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Advancements in Aptamer Discovery Technologies

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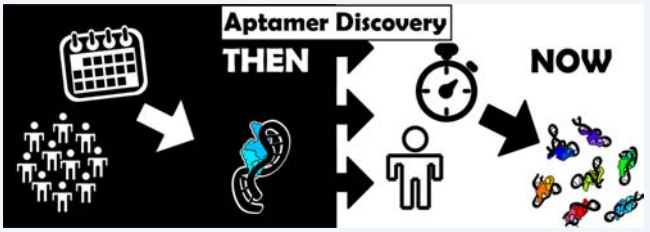
CONSPECTUS: Affinity reagents that specifically bind to their target molecules are invaluable tools in nearly every field of modern biomedicine. Nucleic acid-based aptamers offer many advantages in this domain, because they are chemically synthesized, stable, and economical. Despite these compelling features, aptamers are currently not widely used in comparison to antibodies. This is primarily because conventional aptamer-discovery techniques such as SELEX are time-consuming and labor-intensive and often fail to produce aptamers with comparable binding performance to antibodies. This Account describes a body of work from our laboratory in developing advanced methods for consistently producing high-performance aptamers with higher efficiency, fewer resources, and, most importantly, a greater probability of success.



We describe our efforts in systematically transforming each major step of the aptamer discovery process: selection, analysis, and characterization. To improve selection, we have developed microfluidic devices (M-SELEX) that enable discovery of high-affinity aptamers after a minimal number of selection rounds by precisely controlling the target concentration and washing stringency. In terms of improving aptamer pool analysis, our group was the first to use high-throughput sequencing (HTS) for the discovery of new aptamers. We showed that tracking the enrichment trajectory of individual aptamer sequences enables the identification of high-performing aptamers without requiring full convergence of the selected aptamer pool. HTS is now widely used for aptamer discovery, and open-source software has become available to facilitate analysis. To improve binding characterization, we used HTS data to design custom aptamer arrays to measure the affinity and specificity of up to ~10^4 DNA aptamers in parallel as a means to rapidly discover high-quality aptamers. Most recently, our efforts have culminated in the invention of the "particle display" (PD) screening system, which transforms solution-phase aptamers into "aptamer particles" that can be individually screened at high-throughput via fluorescence-activated cell sorting. Using PD, we have shown the feasibility of rapidly generating aptamers with exceptional affinities, even for proteins that have previously proven intractable to aptamer discovery. We are confident that these advanced aptamer-discovery methods will accelerate the discovery of aptamer reagents with excellent affinities and specificities, perhaps even exceeding those of the best monoclonal antibodies. Since aptamers are reproducible, renewable, stable, and can be distributed as sequence information, we anticipate that these affinity reagents will become even more valuable tools for both research and clinical applications.

1. INTRODUCTION

Affinity reagents represent a critical tool for biomedical research, clinical diagnostics, and targeted therapies; indeed virtually every area of biomedicine relies to some degree on their capability to specifically bind to target molecules. Nucleic acid-based aptamers offer many advantages in this context because they can be reproducibly synthesized using well-established chemistries and can therefore be shared as sequence information rather than as a physical entity, which leads to improved reproducibility relative to monoclonal antibodies.[1,2] Aptamers are also thermostable and can fold reversibly, which renders them more tolerant to environmental variability. Finally, aptamers are economical, costing far less per mole than monoclonal antibodies. Despite these compelling features, aptamers are currently not widely used. This is partly because conventional aptamer discovery techniques (such as SELEX) are time-consuming and labor-intensive and often fail to produce aptamers with comparable binding performance to antibodies.

Over the past decade, our lab has focused on developing new experimental strategies to facilitate the consistent production of high-performance aptamers. We have systematically transformed each major step of the aptamer discovery process, selection, analysis, and characterization, so that each can be performed with higher efficiency, fewer resources, and, most importantly, a greater probability of success (Figure 1). In this Account, we chronicle our efforts in developing these methods. First, we examine how controlling the target concentration and washing stringency through microfluidics technology can improve the selection process. Next, we describe our experience in using high-throughput sequencing (HTS) to effectively identify and predict high-quality aptamers. We then explain

