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Review

Strand Displacement Strategies for Biosensor Applications

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DNA has many unique properties beyond encoding genetic information, one of which is its physicochemical stability based on Watson–Crick base pairing. Differences in sequence complementarity between multiple DNA strands can lead to the strand displacement reaction (SDR). SDRs have been regularly applied in synthetic biology, drug delivery, and, importantly, biosensing. SDR-based biosensors have high controllability, high sensitivity, and low interference, and can be used for multiplexed detection. Such biosensors have been demonstrated to detect nearly every class of biomolecule. As the field continues to mature, such platforms can be used as an integral tool for the manipulation of biomolecular reactions, bringing biosensors one step closer to the ultimate goal of point-of-care systems.

Highlights

SDRs directly connect a biosensing recognition event to its signaling event, producing simple, integrated platforms.

Combining SDRs into cascades provides inherent amplification of target signals, thus boosting the sensitivity of the biosensing system.

The incorporation of orthogonal strand displacement cascades allows multiplexed detection of multiple targets in one sample.

SDRs can be applied universally to a diverse type of targets, including nucleic acids, proteins, and small molecules.

Dynamic Nucleic Acid Hybridization for Strand Displacement

Although nucleic acids primarily function to transfer genetic information between generations of organisms, their physiochemical properties, including stable secondary structure and the dependable encodability of the Watson–Crick base pair, provide a multitude of opportunities for applications in nanotechnology [1–4]. One such application is the thermodynamically driven SDR (Figure 1, Key Figure) [5]. Typically, this reaction involves three elements: a substrate strand, an initiator strand, and an output strand. The substrate and output strands are specifically designed to partially hybridize to one another, leaving an exposed region on the substrate strand. This overhanging region is known as a toehold, which guides hybridization with the initiator strand. Upon introduction of the initiator strand, which is fully complementary to the substrate strand, the output strand is displaced from the substrate in a thermodynamically-driven migration process. This process results in the formation of a substrate–initiator duplex. The thermodynamic basis of the SDR has been comprehensively described in previous reviews [6,7]. Complex nucleic acid circuits can be readily constructed through multiple SDRs combined into cascades. Importantly, the constructed circuits are enzyme-free reaction networks that can be dynamically controlled through slight alterations to the design of the input and output strands. Therefore, SDR cascades have found applications ranging from artificial gene regulation to synthetic biology [8–13]. Although this review describes recent advances in specific SDR designs for biosensing applications as potential point-of-care systems, biosensing applications using different types of nucleic acid nanotechnologies have been comprehensively reviewed [14,15]. In reviewing current efforts in the selected area, we provide general guidelines for the development of SDR-based biosensing strategies for the real-world implementation of such systems.

Biosensing Applications of SDRs

The dynamic nature, high degree of control, and freedom of the SDR design make this a powerful tool for biomolecule detection and, therefore, for biosensors. One key element of a biosensor is a specific recognition element to preferentially bind to the target analyte. Recognition elements can be antibodies, proteins, DNA or RNA aptamers, or single-stranded nucleic acids. Binding between the recognition element and the analyte is converted to an observable signal by signal transducers. Common signal transducers include an electrode for electrochemical readout [16], a photon counter for light-based readout [17], and a piezoelectric device for mass change [18]. Strand displacement cascades can be designed to connect the recognition element to the signal transducer directly, delivering an integrated and efficient biosensing system.

The versatility of SDRs allows them to overcome common challenges associated with biosensors, including operation complexity, low sensitivity, matrix interference, and difficulty with multiplexing (Table 1) [19]. With the goal of applying biosensors to point-of-care applications, the simplicity of

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the detection strategy is crucial. With properly designed displacement cascades, SDRs regulate the target detection process directly in the sample matrices, minimizing the materials required for biosensing [20,21]. In addition, owing to the low abundance of many biomarkers in biological fluids, high sensitivity and low detection limits are essential for the success of a biosensor. By connecting SDRs in series within displacement circuits, non-enzymatic cyclic amplification is achieved, enabling the tuning of the limit of detection [22]. Furthermore, because of the speed of SDRs, displacement outputs can be monitored during the reaction process, enabling time-resolved detection of dynamic changes in target concentrations [23]. Finally, because of the sequence specificity of nucleic acid hybridization events, each SDR is unique. Hence, multiple displacement circuits can be designed and monitored simultaneously to achieve multiplexed detection of multiple biomarkers in a single sample, which is known to increase the accuracy of disease diagnostics [24].

Optical Sensors

Optical sensors function as homogeneous assays, directly performing target analysis in a single container containing the sample specimen. Readout from such assays is based on a change in absorbance or fluorescence due to the binding of the analyte of interest to a recognition element. Therefore, optical readout negates the need for engineering and fabrication of complex sensing devices.

Detection of Nucleic Acids

The extremely low abundance (often sub-pM) of nucleic acid biomarkers in biological samples typically requires signal amplification techniques for effective detection [25]. These can include polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP), self-sustained sequence replication (3SR), and rolling circle amplification (RCA), which are reagent-redundant and time-consuming methods [26–31]. Using SDRs to construct a cyclic cascade, non-enzymatic amplification of the signal with high **signal-to-noise ratios** (see Glossary) can be achieved without the need for complex amplification strategies. Because of the high sensitivity, ability to multiplex, and ability to operate in complex matrices, optical detection of nucleic acids with SDR-based biosensors is a powerful method for point-of-care analysis.

A Paper-Based Toehold Switch

A colorimetric, paper-based biosensor was developed for the detection of RNA through a target-initiated SDR (Figure 2A) [32]. The platform comprises a paper substrate modified with a freeze-dried toehold switch-based riboregulator [33]. Signal transduction occurs through ribosomal translation of the ***lacZ* gene** to generate active β-galactosidase enzyme. This enzyme generates a colorimetric change from yellow to blue by catalyzing the cleavage of chlorophenol red-β-D-galactopyranoside on the paper substrate. When the target RNA is introduced into the system, the designed toehold region of the riboregulator recognizes the sequence. Hybridization between the target and the toehold induces opening of a hairpin region in the ribosome binding site (RBS). Protein expression is then initiated from the AUG codon, generating the enzyme responsible for the colorimetric change. The color change is directly quantifiable using light-emitting diode (LED) light. This detection strategy has been further extended to point-of-care detection of **Zika virus** with the incorporation of **CRISPR/Cas9** [34]. This approach utilizes a paper substrate and a simple optical transducer, and does not require conventional fabrication of an essential sensing element and a laboratory-based transduction element, achieving a cost-effective detection platform. In addition, the freeze-dried recognition element ensures stable sensing performance over a long period of time, providing a portable integrated sensing platform.

