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Perspective

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Perspective on the Future Role of Aptamers in Analytical Chemistry

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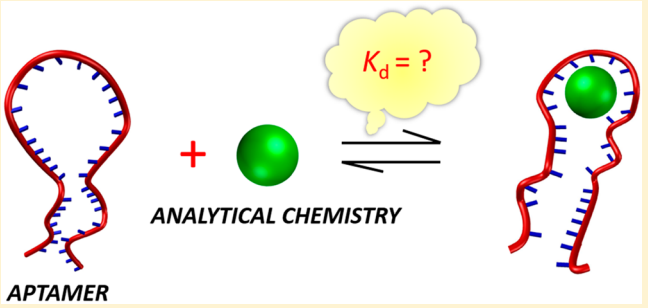
Supporting Information

ABSTRACT: It has been almost 30 years since the invention of Systematic Evolution of Ligands by Exponential Enrichment (SELEX) methodology and the description of the first aptamers. In retrospect over the past 30 years, advances in aptamer development and application have demonstrated that aptamers are potentially useful reagents that can be employed in diverse areas within analytical chemistry, biotechnology, biomedicine, and molecular biology. While often touted as artificial antibodies with an ability to be selected for any target, aptamer development, unfortunately, lags behind development of analytical methodologies that employ aptamers, hindering deeper integration into the application of analytical tool development. This perspective covers recent advances in SELEX methodology for improving efficiency of the SELEX procedure and enhancing affinity and specificity of the selected aptamers, what we view as a critical barrier in the future role of aptamers in analytical chemistry. We discuss postselection modifications that can be used for enhancing performance of the selected aptamers in an analytical device by including understanding intermolecular interaction forces in the binding domain. While highlighting promising properties of aptamers that enable several analytical advances, we provide discussion on the challenges of penetration of aptamers in the analytical field.



Nucleic acid aptamers are short, single strands of RNA or DNA molecules with a stable three-dimensional (3D) structure selected by a well-known process, Systematic Evolution of Ligands by Exponential Enrichment (SELEX).[1,2] In this process, an initial oligonucleotide library (~10^{12}−10^{15} sequences), tailored by flanking primer-binding regions on 5′ and 3′ ends of a randomized region (30- to 50-mer nucleotide), is designed for selection.[3] Typically, selection is performed in a binding buffer containing common monovalent and divalent ions (Na^+, K^+, Mg^{2+}, Ca^{2+}, etc.) to screen the negatively charged phosphate backbone of the oligonucleotides. As such, secondary or tertiary oligonucleotide structural motifs, including G-quadruplexes, stem-loops, pseudoknots, kissing complexes, three-way junctions, bulges, and hairpins,[4−8] are promoted to recognize target molecules through intermolecular interactions including hydrogen bonding, electrostatic interactions, van der Waals forces, $\pi-\pi$ stacking, etc.[9−12] Subsequently, the bound sequences are separated from the unbound sequences, eluted, and amplified by polymerase chain reaction (PCR). Iterative rounds of selection-amplification cycles are performed to enrich the oligonucleotide pool until desired target binding activity dominates the pool.

When considering aptamers as affinity reagents, intuitively, the counterpart, antibodies, come to mind, of which a rich analytical history exists. Discovered almost a century later than antibodies, aptamers have not yet fully transitioned from proof-of-concept demonstrations to clinical applications. As diagnostic and research reagents, antibodies have provided clinicians and researchers an extraordinarily powerful and ubiquitous tool in a variety of medical and scientific disciplines through the use of, for example, enzyme linked immunosorbent assays (ELISAs) among others. This in part due to the capability of antibodies to bind specific epitopes present on carbohydrates, nucleic acids, proteins, or cell membranes.[13−15]

When compared to antibodies, synthetic affinity ligands such as aptamers are relatively straightforward and inexpensive to synthesize, display prolonged shelf life, are stable under nonphysiological conditions, provide high batch-to-batch reproducibility, and offer flexible modification for improved functionality. Given these merits, aptamers display additional advantages in diagnostics for specific and sensitive detection of target molecules over their analogue antibodies especially when the analytes are small molecules (<~1700 Da). For instance polydisperse polysaccharides in animal tissue,[16] glycosaminoglycans (GAGs), are nonimmunogenic and thus it has been challenging to harvest antibodies that display high affinity and specificity against GAGs. On the contrary, RNA aptamers against two unmodified GAGs, heparosan and chondroitin,

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respectively, were selected successfully through bead-based SELEX.[17] Furthermore, aptamers for small molecules recognition have been selected and used for monitoring a variety of other small molecules.[18−24] Since the invention of SELEX in the early 1990s, hundreds of aptamers have been selected. Typically, aptamers have been categorized based on their binding partner in the following five categories of targets: proteins, small molecules, cells, viruses, and bacteria. It should be noted here, however, that "cell," "bacteria," and "virus" binding aptamers is a misnomer, as the aptamer is likely specific to some entities (e.g., protein, carbohydrate, etc.) expressed on the particle surface. Thus, identifying these entities is crucial for improving specificity of the aptamers toward these categories of targets and wider applications of the aptamers. Nonetheless, aptamers are typically classified into these categories. Here we list the most well-studied aptamers based on the number of articles employing the listed aptamers (Table S1). Readers are encouraged to read refs 25−29 for more comprehensive lists of aptamers.[25−29]

Model aptamers, such as the thrombin-binding, streptavidin-binding, adenosine triphosphate (ATP)-binding, and cocaine-binding aptamers, are significant for demonstrating proof-of-concept aptamer-based analytical methods. However, with the overwhelming number of publications utilizing such a small set of aptamers, questions arise such as what challenges hinder or prevent the analytical aptamer community from integrating hundreds of existing aptamers into analytical platforms for real-world applications?

