Particle Display: A Quantitative Screening Method for Generating High-Affinity Aptamers**
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Abstract: We report an aptamer discovery technology that reproducibly yields higher affinity aptamers in fewer rounds compared to conventional selection. Our method (termed particle display) transforms libraries of solution-phase aptamers into “aptamer particles”, each displaying many copies of a single sequence on its surface. We then use fluorescence-activated cell sorting (FACS) to individually measure the relative affinities of >10^6 aptamer particles and sort them in a high-throughput manner. Through mathematical analysis, we identified experimental parameters that enable optimal screening, and demonstrate enrichment performance that exceeds the theoretical maximum achievable with conventional selection by many orders of magnitude. We used particle display to obtain high-affinity DNA aptamers for four different protein targets in three rounds, including proteins for which previous DNA aptamer selection efforts have been unsuccessful. We believe particle display offers an extraordinarily efficient mechanism for generating high-quality aptamers in a rapid and economic manner, towards accelerated exploration of the human proteome.

Complete functional exploration of the proteome will require access to comprehensive sets of well-characterized affinity reagents that specifically bind to their respective target proteins with high affinities. Accordingly, research communities in the U.S., Europe, and Asia have recently mounted international efforts to generate high-quality reagents for targeting the human proteome. The technical challenges are considerable: the proteome consists of multiple closely related protein variants arising from alternative splicing and posttranslational modifications (PTMs), yielding complexity orders of magnitude greater than that of the genome. Unfortunately, existing antibodies only target a small fraction of the proteome, and there is an urgent need for novel technologies that can efficiently generate reagents for the large number of unaddressed protein targets.

Nucleic acid aptamers have garnered significant interest over the past two decades as a promising alternative to antibodies, as they are chemically synthesized and their sequence information rather than as a physical entity, greatly accelerating reagent dissemination throughout the research community. Aptamers are also thermostable, fold reversibly, and can be distributed as sequence information rather than as a physical entity, greatly accelerating reagent dissemination throughout the research community. Finally, aptamers are inexpensive and can be readily synthesized chemically using standard laboratory techniques such as PCR. Despite these useful features, the number of published aptamers with sufficient affinity and specificity for proteomic analysis is extremely limited in comparison to antibodies. Two reasons have been previously proposed to explain this shortage of high-quality aptamers. First and foremost, there is anecdotal evidence that natural nucleic acids may not possess the full spectrum of chemical functional groups and conformational space needed to yield high-quality aptamers for many proteomic targets. In fact, recent reports have suggested that it may only be possible to generate natural DNA aptamers for less than 30% of the human proteome. This problem has been addressed to some extent by adding chemical diversity through the introduction of modified nucleotides, and several efforts have already yielded aptamers with improved specificity and affinity. The efficiency of SELEX-style methods is constrained by the limited enrichment that can be achieved in a single round. Irvine and others have shown that the theoretical maximum enrichment that can be achieved for a given aptamer relative to another lower-affinity aptamer in a single round is equal to the ratio of their equilibrium dissociation constants (K_d). For example, 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 selection. Given that SELEX typically begins with a large, diverse library (typically in excess of 10^12 molecules), this necessitates many rounds of selection (typically 8–15 rounds), which in turn introduce undesired biases including loss of rare sequences, PCR bias, and parasitic amplification of low-affinity or non-specific sequences. Accordingly, many SELEX experiments ultimately fail or yield low-quality aptamers, and there is an urgent need for alternative aptamer discovery technologies that can overcome the fundamental limitations inherent to affinity-based selection.

To this end, we describe a screening-based method for aptamer discovery (termed particle display), in which we quantitatively measure the relative affinity of every aptamer candidate sequence in a library and individually sort them in a high-throughput manner. Drawing inspiration from yeast and bacterial display techniques used in protein engineering, our particle display method transforms individual aptamers into aptamer particles (APs), wherein each particle presents many copies of a single nucleic acid sequence on its surface. We then individually measure the relative affinities of each of these APs by fluorescence-activated cell sorting (FACS) and isolate only those with the highest affinities. Although FACS has been previously used for


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aptamer discovery,[17] our approach is distinct from those in the literature, because it enables the sorting of individual aptamers after their affinity has been measured. In this way, particle display achieves enrichment performance that far exceeds the theoretical maximum achievable with any selection method by many orders of magnitude.

