to replace antibodies in many circumstances.16-19 More specifically, aptamers are structurally more flexible and are magnitudes smaller than antibodies, facilitating their ability to recognize regions of the antigen that are otherwise inaccessible to antibodies. Their smaller size also aids in higher cell entry for in situ diagnostics, imaging, and disease treatment. Aptamers can be synthetized in large scale using phosphoramidite chemistry at a low cost. Further, aptamers can recognize a wide range of targets, tolerate various storage and usage conditions at different temperatures, and can return to their original conformation after denaturation and annealing, allowing repeated use.20,21 The in vitro selection process of specific aptamers is less time-consuming and cheaper compared to antibody production, where generation and screening of monoclonal antibodies is laborious and requires highly specialized facilities. To date, aptamers have been selected with nanomolar to micromolar $K_D$, targeting

Received: October 17, 2021
ions, small molecules, as well as biological molecules and cells in both buffer and physiological conditions, including blood plasma and serum, and employed in environmental sensing, bioimaging, disease diagnosis, and treatment. Despite these advantages of aptamers over antibodies, it has to be pointed out that compared to antibodies, aptamers are easier to degrade in vivo and thus need to be modified either chemically or enzymatically for enhanced biostability. Also, aptamers are quickly excreted by renal filtration from the bloodstream and thus need to be attached to other higher-molecular-weight molecules such as cholesterol or polyethylene glycol for prolonged bioavailability when used as diagnosis/imaging reagents or as part of drug complexes. In Table 1, we briefly summarize the pros and cons of using aptamers for virus detection and inhibition when compared with antibody-based methods.22−24

![Table 1. Comparison of the Properties of Antibodies and Aptamers in Virus Diagnostics and Therapeutics](image)

Aptamer structures consist of a diverse set of secondary motifs such as stem-loop, hairpin, pseudoknot, kissing loop, three-way junction, G-quadruplex, and internal bulge structures.25 Aptamer−target binding occurs through a variety of noncovalent interactions such as hydrogen bonding, electrostatic, van der Waals, and hydrophobic interactions. The binding affinity of aptamers to their targets is also mediated by the environment, including buffer and ion composition and concentration, pH, and temperature.26 The overall three-dimensional shape and conformation of the target molecule defines the strength of the binding affinity of the selected aptamer27 (Figure 1). For example, aptamers can differentiate between closely associated molecules and even between different chirality and recognize a specific epitope of a target molecule.28 Previous studies have established that aptamers can bind to a variety of targets such as bacteria and viruses,29 proteins,30 prions,31 and other small molecules.32,33 Specifically, various aptamers have been developed to recognize key structural or metabolic determinants associated with bacterial and viral pathogens.34 This specificity toward clinically relevant biomarkers makes aptamers useful in biosensing. Recent studies have developed a range of aptamers for different viruses including vaccinia virus, dengue virus, severe acute respiratory syndrome (SARS), hepatitis C virus (HCV), human immunodeficiency virus (HIV), apple stem pitting virus, norovirus, rabies virus, bovine viral diarrhea virus, hepatitis B,Ebola, and influenza.32,33,35 For these interactions to be used in sensing, aptamers are coupled with a variety of detection formats such as fluorescence, radioisotope, electrochemical, optical, colorimetric, and enzyme-linked assays. In addition to detection capabilities, aptamers can block proteins or molecules from binding their target and inhibit viral infection by stopping further replication, thus preventing the
infection from spreading. This aptamer recognition can also act as a targeting tool for drug delivery (for example, using an aptamer that is specific to a tumor cell receptor). In this review, we discuss the structure, function, and development of aptamers as well as their use in viral detection, inhibition, and therapy. We also discuss an outlook into promising developments in DNA nanotechnology that has the potential to use aptamers for sensing and therapeutic applications. We have provided a detailed index of existing virus-targeting aptamers with corresponding sequences in Table S1.

■ APTAMER SELECTION BY SELEX

Aptamers can be selected specifically for single atoms/ions, molecules, viruses, bacteria, eukaryotic cells, and tissues using different types of systematic evolution of ligands by exponential enrichment (SELEX) strategies and modifications to the incubation conditions (e.g., pH, temperature, buffer, etc.) (Figure 2).

APTAMER SELECTION BY SELEX

Aptamers can be selected specifically for single atoms/ions, molecules, viruses, bacteria, eukaryotic cells, and tissues using different types of systematic evolution of ligands by exponential enrichment (SELEX) strategies and modifications to the incubation conditions (e.g., pH, temperature, buffer, etc.) (Figure 2). Aptamer selection relies on the distinctive secondary and tertiary structures that assist in target binding. The SELEX method starts with a combinatorial library that consists of two constant primer regions flanking a randomized segment of 20–50 nucleotides. Iterative rounds of incubation, separation, and amplification enrich oligonucleotides that bind the intended target from the initial library. Incubation of the libraries with the target can be carried out in several ways. Usually, the target of interest is immobilized on a surface that can be washed or separated from the bulk liquid (centrifugation or magnetic separation of particles). The bound sequences that remain after binding and washing are then released chemically and/or thermally. This provides a phenotype–genotype linkage, where the desired binding sequences can then be amplified using polymerase chain reaction (PCR) (for DNA-based libraries) or reverse transcription PCR (for RNA-based libraries). This process is repeatedly performed for 8 to 15 rounds to get a desired pool of target-specific aptamers. Increasingly stringent conditions during library binding are utilized to obtain high-affinity aptamers, and negative (against the solid support) and counter (against unintended targets) selections are performed to increase the specificity of the evolved pool. At the end of the SELEX procedure, the identity of the enriched aptamer pool is determined by cloning and sequencing. The SELEX technique is a potential method for assessing aptamers against a variety of target molecules and is crucial for developing novel aptamer-based detection procedures.

Cell-SELEX. Since its inception in 1990, there have been many advances to the SELEX technology. Cell-SELEX is a modified form of SELEX used to develop aptamers against a whole cell. Cell-SELEX provides a wide flexibility to target unknown cell particles and detection of various cell types (bacteria, viruses, and tumor cells), primarily targeting the extracellular proteins present on the outer membrane of the cells or the characteristic structures specific to the cells. Cell-based SELEX processes have an additional step such as centrifugation or washing depending on the nature of the cells (adhesive or suspension). The bound sequences are collected and incubated with a negative control cell where the unbound nucleotides are then collected and used for negative selection.

Figure 2. Outline of SELEX. (a) Degenerate nucleic acid sequence library is incubated with the target molecule under defined solution conditions. (b) Target-bound nucleic acids are partitioned. (c–e) Species with lower binding affinity are removed, and the bound species are eluted, allowing preferential amplification of higher affinity species. This enriched pool is then used as the starting point in subsequent cycles. Typically, 10–20 cycles are carried out before aptamer characterization. In early rounds, species with no affinity are competed out of the pool. In later rounds, molecules with affinity compete for binding sites on the target. Such competition results in enhancement of the pool binding affinity in a manner similar to Darwinian evolution. Recent technical developments described in the text are listed alongside each step in brackets. CE, capillary electrophoresis; SELEX, systematic evolution of ligands by exponential enrichment; SPR, surface plasmon resonance. Image reproduced with permission from ref 75. Copyright 2006 Springer Nature.
round.\textsuperscript{40} Once the aptamers are developed, they can be used for diverse applications such as drug delivery, cell-specific therapy, and cell surface diagnostics.\textsuperscript{41,42} As the aptamers developed by Cell-SELEX may target molecules that have not been characterized as cell-specific surface molecules, they might be novel biomarkers. As a result, Cell-SELEX may be used to explore new biomarkers for a specific cell. Further, Cell-SELEX may be used to manufacture aptamers against a particular target protein present on living cells such as transmembrane proteins (receptor kinases, G-protein-coupled receptors, and ion channels).\textsuperscript{43,44} For example, Tang et al. used Cell-SELEX to produce aptamers against adenocarcinoma epithelial cells infected with the vaccina virus (AS49).\textsuperscript{45}

\textbf{Complex—Target SELEX.} This process involves the use of genetically modified cells. The whole cell is used as a target, and these cells include genetically modified cells which overexpress a target recombinant protein on the cell surface. Using a parallel selection process, multiple aptamers are selected for multiple targets in a single experiment and sequential target selection (X-SELEX) selects aptamers that bind to the multiple forms of a single target. The main advantage of this methodology is the ability to target and specifically differentiate microbial strains without knowing the details of the membrane structure or molecules present in any particular micro-organism.\textsuperscript{46} Pan et al. isolated aptamers for Rous sarcoma viruses, an enveloped avian retrovirus, using this method.\textsuperscript{47} Aptamers were specific to the Rous sarcoma viruses surface glycoproteins that are necessary for binding and entry into host cells. Inhibition of viral infection was identified after 12 rounds of selection by chosen pools.