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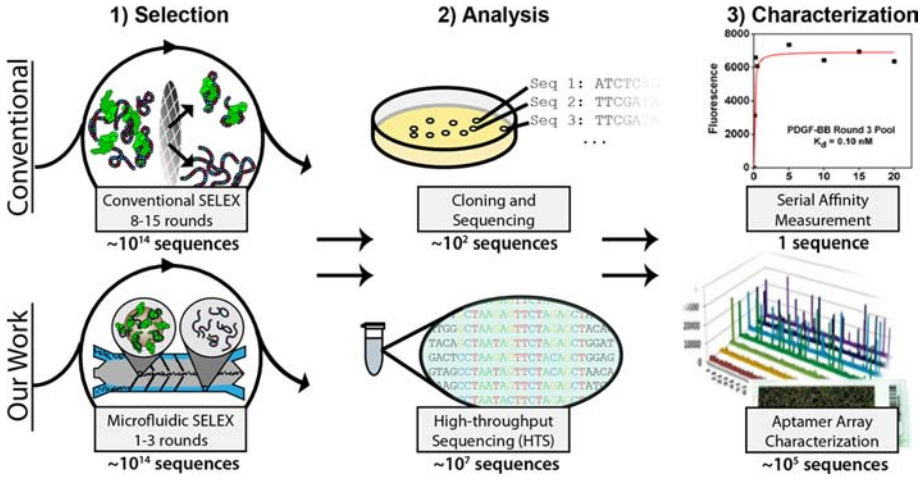

Figure 1. We have systematically overhauled all three major steps of aptamer discovery: selection, analysis, and characterization. We began by rethinking the selection process with microfluidic devices that allow rapid and efficient partitioning of aptamer candidates from random nucleic acid libraries. Next, rather than identifying aptamers by individually choosing bacterial clones for sequencing, we utilized high-throughput sequencing (HTS) to rapidly analyze much larger pools of aptamers. Finally, we moved beyond the labor-intensive process of individually measuring aptamer affinity and specificity by using DNA microarrays to characterize thousands of aptamer candidates simultaneously.

how we use HTS data to direct the synthesis of aptamer arrays in order to characterize the affinity and specificity of large numbers of aptamer candidates in parallel. Lastly, we describe a breakthrough selection strategy called particle display, which allows us to measure the binding affinities of individual aptamers at high-throughput, isolating high-quality aptamers with unprecedented efficiency.

2. CONTROLLING SELECTION STRINGENCY WITH MICROFLUIDICS

Most aptamers described in the literature have been discovered via systematic evolution of ligands by exponential enrichment (SELEX) (Figure 2A).[3,4] The SELEX process is elegant in its simplicity. It applies the three principles of evolution—heredity, variation, and selection pressure—to discover aptamers that specifically bind a target ligand. SELEX begins with a diverse library of single-stranded DNA or RNA sequences, each containing a region of randomized nucleotides that gives it unique structural and biochemical characteristics. A partitioning process is then performed, in which this library is exposed to a target ligand; target-binding molecules are collected, and nonbinders are discarded. Binders are amplified, and the partitioning process is repeated until the library converges to a small number of sequences that can be readily synthesized and characterized in terms of their affinity and specificity. A typical SELEX experiment concludes with the characterization of fewer than 100 such sequences.

SELEX, however, is limited by the inherent inefficiencies in the partitioning process. Previous theoretical work by Irvine and others has shown that the maximum enrichment that can be achieved in a single round of SELEX for a given aptamer relative to another is equal to the ratio of their equilibrium dissociation constants (K_d).[5−7] In other words, a high-affinity aptamer with a K_d of 1 nM can only be enriched 100-fold relative to another aptamer with a K_d of 100 nM in a single round of SELEX under equilibrium conditions. Since a typical SELEX experiment starts with ∼1 nmol (6×10^{14}) of molecules, this means that many rounds of selection (typically 10 or more) are needed to achieve convergence to a

