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Aptamers 101: aptamer discovery and *in vitro* applications in biosensors and separations

Introduction to aptamers, which are short, single-stranded nucleic acids that specifically recognize and bind targets. Students will develop an understanding of how aptamers are discovered and their *in vitro* applications. Explores aptamer applications in biosensing, such as electrochemical aptamer-based biosensors and lateral flow assays, through the lens of COVID-19 diagnostics. Investigates aptamer-based separations, such as label-free cell separation for CAR T cell therapy. Equips students to understand cutting-edge aptamer research and develop new aptamer technologies. *Pre-requisite: None. No textbook required. Offered: fall, winter, spring, summer.*

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REVIEW

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Aptamers are single-stranded nucleic acids that bind and recognize targets much like antibodies. Recently, aptamers have garnered increased interest due to their unique properties, including inexpensive production, simple chemical modification, and long-term stability. At the same time, aptamers possess similar binding affinity and specificity as their protein counterpart. In this review, we discuss the aptamer discovery process as well as aptamer applications to biosensors and separations. In the discovery section, we describe the major steps of the library selection process for aptamers, called systematic evolution of ligands by exponential enrichment (SELEX). We highlight common approaches and emerging strategies in SELEX, from starting library selection to aptamer-target binding characterization. In the applications section, we first evaluate recently developed aptamer biosensors for SARS-CoV-2 virus detection, including electrochemical aptamer-based sensors and lateral flow assays. Then we discuss aptamer-based separations for partitioning different molecules or cell types, especially for purifying T cell subsets for therapeutic applications. Overall, aptamers are promising biomolecular tools and the aptamer field is primed for expansion in biosensing and cell separation.

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Introduction

Aptamers are short, single-stranded nucleic acid molecular recognition agents that fold into 3D conformations and specifically bind to targets like proteins, peptides, small molecules, and metal ions. Most aptamers are discovered through a library selection process called systematic evolution of ligands by exponential enrichment (SELEX), which uses a desired target to enrich for binders among many random sequences. Utilized in research fields from biosensing to therapeutics, aptamers

have versatile applications but have yet to reach their full potential.

Aptamers are similar in function to antibodies, which are currently the most widely used molecular recognition agent. Aptamers can have very high affinity (as low as ~ 10 picomolar¹) and specificity (e.g. with the ability to distinguish between single amino acid differences in proteins²) for their targets. However, unlike antibodies, aptamers are chemically synthesized and thus are less expensive and faster to produce, more homogenous with less batch-to-batch variation, and more amenable to controlled chemical modifications.^{3–6} Moreover, DNA aptamers are more stable at a range of ionic conditions, pH, temperatures, and other storage conditions compared to

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		Aptamer	Antibody
Intrinsic qualities	Affinity	10 pM to 10 μ M	10 pM to 10 μ M
	Specificity	Highly specific	Highly specific
	Molecular weight	10–20 kDa	150 kDa (IgG)
	Synthesis method	Chemical	Biological
	Stability	pH 5 to 9, –80 to 100 $^{\circ}$ C, liquid or dry	pH 5 to 8, sequence specific, empirically determined, –80 to 4 $^{\circ}$ C
Application-related qualities	Selection speed	Days to weeks <i>in vitro</i>	Weeks <i>in vivo</i>
	Variability	Very uniform	Batch to batch
	Commercial cost of 1 mg	~\$50 ^a	~\$2000–5000
	Chemical modifications	5' end, 3' end, internal; controlled	Primary amine, carboxylic acid, thiol chemistries; difficult to control

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In this review, we will describe aptamer selection and optimization strategies and then examine biomedical applications of aptamers in *in vitro* settings such as biosensing and cell separation. Overall, this review will prepare readers for entering the world of aptamers as it stands today, especially in light of the COVID-19 pandemic. For further reading, we recommend many other excellent general aptamer reviews^{22–25} as well as more focused reviews.^{26–30}

Panning for gold: aptamer sequence discovery & optimization

As the primary method for discovering new aptamers, SELEX is the heart of aptamer research (Fig. 1). In SELEX, a starting library is incubated with desired molecular targets. In each selection round, bound sequences are recovered and PCR-amplified to create a new pool of aptamer sequences, which are used in the subsequent selection round. Typically, between five to 20 rounds are necessary to enrich the aptamer pool to find target-binding aptamers. In this section, we will cover basic concepts of and considerations for SELEX.

Starting library selection

The properties of the aptamer library are important to optimize for successful selection. In this section, we will focus on (A) the constant region, (B) the random region, (C) the library length, (D) pre-structured libraries, and (E) the type of nucleic acid.