NanoFlare – A Spherical Nucleic Acid (SNA) Gold Nanoparticle (AuNP)

Utilization of nucleic acids for biosensing in human biological matrices commonly faces non-specific nuclease degradation, resulting false-positive/false-negative results. The construction of NanoFlare, an SNA AuNP-based sensing platform, provides nanoparticles coated with a high-density of orientated oligonucleotides with enhanced resistance to degradation (Figure 2B) [35]. The monolayer of single-stranded (ss) DNA (fully complementary to the target sequence) tethered to the AuNP

Glossary

CRISPR/Cas9: clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) constitute an RNA-guided endonuclease that targets its complementary sequence through recognition of protospacer adjacent motif (PAM) by Cas protein and complementarity confirmation by guide RNA.
Circulating nucleic acids (CNAs): DNA/RNA segments that are released from dying cells into the bloodstream.
DNA origami: folding of DNA to construct 2D or 3D shapes through computational design of complementary sequences.
Epithelial cell adhesion molecule (EpCAM): a transmembrane glycoprotein that is involved in multiple cell metabolism processes.
Fluorescence resonance energy transfer (FRET): energy transfer between donor chromophore and acceptor chromophore.
Gas vesicles: a hollow structure made by protein that allows gas permeation.
G-quadruplex: a secondary structure of nucleic acids with guanine-rich sequences.
***lacZ* gene**: part of the *lac* operon, which contains genes for lactose metabolism in *E. coli*. Expression of the *lacZ* gene leads production of β-galactosidase that metabolizes lactose into monosaccharides.
miRNA-21: mammalian microRNA encoded by *MIR21* gene.
Neural recording electrodes: these enable extracellular monitoring of neural activity through the detection of low-frequency local field potential oscillations and high-frequency action potentials of single units.
Plasmonic effect: the interaction between electrons on metal nanoparticles and light.
Phenotypic screening: the identification of biomolecules that induce a desirable change in the phenotype of cells or organisms.
Signal-to-noise ratio: the ratio of specific signal to non-specific signal.
Tau protein: a protein that stabilizes microtubules in central nervous system; Tau is an important biomarker for neurodegenerative

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prehybridizes with a partially complementary ssDNA containing a fluorescent reporter. The activity of the fluorescent dye is quenched due to the proximity of the noble metal. With the presence of the target mRNA, the reporter strand is displaced from the AuNP, releasing the fluorescent signal for target quantification. More impressively, owing to the high-density nucleotide scaffold on the noble metal particles, Nanoflares are able to be taken up across the cell membrane, enabling *in vivo* multiplexed profiling of genetic information [15,36–38]. This sensing strategy delivers a simple nucleic acid-sensing scaffold that can resist enzymatic degradation owing to the steric protection by high-density probes on noble metal nanoparticles. Furthermore, the one-step displacement setup on nanoparticles provides an easily generalizable and programmable platform for different nucleic acid targets.

disorders such as Alzheimer's disease and Parkinson's disease.
Uracil-DNA glycosylase: removes uracil from DNA molecules by cleaving the *N*-glycosyl bond.
Zika virus: a virus transmitted mainly by *Aedes* mosquitoes.

Signal Amplification with Functionalized Nanoantenna

Although SDR-based sensing strategies are inherently sensitive, the nature of optical transduction limits the signal-to-noise ratio. In combination with signal amplification methods using the **plasmonic effect** of nanoantenna, SDR-based sensing platforms can reach unparalleled detection limits. Recently, a **fluorescence resonance energy transfer (FRET)**-based signal amplification strategy successfully converted a single nucleic acid binding event into hundreds of emissions from fluorescent dyes (Figure 2C) [39]. The sensing element is a poly-metyhlmethacrylate-co-methacrylic acid (PMMA-MA)-functionalized nanoantenna loaded with the ion pair rhodamine (R18) (green) and tetrakis(pentafluorophenyl)borate (F5-TPB). R18 acts as the fluorescence energy donor, and F5-TPB prevents the aggregation of R18 to maintain it as an active dye. Cy5-modified nucleotides act as the FRET receptor. The recognition element is a toehold-based half-complementary nucleic acid pair. Hybridization between the exposed toehold and the target strand initiates the branch migration displacement process of the original complement. Therefore, upon introduction of the target sequence, SDR releases the Cy5-modified strand, preventing FRET transmission. However, in the absence of the target, FRET occurs between the R18 and Cy5, and this can be used for quantification. Utilizing the plasmonic effect of metallic nanoparticles, another study presented a **DNA origami**-based optical antenna tethered with a fluorescence-quenching hairpin (FQH) molecular beacon for Zika virus detection [40]. Zika virus DNA displaces the molecular beacon, releasing the fluorescent signal. By incorporating DNA origami to immobilize the nanoantenna and the recognition hairpin, the non-specific signal generated by unspecifically-opened FQH can be discriminated by its location relative to the DNA origami, providing a highly selective detection platform. By utilizing the plasmonic effects of noble nanoparticles, signal amplification can be achieved without construction of a complex sensing interface such as an affinity sensor. Moreover, the adaptability of nanoparticles ensures their wide use in various biosensing systems.

Detection of Proteins

The application of SDRs to the detection of proteins is less straightforward than for nucleic acids. To apply the SDR to protein detection, a specific interaction between the target protein and a nucleic acid sequence is necessary. Generally, a reaction or binding event is necessary to provide an impetus for the displacement process. Such interactions include a change in the conformation of an aptamer [41] or enzymatic cleavage of a specific nucleic acid sequence [42].

Enzyme Recognition Sites for SDRs

Recently, a toehold-mediated SDR was used for the detection of **uracil-DNA glycosylase**, an enzyme involved in the repair of DNA lesions (Figure 3A) [43]. Detection of this enzyme is based on a cascade reaction that generates a **G-quadruplex** that in turn binds to the fluorophore *N*-methyl-mesoporphyrin (NMM). Uracil-DNA glycosylase removes two uracil bases incorporated into the ssDNA probe sequence, generating two apurinic/apyrimidinic sites, which are now mismatched bases. With mismatches present, the ssDNA probe does not undergo strand displacement with a hairpin probe. Instead, this ssDNA directly hybridizes to the reporter probe, generating a G-quadruplex. In the absence of the enzyme target, the probe undergoes SDR with the hairpin probe, preventing hybridization with the reporter. It is worth noting that this detection strategy is based on the conversion of protein activity into a DNA hybridization-based readout and should be generalizable [44].