To experimentally demonstrate the effectiveness of our method, we generated high-affinity natural DNA aptamers for four different proteins within three rounds of screening, including two proteins for which previous DNA aptamer selection attempts have been unsuccessful without resorting to the use of chemically modified nucleotides.[8] These results indicate that particle display offers an effective means for generating superior aptamers, and suggest that a broader swath of the proteome may be accessible to DNA aptamers than previously envisaged.

Particle display enables us to measure the binding affinities of more than 100 million aptamers by converting a library of solution-phase aptamers into APs, and individually sorting them in a high-throughput fashion using FACS (Scheme 1). We synthesize these APs using emulsion polymerase chain reaction (ePCR)[18] (for a detailed description, see the Supporting Information). Briefly, we prepare water-in-oil emulsions with PCR reagents, such that each droplet contains (in most cases) one DNA template and one magnetic bead coated with forward primer (FP) (Scheme 1, step 1). We then perform PCR amplification within the droplet, yielding particles that display roughly $2.4 \times 10^{5}$ copies of the aptamer on their surface (Scheme 1, step 2). After breaking the emulsion and removing unreacted PCR reagents, we denature and release the reverse strands by treatment with NaOH and collect the APs through magnetic separation (Scheme 1, step 3). We then incubate these APs with fluorescently labeled target protein (Scheme 1, step 4). It is important to note that the fluorescence intensity from proteins captured by the APs is proportional to the binding affinity of the aptamer (see theoretical analysis below). This direct correlation between fluorescence and aptamer affinity enables us to quantitatively identify and sort the aptamers with highest affinities by FACS (Scheme 1, step 5). After FACS, we perform PCR directly from the sorted APs (Scheme 1, step 6) to generate an enriched pool of aptamers for the next round of screening or for sequencing (Scheme 1, step 7).

Particle display offers many important advantages over conventional selection. First and foremost, because each aptamer is individually measured and sorted based on its binding affinity to the target protein, enrichment performance is not subject to the same theoretical upper bound that limits conventional selection. This makes it possible to achieve finer discrimination between aptamers with similar affinities, enabling isolation of the highest-affinity aptamers in far fewer rounds (see analysis below). Second, particle display can virtually eliminate unwanted enrichment of nonspecific aptamers as a result of stochastic binding events. In conventional selection, a substantial percentage of low-quality aptamers is inevitably enriched due to random, nonspecific binding.[21] With an AP that displays $10^{5}$ copies of the same low-affinity aptamer, however, such random binding events will yield low overall fluorescence and these APs will therefore be eliminated during FACS. Finally, FACS enables users to visualize the screening process, and thus makes it possible to finely control the stringency of the screen and thereby designate the desired aptamer affinity threshold in a quantitative and reproducible manner.

There are two experimentally adjustable parameters that govern the stringency of particle display: the concentration of the target protein and the placement of the sort gate during FACS. We used mathematical modeling to explore how these two parameters affect the screening process, and to identify the optimal values that yield the highest affinity aptamers. Since the fluorescence intensity of an AP is directly proportional to the aptamer affinity [see Eq. (S1)], we first sought to identify the target concentration that maximizes the difference in fluorescence signal between APs displaying aptamers with differing affinities. To do so, we calculated the ratio of the mean AP fluorescence for the highest-affinity aptamer relative to other lower-affinity aptamers in the pool [Eq. (S2), see the Supporting Information for derivation]. We focused our analysis on aptamers with $K_{d}$ values that are that are two to ten times higher (i.e. lower affinity) than that of the highest-affinity aptamer, because successful isolation of these aptamers automatically ensures the exclusion of aptamers with poorer affinity.

The resulting analysis revealed an optimal target concentration that yields the maximum difference in fluorescence between the AP with the highest affinity and the rest of the population (denoted by * in Figure 1a). This optimal target concentration can be closely approximated as one-half of the $K_{d}$ value of the highest-affinity aptamer [Eq. (S3), see the

![Scheme 1](https://example.com/scheme1.png)
that particle display can achieve a 1.7 \times 10^9-fold enrichment of target concentration into Equation (S1), we found that APs displaying the highest-affinity aptamers exhibit a mean fluorescence of roughly 1 nm fluorescence that far exceeds the theoretical maximum that can be achieved with affinity-based selection by many orders of magnitude.