Aptamers typically consist of a random region flanked by two constant regions, which allow PCR amplification *via* complementary primers (Fig. 1, top left inset). The random region is theoretically unique for each aptamer in a starting library, and the longer the random region, the more possible candidate sequences there are. For example, a complete library containing a 40-nt random region would have 4^{40} or $\sim 10^{24}$ unique sequences, which would weigh ~ 50 kg in its entirety. Practically

speaking, selection libraries are smaller and usually contain 10^{14} – 10^{15} unique sequences.^{31–33}

The choice of aptamer length and random region length may influence SELEX success. Aptamers have been discovered in libraries with random regions as large as 220-nt and as short as 22-nt.³¹ One study compared the effect of six different random region lengths (16, 22, 26, 50, 70, 90) on target-binding sequence abundance and found that the target motif was most abundant in the 50-nt and 70-nt selections.³⁴ In recent publications, a random region of 36- to 52-nt in length have been the most common, which is about 70- to 90-nt in total aptamer length. DNA synthesis costs also play a factor, as longer syntheses become more expensive and less efficient. For example, if the efficiency of adding a nucleotide base is 99.5%, an 80-mer aptamer is produced with 69% yield but a 160-mer aptamer is produced at 45% yield.

Pre-structured libraries are one strategy for increasing the probability of successful selection. One pre-structured library stabilizes the aptamer structure by incorporating a double-stranded stem formed by the constant regions. This predictable final structure also simplifies aptamer truncation designs.³⁵ Another library strategy is guanine (G)-rich sequences in the random region to increase the probability of G quadruplexes,³⁶ which are stable secondary structures formed by stacks of four guanidine units. Aside from their unique structure, G quadruplexes are of interest because they have important functions in DNA replication and repair, epigenetics, and other pivotal cellular processes.³⁷ G quadruplexes are also often found in high-affinity aptamers,^{38,39} therefore increasing their frequency in the starting library may lead to higher chances of success.

Although early aptamer research focused on RNA aptamers, the majority of recently discovered and applied aptamers are DNA-based. Both DNA and RNA can form secondary and tertiary structures, though RNA backbone is more flexible. Compared to RNA, DNA is more stable and does not require reverse



Fig. 1 Systematic evolution of ligands by exponential enrichment (SELEX). Aptamers are composed of a random region and constant regions. After annealing, aptamers fold and adopt secondary structures that may allow them to bind to targets. (1) The starting aptamer library contains 10^{14} – 10^{15} unique sequences. (2) Then the aptamer library is incubated with target during selection. (3) Unbound aptamers are removed, and target-binding aptamers are eluted. (4) After conducting PCR on aptamers, sense and anti-sense strands are separated. (5) The enriched aptamer pool is used in the next round of selection and evaluated for binding.



Another non-natural nucleic acid strategy is to use mirror image aptamers made from L-RNA or L-DNA, called spiegelmers.⁴³ Unrecognized by nucleases and DNA processing enzymes, spiegelmers are more stable *in vivo* but also are not easy to PCR amplify in selection. To circumvent the amplification problem, Williams *et al.* conducted SELEX with the enantiomer of the target peptide and D-RNA then synthesized the spiegelmers based on the discovered D-RNA sequence.⁴⁴ However, challenges in generating target enantiomers limit the discovery of spiegelmers. The need for non-natural enantiomers is bypassed if the target is a symmetric molecule, such as biphenol A (BPA). In one recent work, Ren *et al.* developed BPA-binding spiegelmer for gold nanoparticle-based colorimetric detection of BPA.⁴⁵

A plethora of cell types have been used as selection targets,⁴⁶ including cancer cells,^{39,47,48} T cells,³³ and bacteria.⁴⁹ The protocol for cell-SELEX is straightforward to perform and does not require many specialized tools.⁵⁰ Cells are easily partitioned by centrifugation, leaving unbound aptamers in suspension. The cell-bound aptamers are eluted by incubating at high temperatures then separating by centrifugation. Aside from its simple protocol, cell-SELEX is advantageous because of its authentic target presentation on the cell surface and variety of

Another issue with protein-SELEX is that typically embedded or inward-facing portions of membrane proteins can be exposed during selection, potentially allowing aptamers to bind to an inaccessible location in the native state. One strategy to avoid this issue is to conduct selection with enveloped viruses. In “viro-SELEX”, the target membrane proteins are expressed on the viral envelope of a surrogate baculovirus, allowing native