APTAMER SELECTION STRATEGIES

In 1990, the SELEX method was described for the first time by Tuerk and Gold while exploring the interaction between the bacteriophage T4 DNA polymerase and its messenger mRNA.[1] When randomizing one eight-base region of the mRNA, two different sequences displaying equivalent binding constant toward the T4 DNA polymerase were selected from a random pool of approximate 65 536 species. Concurrently the term aptamers, borrowed from the Latin word "*aptus*" and the Greek word "*mers*", were first introduced by Ellington and Szostak who isolated RNA molecules capable of creating binding "pockets" for organic dyes.[2] From then on, aptamers have been known as short single-stranded nucleic acid sequences that are selected through a directed evolution process for binding to a specific target with high affinity. One critical hurdle to the broader penetration of aptamers in analytical chemistry is specificity or how well the aptamer binds a specific target analyte. The following discusses how the SELEX procedure has advanced to tackle this very issue.

Brief Introduction to Conventional Aptamer Selection: SELEX. In order to yield aptamers with high specificity and affinity to target molecules, the SELEX method involves steps of iterative binding, partitioning, dissociation, and amplification. Despite that more than 32 variants of SELEX process have been described in the past 3 decades, the fundamental steps of the procedure remain in most variations. Among these steps, immobilizing target molecules on a solid phase such as magnetic beads, glass coverslips, agarose-based resin, etc. is a common strategy that is used to enhance efficiency of the selection of aptamers for small molecules including drugs, toxins, antibiotics, and molecular markers.[30]

When performing SELEX, the choice of nucleic acid library is the first of many steps that can be tailored to address the downstream application of the selected aptamer. In the first decade of implementing SELEX technology, RNA libraries were used more frequently than DNA libraries, as the general belief was more structural motifs could be generated from RNA.[31] Despite RNA libraries having been wildly used in the early stage of SELEX, their stability and additional cost on extra modifications largely restrict wide applications of RNA aptamers. As a result, DNA libraries are being preferentially used in SELEX studies since 2008.[32] In the initial library, $\sim 10^{12}-10^{15}$ nucleic acids containing a randomized region and two primer-binding sequences are exposed to the target. Through positive selection, the unbound nucleic acids are removed from the bound and those bound sequences are eluted for counter selection. During this process, structurally similar nontarget compounds are introduced to incubate with the eluted bound sequences to effectively discriminate nonspecific nucleic acids. The recovered sequences are amplified by PCR (DNA SELEX) or reverse-transcription PCR (RNA SELEX). Enriched PCR products are applied in the next cycle of selection. To obtain the selected nucleic acids to display high affinity and specificity toward the target, iterative SELEX cycles are performed (Figure S1). Ultimately, the success of the conventional SELEX process depends heavily on the isolation of the bound nucleic acids from the unbound.

Improving Performance of Aptamers Through New Selection Strategies. The major disadvantages of conventional SELEX rest with tedious selection cycles, requirements for large quantities of reagents, or the selected aptamers display low affinity and specificity. On the basis of the fundamental steps of conventional SELEX, current SELEX technology has evolved significantly by integrating advancements achieved in disciplines such as synthetic chemistry, material science, engineering, molecular biology, and bioinformatics to improve efficiency of the SELEX procedure and enhance performance of the selected aptamers.

The limited chemical diversity originating from the finite combinations of nucleotides brings challenges for conventional SELEX. A selected aptamer normally comprises 10−40 nucleotides of 4 different nucleotide species.[33] In contrast, the binding sites of an antibody consist of 110−130 amino acids, and each amino acid could be 1 of the 20 different amino acids.[34] In view of the possible combinations, structure variations formed by aptamers are far from comparable to their proteinaceous counterpart antibodies. Perhaps the less chemical diversity of aptamers lowers the success rate of developing high-performance aptamers.[35] Improving the SELEX method typically focuses on four aspects: reduce the time for aptamer development,[36−40] incorporate novel designs and functions prior to the SELEX process for improving chemical diversity,[41,42] increase the selection stringency,[43,44] and improve the process throughput of aptamers (Table S2).[45−47]

Presumably, through increasing the interactions of DNA/RNA libraries with targets, higher affinity aptamers can be selected. To this end, various strategies have focused on endowing initial DNA/RNA libraries with more proteinlike properties to enhance interactions with targets, since aptamers lack a variety of physiochemical properties such as charge, hydrophobicity, pK_a that proteins present.[48] A novel evolutionary engineering method ExSELEX based on inducing hydrophobic interactions between DNA and targets was reported by Hirao and co-workers (Figure S2a).[49] In this approach, randomized sequences comprising five bases: four