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Key Figure

Strand Displacement Reactions (SDRs) Applied to Biosensors

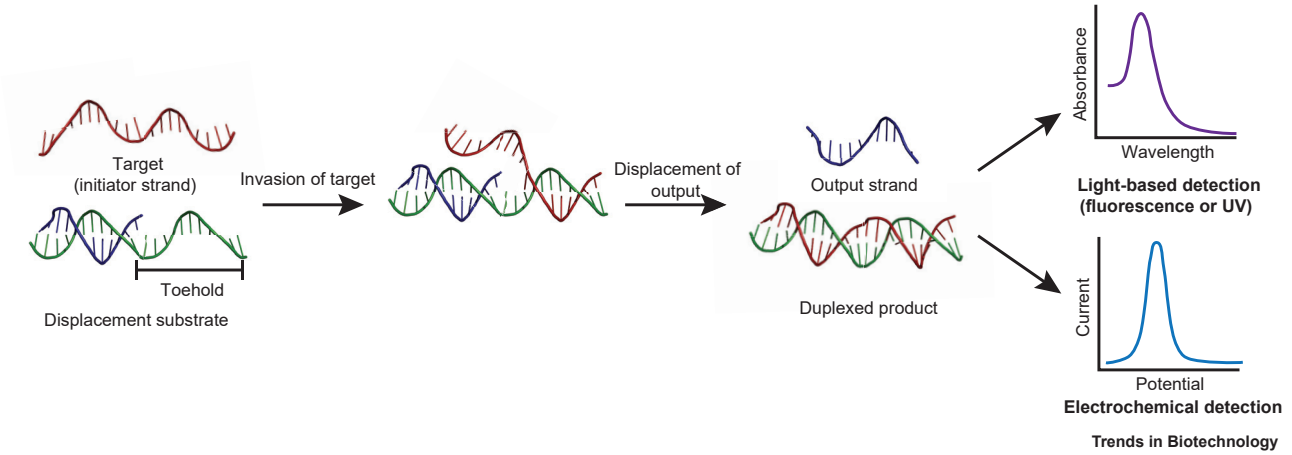

Figure 1. The SDR commences with a target (initiator strand) and a displacement substrate (containing two partially complementary strands). Invasion of target onto the toehold region of the substrate leads to strand migration and eventual displacement of the output strand from the substrate. The initiator is now hybridized to the toehold-containing strand of the substrate, and the output strand is released for detection. Generally, biosensor-based detection using SDRs have either optical or electrochemical readouts.

Relying on a similar sensing strategy, researchers have developed a sensor for telomerase, an enzyme marker of bladder cancer [45]. This sensor is based on changes in the UV absorption characteristics of AuNPs as a result of changes in their surface plasmon resonance. SDRs occurring on the surface of AuNPs cause changes in the aggregation of the particles, leading to variations in average diameter and UV absorbance. The SDR is triggered by the reaction between a specific DNA probe sequence and the telomerase. This platform successfully detected bladder cancer from human urine samples [46]. As in the aforementioned examples, detection principles based on the conversion of enzymatic activity into nucleic acid hybridization events are generalizable to nearly any enzyme with a specific nucleic acid substrate.

Recognition Element-Linked ssDNA

Another method for utilizing an SDR for protein detection is to conjugate a recognition element for the protein of interest directly to an ssDNA sequence. Recently, a detection strategy was reported based on a shift in the equilibrium of an SDR caused by the binding of a target protein to a ligand-conjugated nucleic acid sequence (Figure 3B) [47]. In the absence of the target, two strands (A and B), each conjugated with an orthogonal recognition molecule for protein targets, reach a dynamic equilibrium with strand S. However, in the presence of the target, the equilibrium shifts to hybridization between the A strand and the S strand. This hybridization event is detectable by changes in the FRET signal. Biotin-conjugated DNA strands were used for the specific detection of streptavidin. A fluorescent signal is generated when streptavidin binds to the biotin on strand B, increasing the formation of the AS duplex. The AS duplex brings together a fluorescent donor and acceptor, thus enhancing the FRET signal that provides a quantitative measurement of the target protein concentration. This platform enabled the detection of streptavidin with a real-time FRET response.

Antibody Detection

Antibodies are key biomarkers of disease because they are some of the best physiological indicators of the human immune response [48,49]. Owing to the exceptionally large size of a typical antibody compared to other proteins, in addition to their relative instability, the specific detection of

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Target	Detection limit	Sensing element	Recognition element	Transduction element	Refs
Ebola virus	nM	Paper	Riboregulator-based toehold switch	Expression of the *lacZ* gene induces a color change	[32]
Zika virus	fM	Paper	Riboregulator-based toehold switch + Cas9	Expression of the *lacZ* gene induces a color change	[34]
mRNA	pM	Nanoflare	Complementary nucleic acid	Cyanine 5 fluorophore	[35]
ssDNA encoding survivin	pM	Nanoantenna	Complementary nucleic acid	FRET: F5-TPB + R18 pair	[39]
Zika virus	nM	Nanoantenna	Hairpin DNA	ATTO 647N fluorophore	[40]
Uracil-DNA glycosylase	nM	Homogeneous assay	DNA	N-methyl mesoporphyrin IX (NMM) fluorophore	[43]
Telomerase	pM	AuNPs	DNA	Optical	[45]
Streptavidin	nM	Homogeneous assay	Small molecule	FRET: Alexa647 + 555 pair	[47]
HIV antibody	nM	Homogeneous assay	Antibody-initiated formation of the displacement setup	Fluorophore + quencher	[50]
Enantiomeric molecules	μM	Homogeneous assay	Reciprocal chiral aptamer	Fluorophores	[58]
MicroRNA-21	fM	NCS/MoS_2 electrode	Target-initiated SDR	Ferrocene	[75]
MicroRNA-155	fM	AuNPs@Cu-MOFs + gold electrode	Target-initiated SDR	Glucose	[76]
MicroDNA-21	fM	Gold electrode	Target-initiated SDR + endonuclease nicking	Ferrocene	[77]
DNA	fM	Indium tin oxide electrode	PNA	Ferrocene	[79]
EpCAM	nM	Gold electrode	Aptamer-based displacement substrate	Methylene blue	[81]
Anti-Dig	nM	Gold electrode	Digoxigenin (Dig) epitope	Methylene blue	[84]
Tau	nM	Gold electrode	Peptide-based inhibition sequence	Ferrocene	[88]

Table 1. Summary of Selected Biosensing Strategies

antibodies can be a challenge. Thus, conventional SDRs are insufficient for the detection of this class of biomarkers. Recently, however, a clever construct was developed for strand displacement-based antibody detection that relies on the unique 'Y' shape of antibodies as a displacement scaffold (Figure 3C) [50]. In this assay, the antigen for the target antibody is conjugated to ssDNA strands. When the antibody target is present, one antigen conjugated to the input ssDNA sequence binds to each of the two antigen binding sites (Fab) on the immunoglobulin G. This binding increases the local concentration of the ssDNA sequence significantly. The SDR is then initiated through binding to an exposed loop region in a hairpin sequence. One end of the hairpin is modified with a fluorophore moiety, and the distal terminus is modified with a quencher molecule. Upon opening of the hairpin in the presence of the antibody target, the quencher moves away from the fluorescent probe,