For both cell- and protein-SELEX, negative selection or counter-selection is a common strategy used to reduce enrichment of off-target binders. During a round of selection, the aptamer pool is incubated with an undesired target, and those binders are depleted from the pool. For example, in some of our successful selections, the aptamer library is incubated with His-tagged non-target protein (negative selection), then after partitioning, the remaining sequences are incubated with His-tagged target protein (positive selection).^{35,60}

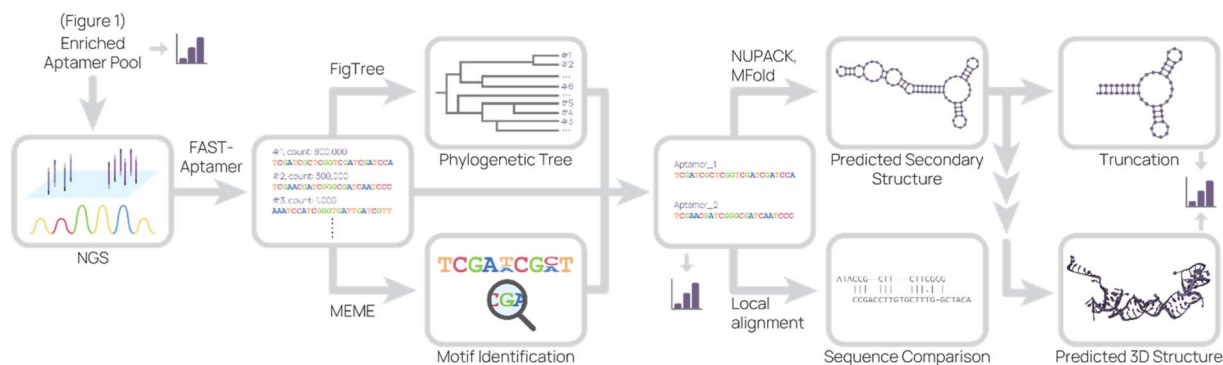
Another approach for protein-SELEX is immobilization of protein onto magnetic spheres for magnetic separation. This technique was first used in 2005 as a less wasteful alternative to immobilization on columns,⁶⁵ and today many SELEX strategies use magnetic spheres. Polyhistidine tags (His-Tag) on proteins are immobilized onto nickel-nitriloacetic acid (Ni-NTA) or cobalt-based magnetic spheres.^{32,35,66} Since this method only requires cheap, commercially available components like magnetic spheres and magnetic rack, protein SELEX with magnetic spheres is very accessible. Some drawbacks of

Altogether, aptamer researchers today can choose from a diverse cast of SELEX targets and partitioning methods, not to mention combinations of multiple approaches and emerging techniques.

For each selection iteration, “knobs” can be adjusted to tune the stringency and desired results. For example, the stringency can be increased through: a decrease of aptamer library concentration; a decrease in incubation time or concentration of target; addition or increased concentration of competitors, such as bovine serum albumin (BSA), yeast tRNA, salmon sperm DNA, and dextran sulfate; addition or increased concentration of surfactants, such as Tween-20; an increase in wash steps; addition or increased concentration of counterselection target; a change in target, for instance to a smaller portion of the target protein or alternative cell line. Generally, these selection

In our experience, we have found that simple primary and secondary structure predictions are sufficient to continue aptamer characterization and applications. However, visualizing the aptamer binding site is important for understanding the binding mechanism. To that end, more computationally intensive methods are needed for tertiary structure prediction, which results in predicted 3D aptamer structures for *in silico* docking studies. There are several methods for tertiary structure prediction. In general, the aptamer sequence is converted to RNA for secondary and tertiary structure prediction, converted back to DNA, then refined by structural energy minimization.⁸⁵ Molecular docking-based and machine learning-based methods are best reviewed in the cited articles due to the complex nature of these analyses.^{85,86} Overall, *in silico* experiments are useful to generate hypotheses, but further experiments are still necessary to define predicted aptamer–target interactions.

Lastly, the aptamer round libraries are sequenced and analyzed (Fig. 2). Next generation sequencing (NGS) is the primary tool for sequencing because it is commercially available, requires very little aptamer library relative to binding studies, and accurately outputs sequences. In our process, we typically read 100 000 to 500 000 complete aptamer sequences per library pool. To analyze the primary sequence, several software and web tools are commonly used. FASTAptamer ranks aptamer sequences by frequency and fold enrichment,⁷⁸ Multiple Em for Motif Elicitation (MEME) Suite groups aptamers by motifs,⁷⁹ G-quadruplex prediction software “Quadruplex forming G-Rich Sequences” (QGRS) predicts the presence of G-quadruplexes,⁸⁰ FigTree visualizes sequences in a phylogenetic



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itself. In this review, we focus on recent *in vitro* applications in two main sections: biosensing (with an emphasis on SARS-CoV-2 applications) and separations.