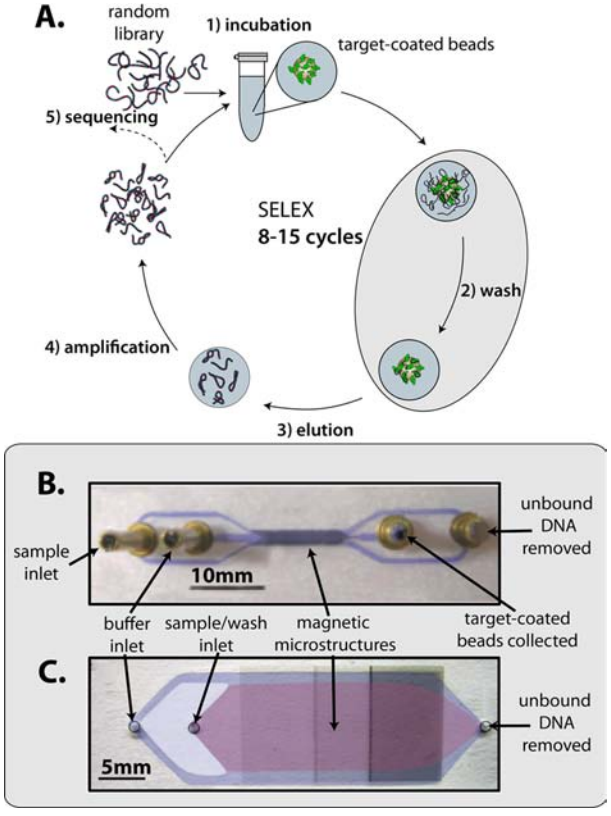

Figure 2. Improving the efficiency of selection through the use of microfluidic devices. (A) Conventional SELEX requires numerous rounds to converge to a final aptamer pool, rendering it vulnerable to biases and artifactual amplification of nonspecific binders. (B) The continuous-flow magnetic activated chip-based separation (CMACS) device uses a laminar-flow system to separate out aptamers that bind to target-conjugated magnetic beads and allows us to improve selection efficiency by working with far smaller target concentrations. (C) We achieved further gains in performance with our micromagnetic separation (MMS) chip, which uses magnetic trapping to enable high-stringency washing that efficiently purges aptamer candidates with low affinities.

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manageable number of aptamers that can be experimentally characterized.

This requirement for multiple rounds of selection becomes problematic because every selection round is inevitably prone to undesired biases, including the loss of rare sequences,[8] PCR bias,[9] and parasitic amplification of non-target-specific sequences.[5,6] The cumulative effects of these biases over multiple rounds can lead to failed selections or yield only aptamers with poor binding performance. To avoid these confounding factors, we sought to develop aptamer discovery strategies that allow us to rapidly converge a starting library in as few selection rounds as possible. We therefore looked to the law of mass action, which states that the average K_d value of aptamers that bind to a target under equilibrium can be analytically expressed using eq 1:

$$K_d = \frac{[A][S]}{[AS]} \quad (1)$$

where [S] is the concentration of unbound target, [A] is the concentration of unbound aptamers, and [AS] is the concentration of aptamer bound to the target. Based on eq 1, it becomes clear that K_d is minimized and selection efficiency is improved as $[S] \rightarrow 0$ and as $[A] \rightarrow 0$.[6,10] In other words, using the smallest feasible amount of target and stringently removing unbound aptamers will yield the best-performing aptamer sequences. Although this is theoretically straightforward, practical implementation requires delicate handling of a minuscule amount of target molecules without loss.[11]

To overcome these challenges, we designed microfluidic devices that allow us to maintain rigorous control over a small amount of target in a reproducible manner. The first generation of our microfluidic devices, which we called the "continuous-flow magnetic activated chip-based separation" (CMACS) device,[10] uses a continuous, laminar-flow buffer stream to efficiently manipulate a very small number of target-coated magnetic particles. Within the CMACS device, we employ a pattern of highly localized magnetic-field gradients to efficiently transfer the beads and target-bound aptamers, but not unbound DNA, into a buffer stream from which they are subsequently collected, enabling efficient separation of target-bound from unbound aptamers (Figure 2B). We tested whether CMACS could enrich high-affinity aptamers in a single round of selection using a minimal amount of target by flowing a mixture of $\sim 10^{14}$ random DNA sequences and $\sim 10^{10}$ bead-immobilized target molecules into the device. The target in question was a recombinant form of an important neurotoxic protein, botulinum neurotoxin type A (BoNT/A-rLc). After just a single round of CMACS selection, we were able to generate high-affinity aptamers, with K_d ranging from 34−86 nM. We attribute this greatly improved efficiency to the low amounts of target used in this selection compared with conventional SELEX. For example, in this experiment the molar ratio between the DNA library and protein target ($R_{library/target}$) was 1×10^6, whereas in conventional SELEX the $R_{library/target}$ values typically range between 10 and 1000.[10]