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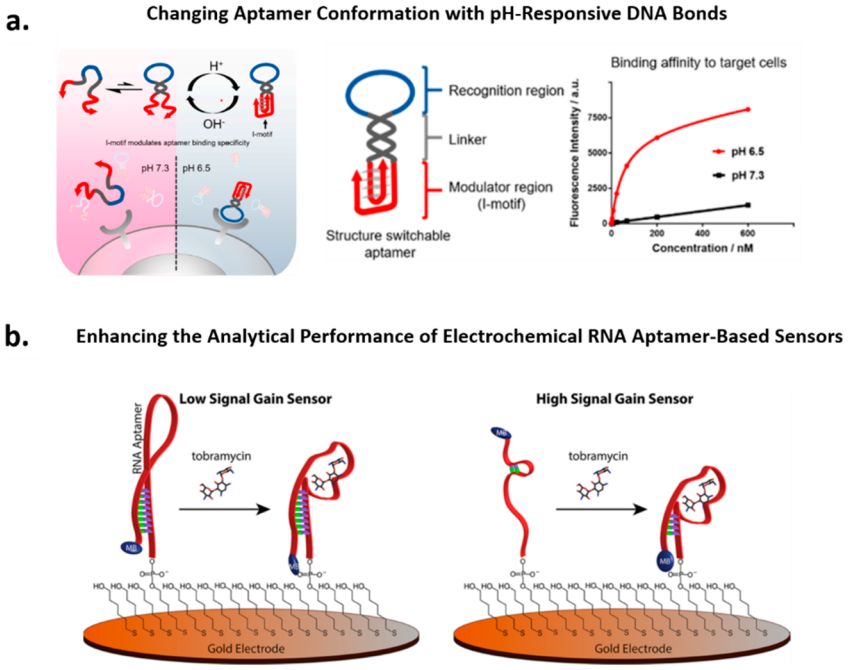

Figure 1. (a) Schematic representation of a structure-switchable aptamer (SW-Apt), achieved by conjugating pH-responsive i-motifs with tyrosine kinase-7 (PTK7)-binding aptamer sgc8. At acidic pH 6.5 (red), the i-motifs of the SW-Apt fold into quadruplex structures, enabling the recognition loops to bind receptors, whereas at physiological pH 7.3 (black), the i-motifs of the SW-Apt is unstructured, preventing the recognition loops from binding to receptors. Reproduced with permission from ref 87. Copyright 2018 American Chemical Society. (b) Electrochemical structure-switching aptamer-based sensors for the detection of aminoglycoside antibiotics, low signal gain sensor, and high signal gain sensor are shown on the left and right, respectively. High gain sensor was achieved by rationally engineering the secondary structure of the aptamer to create large conformation changes upon target binding. Reproduced with permission from ref 88. Copyright 2014 American Chemical Society.

natural bases and one artificial hydrophobic base, 7-(2-thienyl)imidazo[4,5-*b*]pyridine (Ds), were used as the improved library, from which a Ds-containing DNA aptamer was selected to display significantly higher affinity (K_d = 75 pM) compared to the known DNA aptamer selected from natural DNA libraries. In addition to adding an extra artificial hydrophobic base in the initial library, base modification has also been conducted for initial library design by Gold et al. (Figure S2b).[50] In their report, DNA libraries incorporated one of four dUTPs modification at the 5-position was used as initial libraries for selecting modified aptamers. As a result, modified DNA SELEX was able to select aptamers against 13 "difficult" proteins whereas unmodified DNA SELEX failed.

When evaluating performance of the selected aptamers, specificity is another critical parameter. Because of the negatively charged phosphate backbone, aptamers may non-specifically attract cationic molecules. Strategies using salt gradients, denaturing detergents, and cation-chelating additives have been implemented in the elution buffer to minimize nonspecific interactions.[51−53] It is suspected that nonspecific binding of other molecules can also be contributed to interactions with the two primer-binding sequences that flank the randomized DNA/RNA sequences. To alleviate this, primer-free libraries were employed in some SELEX procedures; however, the complexity and cost by introducing restriction and ligation enzymes largely prevent researchers from pursuing this path.[54−56] Along with minimizing non-specific interactions, counter selection using structurally similar targets to incubate with initial nucleic acid libraries prior to/simultaneously with successive SELEX rounds being performed.[57−59] For instance, a RNA aptamer that can distinguish mutated p53 against wild-type (WT) p53 was isolated via contrast SELEX.[60] Positive selection and counter selection were performed simultaneously in a pool of the agarose beads and magnetic beads conjugated with WT and mutant proteins, respectively.