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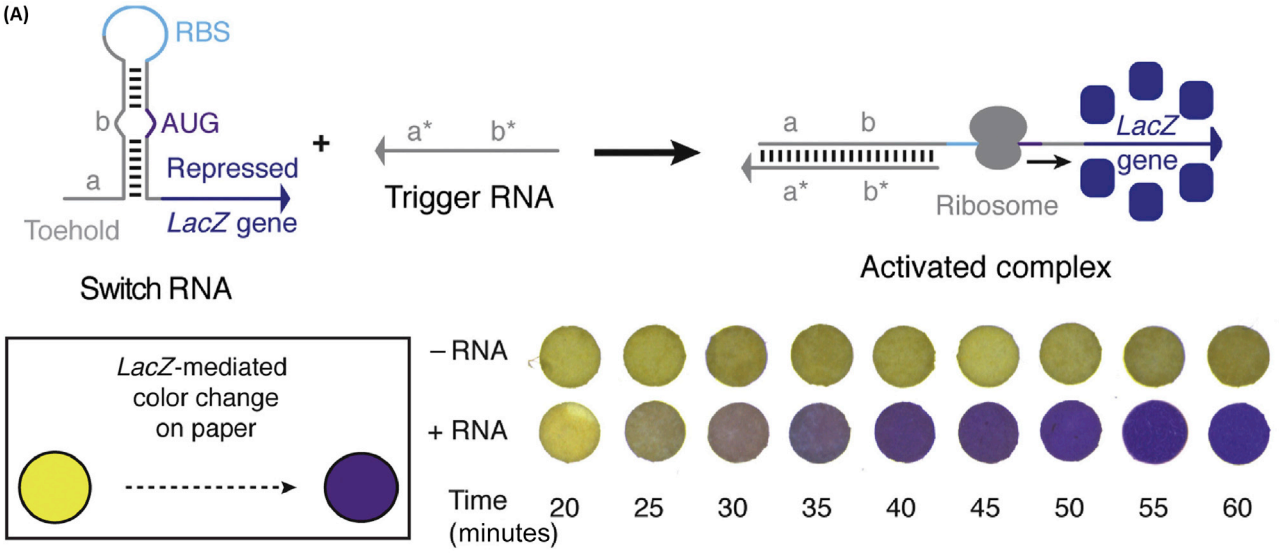

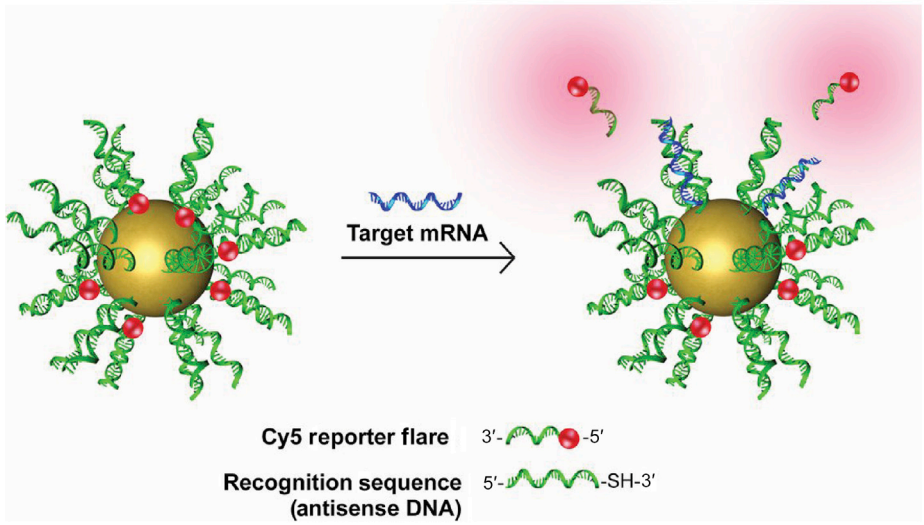

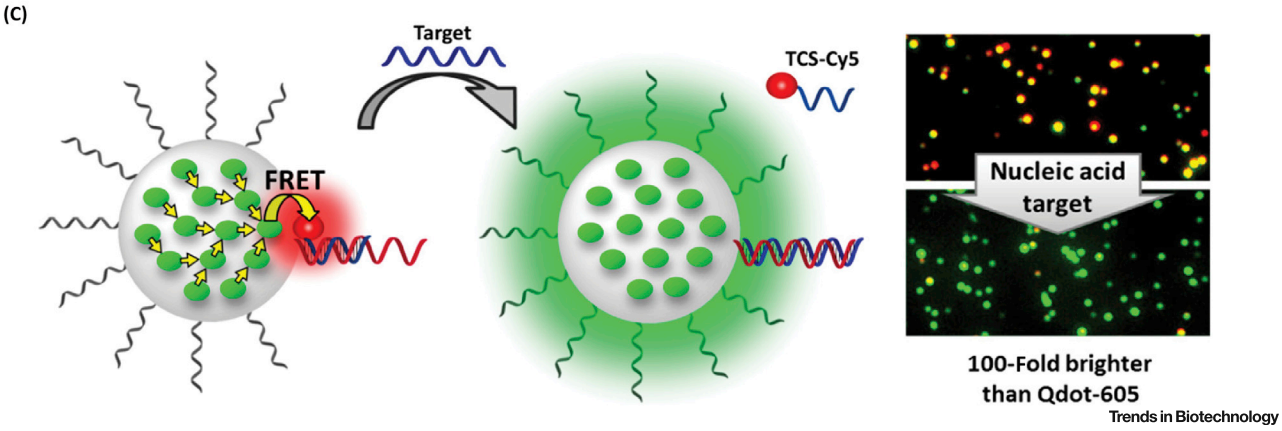

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increasing the fluorescent signal. The design of the SDR sequences for this sensor is such that, in the absence of the antibody, the hybridization efficiencies of the antigen-modified ssDNA strands are low. Therefore, without the antibody scaffold, these sequences would not hybridize efficiently.

As demonstrated in two aforementioned examples, the advantage of the conjugated protein–nucleic acid sensing setup is that it utilizes the natural protein-binding pairs (e.g., antibody–antigen), therefore eliminating the need to discover new protein–nucleic acid interaction pairs. However, such protein–nucleic acid conjugation pairs might lead to decreased thermal stability of the constructed double-strand (ds) DNA displacement setup, leading to potential false-positive results.