To further push the limits of selection stringency, we devised a second-generation microfluidic device called the "micro-magnetic separation" (MMS) device, which combines the ability to reproducibly manipulate small amounts of target with the capacity for highly stringent washing to discard weakly and nonspecifically bound aptamers.[12] MMS employs a set of ferromagnetic structures within the device to achieve stationary trapping of target-coated paramagnetic beads (Figure 2C). Once the beads are trapped, wash buffer is directed through the device channel, and the beads subsequently undergo highly stringent washing. These features of MMS allowed us to wash away the vast majority of unbound DNA within a few minutes.[12] We used this device to isolate aptamers that bind to streptavidin with K_d values as low as 25 nM after only three rounds of selection. In contrast, the highest affinity DNA aptamers for streptavidin,[13] obtained after 13 rounds of conventional SELEX, exhibited a 2-fold higher K_d value of 56 nM. Our work with these two microfluidic devices conclusively shows that the use of small amounts of target combined with stringent and tightly controlled washing conditions can generate higher affinity aptamers after far fewer rounds of selection relative to conventional methods.

3. HIGH-THROUGHPUT SEQUENCING TO IMPROVE APTAMER ANALYSIS

Conventional SELEX is typically carried out until the pool of aptamers converges to a relatively small number of sequences so that the most abundant sequences containing "consensus motifs" can be identified via bacterial cloning and Sanger sequencing. Unfortunately, due to the small number of sequences that can be realistically obtained using Sanger sequencing (fewer than 100 in a typical SELEX experiment) there is cause to believe that these enriched sequences are not truly representative of the best binders in a pool. This was supported by considerable anecdotal evidence that the final enriched pool may contain a highly diverse population of high-affinity aptamers greatly exceeding what can be identified using Sanger sequencing.[8,14]

With this in mind, we employed HTS to overcome the inherent throughput limitations of Sanger sequencing and enable better analysis of enriched aptamer pools. With HTS, we are able to address questions about the selection process that had been previously unanswerable. For example, we can determine the absolute diversity of sequences in a final pool, track the enrichment trajectory of individual sequences over the course of selection, identify the effects of undesired biases during the selection process, and, perhaps most importantly, attempt to predict the best-performing sequences in early selection rounds without having to fully converge the library.

Our "quantitative selection of aptamers through sequencing" (QSAS) method[15] relies on interpretation of the trends in HTS data to identify high-performing aptamers without requiring full convergence of the selected aptamer pool. In an initial demonstration, we performed three rounds of selection using our MMS device against a known cancer biomarker: platelet-derived growth factor BB (PDGF-BB). With HTS, we obtained data for $>1.7 \times 10^7$ sequences from each round of selection, and our analysis of these data revealed a number of surprises that contradicted long-held assumptions. First, we confirmed that the most highly represented sequences are not necessarily the best binders; rather, aptamers that exhibit the greatest enrichment between successive rounds regularly outperform sequences that are most abundant at the end of selection. We demonstrated this by tracking the enrichment trajectory of each sequence across all three rounds of selection and found that the sequence exhibiting the highest-fold enrichment between the second and third round of selection showed greater affinity in comparison to the aptamer with the highest copy number in round 3 (Figure 3). This ability to track the enrichment trajectories of sequences across several rounds also proved valuable in identifying sequences that enrich due to selection

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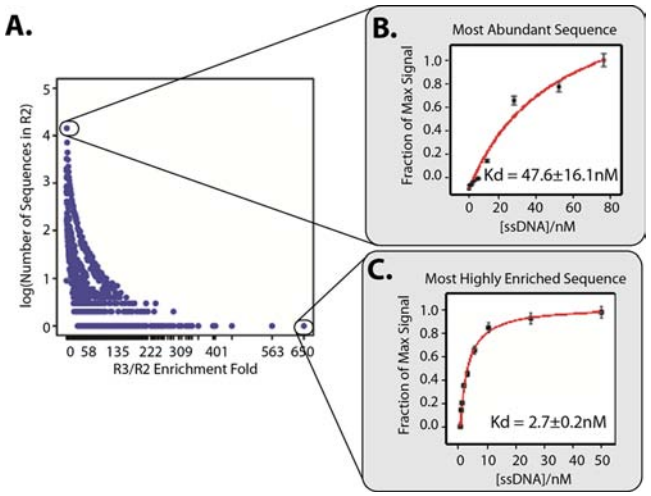