Downstream Application of the Aptamer and Properties of the Target Analyte Should Drive Selection Strategy. To increase the probability of selecting suitable aptamers for analytical application, the downstream analytical device for incorporating the selected aptamers should be considered prior to perform the SELEX process. An example demonstrating that functionalities of the aptamer for the downstream application should be contemplated prior to SELEX is the selection of structure-switching aptamers for developing biosensors. Structure-switching aptamers provide a means to convert a binding event into a change in aptamer structure or conformation which can ultimately provide analytical readout using optical[61−65] and electrochemical methods.[66−71] To select aptamers able to induce detectable signal upon target binding, Xiao and co-workers developed an alternative selection strategy relying on binding-induced release of aptamers from duplexed nucleic acids. More specifically, the authors reported a generalizable approach for isolating small-molecule-binding aptamers with intrinsic dye-displacement functionality.[72] In their approach, an aptamer against 3,4-methylenedioxypyrovalerone (MDPV) was isolated from a randomized DNA library pool which was found to generally bind the dye diethylthiatricarbocyanine (Cy7) within their three way junction domain. Based on the small-molecule-binding aptamer, they successfully designed a colorimetric sensor for the detection of the small molecule MDPV.

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POSTSELECTION MODIFICATIONS AS STRATEGY TO IMPROVE PERFORMANCE OF APTAMER IN ANALYTICAL DEVICE

As described above, the performance requirements of aptamers to be employed in analytical devices lie in the sensitivity and specificity of the aptamer−target interaction. While selection strategies continue to improve these features, there are also several strategies that can be employed postselection to improve the performance of aptamers for use in analytical devices. Key to the success of such strategies is the understanding of how interaction forces between aptamer and target affect binding. To this end, reliable analytical approaches, such as nuclear magnetic resonance spectroscopy (NMR), X-ray crystallography, and docking and molecular simulations, have been used to provide quantitative information about aptamer−target interactions.

Understanding Intermolecular Interactions in the Binding Domain as a Method for Improving Aptamer Performance. Formation of a specific and tightly bound aptamer−target complex represents a delicate balance, as it requires coordination of the following three participants: the binding buffer, the nucleic acid sequence, and the properties of the target analyte. The optimal binding buffer conditions can be obtained by controlling the composition and concentration of monovalent and divalent salts, pH, and buffer identity. In the optimal binding buffer, secondary or tertiary structure motifs of the selected DNA/RNA aptamers can be promoted to interact with the target analytes including small molecules, macromolecules, cells, etc.[73−85] However, the postselection use environment must be considered as oftentimes solution conditions are not identical. In fact, this caveat was pointed out in a 2009 *Annual Review in Analytical Chemistry* article by Cho, Lee, and Ellington.[86] The authors state "...knowledge of the sequence of an aptamer is often taken as an indication that only this sequence itself is necessary for experimental repetition. In fact, it is not only the sequence, but many other variables, such as buffer conditions, temperature, purification method, and conformational state, that affect the function of an aptamer. If an analytical researcher does not know or heed these important variables, then their experiments may be meaningless."

An in-depth understanding of the intermolecular interactions between the binding domain of the selected aptamer and the target analyte is useful for improving aptamer performance in the downstream analytical device. If the binding domain of a selected aptamer is known, one can incorporate the binding domain into rational designs to improve aptamer performance. Tan and co-workers recently reported a smart strategy to improve aptamer specificity (Figure 1a).[87] In their work, a structure-switchable aptamer (SW-Apt), achieved by conjugating pH-responsive i-motifs with tyrosine kinase-7 (PTK7)-binding aptamer sgc8, is capable of distinguishing identical target antigens on both tumor and healthy cells. At slightly acidic pH (tumor microenvironment), the i-motifs of the SW-Apt fold into quadruplex structures, enabling the recognition loops to bind receptors; whereas at physiological pH (healthy cells), the i-motifs of the SW-Apt are unstructured, preventing the recognition loops from binding to receptors. Furthermore, in another rational design method (Figure 1b),[88] we introduced structural approaches to enhance the observed binding affinity and sensitivity of the aminoglycoside targeting RNA aptamer. Specifically we bimolecularly engineered several aptamers based on the parent RNA aptamer to create largest changes in aptamer conformation and thus signaling based on our sensor mechanism. Since the binding domain is known, by conserving the bases involved in the binding of tobramycin and destabilizing the secondary structure through deletion of bases in the parent aptamer, we demonstrated a semirational design strategy for improving the analytical performance.

Semirational Postselection Strategies to Improve Aptamer Performance Downstream. The analytical approaches introduced above have been used in postselection strategies to provide quantitative information about aptamer−target interactions and the binding domain. Reportedly, ∼400 publications have performed the above-mentioned three techniques; however, compared with thousands of papers published on the aptamer, characterization of the aptamer-target structures and the binding mechanism is still lacking. Quantitative information about aptamer−target interactions and the binding domain could guide postselection modifications to improve aptamer performance downstream.