Biosensing

Typically, SELEX is conducted by manual pipetting, but microfluidic and computational approaches are under investigation for automating the process. Initially prototyped in 2006,⁸⁷ microfluidic SELEX platforms can precisely control fluids and temperature in order to mix, incubate, and partition SELEX reagents from aptamer library introduction to secondary library generation. Several microfluidic systems that automate part of the SELEX process have been proposed,⁸⁸ including systems that utilize magnetic particles or a class of molecularly imprinted polymers (MIPs) called sol-gel. Recently, Sinha *et al.* developed a microfluidic system capable of automating most of the process on-chip (negative selection, positive selection, PCR, competitive assay).⁸⁹ Despite these advances, microfluidic SELEX requires substantial technical know-how and investment to set up, such as master molds for pouring polydimethylsiloxane (PDMS) and controllers for air valves and temperature regulation. In contrast, traditional SELEX uses tools readily available in most molecular biology labs (thermocycler, magnetic rack, *etc.*) therefore, additional investigation is needed to commercialize and disseminate the technology.

Not only are aptamers capable of molecular recognition like antibodies, but also they are smaller in size, easier to chemically modify, robust across various sample conditions, and more affordable to produce. These properties uniquely position aptamers as a recognition agent of choice in biosensing. The aptamer's compact size ($\sim 10^1$ kDa) is essential to decrease the distance between an electrode and the binding event, allowing for sensitive detection of electrochemical signal changes. Addition of functional groups and molecules onto nucleic acids is straightforward and controlled, and therefore less poly-disperse and more consistent than chemistries conducted on antibodies.³⁻⁶ Lastly, aptamers can withstand harsh cleaning treatments and reuse unlike antibodies,⁹⁶ allowing for repeated measurements on the same device.

Due to the aforementioned advantages, aptamers have been used in biosensors across many fields of study. In particular, the COVID-19 pandemic has shined a spotlight on biosensors for disease detection.^{97–100} Although PCR testing has been the gold standard, the need for rapid, point-of-care results led to the dominance of lateral flow assays and investigation into other rapid testing technologies, including aptamer-based detection methods. In this section, we will focus on biosensors organized by detection method through the lens of SARS-CoV-2 antigen detection.

Computational approaches to SELEX have also been investigated. *In silico* binding design can save time and reagents by performing docking simulations instead of tedious benchwork. This concept has been readily applied to *de novo* protein switches and biosensors.^{90,91} In addition, *in silico* SELEX can circumvent some inherent flaws in the process, such as avoiding the amplification of sequences that are preferentially PCR amplified or transcribed.⁹² Computationally-aided⁹³ and combined computation and high-throughput array SELEX schematics⁷² have recently been conducted. However, *in silico* methods are hampered by the difficulties in accurately predicting aptamer structure, especially 3D DNA structures. A more accurate method for modeling is cryogenic electron microscopy (cryo-EM), which is difficult due to the flexibility and small size of short single-stranded DNA. For example, we recently developed a cryo-EM density-guided model of the DNA aptamer tJBA8.1 binding to its target transferrin receptor 1.⁹⁴ However, our attempt to model the 3D structure of tJBA8.1 from its 2D prediction failed to match the cryo-EM map, indicating that *in silico* tools need further improvement to accurately predict structures and binding.

Electrochemical aptamer-based (E-AB) sensors are one type of label-free biosensor that employs conformational change of the aptamer upon binding to produce an electrical signal change (Fig. 3A). Typically, E-ABs require three electrodes, one of which is the aptamer-modified gold working electrode. A region of the aptamer containing redox reactive molecules, such as methylene blue or ferrocene, moves relative to the electrode after target binding. A “signal-on” E-AB produces the signal after binding, while a “signal-off” E-AB interrupts the signal after binding. The electrical signal change can be a change in potential, current, or impedance depending on the setup.^{101,102} Potential for high sensitivity, label- and sample processing-free prep, real-time detection of signal, and reusability give E-ABs distinct advantages over other diagnostic platforms.