Figure 3. HTS overcomes the inherent throughput limitations of Sanger sequencing and enables better analysis of enriched aptamer pools. (A) For each aptamer, $\log_{10}$(number of sequences) in round 2 of MMS selection is plotted versus the relative extent of that sequence's enrichment from round 2 to round 3. (B) The aptamer with the highest copy number in round 2 had a significantly lower affinity than (C) the aptamer that was most highly enriched between these two selection rounds. The most abundant sequence in round 2 is the same as that in round 3.

bias rather than true target binding. We also found that a final pool that appears to be fully converged based on Sanger analysis may actually contain many thousands of unique sequences. Our results showed that conventional analytical methods are likely to be insufficient for discovering the best aptamer sequences in a given pool and that bioinformatic analysis of HTS data is a superior means for predicting aptamer performance.

Since the QSAS work was published, HTS has become widely used for aptamer discovery.[16−22] In agreement with our findings, many groups have confirmed that the sequence showing the highest copy number in a final pool is not necessarily the best binder; rather, the best binders are usually those that enrich most rapidly in the very early rounds of selection.[20,21,23] In fact, Schütze et al. have shown that library convergence with SELEX can result in well-performing sequences being outcompeted by weaker-performing sequences that amplify more efficiently during PCR.[20] As HTS becomes more widely used for aptamer discovery, a number of data analysis tools have become available to enable researchers to explore the resulting vast troves of sequence information[23−26] and to help identify and classify potential aptamers from HTS data sets. Two such tools, AptaCluster[23] and FASTAptamer,[25] are open-source and allow the user to rank sequences by read counts, cluster sequences into families, and determine round-over-round enrichment across many rounds of selection. As the price of HTS continues to decline,[27] it is likely to become an even more ubiquitous tool for aptamer discovery.

4. PARALLEL BINDING MEASUREMENTS USING AN APTAMER ARRAY

Using conventional methods, binding characterizations of selected aptamers require the most time and resources because the affinity and specificity of each aptamer must be measured serially. This typically entails the synthesis of individual aptamer sequences, with binding affinity determined via analytical techniques such as electrophoretic mobility shift assays, filter-binding assays, flow cytometry, and surface plasmon resonance.[28] All of these methods, however, require a titration of the aptamer with its target, making the characterization of individual aptamer sequences burdensome and time-consuming.[29] This problem has become especially salient with the growing use of HTS, which can generate thousands of candidate sequences. Serial characterization becomes impossible at this scale, creating an urgent need for multiplexed