\textbf{Genomic SELEX.} The conventional DNA library uses a chemically synthesized library, whereas the genomic library uses the genomic DNA library.\textsuperscript{48} Genomic SELEX is prepared via random priming, PCR amplification, and in vitro transcription, which creates an initial library.\textsuperscript{49} This library is then transcribed into RNA and used for the selection process. At first, a counter selection method is performed at the immobilization matrix level. Due to the reversal of the transcription in several rounds, it can cause severe effects on highly structured RNA and is more acute in the case of genomic SELEX. Nucleic acid binding proteins with diverse specificities and affinities are the most prevalent baits used in genomic SELEX. Some examples of such nucleic acid binding proteins (associated to regulatory noncoding RNAs) are those involved in transcriptional and post-transcriptional silencing, chromatin remodeling, and components of machinery that participate in transport, RNA processing, and translation.\textsuperscript{50–53} The utility of such proteins as targets enhances the potency of genomic SELEX for analyzing RNA—protein interactions.

\textbf{Microfluidic SELEX (M-SELEX).} This process generates DNA aptamers by employing conventional SELEX within a microfluidic system.\textsuperscript{54} M-SELEX increases the stringency of the selection by utilizing a minimum amount of target molecules. It is a cost-effective method that consumes a small number of reagents, can be automated and multiplexed, and reduces the time required for the selection of new aptamers. For example, M-SELEX was used to isolate aptamers against hepatitis C virus (HCV) RNA polymerase.\textsuperscript{55} A wide variety of M-SELEX methods have now emerged from employing microfluidic incubation, amplification, and separation techniques.

\textbf{Magnetic Bead-Based Microfluidic SELEX.} As one of the most widely used methods, this process uses a micro- or nanosized magnetic bead as a solid support for target binding. Here, a magnetically activated chip-based separation takes place in a continuous flow.\textsuperscript{56–58} The screening of aptamers in magnetic bead-based M-SELEX takes place by incubating a target-immobilized magnetic bead with a random nucleic acid library. Afterward, unbound nucleic acids are separated from target-bound aptamers by performing a stringent washing in the microfluidic channel. After separation, the external magnet is removed, and the attached, selected aptamers are collected for further PCR amplification. Small molecules, proteins, and cell surfaces have been used as effective targets for magnetic bead-based selection approaches.\textsuperscript{56} For example, Soh’s team designed a high-efficiency continuous-flow magnetic-activated chip-based separation (CMACS) system that combines microfluidics technology with magnetic bead-assisted SELEX. The highly localized magnetophoretic forces and magnetic field gradients present in this system allow separation of the target protein with high purity. In addition, nonspecific binding was reduced using carboxylic-acid-coated beads on negatively charged oligonucleotides, further increasing the efficiency of the selection process.\textsuperscript{59}

\textbf{Capillary Electrophoretic (CE) SELEX.} This method was the first microfluidic technique to yield a highly rapid SELEX process.\textsuperscript{60–65} The difference in the electrophoretic mobility of the components in a mixture is used as the separation tool in capillary electrophoresis. The change in the size and charge of the target—aptamer complex decreases their mobility compared to unbound DNA or RNA sequences with high negative charge density. Several aptamers targeting HIV-1 reverse transcriptase, \textit{Lactobacillus acidophilus}, and adenosine have been isolated by CE SELEX.\textsuperscript{64–66}

\textbf{Sol–Gel Method.} This is another prominent microfluidic-based SELEX that overcomes the uncertainty behind the effects of target immobilization on its conformation as well as blockage of binding sites.\textsuperscript{67} Here, the sol consists of silica derivatives, and the addition of chemical additives solidifies the sol into a nanoporous framework which enables the trapping of the target molecule within the gel. The nanoporous gel provides an aqueous domain to conserve the biological activity and stability of the entrapped target. Park et al. developed the first nanoporous sol–gel protein microfluidic array for entrapping target molecules and enabled the selection of RNA aptamers against multiple target molecules.\textsuperscript{68} Ahn et al. used a sol–gel M-SELEX for high-throughput characterization and selection of RNA aptamers. This microchip used a sol–gel network to immobilize the targeted protein and a localized heat source to selectively extract the RNA aptamers from the targeted protein.\textsuperscript{69}

\textbf{AFM-SELEX.} In this method, the aptamer is immobilized on a cantilever via biotin–streptavidin, and target molecules are immobilized on a gold chip.\textsuperscript{70} If the target–aptamer affinity is very strong, the biotin–streptavidin interaction of the aptamer to the cantilever breaks and the aptamer remains on the gold surface. The DNA is then recovered by heat elution followed by PCR for amplification.\textsuperscript{70} Such a method has been used to select a DNA aptamer against thrombin with very strong affinity ($K_{d} = 200$ pM).\textsuperscript{71}

\textbf{Toggle-SELEX.} Toggle-SELEX is a selection method for aptamers that can bind to a particular target present in different organisms. For instance, RNA aptamers that can bind to both porcine and human thrombin are selected by “toggling” the target between the human and porcine thrombin during alternate rounds of selection. In the first round, the
library is incubated with both porcine and human thrombin. Aptamers that are bound to the protein are then recovered and amplified.72 Using this process, Derbyshire et al. isolated aptamers capable of targeting several aminoglycosides.73 While toggle-SELEX is useful to develop cross-reactive aptamers, sometimes affinity may be compromised during the selection process.74

**APTASENSOR-BASED DETECTION OF VIRAL PATHOGENS**

As evidenced by the ongoing COVID-19 pandemic, diagnosis of viral pathogens at early stages of infection is crucial for the prevention and early treatment of viral infections. Current gold standard methods for detecting viral infections include nucleic acid testing (NAT)76 and antigen–antibody-based ELISA tests,77 and other common methods include viral plaque assay,78 flow cytometry,79 and hemagglutination assay.80 NAT methods are amplification-based enzymatic assays that detect viral genetic material (DNA or RNA) typically using PCR. Although NAT is sensitive, it requires labor-intensive, laboratory-based sample preparation protocols for viral lysis, extraction of genetic materials, purification of the isolated materials, thermal cycling for enzymatic amplification of viral nucleic acid sequences, and interpretation of complex results by skilled personnel. Immune assays test for viral antigens or antibodies and are, in general, rapid but less sensitive. There have been tremendous efforts in the development of alternate methods for faster and low-cost methods for detecting cellular and disease biomarkers.81,82 In this section, we discuss aptamer-based sensors (called aptasensors) that are surface-based assays (where the aptamer is immobilized on a surface) or solution-based assays (where they are mixed with analytes in solution). In such assays, aptamer–analyte binding transduces a detectable signal which can be read out by electrochemical, optical, or enzyme-linked methods.

**Electrochemical Aptasensors.** Electrochemical aptasensors rely on the immobilization of an aptamer ligand on a conductive surface. Gold- or carbon-based electrodes are commonly used for this purpose, as their surfaces are hydrophobic, inert, and easily functionalized to provide robust attachment of many aptamer ligands. Different chemical strategies have been developed to immobilize aptamers on electrode surfaces. These include chemisorption (attachment of the aptamer to the surface by noncovalent interactions such as electrostatic, hydrogen bonding, and π−π stacking), biotinylation of aptamers to bind avidin-modified surfaces, covalent linkage mechanisms such as click chemistry (azide-modified aptamer to the alkyne-modified surfaces), and chemical cross-linking (coupling of the amine-modified aptamers to a carboxyl-modified surface).83 Electrochemical aptasensors use electrochemically active species to provide a readout, where an electrode-immobilized aptamer serves as a transducer. Some examples of electrochemically active species include organic molecules such as ferrocene, methylene blue (MB), and thionine, which are redox-active molecules that interact with DNA via intercalation and electrostatic interactions. Their presence facilitates the transfer of electrons from the aptamer–target binding site to the electrode surface and provides a more sensitive electrochemical output.83,84 Electrochemical changes that result from the formation of an aptamer–analyte complex can be detected in the form of

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**Figure 3.** Electrochemical aptasensors. (a) Thiolated norovirus-specific DNA aptamer self-assembled onto a gold-nanoparticle-modified screen-printed carbon electrode. Binding of the virus to the immobilized aptamer causes a decrease in the redox current, measured via square wave voltammetry. Reproduced with permission from ref87. Copyright 2013 Public Library of Science. (b) Use of glassy carbon electrode with graphene quantum dots for HCV core antigen detection. Reproduced with permission from ref 90. Copyright 2017 Elsevier. (c) Schematic structure of diamond-FET-based RNA aptamer for HIV-1 Tat protein detection based on changes in the surface charge. Reproduced with permission from ref 92. Copyright 2013 Elsevier.
current, potential, and impedance, typically measured by amperometric, potentiometric, and conductometric techniques. These include electrochemical impedance spectroscopy, cyclic voltammetry, differential pulse voltammetry, square wave voltammetry, field-effect transistor, linear sweep voltammetry, and potentiometry. Electrochemical aptasensors present advantages such as repeatability, accuracy, high sensitivity, low cost, easy miniaturization, and robustness. These detection methods can also be transformed into a chip-based platform for use as portable devices at point-of-care.