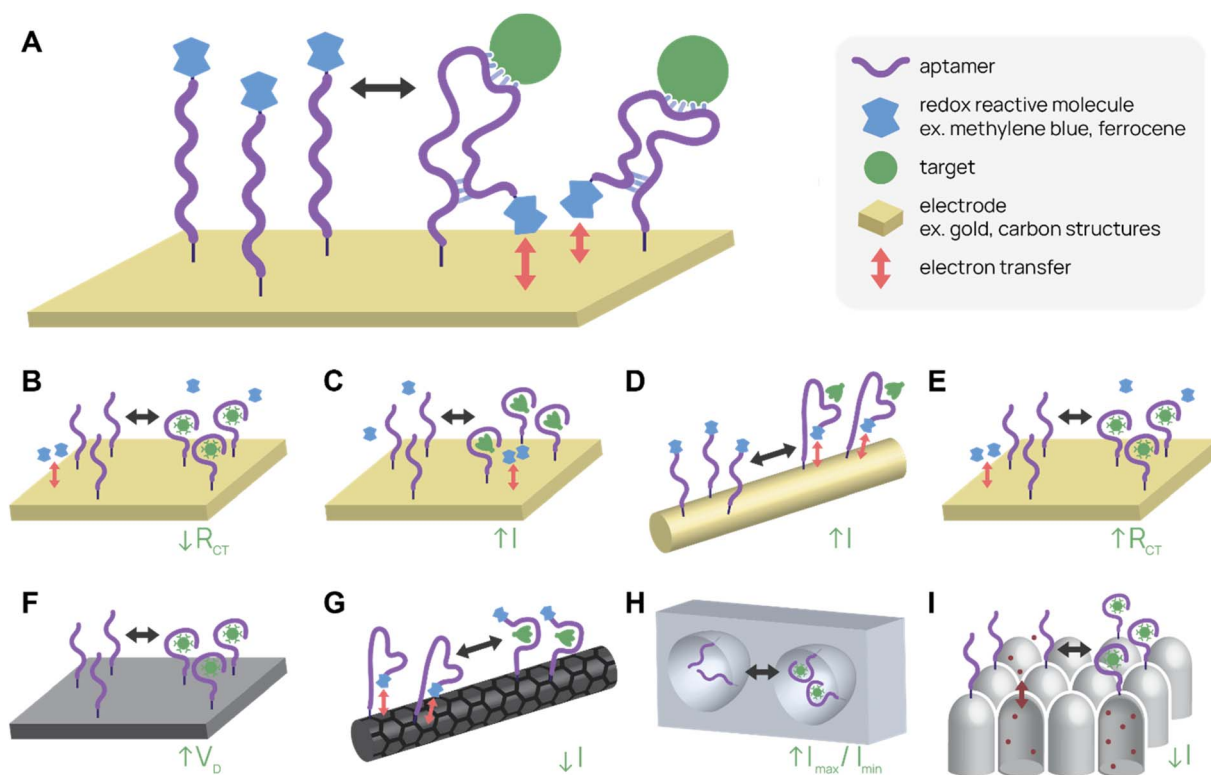


Fig. 3 Electrochemical aptamer-based (E-AB) biosensor diagrams. (A) In a typical “signal-on” E-AB, aptamers change conformation after target binding, resulting in an increase in electrical signal. (B–I) SARS-CoV-2 detecting E-ABs. (B) Virus detection from Lasserre *et al.*¹¹⁰ (C) Spike protein detection from Martínez-Roque *et al.*¹¹¹ (D) Spike protein detection from Idili *et al.*¹¹² (E) Virus detection by “Cov-eChip” from Zhang *et al.*¹¹³ (F) Virus detection from Ban *et al.*¹¹⁴ (G) Spike protein detection from Curti *et al.*¹¹⁵ (H) Virus detection from Peinetti *et al.*⁵⁹ (I) Virus detection from Shi *et al.*¹¹⁶

One of the most-cited E-AB platforms is a thrombin-detecting “signal-off” E-AB from the Plaxco group.¹⁰³ A thrombin-binding aptamer was functionalized with methylene blue, a redox agent, and conjugated to a gold working electrode. When the aptamer bound thrombin, its new conformation shifted the methylene blue away from the electrode, reducing electron transfer and signal. This thrombin E-AB demonstrated rapid detection, nanomolar sensitivity, and reusability, igniting further research into E-ABs. Today, research groups have developed different electrode set ups and detection schemes, as well as expanded the menu of analytes, which now includes small molecule drugs (cocaine,¹⁰⁴ ampicillin¹⁰⁵), proteins (CRP,¹⁰⁶ insulin¹⁰⁷), and viruses (zika virus,¹⁰⁸ avian flu,¹⁰⁹ SARS-CoV-2 (ref. 59 and 110–116)).