Aptamer-Based Displacement to Detect Small Molecules

Aptamers are short, ssDNA and ssRNA sequences that bind to a particular target [51–54]. They have recently garnered significant interest because of their specificity and versatility [55–57]. Importantly, both enantiomers (L and D) of a particular DNA sequence are readily accessible artificially. This feature has enabled the application of aptamer-based SDRs to detect the enantiomeric excesses of small molecules (Figure 3D) [58]. Two aptamers with identical sequences but of opposite chirality, each modified with a unique fluorophore, form the basis of this sensor. A partial complement modified with a quencher is hybridized to each aptamer. Upon small-molecule binding to one enantiomer of the aptamer, the quencher-modified sequence for one chirality is released. Because orthogonal fluorophores are used, enantiomeric excess can be determined by the relative intensity of each of the fluorophores. Importantly, the simultaneous operation of dual displacement cascades enabled multiplexed biosensing in a single sample.

Optical transduction for SDR-based sensors is a powerful tool. This method enables real-time monitoring of the concentration of a nucleic acid, protein, or small-molecule target in a crude sample matrix. The combination of the dose-dependent nature of SDRs and the direct signal transduction mechanism of optical techniques enables rapid target analysis, satisfying the requirement for quick turnaround time in clinical applications. In addition, multiplexing sensors is relatively simple because all that required is orthogonal fluorophores and unique SDR cascades. Despite all of the benefits, challenges do remain. In many cases, to achieve high sensitivity, relatively complex amplification systems are necessary, especially to generate sufficient signals for FRET-based measurements, which might lead to an increase in sensor fabrication cost. Furthermore, careful selection of the FRET donor and acceptor pair is necessary to prevent emission signal bleed-through, resulting in inaccurate measurement. In addition, optical devices typically require costly instruments for signal transduction. Therefore, future efforts to develop low-cost, portable optical transduction devices will facilitate development of these sensors for point-of-care applications.

Electrochemical Biosensors

Electrochemical readout is one of the best-established and most reliable technologies for point-of-care sensors, and the electrochemical glucose sensor sets the standard for such devices [59,60]. These biosensors have continued to increase in popularity over the past decade [19,42,54,61–71]. Their continued success can be attributed to the simplicity and low cost of the instrumentation required [66]. However, limitations remain for some types of electrochemical biosensors, such as affinity-based immunosensors, which require a lengthy target absorption process by the recognition element onto the sensing element.

Figure 2. Optical Nucleic Acid Detection Using Strand Displacement.
(A) Paper-based synthetic riboregulator for RNA detection. Target RNA initiates the synthetic riboregulator through a toehold-mediated migration process, opening the ribosome binding site and leading to *lacZ* translation. The *lacZ* gene product catalyzes an enzymatic reaction on the paper substrate with a color change from yellow to blue as the signal output. Image adapted, with permission, from [32]. (B) NanoFlare is constructed by a target complementary DNA for target capture and a Cy5 fluorophore-conjugated DNA reporter. The presence of an mRNA target leads to displacement of the DNA reporter, producing a fluorescent signal. Image adapted, with permission, from [35]. (C) Additional signal amplification can be obtained by the incorporation of fluorescence resonance energy transfer (FRET) into SDRs. A target nucleic acid displaces the Cy5 (FRET receptor)-linked nucleic acid from a nanoantenna, decreasing FRET transmission from the donor to the acceptor. This changes the overall color of the nanoantennae (right), enabling enhanced detection of the target nucleic acid. Image adapted, with permission, from [39]. Abbreviation: RBS, ribosome binding site; TCS, target-competitive sequence.

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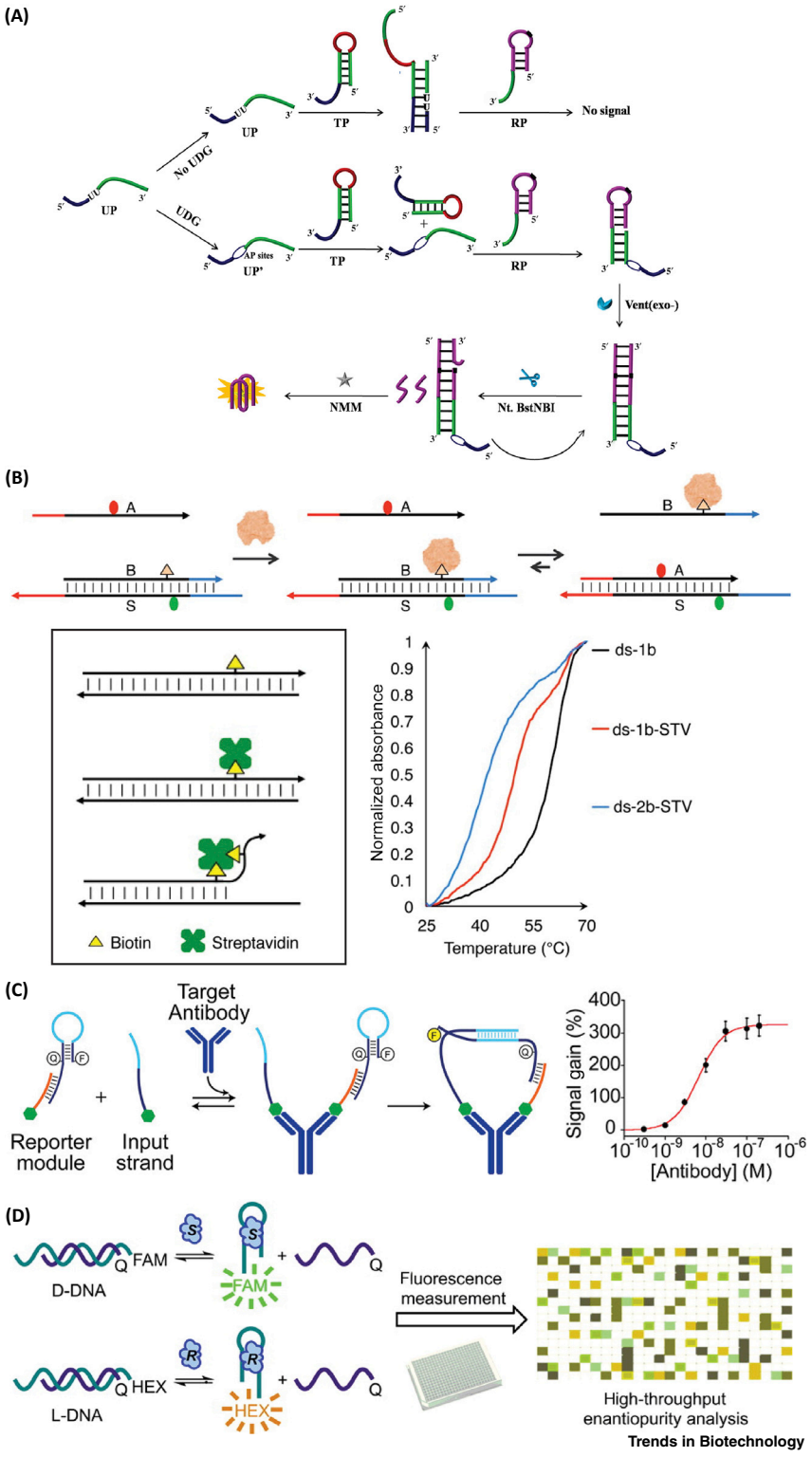

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Significant interference occurs from other molecules in complex sample matrices during this absorption process, resulting non-specific absorption of biomolecules from body fluids to electrode surfaces. Hence, these unspecific immobilized molecules would significantly disrupt electron transfer during the indirect electrical quantification process. However, SDRs typically integrate the recognition and transduction processes together, providing rapid and direct target-initiated dose-dependent responses. Therefore, applying SDRs to electrochemical platforms can circumvent many of these time-dependent issues, enabling progression of these platforms to point-of-care applications [61,72,73].