**Voltammetry and Electrical Impedance Aptasensor.** Giamberardino et al. developed an ultrasensitive electrochemical norovirus detection system using aptamers evolved to bind both murine norovirus (MNV) and human norovirus (HuNoV) with picomolar affinity (Figure 3a). Aptamer AG3 that selectively binds MNV and the HuNoV strain GII.3 was modified with a thiol group at the 5′ end and subsequently immobilized on gold-nanoparticle-modified carbon electrodes. Using the square wave voltammetry readout technique and a ferricyanide/ruthenium hexamine redox reporter system, the norovirus aptasensor exhibited a limit of detection (LoD) of 10 aM, or 180 virus particles for MNV. Lum et al. developed an impedimetric aptasensor for the detection of the avian influenza virus (AIV) H5N1. A biotin-labeled, H5N1-specific DNA aptamer was immobilized on streptavidin-modified gold interdigitated microelectrodes that were embedded in a microfluidics chip. The virus was allowed to interact with the aptamer-coated electrodes for 30 min before impedance was measured. The difference in the impedance of the virus:aptamer-electrode complex and the aptamer-electrode alone indicates the presence of the virus, with an LoD of 0.0128 hemagglutinating units (HAU). Detection methods for influenza A and vaccinia virus were also constructed using such an electrochemical aptasensor approach. A similar technique was used for detecting HCV using an aptamer evolved to bind the HCV core antigen that was chemisorbed on graphene quantum dot (GQD)-coated electrodes (Figure 3b). Electrochemical impedance spectroscopy was used to monitor changes in electrical signals upon aptamer interaction with HCV antigen. The purported mechanism is that the aptamer:HCV antigen complex increases the ability of the

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**Figure 4.** Enzyme-linked electrochemical aptasensors. (a) Enzyme catalysis in ultralow ion strength media to develop an ion strength increase-based impedance biosensor for H5N1 virus. Reproduced from ref 96. Copyright 2014 American Chemical Society. (b) Schematic diagram of H5N1 viral protein detection using the enzymatic reaction of the substrate 4-amino phenyl phosphate with the surface formed aptamer/H5N1/antiH5N1-alkaline phosphatase on gold-nanoparticle-modified screen-printed carbon electrode. Reproduced with permission from ref 97. Copyright 2015 Elsevier. (c) Working principle of the norovirus nanozyme aptasensor. Reproduced from ref 100. Copyright 2019 American Chemical Society.
redox species in solution to reach the electrode. This "signal-on" mechanism provided an LoD of 3.3 pg/mL.

**Field-Effect Transistor-Based Aptasensors.** A field-effect transistor (FET) is a voltage-controlled three-electrode (source, gate, and drain) system that acts as a transducer and converts signals generated by the detected target to an electrical readout. Aptamers are immobilized on a FET electrode surface that measures the charge distribution when the target binds to the aptamer. Various biomolecules such as proteins, viruses, and nucleic acids have been detected by FET-integrated aptasensors. Rahim Ruslinda et al. demonstrated diamond FET for the detection of HIV-1 using RNA aptamers as the sensor element (Figure 3c). The group used the RNA−TAT aptamer to detect the HIV-1 transactivator Tat protein that contributes to several pathological symptoms of HIV-1 infection and plays a critical role in virus replication.

**Piezoelectric Aptasensors.** Certain materials possess the ability to generate electric charge in response to applied mechanical stress, known as the piezoelectric effect. Quartz...
crystal microbalance (QCM) is the most common piezoelectric sensor used for the detection of biomarkers. QCMBased strategies have also used aptamers immobilized on gold-coated quartz as sensing elements. Binding of the target to the aptamers increases the mass on the surface of the crystals, which generates a detectable signal due to the decrease in the resonance frequency of the crystal. Wang et al. developed a ssDNA cross-linked polymeric hydrogel to form a network of water-insoluble polymer chains in a QCM aptasensor for the rapid detection of AIV H5N1. An aptamer specific to the AIV H5N1 surface protein was hybridized to the ssDNA, thus cross-linking the hydrogel in a shrunk state. The aptamer—hydrogel complex was fixed on the gold surface of the QCM sensor using a self-assembled monolayer method. When the surface protein binds to the aptamer, the aptamer is released from the hydrogel complex, causing the hydrogel to swell, with the changes transduced to a detectable decreased frequency. The assay time for this method was 30 min with a detection limit of 0.0128 HAU.

**Enzyme-Linked Electrochemical Aptasensors.** Enzyme-based biosensors have also been used in combination with aptamers to detect viral pathogens. For example, an electrochemical aptasensor was coupled to a glucose oxidase (GO) enzyme-based readout for the detection of H5N1 (Figure 4a). They used a complex consisting of gold nanoparticles (AuNPs), glucose oxidase, and concanavalin A (AuNPs-GOx-ConA), and the capturing aptamers were immobilized on magnetic beads. The complex triggered an enzymatic catalysis that, in turn, increased the ion concentration and decreased the impedance, with the changes measured by electrical impedance spectroscopy. The LoD for the technique was $8 \times 10^{-4}$ HAU in a 200 $\mu$L reaction. Another study involving enzymatic electrochemical detection of H5N1 utilized AuNP-modified electrodes which were coated with the capturing aptamers (Figure 4b). The functionalization of the electrodes with 3-mercaptopropionic acid and the presence of the anti-H5N1 antibodies modified with alkaline phosphatase generate an electrochemical signal with an LoD of 100 fM.

Nanozymes, artificial nanomaterials that imitate properties of natural enzymes, have also been used in aptasensors. The chemical makeup of nanozymes include simple metal and metal oxide nanoparticles, metal nanoclusters, quantum dots, nanotubes, nanowires, or a metal–organic framework. Compared to conventional enzymes, nanozymes exhibit improved catalytic activity, lower cost of production, greater robustness and modification capabilities, and long-term storage and shelf life. Such a nanzyme-based aptasensor was used to detect norovirus (HuNoV). This aptasensor employs the murine norovirus (MNV)-specific aptamer (AG3) as the molecular recognition element, with a detection limit of 200 virus particles/mL in 10 min (Figure 4c).

**Optical Detection-Based Aptasensors.** The aptasensor field is replete with optical sensors that rely on fluorescence, luminescence, colorimetry, and plasmon or refractive index-related changes for analyte detection. Optical biosensing is divided into two modes: label-free, where target binding is analyzed by a coupled material that transduces an optical signal, and label-based, where colorimetric, luminescent, or fluorescent labels on either the aptamer or the target or both generate an optical signal. Optical biosensors use various molecular recognition elements such as nucleic acids, enzymes, antibodies, whole cells, and tissues.

**Surface Plasmon Resonance Aptasensors.** Surface plasmon resonance (SPR) aptasensors are based on the change in the refractive index of a metal, typically gold, surface due to the resonant oscillation of free electrons. In the SPR technique, polarized light of a specific wavelength and incident angle passes through a prism and is reflected off the gold surface. SPR aptasensors are label-free and have recently been developed to be miniaturized, portable, and automated. For example, Tombelli et al. developed an SPR aptasensor for HIV-1 detection with an LoD of 0.25 ppm. Here, a biotinylated RNA aptamer that recognizes the Tat protein was immobilized on an avidin-modified gold surface. Bai et al. developed a portable and fast SPR-based aptasensor for AIV H5N1 (Figure 5a). Using the streptavidin–biotin interaction, a DNA aptamer that targets the glycosylated hemagglutinin (HA) viral protein was immobilized on the gold surface. The resulting sensor was shown to detect in vitro isolated AIV H5N1 as well as AIV H5N1 from poultry swabs with an LoD of 0.128 HAU. This sensor allowed rapid detection within minutes, and with poultry swab preparation, it takes a total time of only 1.5 h.

Localized SPR (LSPR) is an optical phenomenon that occurs in metallic nanostructures (nanospheres, nanodiscs, and nanorods). Normally these localized metallic nanostructures are designed within microfluidic channels to detect ssDNA in low nanogram/milliliter range, giving a cheaper alternative for biomolecule sensing. Incident light at the specific plasmonic wavelength irradiates the metallic structure, which induces collective electron charge oscillations. This ultimately leads to a shift in absorbance in the ultraviolet–visible region, which can be used to detect target binding. Klinghammer et al. developed an aptasensor based on this mechanism using arrays of gold nanorods (Figure 5b). They used different complementary DNA (cDNA) strand hybridization kinetics to monitor the optical nanostructure resonance of densely packed gold nanorods upon binding of biomolecules. Red shifts of 2 and 5 nm were detected upon binding of 25 and 100 bp, respectively.