Several recent publications describe aptamer-functionalized gold electrode E-ABs for detection of SARS-CoV-2 proteins and virus. Lasserre *et al.* used thin film gold electrodes (used in blood glucose test strips) functionalized with SARS-CoV-2 spike protein-binding aptamers to detect target by electrochemical impedance spectroscopy (Fig. 3B).¹¹⁰ The system detected 80 ng mL⁻¹ (~1 nM) of SARS-CoV-2 spike subunit 1 (S1) protein and distinguished between SARS-CoV-2 positive and negative clinical samples. In a different report, Martínez-Roque *et al.* described an aptamer-functionalized gold electrode E-AB to detect SARS-CoV-2 spike protein with sub-femtomolar

sensitivity (0.007 to 700 fM dynamic range) (Fig. 3C).¹¹¹ Idili *et al.* achieved picomolar precision (10⁻¹¹ to 10⁻⁹ M dynamic range) detection of SARS-CoV-2 spike protein or receptor binding domain (RBD) with an aptamer-functionalized gold wire electrode (Fig. 3D).¹¹² Lastly, Zhang *et al.* functionalized dimeric DNA aptamers to gold electrodes for SARS-CoV-2 protein and virus detection, named Cov-eChip (Fig. 3E).¹¹³ Notably, Cov-eChip detected SARS-CoV-2 with 80.5% sensitivity and 100% specificity in 73 unprocessed patient saliva samples, which outperformed all commercial and published rapid tests available.

There are also non-gold electrode E-ABs with similar detection strategies. Ban *et al.* recently reported a DNA aptamer-conjugated graphene field-effect transistor (GFET) platform for detecting SARS-CoV-2 spike protein and intact inactivated virus at <2 PFU mL⁻¹ (Fig. 3F).¹¹⁴ This system was able to accurately distinguish between 10 positive and negative wild-type SARS-CoV-2 patient samples with 100% sensitivity and specificity. Curti *et al.* developed DNA aptamer-functionalized single-walled carbon nanotube screen-printed electrodes (SWCNT-SPEs) for detecting SARS-CoV-2 spike protein (Fig. 3G).¹¹⁵

In addition to utilizing electrical properties, E-ABs can use shape to specifically amplify detection signals. Peinetti *et al.* use aptamer-functionalized nanopores to confine and detect



pseudotyped SARS-CoV-2 particles at as low as 10^4 copies per mL, even in undiluted human saliva (Fig. 3H).⁵⁹ Remarkably, the platform can distinguish between UV-inactivated and intact SARS-CoV-2 pseudovirus because the pore entrance is narrow, increasing the variation in current signal upon virus binding. Shi *et al.* uses aptamer-functionalized nanochannels to detect as low as 1 fM of SARS-CoV-2 S1 protein and SARS-CoV-2 in clinical pharyngeal swabs (Fig. 3I).¹¹⁶ The analyte obstructs ion transport across the nanochannel and increases the zeta potential of nanochannel surface, resulting in a change in current.

Colorimetric biosensors

Colorimetric biosensors rely on color change to indicate detection. The most well-known example is the lateral flow assay (LFA), which were first pioneered in urine pregnancy tests¹¹⁷ and recently in COVID-19 rapid antigen tests. LFAs are portable, single-use paper devices that rely on molecular recognition agents, such as antibodies and aptamers, to capture and detect antigens. Typically, sample is applied to an absorbent pad and travels across the test strip by capillary action. When the target antigen is present, a test band appears because of antigen-specific accumulation of nanoparticles (Fig. 4A). LFAs are low-cost and easy to use, making them an effective tool for point-of-care diagnostics.^{118,119} Using aptamers in place of antibodies can further improve LFAs. Aptamers are easier to chemically modify, cost less to synthesize, are more consistent batch-to-batch, and possess longer shelf life.¹¹⁹ For example,

aptamers can be commercially ordered with a thiol end group, which conjugates to gold nanoparticles with simple chemistry and consistent orientation. In contrast, antibodies need to be functionalized at existing chemical groups for covalent conjugation, which requires more complex chemistry and inconsistency in antibody orientation and number of functionalized sites.^{4,56}