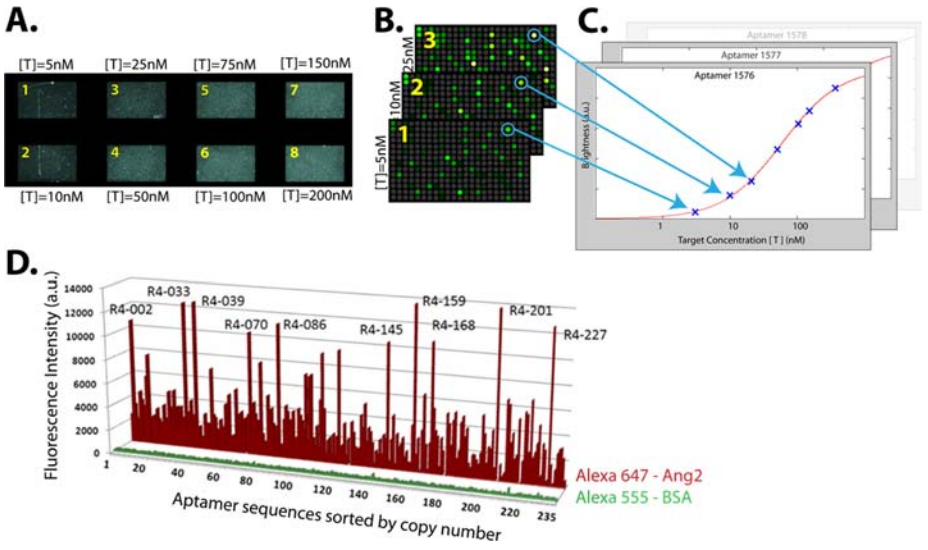

Figure 4. Schematic of the QPASS process. (A) The QPASS chip comprises eight identical subarrays, representing thousands of candidate aptamer sequences. Each subarray is incubated at a different concentration of fluorescently labeled target. (B) Measurements of the brightness of each aptamer "feature" across each subarray can be used to plot (C) a binding isotherm for every displayed aptamer. (D) Quantitative comparison of target (red) versus nontarget (green) binding for aptamer candidates in a QPASS experiment with the protein angiopoietin-2 (Ang2). Nontarget measurements were collected in the presence of a 20-fold excess of bovine serum albumin (BSA). Virtually every aptamer screened has low affinity for BSA, and 10 aptamers exhibit especially high affinity and specificity for Ang2.

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testing methods that can characterize large numbers of aptamers in parallel.

We therefore devised a system called "quantitative parallel aptamer selection system" (QPASS),[30] which makes it possible to measure the affinity and specificity of up to ~10^4 DNA aptamers simultaneously. QPASS utilizes commercially available custom microarrays, such as those typically used for mRNA analysis, to display DNA aptamers. Aptamer arrays had previously been used for the analysis of known aptamer sequences and their derivatives,[31,32] but QPASS was the first method to link HTS with parallel aptamer-binding characterization for the discovery of new aptamers. In QPASS, aptamer candidates are chosen based on HTS analysis and then synthesized *in situ* onto a DNA microarray. All of the selected sequences are patterned into eight identical subarrays on a single chip (Figure 4A). Each subarray is then incubated with a different concentration of fluorescently labeled target, and the fluorescence intensity of each array feature (representing a distinct aptamer sequence) is measured using a microarray scanner. This allows us to directly determine the K_d for each aptamer by plotting the fluorescence intensity of each feature as a function of target concentration (Figure 4C) and enables binding characterization for many thousands of defined sequences in parallel.

To confirm the effectiveness of QPASS, we characterized aptamers targeting the vascular growth factor angiopoietin 2 (Ang2). We performed four rounds of selection using the MMS device and obtained HTS data for ~3×10^7 sequences from each round. We then took several hundred candidate sequences from each round and displayed them on the aptamer array. Based on the resulting array data, we identified a subset of 60 high-affinity aptamers, with K_d values as low as 20.5 nM. QPASS can also be used to characterize the target specificity of aptamers in parallel, and we showed this by performing binding characterization in the presence of the fluorescently labeled nontarget protein bovine serum albumin (BSA), the most abundant plasma protein. This analysis revealed a smaller set of 10 aptamers that exhibit both high affinity and specificity for Ang2 (Figure 4D). In principle, any off-target protein or combination of proteins could be used in the same assay to determine specificity. These results help demonstrate the usefulness of our array-based, high-throughput approach for rapidly identifying the top-performing candidates in large aptamer libraries.

Several other groups have subsequently demonstrated an alternate high-throughput strategy for binding characterization, wherein modified HTS equipment is used to interrogate the affinities of aptamer sequences in the same flow cell used for sequencing.[33−35] These systems offer several advantages including higher sequence throughput (>10^7), synthesis of longer aptamers, and no processing between obtaining raw sequence data and characterizing the binding affinity of those sequences. However, QPASS offers two distinct advantages. First, since the sequences displayed on the array are generated after HTS analysis is complete, this population can be expanded to include variants of those sequences that were not present in the original pool. For example, one might want to look at structural variations, substitutions, minimizations, or combinations of a given sequence on the same microarray. Second, the array synthesis process gives users tight control over the spatial arrangement of each aptamer feature. As described above, this enables the production of the multiple identical subarrays on a single chip, which creates the opportunity for multiplexed binding characterization at multiple target concentrations for every sequence simultaneously. Both HTS- and aptamer-array-based systems clearly offer distinct benefits over serial characterization, and the increasing interest in high-throughput affinity reagent characterization suggests that even more efficient and sophisticated screening tools are likely to emerge in the future.