In many cases, the selected full aptamers, including primer regions, are not needed, as only the binding domain is essential for target recognition.[89−97] Truncating the nonessential binding component of an aptamer may lead to improved specificity, sensitivity, and cost-effectiveness of the oligonucleotide sequence, as nonspecific interactions could potentially be reduced.[98−100] In an example of the benefits of truncation, Soontornworajit and co-workers designed a successful truncation strategy based on oligonucleotide hybridization combined with competitive antibody binding (Figure S3a).[101] Tailored by the immunoassay results, which does not require knowledge of the binding site, the parent platelet derived growth factor (PDGF-BB) aptamer comprising 86 nucleotides (nt) was reduced to truncated aptamers comprising 36−40 nt. The truncated aptamers displayed picomolar K_d values, among which, the 36 nt truncated aptamer displayed the highest affinity toward PDGF-BB, with a 150-fold increase compared with the parent aptamer. In another rational design reported by Xiao and co-workers (Figure S3b),[102] structure-switching functionality was introduced to both the cocaine and ATP aptamer via exonuclease III (Exo III)-directed truncation. The parent cocaine aptamer prefolds into a three-way-junction (TWJ) structure while the parent ATP aptamer prefolds into a stem-loop structure. As Exo III-directed truncation halts four bases prior to the target-binding domain, the resulting truncated aptamers displayed a target-induced structure-switching functionality, which can be used in the downstream application of constructing structure-switching aptamer-based biosensors. The strength of this technique is that it does not require knowledge of the binding domain.

Similar to the postselection strategy truncation, site-directed mutagenesis is a common method for identifying certain crucial nucleotides primarily involved in the aptamer−target interactions.[103−105] Very recently, Willner and co-workers examined eight mutants of ATP-binding aptamers, where the thymine bases within the binding domain were substituted with cytosine bases or the A-T base pairs were replaced with G-C. (Figure S4a).[106] Among the eight mutants, the ATP-aptamer mutant 7 displayed improved affinity (K_d = 15 ± 1 μM) over the parent ATP-aptamer (K_d = 31 ± 3 μM), presumably due to more favorable hydrogen bonding and $\pi-\pi$ interactions between the mutant ATP-binding aptamer and the target ATP. In addition to identifying crucial nucleotides on

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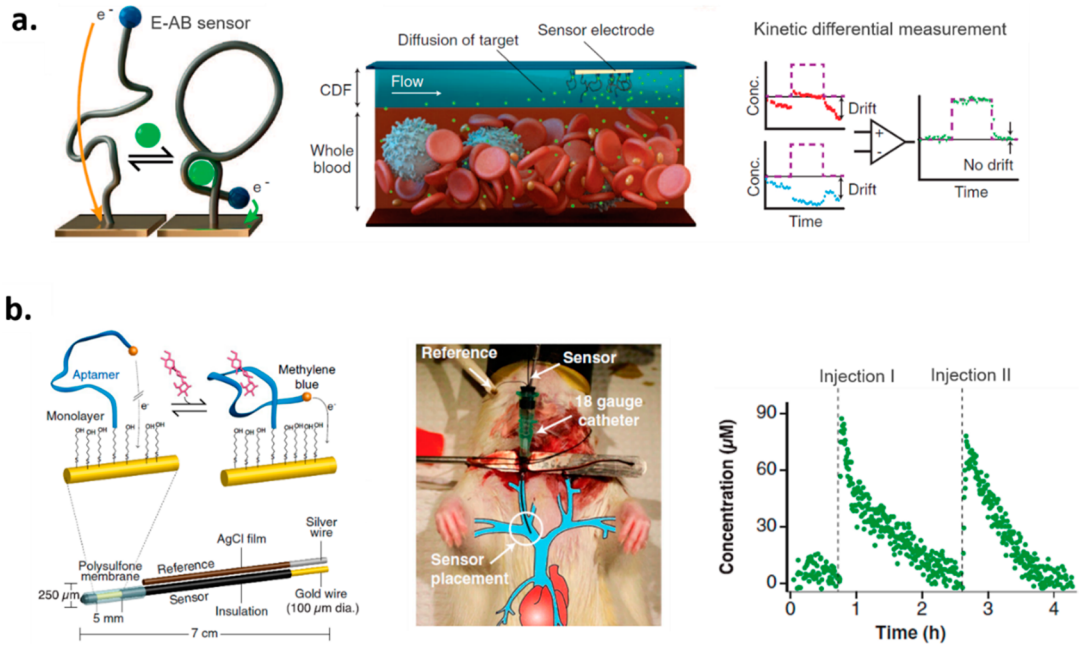

Figure 2. (a) Schematic representation of real-time measurement of doxorubicin and kanamycin in whole blood. The microfluidic electrochemical detector for in vivo continuous monitoring was developed by including an electrochemical aptamer-based sensor for the measurement and a continuous-flow diffusion filter to protect the aptamer probes from fouling. To minimize current drift and enhance signal-to-noise ratios, the kinetic differential measurement (KDM) was conducted for improving accuracy of real-time measurements. From Ferguson, B. S.; Hoggarth, D. A.; Maliniak, D.; Ploense, K.; White, R. J.; Woodward, N.; Hsieh, K.; Bonham, A. J.; Eisenstein, M.; Kippin, T. E.; et al. Real-Time, Aptamer-Based Tracking of Circulating Therapeutic Agents in Living Animals. *Sci. Transl. Med.* **2013**, *5* (213), 1−9 (ref 126). Reprinted with permission from AAAS. (b) Schematic representation of real-time, continuous measurement of drugs in the bloodstream of awake, ambulatory animals. To protect the sensor from fouling, a biocompatible polysulfone membrane with 0.2-μm pores was used to encase the sensor. The resultant device was emplaced in one of the external jugulars of a rat using an 18-gauge catheter. Two serial injections of the antibiotic tobramycin in the blood of an anesthetized rat was demonstrated. Reproduced with permission from ref 127. Arroyo-Currás, N.; Somerson, J.; Vieira, P. A.; Ploense, K. L.; Kippin, T. E.; Plaxco, K. W. Real-Time Measurement of Small Molecules Directly in Awake, Ambulatory Animals. *Proc. Natl. Acad. Sci.* **2017**, *114* (4), 645−650. Copyright 2017 The National Academy of Sciences.