Several aptamer LFAs have been developed for sensitive SARS-CoV-2 detection. Zhang *et al.* produced an LFA that uses an antibody to capture and two different nanoparticle-conjugated aptamers to detect as low as 20 pM SARS-CoV-2 nucleocapsid protein (Fig. 4B).⁷⁷ We (the Pun group) developed several aptamer-based LFAs for SARS-CoV-2 detection by the spike protein.^{35,56,60} Notably, the antibody-free aptamer LFA (AptaFlow) uses two different SARS-CoV-2 spike protein binding aptamers for detection and capture, and can detect as low as 10^6 copies per mL of intact SARS-CoV-2 virus (Fig. 4C).⁵⁶ In addition, we developed a multiplexed LFA that distinguishes between two variants of SARS-CoV-2 *via* variant-specific spike protein-binding aptamers (Fig. 4D).⁶⁰

Other colorimetric biosensors have also been developed with aptamers in place of antibodies. Enzyme-linked immunosorbent assays (ELISAs) and enzyme-linked apta-sorbent assays (ELASAs) use antibodies or aptamers, respectively, to capture antigens. The molecular recognition agent is conjugated to an enzyme, such as horseradish peroxidase, to amplify signal by color-changing substrate. ELASAs are used to screen and



Fig. 4 Aptamer lateral flow assay (LFA) diagrams. (A) In a typical aptamer sandwich LFA, target molecules within a sample bind both the aptamer-conjugated gold nanoparticle (NP) detection agent (green) and the aptamer capture agent (pink), turning the test band dark. (B)–(D) Schematics showing only the test bands during detection. (B) Aptamer-antibody LFA detecting SARS-CoV-2 nucleocapsid protein (green) with two different aptamer sequences on the gold NP. Adapted from Zhang *et al.*⁷⁷ (C) Antibody-free aptamer LFA ("AptaFlow") detecting SARS-CoV-2 virus (green) with one aptamer for capture and another for detection. Adapted from Yang *et al.*⁵⁶ (D) Multiplexed LFA for detecting two variants of SARS-CoV-2 spike protein (green and teal) using two test bands with unique aptamers. Adapted from Yang *et al.*⁶⁰



measurements. When conjugated to the surfaces of microcantilevers, aptamers can immobilize their targets and cause a signal change in the microcantilever. In stress mode, the additional mass on the cantilever surface causes the microcantilever to bend, which can be measured by a laser and detector.¹²⁸ Aptamer-functionalized microcantilevers have recently been applied to detect liver toxin microcystin-leucine-arginine (MC-LR),¹²⁹ epithelial tumor marker Mucin 1,¹³⁰ and tumor biomarkers (carcinoembryonic antigen and alpha-fetoprotein).¹³¹

Summary

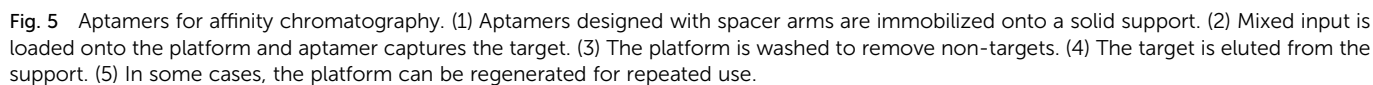
The field of aptamer-based biosensors is expanding rapidly with a wide variety of application strategies. E-ABs take advantage of the facile chemistries and small size of aptamers for real-time, label-free, sensitive detection of analytes. Despite these advantages, E-ABs have yet to reach their full potential in clinical translation. In future work, streamlining of E-AB manufacturing and miniaturization of the equipment required may make E-ABs more amenable to real-world use cases. As evident in the COVID-19 pandemic, LFAs are a powerful tool for home diagnostics as healthcare shifts focus to mobile care instead of facility-based care.¹³² Aptamer-based LFAs can retain the sensitivity of antibody-based ones while also reducing production costs and increasing shelf life. However, challenges still remain for non-LFA colorimetric and fluorescence biosensors because they often rely on instruments to readout sensitive detection. Another concern for many biosensors is decreased sensitivity in non-optimal buffers. For example, aptamer binding is sensitive to ionic strength,¹ and many biosensors have lower detection signals in biological fluids and complex matrices (saliva,¹¹² viral transport media,^{110,115} nasal swab⁵⁶) compared to buffer optimized for the aptamer. Some aptamer biosensor platforms dilute the sample at the cost of losing sensitivity,¹¹³ while others perform well with environmental and biological samples with no pretreatment or dilution.^{59,116} Aside from improving aptamer technology, other factors can improve point-of-care COVID-19 tests, including internet integration, expanded testing sites, and big data analysis.¹³³

The SARS-CoV-2 aptamer biosensors discussed have future applications beyond the immediate needs of the COVID-19 pandemic. Because aptamers are modular, the aptamers used in biosensors can be replaced with other sequences without needing new chemistries or bioconjugation strategies. Aptamers that bind another SARS-CoV-2 variant or another virus target can be substituted in, rapidly re-tooling the biosensor for other variants or diseases. Overall, ongoing investigation into aptamer biosensors can improve their cost effectiveness and ease of use for clinical translation.