Target-Induced Strand Displacement Cycle

Some sequences of **circulating nucleic acids (CNAs)**, short DNA and RNA sequences in the blood, are markers of cancer [25]. The use of CNAs as indicators of malignancy progression provides a minimally invasive and rapid method to monitor the health of a patient without requiring numerous, painful biopsies. However, in most cases, the CNAs of interest are present in only trace amounts in the blood (<pM) [25], limiting the detection strategies that can be applied. Often, sensors cannot achieve this required sensitivity without external amplification methods [31] which can lead to false-positive results [74]. Construction of an SDR-based cyclic cascade circumvents external enzymatic amplification, providing an accurate strategy for the detection of such low-abundance species.

One of the simplest methods to construct a displacement reaction is the use of a hairpin structured probe [52], a single unit that undergoes a conformational change upon displacement. A recent study applied SDRs to the electrochemical detection of **miRNA-21** based on the application of multiple hairpin probes (Figure 4A) [75]. miRNA-21 is often upregulated in solid tumors and is also implicated in the development of cardiac disease [28]. This sensor, based on an *N*-carboxymethyl chitosan (NCS) MoS_2 electrode, involves the capture of a ferrocene-modified DNA strand. An SDR-induced duplex is captured by a probe tethered to the electrode. The SDR cascade is designed based on the opening and subsequent hybridization of two DNA hairpins. miRNA-21 forms a complex with one hairpin (HDNA1). A second ferrocene-modified hairpin, HDNA2, disrupts the HDNA1–miRNA-21 complex, displacing the target from the HDNA1. The HDNA1–HDNA2 duplex is captured by the tethered probe on the electrode, and quantification is possible based on the amount of ferrocene signal. This technique is sufficiently sensitive to detect microRNA in cell lysates. Similar target-induced strand displacement cycling has been applied to AuNP-modified copper metal organic frameworks for the detection of microRNA [76]. The advantage of this type of sensing setup is that the displacement reaction can be first performed in a test tube, limiting the possibility of contaminating the electrode surface with non-specific molecules by reducing the on-electrode incubation period.

Figure 3. Strand Displacement Reactions (SDRs) for Detecting Proteins and Small Molecules.
(A) An enzyme target generates the initiator strand for the SDR in this optical biosensor. The uracil-DNA glycosylase (UDG) target creates mismatches on the UP strand, preventing a SDR from occurring with the TP strand. This facilitates hybridization with the RP strand, generating a detectable optical signal. Image adapted, with permission, from [43]. (B) Nucleic acids modified with the ligand for the target protein serve as initiators for this SDR. Protein binding induces a shift in the displacement equilibrium. In one example, streptavidin (STV) binds to the biotin-conjugated nucleic acid sequence, decreasing the melting temperature (Tm, thermal stability) of the double-stranded (ds) DNA. Upon STV binding to two biotins, the thermal stability of the dsDNA is further diminished, providing an exposed region at which the SDR can occur. Image adapted, with permission, from [47]. (C) The target antibody acts as a scaffold to enhance the SDR rate. Antibody recognition motifs are conjugated to the reporter module and the input strand. Binding of the antibody to these antigens brings the reporter module and the input strand into proximity with each other, increasing the probability of SDR initiation. Image adapted, with permission, from [50]. (D) The enantiomeric excess of small molecules can be determined using enantiomers of a DNA aptamer specific for the small-molecule target. The small molecule initiates the SDR based on the chirality of the aptamer (either L or D). A florescent probe and a quencher molecule are both tethered to the displacement substrate. The SDR initiated by the aptamer releases the quencher, generating a fluorescent signal. This method is compatible with high-throughput analysis performed in 96-well plates. Image adapted, with permission, from [58]. Abbreviations: FAM, carboxyfluorescein; HEX, hexachloro-fluorescein; NMM, *N*-methyl-mesoporphyrin; Nt. BstNBI, nicking endonuclease; Q, quencher; RP, reporter probe; STV, streptavidin; TP, hairpin probe; UP, single-stranded DNA probe; Vent(exo-), polymerase.

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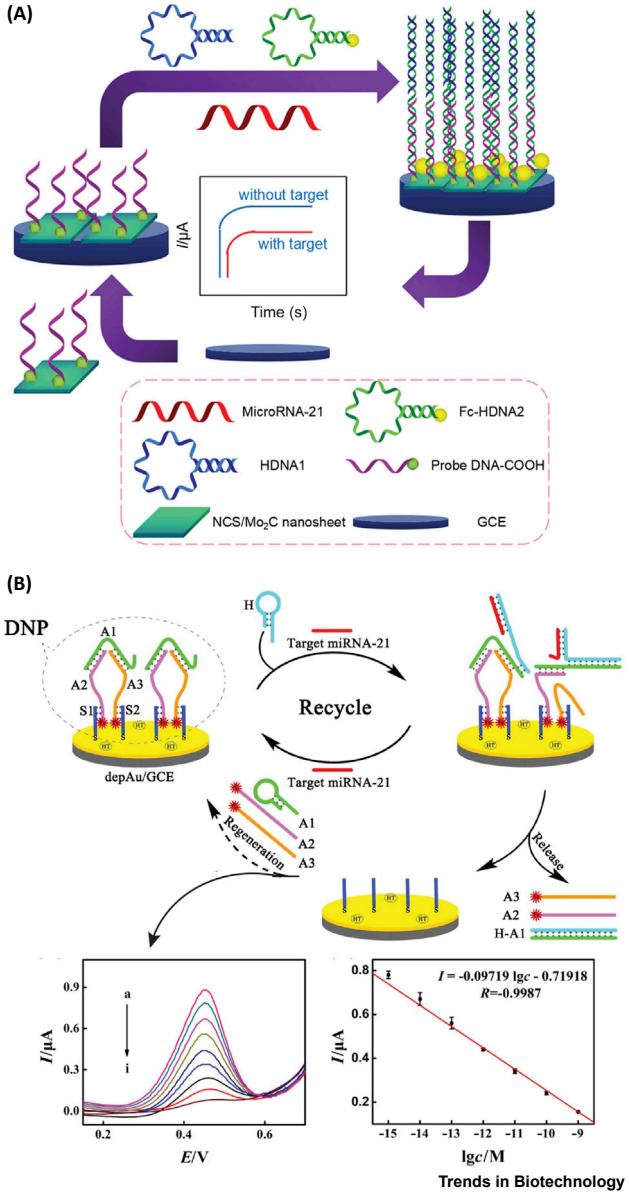