the aptamer to improve binding affinity, alternatively, Juncker and co-workers tuned binding affinity of aptamers by introducing single-base mismatches into complementary strands through an induced fit binding pathway (Figure S4b).[107] In this pathway, a duplexed aptamer (DA) actively senses the ligand from the duplexed state. Subsequentially, target binding catalytically dissociates the DAs. To systematically and comprehensively profile DAs target-binding landscapes, aptamer-complementary element scanning (ACE-Scan) was used to study the binding kinetics of thousands of surface-assembled DAs. A combined 5′ and 3′ enantio heat map was obtained for identifying mutant DAs that displayed higher dissociation rates (k_{off}) toward target binding. These two examples, however, typically require knowledge of the binding domain, as changes to this domain severely hinder binding.[108]

When the downstream application of selected aptamers is for ability to function in complex media, stability of the selected aptamers are major concerns, as nuclease degradation create practical barriers for the in vivo application of aptamers, particularly for RNA.[109−114] Perhaps replacing the RNA sequence with a deoxy version is the simplest way one can envision to improve stability of RNA. Indeed, there was an attempt to increase the stability of RNA aptamer by employing the DNA version of the aptamer. Although it was claimed that the function of the RNA aptamer was retained in the DNA version,[115] it was demonstrated later that the DNA dopamine aptamer responded to both dopamine and other structurally related catecholamine neurotransmitters.[116] Given that simple replacement approach is not feasible for improving stability, various chemical modifications have been utilized to improve the stability of aptamers, among which, 3′-end-capping with inverted thymidine has been shown to combat 3′-exonuclease in human serum.[117] Other existing chemical modifications able to improve the stability include using LNA (locked nucleic acid) to substitute ribonucleotide, introducing 3′-biotin to the 3′-terminal of the aptamers,[118−120] modifying the sugar rings with 2′-fluoro (2′-F) or 2′-amino (2′-NH_2) ribose groups,[121,122] replacing phosphodiester linkages of DNA with phosphorothioate analogues or triazole linkages,[123−125] etc.

ANALYTICAL APPLICATION OF APTAMERS: HIGHLIGHT THE ADVANTAGES OF USING APTAMERS AS WELL AS THE HURDLES

Functioning as affinity reagents, both aptamers and antibodies have received great attention in diverse fields, including affinity isolation, biomarker discovery, food safety and environmental monitoring, and in particular clinical diagnostics. However, the analytical application of aptamers is still in its infancy while antibody-based methods dominate in clinical diagnostics. Nonetheless, since the invention of aptamers in the early 1990s, the field has witnessed significant advances in analytical methodologies that employ aptamers. In fact, we believe that aptamer-based methodologies can tackle challenges not accessible with antibody-based detection, particularly small molecule dynamics monitoring.

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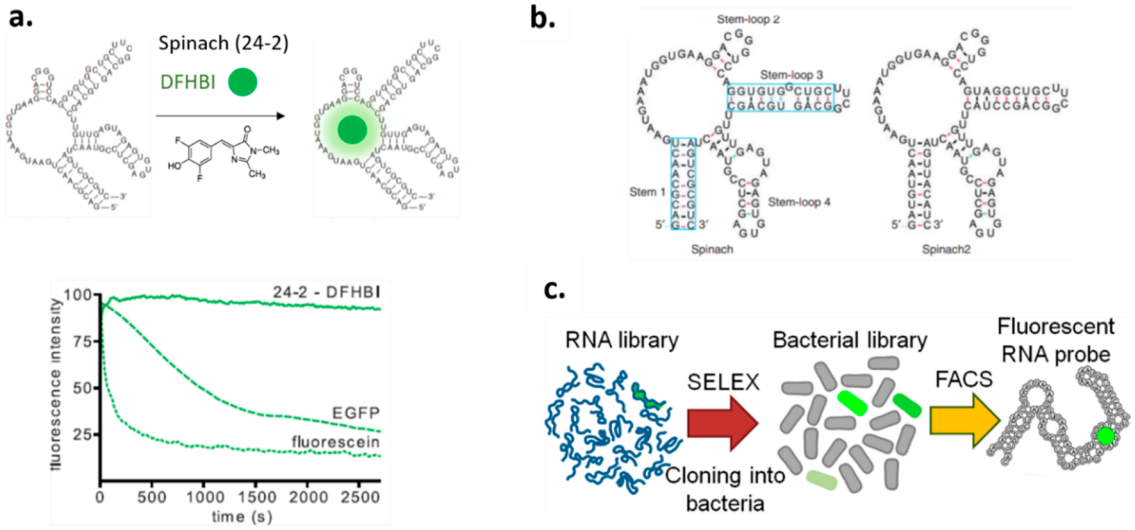