Another process of metal-enhanced fluorescence occurs when there is an increased emission around specific metallic materials. When fluorophores are close to the surface of metal nanoparticles, the LSPR of the metal nanoparticles coupled with the excited fluorophores improves the detection sensitivity significantly. The signals can be detected by surface plasmon field-enhanced fluorescence. Pang et al. developed an aptasensor using this principle for detecting H5N1 virus (Figure 5c). A guanine-rich DNA aptamer that recognizes recombinant hemagglutinin (rHA) was immobilized on core–shell AgSiO$_2$ nanoparticles, and thiazole orange was added as a fluorescence amplification reporter. Target binding causes the aptamer to fold into a G-quadruplex structure. The thiazole orange previously in solution then binds to the G-quadruplex, and its interaction with the core–shell nanoparticle causes an increase in fluorescence. Using this assay, H5N1 rHA could be detected in 30 min in both buffer and when spiked in serum. Another study used aptamer-based fluorescent nanoparticles for the detection of the respiratory syncytial virus (RSV) (Figure 5d). This method could detect viral particles that are 80–100 nm.
an enhanced electromagnetic field on a rough metallic surface which amplifies the incident light and therefore the Raman modes being detected and (ii) direct enhancement of the Raman signal by the resonant surface. Together, these mechanisms can provide signal enhancements of $10^{10}$ to $10^{12}$, potentially allowing for single-molecule detection. SERS-based biosensing can be categorized as direct or indirect. In the direct technique, detection is based on the Raman spectrum of the analyte itself without any reporter molecules (label-free techniques). This technique has been used for detecting hepatitis B virus (HBV), adenovirus, rhinovirus, and HIV. The indirect SERS-based technique involves the use of molecular recognition elements such as antibodies, aptamers, or other specific binding molecules placed close to the “hot spots” (interparticle gap in a nanocluster) on the surface. These methods rely on the change in Raman signals from the recognition element (e.g., aptamers) or reporter molecules and not the analyte itself. The reporter molecules used in SERS-based methods are water-soluble and easily conjugated or intercalated to oligonucleotides. For example, Chen et al. developed a SERS-based aptasensor using a Cy3-labeled DNA aptamer for the detection of influenza A (H1N1) virus with high sensitivity. The biosensor consisted of a three-dimensional (3D) nanopopcorn plasmonic substrate fabricated by depositing gold layers on a polyethylene naphthalate (PEN) polymer substrate. On binding the virus, the Cy3-labeled DNA aptamer is released from the nanopopcorn substrate surface, causing a decrease in the Raman peak intensity. In another example, Kukushkin et al. developed an aptasensor for the detection of influenza virus including H1, H3, and H5 hemagglutinin subtypes. The aptamer RHA0385 showed strain specificity to both recombinant hemagglutinins and whole cell viruses. The aptasensor consisted of a primary aptamer attached to the metal particles of the SERS substrate and a secondary aptamer labeled with Raman-active molecules. The influenza virus was captured and bound to the labeled secondary aptamer. The LoD for this aptasensor was $10^{4}$ virus particles per sample or $10^{-4}$ HAU per sample.

Chemiluminescent Aptasensors. The energy transition between the molecular orbitals emits light which is termed as luminescence. When this phenomenon occurs in the presence of a chemical reaction, it is termed as chemiluminescence. Chemiluminescent methods have often been implemented as signal amplifiers to provide detection limits of $10^{-12}$ to $10^{-21}$ mol. This technique has been used for detecting the nucleocapsid protein of SARS coronavirus (SARS-CoV). This aptasensor utilizes an aptamer–antibody sandwich method, where the nucleocapsid proteins are recognized by surface-immobilized aptamers. This assay provided detection at a limit of 2 pg/mL. Xi et al. developed a chemiluminescent aptasensor for the detection of the hepatitis B surface antigen. This aptasensor used aptamers immobilized on Fe$_3$O$_4$–SiO$_2$ magnetic nanoparticles that allowed for detection of the target biomarker with a linear range of 1–200 ng/mL. The detection limit for the aptasensor was 100 pg/mL and worked well even in the presence of interfering substances present in blood.

Fluorescent Aptasensors. Fluorescent biosensors rely on changes in fluorescence polarization, wavelength, or intensity as a means of detection. These changes are produced upon the interaction of the target analyte and fluorescently labeled aptamers. Percze et al. developed a fluorescence polarization assay for the detection of RSV, a viral pathogen affecting young infants. Wang et al. constructed an integrated microfluidic device for fluorescence-based multivirus detection of influenza A H1N1, H3N2, and influenza B virus. This aptasensor consisted of aptamer-modified magnetic beads to detect RSV, and another fluorescent-labeled aptamer was used for counter selection against human rhinovirus (HRV).

Some fluorescence-based methods use Förster resonance energy transfer (FRET). FRET involves two fluorophores of particular electromagnetic properties, where the emission spectrum of a donor molecule overlaps with the excitation spectrum of an acceptor molecule. Distance changes between the fluorophore pairs can therefore be monitored based on the wavelength and intensity of the emitted light, providing a detectable output for target recognition. FRET-based systems can be incorporated into aptasensors in a variety of ways. Frequently, an aptamer is modified with both a donor and an acceptor molecule such that when there is no target present, the donor and acceptor are too far for FRET to occur. When the target is present, the donor and acceptor come closer together, causing a change in FRET signal. In another method, the target molecule and the acceptor are both modified with either part of the donor/acceptor pair. The binding of the aptamer to the target brings the pair in proximity to undergo FRET.

Quantum dots (QDs) are spherical, inorganic, fluorescent nanocrystals which are extensively used as fluorescent probes. Compared to traditional organic dyes, QDs exhibit greater stability, reduced susceptibility to photobleaching, and greater and more precise spectral properties for multispectral detection. Ghanbari et al. used RNA aptamers conjugated to QDs to detect the HCV NS3 protein. HCV NS3 was produced in vitro, immobilized on a glass slide, and then probed with the aptamer–QD complex to detect HCV at a limit of 5 ng/mL. Another study involved aptamers modified with QDs for detecting H1N1 with a detection level of 3.45 nM. This study involved a protein binding bifunctional aptamer and DNA-functionalized QD probe. The aptamer is complementary to part of the target DNA sequence of H1N1 influenza virus, and the rest of the sequence is a recognition sequence for streptavidin. In the absence of the target molecule, the QDs and the aptamer–DNA–streptavidin complex are free in solution and do not hybridize. In the presence of the target, the target hybridizes with aptamer–DNA, and the aptamer–DNA binds to the DNA–QD, forming a complex and providing a readout.

Colorimetric Aptasensor. Colorimetric detection measures changes in color shifts that can be inspected either by the naked eye or a spectrophotometer. While several traditional methods are available for analyzing DNA and RNA biomarkers, they still face a number of drawbacks. For instance, radioactive and fluorescence probe-based Southern blots are criticized for their toxicity and massive cost, whereas PCR-based methods require precise instruments that can be large and expensive and require skilled personnel.
Colorimetric detection has various advantages such as eliminating the use of radioisotopes, reduced costs related to required equipment, and on-site and real-time quantification and detection. The traditional colorimetric aptasensor works by first incubating the aptamer with the virus and then adding catalytically active substances that attach to the trapped virus. To change the color of the sample, appropriate chromogenic reagents are introduced. For example, Chen et al. employed magnetic bead-modified aptamers specific to H3N2 to detect influenza A virus. The sensor consisted of AuNPs modified with concanavalin A and glucose oxidase (ConA-GOx-AuNP). The complex attached to the virus through concanavalin A–glycan interaction, with glucose oxidase transforming a chemical signal into a color change, with an LoD of 11.16 g/mL. Liu et al. developed a colorimetric assay using graphene/AuNP hybrids to detect hepatitis C virus. The ssDNA aptamer reduces the catalytic activity of graphene/AuNPs by preventing the contact between the active interface and peroxidase substrates. Interaction of the virus with the aptamer restores the catalytic activity, with color change produced by the substrate 3,3′,5,5′-tetramethylbenzidine (TMB).

**Non-electrochemical Surface-Imobilized Antibody-Coupled APTASensors.** APTASensors can be coupled with antibodies to be used as direct or sandwich-type immunassays. Enzyme-linked immunosorbent assay (ELISA) is one of the most used diagnostic methods for the detection of proteins and antigens. When aptamers are used in substitution for the antibodies, the method is termed as enzyme-linked oligosorbent assay (ELOSA), enzyme-linked oligonucleotide assay (ELONA), or enzyme-linked aptasorbent assay (ELASA).

The direct method of ELONA constitutes a plate coated with the target and a biotinylated aptamer that binds to the target (Figure 6a, top). Horseradish peroxidase (HRP)-conjugated streptavidin then binds to the biotinylated aptamers, causing chemiluminescence in the presence of the enzyme substrate TMB. In the sandwich-based ELONA, the primary aptamer is immobilized on the surface of the plate and recognizes the target molecule (Figure 6a, bottom).

An enzyme-linked aptamer assay (ELAA)-based study was conducted for detecting the influenza A strain H5N1 using an aptamer that targets the HA protein. This sandwich-based aptasensor comprises amino groups conjugated at the 3′-terminus of aptamers that were immobilized in the wells. The target HA protein or semi-purified influenza virus was then added to the wells followed by addition of streptavidin–horseradish peroxidase. The LoD was 0.1 μg/well, and the assay also discriminated between H1N1 and H3N2 subtypes of the virus. Other analogous ELAA methods have been used for the detection of the human norovirus, Zika virus, and HCV.