Figure 4. Electrochemical Strand Displacement Reactions (SDRs) to Detect miRNA-21.
(A) The miRNA-21 target initiates an SDR to generate an electrochemical signal. The miRNA opens the HDNA1 hairpin. The Fc-HDNA2 strand can then displace the target RNA from the duplex, allowing it to participate in another cycle of target–HDNA1 binding. The HDNA1–HDNA2 duplex then hybridizes with the probe strand on the sensor surface. Quantification is achieved from the electrochemical signal of the ferrocene probe. Image adapted, with permission, from [75]. (B) The miRNA-21 target initiates an SDR cycle to change the surface signaling probe scaffold as a signal-off platform. A target RNA molecule opens the H hairpin probe, which initiates displacement of the signaling duplex from the surface. The sequences involved in the SDR (A1, A2, and A3) can be replenished, leading to cyclic signal amplification. Image adapted, with permission, from [77]. Abbreviations: depAU, deposited gold; DNP, DNA nanoprobe; GCE, glassy carbon electrode; NCS, *N*-carboxymethyl chitosan.

In another recent example of miRNA-21 detection, an SDR was designed as a cyclic system for signal amplification on an electrochemical platform. Application of a toehold SDR to an electrode surface enables enzyme-free target recycling for amplification (Figure 4B) [77]. A 2D DNA nanoprobe is

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constructed on a gold-coated glassy carbon electrode. The presence of the miRNA-21 opens the hairpin probe (H), initiating the displacement of the A1 strand from the toehold area. Hybridization of A1 to H releases the signaling construct (strands A2 and A3 are conjugated with ferrocene), as well as the target strand. The release of the ferrocene-modified strands decreases the electrochemical signal. In addition, because the target strand is also released at this point, another round of release can be initiated. This inherent signal amplification strategy resulted a femtomolar detection limit. The construction logic of the 2D DNA scaffold can be generalized for signal amplification.

Another type of recognition element widely applied for the electrochemical detection of nucleic acids is the peptide nucleic acid (PNA), in which the charged deoxyribose phosphate backbone is substituted with a non-charged pseudopeptide polymer [78]. The neutrally-charged PNA possesses enhanced binding affinity for complementary DNA/RNA because the electrostatic repulsion has been eliminated. The target DNA triggers a change of charge, and a significant change in the electrochemical current can be obtained in the presence of the target [79]. Based on a similar design, with the incorporation of a DNA polymerase, a sequential multiple-cycle PNA-based SDR was utilized to further increase the detection sensitivity [80].

Detection of Proteins

SDRs are also especially useful for the electrochemical detection of protein targets based on the two modes of interaction discussed in the section on optical methods for protein detection. Recently, an electrochemical biosensor for detecting the **epithelial cell adhesion molecule** EpCAM was developed. EpCAM is a transmembrane glycoprotein with oncogenic potential. Detection occurs through an EpCAM-induced aptamer-based SDR (Figure 5A) [81]. A gold electrode is coated with an ssDNA hairpin probe (Hp1) to capture the signaling probe (Hp2), which is modified with an electroactive methylene blue molecule. A displacement substrate with a target-specific aptamer region and a partially complementary strand (probe a) facilitates detection. The aptamer region of the displacement substrate binds to EpCAM, triggering the displacement and release of probe a. This release triggers the opening of Hp1, which hybridizes to the methylene blue-modified Hp2, generating the electrochemical signal.

SDR Based on a Target-Induced Change in Thermal Stability

Antibodies can also be detected using antigen-modified nucleic acid sequences incorporated into SDRs [82,83]. Recently, an on-chip electrochemical SDR cascade was applied to antibody detection (Figure 5B) [84]. A specific domain of the antigen, an epitope, is used to capture the antibody through conjugation to a displacement strand. A signal is generated from a methylene blue molecule conjugated to a ssDNA probe (SP), which acts as an SDR initiator. dsDNA (AP + DP) is immobilized on a gold electrode surface as the displacement substrate. The SDR is triggered by antibody binding to the dsDNA, which decreases the thermodynamic stability of the duplex, forcing a shift in the SDR equilibrium. In contrast to conventional electrochemical immunosensors, which typically function as a signal-off assays, SDR-based biosensors are signal-on assays, and thus ensure specificity and larger dynamic ranges for detection [85]. This one-step detection setup can also be applied to any antibody–antigen pair if the initial displacement substrate possesses high thermal stability.

The major advantage of an electrochemical biosensor is its cost-effective transduction system, which makes the whole detection system deployable and accessible globally. With the incorporation of SDRs into the electrochemical biosensing system, non-enzymatic and highly sensitive detection can be achieved with a rapid turnaround time. Moreover, in contrast to conventional affinity-based electrochemical biosensors, SDRs can be utilized as a sample treatment tool to first allow target-initiated dose-dependent generation of the designed output. The output can then be captured by an electrode and translated into an electrochemical signal, thereby diminishing potential surface-fouling effects by reducing the electrode incubation period required. Although the design and operation of orthogonal SDR cascades make multiplexed detection much easier to achieve as compared to conventional affinity biosensors, a high degree of multiplexed sensing is still limited by the number of redox molecules that are available. Moreover, application of controlled surface molecule immobilization can facilitate