5. TRANSFORMING SELECTION TO SCREENING WITH PARTICLE DISPLAY

Despite considerable advances in high-throughput aptamer analysis and characterization, processes for aptamer enrichment still remain, to some extent, unreliable and inefficient. This is because these SELEX-based selection processes remain fundamentally constrained by the strict enrichment limit discussed by Irvine and others.[5−7] In other words, the enrichment of high-quality aptamers will always be impeded by the presence of nonspecific or weakly bound sequences. Furthermore, each additional round increases the risk that parasitic sequences will become dominant in the pool as a result of the biases described above.

We therefore set out to develop a completely different approach to aptamer enrichment in which we individually measure the performance of every sequence in a pool. This allows us to retain only those that exhibit the properties we want, while actively eliminating low-quality sequences that would otherwise undermine selection-based approaches. We achieve this ability by transforming libraries of solution-phase aptamers into macroscopic "aptamer particles" which can then be individually screened at high throughput via fluorescence-activated cell sorting (FACS). This process draws similarities to other display techniques such as bacterial,[36] yeast,[37] and phage display[38] in that it expresses multiple copies of a monoclonal affinity reagent on the surface of a macroscopic vessel.

In our "particle display" (PD) process (Figure 5), each aptamer sequence is transformed via emulsion PCR into a monoclonal aptamer particle, a magnetic bead that displays

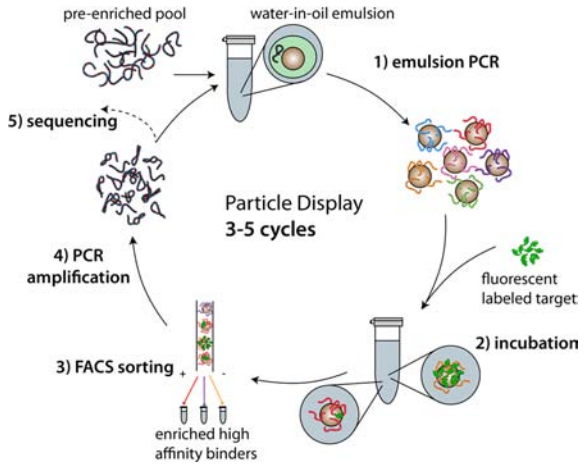

Figure 5. An overview of the particle display (PD) screening procedure. (1) A solution-phase DNA aptamer library is converted into monoclonal aptamer particles through emulsion PCR. (2) The aptamer particles are incubated with fluorescently tagged target molecules and (3) sorted using a fluorescent-activated cell sorter (FACS). (4) The collected aptamer particles are then PCR amplified and either (5) sequenced or subjected to an additional round of screening.

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$\sim 10^5$ copies of a single aptamer sequence on its surface. Each aptamer particle in a library displays an equivalent number of aptamers, enabling us to directly compare the binding affinity of each aptamer particle based on its fluorescence intensity after incubation with a labeled target. FACS allows us to rapidly detect, separate, and collect highly fluorescent aptamer particles that display high-affinity aptamers, while discarding non-fluorescent particles that lack the capacity to bind the target. In this way, PD allows us to directly measure and partition individual sequences that exhibit high binding affinity.

We used PD to discover DNA aptamers for four target proteins (Figure 6), thrombin, apolipoprotein E (ApoE), plasminogen activator inhibitor-1 (PAI-1), and 4-1BB. ApoE and thrombin were chosen as controls to compare aptamers generated via PD against existing aptamers discovered using conventional techniques. The latter two proteins were targets for which previous selections had repeatedly failed and were intended to test the capabilities of PD to isolate aptamers against especially challenging targets.[39] After only three rounds of PD, we generated high-quality, natural DNA aptamers against all four targets, and those selected for ApoE and thrombin exhibited K_d values that were considerably better than those of previously published aptamers.