Lateral flow assays (LFA) are a type of immunochromatographic assay commonly used for the detection and quantification of an analyte. These assays employ layered pads that generate a series of capillary beds to allow for directional fluid movement. They are frequently used to detect a wide range of targets, including viral targets for HIV and HBV. LFA strips consist of different overlapped layers of sample pads, conjugate pads, nitrocellulose analytical membranes, and absorbance pads mounted on a sticky backing sheet. The two most common LFAs are the competitive and sandwich or complementary oligonucleotide assay. In the sandwich assay, aptamers tagged with enzymes/AuNP/fluorescent dyes form a complex with the analyte at the conjugate pad. The complex flows to the test zone via the capillary pull of the strip and is captured by the antibody or another aptamer, which forms a sandwich between the two aptamers or antibody (Figure 6b). This complex results in a visible color change in the test zone, typically a red line. The excess labeled aptamer moves to the control zone, which is captured by another oligonucleotide complementary to the aptamer or protein that binds the antibody and causes another color change (the red line) to validate the assay (as a positive control). Le et al. developed a dual recognition element lateral flow assay (DRELFA) method to detect strain-specific influenza viruses in a multiplexed fashion. Compared to the current antibody-based conventional LFA, this aptasensor can discriminate between different strains of the influenza virus.

Figure 6. Non-electrochemical aptasensors. (a) Mechanism for direct and indirect ELONA where the virus is immobilized on the surface, respectively. (b) Mechanism for aptamer-based lateral flow assay: LFA strip includes positive control line with antibody binding to the target virus and a test line with a streptavidin-immobilized aptamer. Upon binding of the target virus, the AuNP–Ab complex shows the right signal; in the absence of virus, no line is visible in the test region.
Aptamers have shown promising progress in the field of therapeutics, and several aptamers are in preclinical stages. Therapeutic aptamers bind directly to the viral targets and inhibit the downstream signaling of the replication cycle. Aptamers work in one of the following ways: they prevent structural changes in the target molecule, inhibit dimerization through associated molecules, or can phosphorylate the proteins involved in downstream signaling. There are other advantages of using aptamers as antiviral therapeutics. First, aptamers bind to the target tightly via nucleic acid interactions. Second, aptamers can form thermodynamically stable secondary structures by folding itself, regulated by Watson and Crick base pairing. Third, aptamers for a particular target can be identified within a month by the SELEX process and can be adaptable for several chemical modifications. These advantages have brought aptamers into focus and have been extensively studied in the field of drug delivery.

One example of aptamer developed for treatment against viral infections is B40, an RNA aptamer that inhibits the HIV-1 envelope glycoprotein (gp120) from binding the C–C chemokine receptor-5, a T-cell co-receptor. In another example, cell surface SELEX was used to construct a ssDNA aptamer ZE2 that interacts exclusively with E2, a surface glycoprotein of HCV to impede the initial attachment of HCV with the host cells. In vitro studies show that HCV particles are trapped by aptamer ZE2, making the aptamer potentially useful for anti-HCV therapy.

The antigen hemagglutinin, expressed on the surface of influenza viruses, is a prime target molecule for aptamers. As compared to the conventional anti-HA monoclonal antibody, the aptamer P30-10-16 interacts with HA with a greater affinity (15-fold). Kwon et al. synthesized an RNA aptamer that binds to the glycosylated receptor of the HA and neutralizes the receptor binding site of HA, thus restricting the attachment of the virus to the host cell. Yu et al. generated ssDNA aptamers against amino acid residues present in the PA subunit of N-terminus of the polymerase of the influenza A virus. The PAN-2 aptamer they synthesized has an IC50 value of 10 nM and offered crossing protection against influenza viruses (H1N1, H5N1, H7N7, and H7N9). There are only a few studies on aptamers against viruses such as human papillomavirus (HPV), hepatitis B virus (HBV), dengue viruses (DENVs), severe acute respiratory syndrome coronavirus (SARS-CoV), and rabies. Some of these include the HBs-A22 RNA aptamer that targets the HBV surface, S15 tar ssDNA aptamer that targets the envelope protein of DENV-2, and GE54 sar ssDNA aptamer that targets the glycoprotein expressed by viruses (RABV). Valencia-Resendiz et al. reported that RNA aptamers for HPV16 L1 virus inhibits infection at early stages by interacting with the viral particles. Yadavalli et al. isolated a DNA aptamer specific to the gD protein of HSV-1 and demonstrated its superior binding affinity and inhibition of viral reproduction and entry in in vitro, in vivo, and ex vivo studies. The different mechanisms through which aptamers inhibit viral infection are discussed in this section.

Aptamer Suppression of Viral Attachment to Host Cells. Aptamers can obstruct the entry of viral particles into host cells by binding to viral surface proteins, thus affecting the ability of the virus to interact with related receptors on the host cell. Nucleolin, a eukaryotic cellular protein, is one such protein that aids in the attachment and entry of different viruses. Balinsky et al. showed that the interaction of nucleolin with a DENV capsid protein aids in the formation of infectious virus particles. The nucleolin–DENV interaction was hindered by the addition of the RNA aptamer AS1411 that binds nucleolin. Similarly, HCV entry into the host requires the interaction of viral surface E2 proteins with host membrane cell receptors. An aptamer developed against the HCV E2 glycoprotein showed inhibition of viral entry into host cells. The selected aptamer could significantly block the binding of HCV (90%) to the CD81 host receptor, thereby inhibiting the infection of human hepatocytes. The first identified anti-HIV aptamer forms a tetramolecular parallel G-quadruplex (d-(TTGGGGTT)) structure. This aptamer is anionic and strongly interacts with the cationic V3 loop of the HIV envelope glycoprotein, gp120. This interaction inhibits host/virus surface adsorption and cell fusion, inhibiting HIV entry into the host cell. However, HIV entry can still occur through other means, such as by interacting with other cellular receptors including heparan sulfate proteoglycans and nucleolin present on cell surfaces. Application of AS1411, the nucleolin binding RNA aptamer, at low nanomolar concentrations also showed antiviral activity by interfering with HIV attachment via the nucleolin-based pathway. Jeon et al. designed a DNA aptamer A22 against HA regions of influenza virus that blocked the binding of the virus to target cell receptors. Animal studies showed that A22-treated mice lose weight at a rate slower than that of the control group, and infiltration of mononuclear cells in the alveoli was also decreased in the A22-treated group.

One example of an aptamer C7-35M against H9N2 avian influenza virus that
blocks viral infection in a dose-dependent manner. Similarly, Gopinath et al. constructed an aptamer against HIV-1, which impedes viral entry. The IC_{50} value was measured to be 0.8 μM, and it can specifically distinguish HSV-1 from HSV-2.

**Aptamers Inhibiting Virus Replication Cycle.** Aptamers have been employed for viral inhibition and show promise as therapeutic agents by inhibiting the replication of the viral genome. For example, the RNA aptamer B.2 was developed as a therapeutic against HCV. Specifically, the aptamer forms a stem-loop structure and can bind and inhibit the HCV 5B polymerase, a nonstructural RNA-dependent RNA polymerase that catalyzes RNA replication. The aptamer and the template RNA have different binding domains; B.2 noncompetitively binds the RNA polymerase and therefore weakens the polymerase ability to bind the RNA template.

In vitro studies by Bellecave et al. found two aptamers, 27v and 127v, which could inhibit the same polymerase but through competition for the polymerase binding site on the template RNA. The aptamers function at different stages of the replication cycle of viral RNA: 27v blocks both initiation and elongation, whereas 127v blocks initiation and postinitiation events of the viral RNA replication. Similarly, an RNA aptamer developed against the HIV nucleocapsid protein (critical for replication, encapsidation of viral genomes, and assembly of viral particles) hindered viral packaging by competing for psi RNA binding to the nucleocapsid protein. Another truncated RNA ligand, RNA tat, was developed using SELEX and reduced HIV-1 replication by 70%.

Aptamers have also been developed against human cytomegalovirus (HCMV), a member of the beta-herpesvirinae subfamily and affects immunocompromised individuals. It is a major cause of many birth defects, and its impact is increased due to the increase in HIV-infected patients and immunosuppressive treatment. Similarly, RNA aptamers were generated to target the gp120 glycoprotein involved in competing for psi RNA binding to the nucleocapsid protein.

**Inhibition of Viral Enzymes by Activity of Polymerase.** The nonstructural protein-5B (NS5B), an RNA polymerase in HCV replication, is a promising target for aptamer therapy. Biroccio et al. selected an RNA aptamer against the second domain of IRES. Combined blocking in vitro IRES-dependent translation compared to the control group as evaluated by RT-PCR. Gao et al. developed aptamers NS2-1, NS2-2, and NS2-3 to target the nonstructural protein 2 (NS2) of HCV and showed successful inhibition of viral replication.

The 27v aptamer-treated hepatoma cell line (human) infected with the viral RNA template for the polymerase binding site. The 27v aptamer-treated hepatoma cell line (human) infected with the viral RNA template for the polymerase binding site. The interaction between pregenomic RNA with viral polymerase R is an essential part of HBV replication. Feng et al. discovered an RNA aptamer S9 with a strong affinity for viral polymerases of HBV, showing 80–85% suppression of HBV replication in a human cell line infected with HBV. Similarly, DeStefano and Nair showed that a DNA aptamer Targeting the reverse transcriptase of HXB2 strain of HIV suppressed viral replication in vitro. The aptamer prevents the viral replication by competing with natural template for the enzyme’s binding site.