Figure 3. (a) Schematic representation of formation of the 24-2-DFHBI (3,5- difluoro-4-hydroxybenzylidene imidazolinone) complex, termed Spinach. Photobleaching curves for 24-2-DFHBI, enhanced green fluorescent protein (EGFP), and fluorescein demonstrates that Spinach displays fluorescence emission comparable to fluorescent proteins. From Paige, J. S.; Wu, K. Y.; Jaffrey, S. R. RNA Mimics of Green Fluorescent Protein. *Science* **2011**, *333* (6042), 642−646 (ref 129). Reprinted with permission from AAAS. (b) Schematic representation of secondary structure of Spinach and Spinach2. Systematic mutagenesis was conducted on Spinach stem 1 and stem-loop 3 (boxed) to generate Spinach2. Reprinted by permission from Springer Nature. *Nat. Methods*, A Superfolding Spinach2 Reveals the Dynamic Nature of Trinucleotide Repeat-Containing RNA. Strack, R. L.; Disney, M. D.; Jaffrey, S. R. Copyright 2013 (ref 130). (c) Fluorescence-activated cell sorting (FACS)-based directed evolution approach for generating the Broccoli aptamer that displays highly efficient cellular performance. Reproduced with permission from ref 131. Copyright 2014 American Chemical Society.

Illustrative Examples of Analytical Devices That Outperform Antibody-Based Approach. As noted above, we believe that a particular promising application of aptamer-based analytical devices is the ability to monitor small molecule dynamics. For example, aptamer-based sensors capable of measuring real-time pharmacokinetic parameters of drugs can provide feedback information to ensure delivery of optimal drug dose for individual patients. Thus, motivated, Soh and co-workers developed a microfluidic electrochemical detector for in vivo continuous monitoring (MEDIC) of doxorubicin (DOX) and kanamycin in human whole blood and in live rats for up to 4 h (Figure 2a).[126] To achieve this detection, the authors developed a continuous-flow diffusion filter of the MEDIC device to protect the aptamer sensor surfaces from fouling in whole blood. Furthermore, the authors employed a kinetic differential measurement (KDM) to improve accuracy of real-time measurements by minimizing current drift (presumable from surface fouling) and enhancing signal-to-noise ratios. Expanding on this example, Plaxco and co-workers developed electrochemical aptamer-based (E-AB) sensors capable of real-time measurement of four drugs (i.e., doxorubicin, kanamycin, gentamicin, and tobramycin) in the bloodstream of awake, ambulatory animals (Figure 2b).[127] Of note, the aptamer sequences employed are the same as those used in the MEDIC device. In contrast, however, to the complex continuous-flow diffusion filter adopted in the MEDIC invented by Soh and co-workers, the sensors were encased in a biocompatible polysulfone membrane with 0.2-μm pores to reduce fouling of the sensors placed in the jugular veins of anesthetized rats. As such, target molecules were able to reach the sensor surface while larger entities such as cells could not. The membrane-protected E-AB sensors achieved nanomolar precision and 3 s temporal resolution. It should be noted, the specificity afforded by the aptamer for DOX is limited because it also binds the analogue daunomycin.[128] In addition, the aminoglycosides used all bind to the same aptamer due to their structural similarity. These specificity limitations highlight the need for better selection techniques to be developed that can select aptamers capable of discriminating analogues or potentially metabolites of a target if needed. Ultimately, the sensor can only be as specific and sensitive as the aptamer that is used.

Apart from being employed in aptamer-based sensors for real-time measurements of drugs, RNA aptamers have found unique application as "light-up" RNAs, which mimic naturally existing green fluorescent proteins (GFP). The RNA aptamer 24-2 which binds the fluorophore 3,5-difluoro-4-hydroxybenzylidene imidazoline (DFHBI) was selected by Jaffrey and co-workers.[129] Resembling enhanced GFP with fluorescence emission comparable to fluorescent proteins, the RNA-fluorophore complexes, termed Spinach, can be fused with structure-switching nucleic acids for in vivo imaging (Figure 3a). To improve the thermal stability and emission brightness of Spinach, systematic mutagenesis was conducted.[130] When fused to the CGG repeat-containing RNA, the developed Spinach2 displayed improved fluorescence compared to the original Spinach (Figure 3b). Followed by Spinach and Spinach2, a 49-nt RNA aptamer, named Broccoli, was selected against the fluorophore (Z)-4-(3,5-difluoro-4-hydroxybenzylidene)-1,2-dimethyl-1Himidazol-5(4H)-one (DFHBI-1T) using a fluorescence-activated cell sorting (FACS)-based directed evolution approach (Figure 3c).[131] With this direct cell-based selection, Broccoli demonstrates highly efficient cellular performance over Spinach2 for cell imaging, on account that Broccoli displays low magnesium dependence, higher thermostability, ability to fold without a tRNA scaffold, and shorter in size compared with 96-nt-long Spinach2. These recently developed "light-up" RNAs largely enable living-cell imaging for metabolites, proteins, and RNA; however, the

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living cells used in previous studies were mainly bacteria.[132−134]

Reading Between the Lines: Challenges of Aptamers in the Analytical Field. While the above examples are excellent and demonstrate some of the potential promise of using aptamers in analytical chemistry, there still remain challenges that transcend the analytical transduction methodologies presented. For example, typically when an aptamer is examined in the lens of a specific analytical method or device, many times this is done for a proof-of-concept demonstration that the aptamer functions under specific conditions. However, critical readers may ask: "why don't we see more analytical permeation of aptamers in the field?" or "How do we move beyond proof of concept?" or "Why do I keep reading about the same 4 aptamers?"