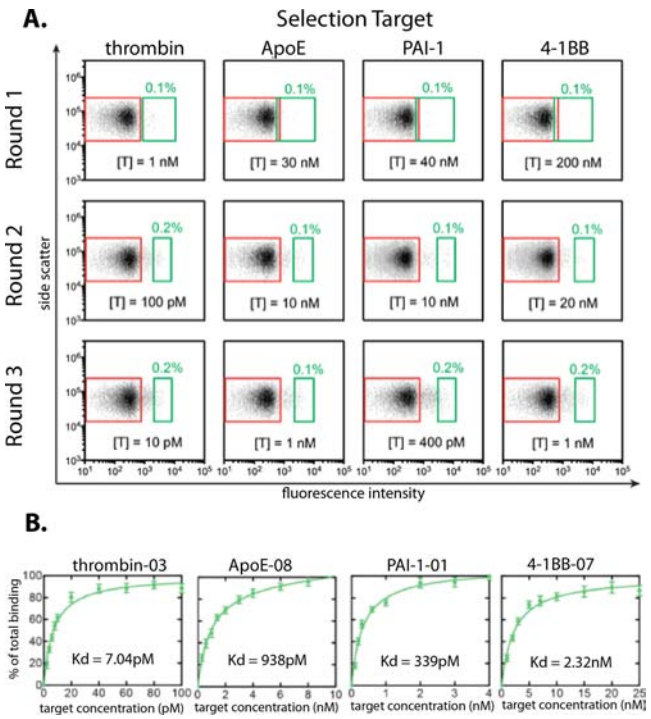

Figure 6. PD screening for four protein targets. (A) FACS plots for three screening rounds with four different protein targets. The target concentration [T] is shown for each round, and each dot represents an individual aptamer particle. Aptamer particles residing within the reference gate (red) display nonbinding aptamers, whereas those within the sort gate (green) display aptamers that bind the target and exhibit strong fluorescence. The sorted aptamer particles from each round were then used as templates for the subsequent round. (B) Binding affinity measurements for aptamers isolated in round 3 of PD screening.

PD offers a number of key advantages over conventional SELEX. First, since the relative affinity of each aptamer is being independently measured and sorted, PD can achieve a much higher level of enrichment in each round than traditional SELEX methodologies.[40] Second, PD enables us to track the selection process in each round; one can easily visualize the performance of individual binders in a given pool and subsequently tune the stringency of partitioning in a manner that is otherwise cumbersome and difficult with traditional selection (e.g., using qPCR, radiolabeling aptamer libraries). For example, one can adjust both the target concentration and the minimum fluorescence threshold required to trigger separation from the pool. Finally, by greatly reducing the number of selection rounds to just three or four, PD greatly diminishes the impact of the amplification and selection biases that inevitably compound over many rounds of selection.

One caveat regarding PD screening is that the throughput of FACS machines can limit the diversity of aptamer particles that can be realistically screened. For example, $\sim 10^8$ aptamer particles can be efficiently sorted using FACS within a few hours, while most SELEX libraries are several orders of magnitude larger. Therefore, we typically begin the process with two rounds of conventional selection to reduce the sequence diversity to $\sim 10^8$ before performing PD. This pre-enriched pool should be minimally affected by the biases that emerge during later rounds of SELEX and can then be subjected to three or four rounds of PD screening to yield high-quality aptamers against a diverse range of protein targets.

6. CONCLUSIONS AND OUTLOOK

It has been more than a quarter century since the initial development of SELEX, and in the decades that have followed, it has been employed with considerable success in generating DNA and RNA aptamers against a broad range of targets.[41] While SELEX methodologies continue to gain in sophistication, the basic process has nevertheless remained time-consuming, labor-intensive, and prone to failure in generating aptamers that are comparable or superior to antibodies. For this reason, we have attempted to systematically overhaul every step of the aptamer discovery process. Our efforts have demonstrated that microfluidic devices can enable discovery of high affinity aptamers in minimal rounds of selection, HTS can rapidly identify high-quality aptamer sequences without requiring convergence of the aptamer pool, and array-based screening can characterize the affinity and specificity of thousands of aptamers in a massively parallel manner. The culmination of our efforts to date has been the development of PD, a powerful method for screening individual aptamer sequences in a high-throughput manner. PD screening has allowed us to identify high-affinity aptamers even for proteins that were previously considered intractable to aptamer generation. While these technologies offer an effective way of generating DNA aptamers against a single target in a rapid, reliable manner, we believe exciting future directions include multitarget aptamer discovery and expanding the selection capabilities to RNA and non-natural nucleic acids.

Based on this recent progress in aptamer discovery technology, we believe that the broader research community is beginning to realize the full potential of aptamers. Aptamers in general demonstrate clear advantages over other affinity reagents, in that they are remarkably easy to synthesize, store, and distribute. We foresee growing appreciation for these benefits, and as advanced discovery platforms continue to be developed, we anticipate aptamers will become even more valuable tools for both research and clinical applications.

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The authors declare no competing financial interest.

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