Separations

Affinity chromatography is a partitioning method widely used to purify molecules and targets from complex mixtures (Fig. 5). Due to their high specificity, high yield, and ease of operation, techniques in affinity chromatography have successfully been

Mass-sensitive detection is another label-free application avenue for aptamers. Microcantilevers are regularly used in microfluidic platforms for real-time, label-free mass



Aptamers have been immobilized to various solid support matrices including resin, magnetic nanoparticles (MNPs), monolithic capillary columns, and microfluidic channels.

In addition to the spacer arm, aptamer grafting densities require optimization. Aptamer concentrations that are too low can result in low binding efficiency. On the other hand, Lönne *et al.* showed that aptamer densities that are too high impaired binding for aptamer-based affinity purification of vascular

An important design consideration in aptamer-based affinity chromatography are the wash and elution buffers. Wash buffers must preserve the structure and binding specificity of the aptamer. For instance, salt-containing SELEX wash buffers are commonly used as the affinity chromatography wash buffer. Elution buffers used at separation must be tested to preserve stability of the target molecule and facilitate dissociation of the

Uniquely, aptamer ligands can be used with complementary reversal strands to separate target cells from a mixture in a label-free manner. Gray *et al.* immobilized EGFR-binding aptamer on magnetic beads or conjugated it to fluorescent dye to purify EGFR+ cells from a cell mixture using magnetic-



Fig. 6 Aptamer-based platforms for label-free cell isolation. A mixed cell population is incubated with aptamer-labeled magnetic spheres, which bind specifically to the target cell. After magnetic separation to remove non-target cells (gray), labeled target cells remain. A reversal agent, or oligonucleotide complementary to a sequence within the aptamer, is added to release aptamer binding, resulting in a label-free target cell population. (A) Separation of EGFR⁺ cells (green) spiked in blood. Adapted from Gray *et al.*¹⁵⁷ (B) Separation of CD8⁺ cells (purple) from PMBCs. Adapted from Kacherovsky *et al.*³³ (C) Serial elution of CD4⁺ (red) and CD8⁺ (purple) cells from PMBCs. Adapted Cheng *et al.*⁹⁴

activated or fluorescence-activated cell sorting, respectively (Fig. 6A).¹⁵⁷ They achieved 99.9% purity and 33% recovery in EGFR⁺ cell purification from a 5% EGFR⁺ cell-spiked blood mixture with the complementary reversal strand. Importantly, they demonstrated that the reversal strand elution restored native EGFR phosphorylation levels, preserving cell viability and function. In another example, we discovered and applied an CD8⁺ T cell-binding aptamer with high affinity and specificity ($K_D \sim 14.7$ nM) to traceless T cell isolation for CAR T cell manufacturing (Fig. 6B).³³ We selected CD8⁺ T cells from human peripheral blood mononuclear cells (PMBCs) through magnetic activated cell sorting with >95% purity and >72% yield. Compared to the standard isolation protocol, aptamers not only reduce manufacturing costs, but also create a less immunogenic product because aptamers can be reversibly denatured. Building on this work, we report in Cheng *et al.* a reversal strand for our CD71-binding aptamer, which we previously used to successfully deplete malignant cells from PBMC product in CAR T cell manufacturing (Fig. 6C).⁹⁴ Upon

combining CD71-binding aptamer and CD8-binding aptamer with their respective reversal strands in a single cell isolation, we successfully sorted out activated CD4⁺ and resting CD8⁺ T cells separately from the same bulk PMBCs with 93% and 85% purity, respectively.¹⁵⁸ This aptamer-based multiplexed cell isolation system could decrease processing times and manufacturing costs and be applied to a diverse set of targets.

Summary

Aptamers show great promise as affinity ligands in chromatographic separations of small molecules, proteins, and cells. With numerous advantages over antibody affinity ligands, such as greater stability, longer shelf-life, cheaper cost of manufacture, and ease of customizability, aptamers could be adapted in the future to industrial downstream purification processes. With complementary reversal sequences, aptamers can also achieve traceless and multiplexed target selection, thus improving product quality and decreasing processing times and manufacturing costs.



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