While contemplating the answer to such questions, inspection of one of the most prolific aptamers, the thrombin-binding aptamer, may provide insight. The proof-of-concept analytical applications of thrombin aptamers have been demonstrated through various examples including designing thrombin biosensors involving various transducers,[135−140] using duplexed aptamers based on thrombin aptamers for exploring the ligand binding dynamics,[107,141,142] improving signal-to-background ratio and sensitivity of the thrombin aptamer by using split thrombin aptamer fragments,[143−146] programming multiprotein nanoarrays with defined nanometer spacing and precision employing thrombin aptamers,[147] constructing target-specific imaging platforms based on thrombin aptamers for in vivo imaging,[148−150] etc. Given these across-the-board applications, we believe the secret of the success of the thrombin aptamer is 2-fold: (1) thrombin aptamers are proven to work via a multidisciplinary array of methods, (2) the binding domain of thrombin aptamers and intermolecular forces between thrombin aptamers and the target are well characterized and the specificity is well documented.

According to the Aptagen online database (https://www.aptagen.com/aptamer-index), there are more than 500 aptamers selected to date. These aptamers are proven to work under specific conditions (e.g., buffer condition, temperature, etc.). Among them, it is highly possible that some of the selected aptamers are better aptamers and display high specificity and affinity that can be applied in the multidisciplinary array of the analytical device. However, the challenges of aptamers in the analytical field lie in figuring out promising aptamers that can pass the test from multidisciplinary examination and concentrating the efforts of the field to rigorously characterize and further improve performance of the promising aptamers. It is up to the aptamer field to decide which are the promising aptamers worth being taken to the next level.

CONCLUSION

The aptamer community is going to celebrate 30 years of the SELEX technology. In retrospect over the past 30 years, advances in the field of aptamers demonstrates that aptamers have the potential to be powerful reagents for use in diverse areas within analytical chemistry as well as biotechnology, biomedicine, and molecular biology. Aptamers have the potential to provide additional advantages over their affinity counterparts, antibodies, as noted from the very beginning of their invention. It is our perspective that one area aptamers can shine in the future of analytical chemistry, when coupled with the right transduction mechanism, is in the specific monitoring of small molecule dynamics whether that will be in the fields of, for example, biomedicine, therapeutic monitoring, or chemistry of the brain applications. While the analytical methodology employing aptamers continues to grow, we see a lag in the application of such affinity reagents beyond proof-of-concept examples. Our perspective highlights potential challenges in using aptamer which include challenges like poor specificity or sensitivity. Even some of the best illustrative examples of the analytical usage of aptamers rely on aptamers that exhibit limited specificity. On the contrary, there are shining examples of aptamers that display exceptional affinity (the theophylline aptamer[151] comes to mind). This often-cited example, however, raises a question that is often encountered in this field, why are some aptamer sequences more prolific than others? This is most likely a result of the fact that the top five or so aptamers have been rigorously characterized by a wide range of researchers. This characterization ranges from structural biochemical characterization, careful analysis of binding specificity, the nature of the biomolecular interactions, and in some cases these aptamers have been subjected to rational postselection modification for improved performance in analytical devices. Rigorous characterization, however, is not always complete. An example of such is the recent publication declaring that the arsenic aptamer does not indeed specifically bind arsenic, even though it has been used in >20 publications describing analytical applications.[152]

In short, aptamers still hold a lot of promise for use as affinity reagents in the development of analytical devices and methods. These methods have advanced considerably, even demonstrating the ability to detect specific small molecules in the bloodstream of a living animal. What will propel aptamers in their future role in analytical chemistry? The field as a whole needs to more rigorously evaluate characterized sequences. This evaluation is a multidisciplinary effort and is critical to the further success of aptamers.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.9b03853.

Table S1, summary of the most well-studied targets in aptamer research; Figure S1, schematic illustration of the conventional SELEX process; Table S2, innovations to improve efficiency of the SELEX procedure; Figure S2, scheme for ExSELEX using the unnatural hydrophobic base and DNA libraries incorporating one of four dUTPs modification at the 5-position was used as initial libraries for selecting modified aptamers; Figure S3, schematic representation of aptamer-complementary oligonucleotides hybridization for the aptamer truncation based on the immunoassay signal from competitive antibody binding and schematic representation of Exo III-directed truncation; and Figure S4, schematic representation of the parent ATP aptamer and ATP-aptamer mutant structure and schematic representation of induced fit binding pathway and enantio heat maps (PDF)

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Notes

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