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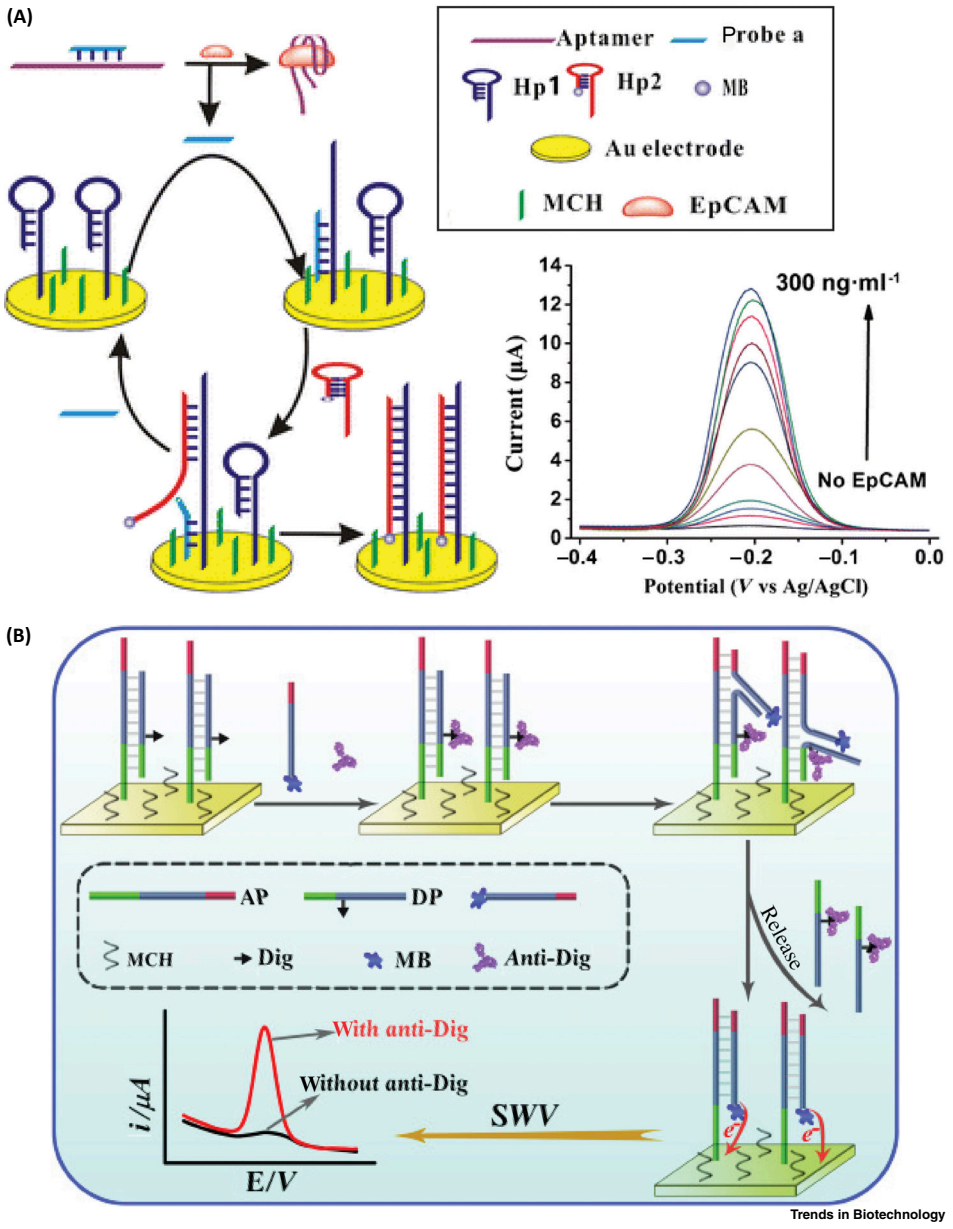

Figure 5. Strand Displacement Reactions (SDRs) to Electrochemically Detect Proteins.
(A) An epithelial cell adhesion molecule (EpCAM) protein target initiates aptamer-based SDR as a signal-on platform. Binding of the EpCAM releases probe a, which opens the Hp1 hairpin on the electrode surface. The opened Hp1 hybridizes with the methylene blue-modified MB-Hp2, producing an electrochemical signal based on the release of probe a by EpCAM. Image adapted, with permission, from [81]. (B) Epitope-conjugated DNA is used to shift the equilibrium of an SDR for an antibody-initiated SDR. A digoxigenin (dig)-conjugated DP strand recognizes the anti-dig antibody target, decreasing the thermal stability of the AP–DP duplex. A methylene blue-modified strand (MB-SP) displaces the DP strand from the duplex, producing an electrochemical signal. Image adapted, with permission, from [84]. Abbreviations: AP, assistant probe; DP, DNA probe; MCH, mercaptohexanol; SP, DNA signal probe; SWV, square wave voltammetry.

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the autonomous and reproducible generation of electrochemical biosensors [86], significantly increasing the applicability of electrochemical biosensors as point-of-care systems.

Concluding Remarks and Future Perspectives

Peptide-Based SDRs

Most current SDR technologies rely on nucleic acids. However, nature has provided another class of programmable biopolymers, based on amino acids, which are more functionally diverse than nucleic acids. Developing SDRs with peptides combines the functionality of amino acids with the controllability of the SDR. To date, fairly simple coiled-coil-based peptide SDRs have been demonstrated [87]. Very recently, a biosensing motor based on a peptide displacement reaction for **Tau protein** detection was developed through the combination of a coiled-coil peptide and a short Tau-capturing (inhibition) peptide as a displacement scaffold that is only initiated in the absence of Tau [88]. Displacement resulted in an electrochemical signal that was used as a readout of Tau concentration. In a similar manner to nucleic acid-based SDRs, we envisage that peptide-based SDRs will provide an important tool for developing the next generation of biosensors.

SDRs as Central Commands

SDRs address many of the most crucial challenges for biosensors, including issues with multiplexing, signal amplification, and signal-to-noise ratios for low-abundance targets. They additionally have unparalleled versatility in the design and control of cascades of these reactions. These advantages suggest that SDRs have potential in the operation of complex logic gates for biosensing [89,90]. For example, instead of simple detection of two or three biomarkers, orthogonal SDRs can be applied as computational commands for profiling a large number of biomarkers for specific diseases or **phenotypic screening**, achieving high-throughput biosensing (see Outstanding Questions). High-throughput multiplexed biosensing also leads to questions regarding the design of various distinguishable signaling probes and enhancement of the kinetics of SDR. Furthermore, SDRs can be designed to control novel biological systems. For example, CRISPR/Cas systems have been recently used for biosensing owing to their high specificity [91–94]. Integrating SDRs into the design of guide RNAs for CRISPR target recognition would enable the generation of a switchable biomachine, affording a more dynamic tool for CRISPR applications. Moreover, incorporation of SDR biosensing systems with novel imaging and probing systems, such as **gas vesicles** for ultrasound imaging [95–97] and **neural recording electrodes** [98,99], may allow these systems to obtain spatial biological information in living organisms, promoting our understanding of cellular and neural functions. In general, SDR can be widely used as a command tool to enhance the programmability and functionality of various biological systems.

Overall, SDR-based biosensing systems have demonstrated outstanding performance in the detection of important biomarkers of disease. The selected examples present improvements over currently-available detection methods either through enhanced biosensing performance or by providing a new capability that is not achievable by conventional techniques. With future efforts focused on the development of more sophisticated SDR circuits, we anticipate the construction of a revolutionary next-generation biosensing system through an SDR network. Combined with the ease of use of SDR-based systems, point-of-care biosensors based on these reactions are on the horizon.

Outstanding Questions

Will SDR-based technologies be applicable to high-throughput disease biomarker profiling beyond simple detection?

What are the best strategies to achieve multiplexed, orthogonal detection of multiple biomarkers simultaneously?

How can we design SDR reactions to be more rapid?

How can SDR be integrated into novel sensing or imaging techniques?

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