**Inhibition of Other Enzyme Activity Associated with Viral Replication.** Certain genomic sequences interact with proteins associated with viral replication, translation, transcription initiation, and assembly. Designing aptamers that can target these genomic sequences is promising for antiviral treatment strategies. The mRNA of HCV has an internal ribosome entry site (IRES) which is associated with viral replication and is a potential target for antiviral therapy. IRES, which has four domains (1–IV) in its 5′ UTR, plays a role in viral replication initiation and mRNA translation. Konno et al. designed an RNA aptamer AP30 consisting of sequences 5′-UAAUCCG-3′ and 5′-UGAUUCG-3′ to target the first domain (SL-D1 and SL-E1 loops) of IRES and showed in vitro suppression of viral replication.

An RNA aptamer (2-02) with sequence 5′-UAUGCU-3′ targeting the domain II of IRES has been synthesized by Kikuchi et al. In a separate study, the same group developed aptamer 3-07, which targets the third domain of the IRES. This mechanism was substantially more potent at suppressing viral infection by blocking in vitro IRES-dependent translation compared to the aptamer against the second domain of IRES. Combined blocking of II and III–IV domains of IRES has also received considerable attention as the IIIe and IIId regions are essential for translation in HCV. Two aptamers (0207 and 0702) which are the combined form of 3-07 and 2-02 reported to have 10-fold higher binding affinity, and the IC_{50} value lowers the translational activity by 10-fold. Romero-Lopez et al. developed a construct (HH363-24) that targets the IIId domain and cleaves the 3′ end of HCV genome sequence.

The HH363-24 construct impedes both the replication and translation of the virus. Other research has focused on aptamers that interact with the long terminal repeats of HIV.

**Aptamers for Delivering Therapeutics to Viral Infected Cells.** Aptamers can also be used to target drugs to specific diseased regions. For example, Liu et al. constructed an RNA aptamer conjugated with fluorescein isothiocyanate (Hbs-A22) to target HbsAg surface antigen present in cells.
infected with HBV. In another example, Zhou et al. created a chimeric construct which contains a siRNA molecule and anti-gp120 aptamer and targeted the mRNA for the tat/rev protein in HIV-1 in the Chinese Hamster Ovary (CHO) cell line. Neff et al. performed a similar study using HIV-1 NL4-3 infected humanized mice (RAG-Hu). 2'-Fluoro modifications further improved the biostability of this siRNA−aptamer chimera in mouse serum. The aptamer-based treatment showed inhibition of viral activity, as confirmed by 75–90% decrease in mRNA transcript level for tat/rev protein in T lymphocytes of mice. Zhu et al. used a different strategy by converting anti-CD4 RNA aptamer to a DNA aptamer and conjugating an siRNA molecule for targeting mRNA of HIV-1 protease. The therapeutic activity was evaluated by qRT-PCR, which quantified the reduction in expression for the mRNA protease in pcDNA-HIV-PR plasmid-transfected CD4+ T-cells.

Other Approaches and Aptamer-Based Therapeutics in Clinical Trials. Different aptamer−siRNA chimeras have been developed against HIV-1 infection such as anti-CD4 aptamer/anti-gag-siRNA and anti-CD4 aptamer/anti-CCR5-siRNA. Bruno et al. developed aptamers against Dengue fever and West Nile virus infections. An RNA aptamer (CL9) containing the cytosolic receptor RIG-I was generated by Hwang et al. and was shown to activate innate antiviral immune response. The increased antiviral response and production of IFNβ is the effect of cytosolic receptor RIG-I which helps in recognizing the pattern for foreign molecular agent in the cells infected with the virus. In vitro studies showed that CL9 inhibits cells from invasion. Table 2 lists the aptamer candidates that are currently evaluated in clinical trials for viral therapeutics.

Table 2. Aptamers in Clinical Trials

| target             | oligonucleotide | functional activity                                   | clinical trial |
|--------------------|-----------------|------------------------------------------------------|----------------|
| HIV-1 Tat          | RNA             | reducing TAT-mediated HIV replication                | phase I        |
| HIV-2 Tat          | RNA             | reducing Tat-2 transactivation 20, and HIV-1 replication | phase I        |
| HIV-1 Rev response | RNA             | HIV replication in vitro and in vivo                 | phase I        |
| hepatitis C NS3    | RNA             | reduction in NS3 activity in vitro                   | phase I        |
|                    |                 | reduction in NS3 protease activity                   |                |
|                    |                 | reduction in MBP-NS3 protease activity               |                |

DNA APTAMERS FOR SARS-CoV-2 DETECTION AND INHIBITION

Since the outbreak of the COVID-19 pandemic, DNA aptamers targeting either SARS-CoV-2 nucleocapsid (N) or spike (S) protein have been selected and employed in the development of viral sensors or inhibitors. Yang and colleagues obtained three effective aptamers, named as CoV-2-RBD-1C, CoV-2-RBD-4C, and CoV-2-6C3, which bind to spike receptor binding domain (RBD) region with nanomolar Kd. Among them, the circular CoV-2-6C3 dimer shows increased stability in human plasma compared to that in the linear monovalent aptamer. In addition, it displays a high antiviral potency with an IC50 of 0.42 nM and reduces the amount of viral genome in the infected cells by 87.1% compared to the viral culture that was not treated with the aptamer. The same group recently constructed a spherical cocktail of neutralizing aptamer–gold nanoparticle (SNAP) decorated by all three aptamers (Figure 7a). Exploiting the synergetic blocking strategy from the multivalent aptamer and steric hindrance effect of the gold scaffold, the cocktail SNAP neutralizes both the wild-type strain and the three variants (commonly called D614G, Alpha, and Beta) with a further improved IC50 at the femtomolar level.Potency of the cocktail SNAP is about 2–3-fold better than the performance of other reported neutralizing aptamers in a monovalent or a circular divalent form. In a separate study by Pun and colleagues, a DNA aptamer, named SNAP1, was selected showing <80 nM Kd and binding to the spike N-terminal domain as revealed by high-resolution cryo-EM imaging. The aptamer detects UV-inactivated SARS-CoV-2 with an LoD of 5 × 10^5 copies/mL when used in lateral flow assay or ELISA, suggesting SNAP1 is a valuable ligand capable of COVID-19 diagnostics in point-of-care settings. Additionally, a dimeric DNA aptamer form, denoted as DSAINS by Li and colleagues, was derived from two previously obtained aptamers, MSA1 and MSA5, in the same group. DSAINS recognizes the spike protein of wild-type, alpha, or delta SARS-CoV-2 strains with a Kd of 120, 290, or 480 pM. After being immobilized onto gold electrodes to produce a sensor rapidly generating electrochemical signals, the aptamer can detect 1 × 10^5 virus particles/mL in 1:1 diluted saliva of both wild-type and alpha/delta variants within 10 min. The study provides the first aptamer for a rapid test of the SARS-CoV-2 delta variant.

In addition to aforementioned aptamers that were selected by targeting specific SARS-CoV-2 surface antigens (N or S protein), Lu and colleagues have evolved an aptamer, called SARS2-AR10, that was selected against intact virions by performing a counter selection using UV-inactivated virus particles. As a result, the aptamer can distinguish active/infectious SARS-CoV-2 virus from the noninfectious form. The SARS2-AR10 aptamer was integrated with a solid-state nanopore system, which renders strong confinement to the virus, to selectively detect intact SARS-CoV-2 containing samples with an LoD of 1 × 10^4 copies/mL (Figure 7b). The same aptamer was recently immobilized onto a customized photonic crystal surface for digital detection of intact SARS-CoV-2 virions with an LoD of 1 × 10^4 copies/mL using a label-free imaging technique called photonic resonator interferometric scattering microscopy (PRISM) (Figure 7c). These sensors offer a tool to minimize or eliminate the chance of false positive results resulting from PCR-based detection of the SARS-CoV-2 RNA genome residue rather than the infectious virions.

DNA APTAMERS IN PLANT VIRAL INFECTION CONTROL

Viral plant infections pose a growing concern in the agricultural field, which can result in low-quality grains, fruits, vegetables, and flowers and lead to huge economic loss. The most common viruses include tobacco mosaic virus (TMV causes infection in Tobacco and Solanaceae plant), tomatospotted wilt virus (TSWV causes infection Nicotiana, groundnut), African cassava mosaic virus (ACMV infects genus Begomovirus such as Datura, Cassava, and Nicotiana), tomato yellow leaf curl virus (TYLCV infects Solanaceae plant family...
such as tomato), cucumber mosaic virus (CMV causes infection in Cucurbitaceae family of plant such as carrot, pepper, bean, spinach), potato virus Y (PVY infects weed plants, pepper, Solanum esculentum, Solanum tuberosum, Nicotiana), plum pox virus (PPV infects peaches, almonds, plums, apricots, nectarines), Potexvirus like potato virus X (PVX infects potato), cauliflower mosaic virus (CaMV infects species of Resedaceae and Brassicaceae), and brome mosaic virus (BMV infects species of poaceae family like barley). The pathogen’s information has a crucial role in the proper diagnosis and therapy of any disease, and it is essential for protecting the crops from infection and saving the farmers from commercial loss.234

Approaches to prevent or inhibit viral infection in plants are based on gene silencing, metabolic regulation, hormones, proteolysis, and immunological receptor signaling.235,236 The few modern methods that can control viral infection in plants are rotation of crop and pathogen-free plant production through tissue culture and through integrated vector management. Traditional breeding techniques can take a long time, and genetic modification with naturally occurring resistance genes (R genes) can produce virus-resistant strains. Alternatively, the use of symbiotic fungal interaction with arbuscular mycorrhizae can improve the natural defense mechanism plants have against plant viruses.237,238 Recent studies have demonstrated the use of peptide and DNA aptamers to control viral plant infection. These aptamers bind specifically to virus coat proteins such as the capsid protein, nucleoprotein, and movement proteins which thereby prevent infection. Specifically, peptide aptamers are highly target-specific and can function in both intracellular and extracellular environments and interfere with viral gene expression or replication.239

Lopez-Ochoa et al. reported a set of peptide aptamers that bind the N-terminus of the Rep protein from the tomato golden mosaic virus (TGMV).240 Peptide aptamers A22 and A64 expressed in transgenic tomato lines have been used for treating viral diseases, specifically by interacting with viral Rep proteins that are involved in the replication, transcription, and infection.241 The two aptamers bind to different regions in the N-terminus of Rep proteins of geminiviruses, such as tomato yellow leaf curl or tomato mottle virus, and interfere with the replication activity of the virus. A22 recognizes the first 35 amino acids of Rep, whereas A64 primarily interacts with residues 64-97, including a highly conserved motif geminivirus Rep sequence (GRS). Another study detected the apple stem pitting virus (ASPV) using a label-free SPR approach.242 DNA aptamers were developed against two viral coat proteins HS-MT32 and HS-PSA-H. Here, thiol-modified aptamers were immobilized on the gold surface of the SPR chip. To avoid nonspecific adsorption, the surface was modified with random oligonucleotides with the same length as that of aptamers. The sensitivity of SPR to the viral fragments was verified using SEM imaging showing aptamer-modified chips binding to the viral fragments. First, $2.2 \times 10^7$ virus fragments/cm$^2$ were visible on the aptamer-modified surface. Using cell-SELEX, Ye et al. produced three DNA aptamers targeting GCRV-infected cells that could be utilized for developing quick detection technologies and antiviral therapeutics for GCRV infection.243 Application of aptamer technology offers a broad spectrum of viral infection resistance. Integrating conventional breeding techniques with peptide aptamers might be a potential route to tackle new variants and virus species. RNA interference (RNAi), which is homology-dependent, plays a role in preventing infections that result in transgenic resistance toward plant infections.244

Figure 7. DNA aptamers selected for SARS-CoV-2 viral detection and inhibition. (a) Schematic of SNAP to block the interaction between the RBD of SARS-CoV-2 and host ACE2 with synergetic strategy of multivalent multisite binding and steric hindrance. Reproduced from ref 230. Copyright 2021 American Chemical Society. (b) Scheme of infectious virus detection using aptamer-functionalized nanopore sensors. Reproduced with permission from ref 231. Copyright 2021 American Association for the Advancement of Science. (c) Working principle of label-free optical detection for intact SARS-CoV-2 using surface-immobilized DNA aptamers and PRISM system. Reproduced from ref 232. Copyright 2022 American Chemical Society.
ROLE OF DNA NANOTECHNOLOGY IN VIRAL DIAGNOSTICS AND THERAPY

Synthetic scaffolds such as polymers, nanofibers, nanoparticles, and liposomes have emerged as advanced platforms for infectious disease detection and treatment. However, these materials do not allow control over surface probe or ligand density for biosensing applications. To address these drawbacks, aptamer-labeled DNA nanostructures have been explored as biosensing and diagnostic platforms. DNA is a spatially controllable and versatile material that can be used for the bottom-up construction of nanostructures. These DNA nanostructures have been robustly self-assembled into 2D and 3D geometries of specific shapes and sizes. Moreover, the chemical nature and predictability of DNA base pairing allow for the precise decoration of DNA nanostructures with ligands at sub-nanometer resolution. Potential ligands include proteins, nanoparticles, oligonucleotides, fluorophores, and other biomolecules. Further, the biocompatibility, biostability and nontoxicity of DNA nanostructures have made them useful in biosensing and drug delivery applications.

On a separate note, flexibility of nucleic acid backbone may prevent aptamers from reaching to optimal folding poses for better binding affinities. DNA nanostructures can provide an ideal platform with excellent addressability to flexible aptamers into a specific configuration. Tan and colleagues recently stabilized an anti-lysozyme aptamer by fixing the termini of the aptamer with a length-optimized triplex structure on a DNA tetrahedron nanostructure. As a result, the target binding affinity of the aptamer increased by ∼10-fold. Additionally, the aptasensor built on the DNA tetrahedron nanostructure achieved a 180-fold better LoD. We expect this emerging aptamer–DNA nanostructure hybridization strategy to have the potential to greatly improve the performance of existing aptasensors.

DNA Nanostructures for Viral Detection. Recently, DNA nanostructures have garnered tremendous interest in biosensing due to their high surface-to-volume ratio, which provides greater space for responsive elements and therefore greater changes in signal generation. This biosensing ability has been explored for the early detection of pathogens in human samples with enhanced target specificity and avidity. In this section, aptasensors utilizing a DNA...
nanostructure scaffold are discussed, categorizing the techniques based on the same output methods discussed previously.

DNA–Antibody Nanostructure as Electrochemical Immunosensors. DNA nanostructures can be employed as electrochemical sensors in which an antibody-labeled DNA nanostructure is attached to a gold electrode (examples in ref 264). For instance, a DNA–antibody nanostructure has been used as an electrochemical immunosensor for the rapid detection of *Streptococcus pneumoniae*. In this study, a hollow structured DNA tetrahedron was assembled and functionalized on the surface of gold electrodes, and the surface was further passivated with bovine serum albumin (Figure 8a). Later, pneumococcal surface protein A (PspA) antibody was tagged onto the top vertex of the DNA tetrahedron via carboxyl group conjugation. Electrochemical detection occurred by the introduction of ferricene carboxylic-acid-conjugated antibodies (FeC-Ab) onto the electrode surface. This electroactive tag reacts with PspA and produces a peak current corresponding to the target concentration, measured using a square wave voltammetry technique. This method allowed detection of PspA peptide and *S. pneumoniae* lysate from synthetic and real human samples from the axilla, nasal cavity, and mouth.

Fluorescent DNA Nanostructures. DNA nanostructure-based biosensors may also utilize FRET as its detectable output, hybridizing specific nanostructure strands with aptamers that are labeled with fluorophore or fluorophore−quencher pairs. Similar to the mechanism employed for regular FRET-based aptasensing, the placement of the FRET pair is rationally designed so that the fluorescence changes when the target is present. Kwon et al. developed a star-shaped DNA nanoarchitecture for the detection and inhibition of dengue virus (DENV). This two-dimensional nanoscaffold consists of five fluorophore−quencher pair which act as molecular beacons as well as 10 DENV envelope protein domain-III (ED3) binding aptamers (Figure 8b). The resulting structure precisely matches the pentagonal arrangement of ED3 clusters on the DENV surface. The molecular beacons are placed such that each edge of the inner pentagonal scaffold consists of a fluoroscene (FAM, fluorophore)-modified ssDNA and a BHQ-1 (quencher)-modified ssDNA hybridized to a hairpin structure that allows for FRET-based sensing capabilities. In the absence of DENV, the hairpin structure is maintained, and fluorescence is quenched due to the close proximity of FAM and BHQ-1. In the presence of DENV, the ED3 aptamers bind the protein targets on the DENV surface and cause a structural expansion of the entire DNA star architecture. To accommodate for this change, the hairpin structure dehybridizes and the FAM and BHQ-1 molecules move apart and allow fluorescence to occur. This platform detected DENV in human blood serum and plasma with high sensitivity of 1 × 10^2 and 1 × 10^3 pfu/mL, respectively.

In another study, DNA dendrimer-based fluorescently labeled barcodes were used for the multiplexed detection of pathogen DNA. This structure contains two kinds of fluorescence dyes (Alexa Fluor 488 and BODIPY 630/650) and a probe that is complementary to the target pathogen DNA used for the specific detection (Figure 8c). The dendrimer DNA structure is formed from a Y-shaped DNA attached to a detection probe and a fluorescent dye. Multiplexed detection is carried out by introducing different nanobarcodes containing specific target probes into a sample containing DNA from four targets: *Bacillus anthracis*, *Francisella tularensis*, Ebola virus, and SARS coronavirus. Binding of the target with the probe yields a particular pseudocolor corresponding to the specific target, generated from the merged images of four possible combinations. The detected pathogen is then identified by decoding with a preassigned barcode library.

Zheng and co-workers used a DNA nanomachine on AuNPs for the selective and ultrasensitive detection of HIV nucleic acids. This strategy combines rolling circle amplification (RCA) and catalytic recycling for a DNA-walker cascade amplification on the AuNP surface (Figure 8d). For the RCA reaction, HIV-specific nucleic acid was taken as a primer DNA, which has a hybridization sequence to the 3’ and 5’ ends of a DNA padlock, which can synthesize long ssDNA from short circular padlock DNA using DNA/RNA primer via RCA. Here, addition of phi29 DNA polymerase helped in driving the RCA reaction. The byproduct of RCA reaction consists of catalysts created by the extended strand containing a recognition sequence that is nicked by the nicking enzyme Nb. BtsI and can be used as triggers for initiating the DNA nanomachine. This catalyst initiates the opening of hairpins to yield signals via duplex formation on the AuNP surface and causes DNA walker cascade amplification. This results in the liberation of FAM-labeled DNA payload and induces fluorescence signals in the presence of HIV targets. The specificity of the DNA nanomachine was confirmed with four different DNA sequences, with an LoD of 1.46 fM.

Atomic Force Microscopy (AFM)-Based readout. AFM has also been utilized in DNA nanostructure-based sensors. This scanning-probe microscopy technique relies on mechanical interactions with a molecular surface to provide a visual image. Biological targets are identified through visual identification of induced structural changes. In one such example, a self-assembled rectangular DNA origami nanochip was constructed through a bottom-up process for the rapid detection of Human Papillomavirus (HPV), an important target for gynecologic diagnosis. Two staples of the DNA origami nanochip were modified to contain single-stranded extensions that act as DNA probes complementary to the HPV target. Binding of the viral DNA to the single-stranded probes causes the formation of a doubled-stranded DNA helix on the origami surface that was visualized using AFM.

Gel-Based Viral Detection Using DNA Devices. Dynamic DNA devices that reconfigure on recognizing biomarkers have also been used in viral detection. Halvorsen and colleagues developed DNA nanoswitches to detect biomarkers such as DNA, miRNAs, and ribonucleases (Figure 8e). The DNA nanoswitch is a long duplex constructed using the single-stranded M13 scaffold and short complementary backbone oligonucleotides. Two of the backbone oligonucleotides were modified to contain single-stranded extensions that are partly complementary to a target nucleic acid. On binding the target, the nanoswitch reconfigures from the linear “off” state to a looped “on” state, and the two states were resolved on an agarose gel. The “on” state of the DNA nanoswitch is universal for any type of target. Recently, the group used this strategy to detect Zika virus, showing a detection sensitivity of ~10^5 copies/μL without any amplification and an LoD of ~100 copies/μL when the strategy was coupled with an enzyme-based isothermal amplification step. The assay was able to discriminate Zika and Dengue viruses, as well as different strains of the Zika virus. They also demonstrated the utility of the assay to detect SARS-CoV-2 virus responsible for the
current COVID-19 pandemic. This DNA nanoswitch-based assay also offers multiplexed detection of several RNA targets, as well as multiplexed barcoded detection of different types of biomarkers such as proteins, DNA, RNA, and antibodies in a single reaction.276

**DNA Nanostructures for Viral therapy.** DNA nanostructures also offer advantageous properties for drug delivery such as high solubility, nontoxicity, and biodegradability. Moreover, DNA nanostructures possess high cellular uptake properties without the utilization of transfection agents, which make them a suitable tool for healthcare applications.277 Many studies have therefore employed aptamer-labeled, drug-conjugated DNA nanostructures as targeted drug delivery vehicles. Mela et al. constructed an aptamer-functionalized DNA origami rectangular frame structure loaded with the antibacterial peptide lysozyme to destroy Gram-positive (*Bacillus subtilis*) and Gram-negative (*Escherichia coli*) bacteria.278 This DNA origami structure contained 14 bacteria-selective aptamers on the origami’s four edges and five wells where the lysozyme is immobilized through a biotin/streptavidin interaction (Figure 9a). The delivery system was efficient against both types of bacteria.

In another study, an antibacterial DNA nanostructure-based hydrogel was developed for the treatment of a cutaneous wound.279 Hydrogel formation occurs based on electrostatic interactions between polyanionic DNA nanostructures and cationic antimicrobial peptides (AMPs) (Figure 9b). Antimicrobial peptide L12 was released from the hydrogel in response to pathogenic *S. aureus* infections. This strategy showed controlled L12 release and superior antimicrobial activity toward methicillin-resistant *S. aureus* infections. Moreover, ex vivo antimicrobial assay of L12 loaded DNA hydrogels against *S. aureus* infected porcine skin showed bioresponsive delivery systems, nuclease sensitive degradation, and significant potency against *S. aureus* and MRSA infections with 24 h of application. Further, the anti-inflammatory effect of the hydrogel was demonstrated in vivo in mice, revealing faster wound healing rates within 10 days of treatment, an approach that can potentially be translated for treating viral infections in humans. In addition to 2D DNA platforms, Dietz and colleagues recently created a programmable icosahedral...
canvas platform for constructing unique DNA origami icosahedral/cage structures whose cavities are large enough to wrap entire virions for effective virus inhibition. Antibodies against adeno-associated virus serotype 2 (AAV2) were attached to the inside of the shells to pilot the in vitro test of antiviral efficacy, which shows that the cages not only decreased the number of infected host cells but also substantially lowered their viral loads when compared to free antibodies (Figure 9c). Decorated with viral antigens on the outer surface of the same shells, the authors created DNA nanodevices that were used for the detection of viral infections as well as the antigen-triggered release of molecular payload. DNA origami platform can provide excellent spatial addressability to enable precise display of multiple virus antigen-targeting aptamers to mirror the spatial arrangement of the target viral surface antigens. Such pattern-matching interactions should be able to further improve viral detection and inhibition performance compared to monovalent aptamers.

■ SUMMARY AND OUTLOOK

In this review, we have summarized the selection, characterization, and use of existing nucleic acid aptamers for diagnosis and treatment of viral infections. Aptamers can be selected against whole cell virus or for viral components such as surface proteins or against blood markers that are upregulated upon viral infection. In response to emerging viruses like SARS-CoV-2, new aptamers can be readily and economically produced using SELEX to target whole viros or epitopes of novel viruses and the mutants. They can then be quickly plugged into existing virus detection platforms that use electrochemistry, fluorescence, optical, or AFM imaging for detection signal readouts. Compared to antibodies, aptamers in general have lower target binding affinities that may compromise the sensitivity of an aptamer-based sensor. However, as nucleic acids, aptamers can be easily docked within designer DNA nanostructures via DNA base pairing to achieve optimal binding poses, multivalency, and/or pattern-matching interactions with targeted viral antigens for greatly enhanced binding affinity and avidity. The resulting higher binding affinity can facilitate the development of rapid, inexpensive, and sensitive strategies for virus sensing early after infection, which is critical for curbing the spread of highly contagious infectious diseases like COVID-19. Additionally, aptamers can be strategically evolved to distinguish infectious virus particles from noninfectious forms, which provides a unique and novel solution to address the problem for people being able to know when they are no longer infectious and can come out of quarantine, as nucleic acid tests are known to generate false positive results from the presence of nucleic acid molecules from degraded viruses. These emerging platforms and technologies are well worth further investigations for the development of better viral sensors to mitigate future epidemics and pandemics.

For applications in therapeutics, aptamers have unique advantages such as their higher penetration in tissues, easy chemical synthesis, high specificity, and ease of conjugation to therapeutic RNAs, proteins, peptides, small drug molecules and nanoparticles. Yet aptamer-based structures are still underdeveloped in the context of viral therapeutics with many areas for improvement and development. For example, due to the small size, aptamers have a shorter duration of renal filtration. Biostability and bioavailability of an aptamer can also be challenged by nuclease action. Recently developed approaches such as backbone or nucleotide modification are promising strategies for reducing aptamer degradation in physiological conditions. Furthermore, aptamers can be easily conjugated with bulk molecules or with DNA nanostructures, which can not only encapsulate the aptamers but also trigger the delivery of drugs upon locking/unlocking mechanism using aptamer–target binding. Thus, molecular platforms built using designer DNA nanostructures have the potential to create the next generation of aptamer-based therapeutics. In terms of using DNA nanostructures for such biological applications, stability against nucleases, robust functionality in a variety of biofluids such as serum and whole blood, and easy readout are some of the aspects that could be addressed in future research. Some of these challenges are already being addressed, including the development of a variety of strategies to enhance nuclease resistance of DNA nanostructures. However, DNA nanostructures also have several advantages. The methods have been shown to be scalable both in terms of the size and amount of DNA nanostructures that can be produced, with minimal cost associated with DNA synthesis and assembly, and new reports have shown clinical utility of DNA nanostructure-based sensors. Developments in aptamers combined with advances in DNA nanotechnology can serve as potential alternatives to traditional methods in viral detection and treatment.

■ ASSOCIATED CONTENT

* Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.1c00546.

Supplementary table and references of virus-targeting aptamers for detection and/or inhibition of viral infections (PDF)

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