Supporting Information for derivation]. By substituting this target concentration into Equation (S1), we found that APs displaying the highest-affinity aptamers exhibit a mean fluorescence of roughly $F_{\text{max}}/3$ (Figure 1a), where $F_{\text{max}}$ is the maximum fluorescence of an AP saturated with labeled target. Taken together, these findings offer useful experimental guidelines for optimizing FACS screening: the highest-affinity aptamers in a given pool can be effectively isolated by setting the FACS sort gate at $F_{\text{max}}/3$ and adjusting the target concentration such that only the particles with the strongest fluorescence signal (e.g., \leq 0.1\% of the population) are collected.

These screening conditions deliver enrichment performance that far exceeds the theoretical maximum that can be achieved with affinity-based selection by many orders of magnitude, and we performed a mathematical analysis to calculate the enrichment that can be achieved with particle display. We assumed a Gaussian distribution of fluorescence intensities (in logarithmic scale) for individual AP populations in our analysis. This is because any given pool of APs is likely to contain various populations of APs that display the same aptamer sequence, and the APs within these populations can exhibit a range of fluorescence intensities due to experimental variability (e.g., differences in aptamer copy number). The resulting distributions (see Figure S1b) allowed us to determine the overlap in fluorescence among populations of APs that each display different aptamer sequences. From this result, we estimated the probability of recovering an AP displaying a given aptamer sequence based on its affinity, and used this estimate to calculate the enrichment of the highest-affinity aptamers in a round of FACS (see the Supporting Information for derivation). As discussed above, in affinity-based selection (i.e., SELEX), the theoretical maximum enrichment that can be achieved for a given aptamer relative to another, lower-affinity aptamer in a single round is equal to the ratio of their equilibrium dissociation constants; thus, aptamers with a $K_d$ of 100 pm can at most be enriched 10-fold relative to a population of aptamers with $K_d$ of 1 nm after a single round. By comparison, we determined that particle display can achieve $1.7 \times 10^{10}$-fold enrichment of the same aptamer population in a single round, enabling dramatically more efficient purification of high-affinity aptamers (Figure 1b).

We experimentally validated the advantages of particle display by using our method to obtain high-affinity aptamers for four different proteins—thrombin, ApoE, PAI-1, and 4-1BB—in only three rounds. We chose thrombin and ApoE in order to compare the affinity of our aptamers to that of previously published aptamers for these targets. PAI-1 and 4-1BB were chosen because although RNA aptamers have been reported previous attempts at generating natural DNA aptamers for these proteins by SELEX were unsuccessful and ultimately required the use of chemically modified bases, suggesting that natural DNA may lack the chemical and/or structural diversity to yield useful aptamers for these two proteins.

Although one can synthesize APs from a naïve random library, due to the practical throughput limitations of our FACS instrument (ca. $4 \times 10^7$ particles per hour) we first performed one round of conventional selection using magnetic beads starting with $10^6$ copies of a random DNA library (Round 0), and used this enriched pool to synthesize an initial AP library consisting of roughly $10^8$ particles (see the Supporting Information). Prior to the screen, we used fluorescently labeled targets to measure nonspecific binding to FP-coated particles. Approximately 80\% of the APs after ePCR are predicted to display only the FP on their surface based on Poisson statistics, and thus should not exhibit significant binding to the protein targets. Thus, FP-displaying particles serve as an excellent negative control to establish the “reference gate” for calibrating nonspecific binding in the particle display screen (Figure 2, red boxes). In contrast, particles with higher fluorescence intensity within the sort gate (Figure 2, green boxes) indicate higher-affinity aptamers. We performed three rounds (R1–3) of particle display screening for each of our four target proteins, and the resulting FACS plots are shown in Figure 2a. In the first round, we exerted low screening stringency to prevent potential loss of high-affinity APs. This was achieved by setting the sort gate close to the reference gate, or with some overlap, such that at least 0.1\% of APs were collected (Figure 2a, row 1). Aptamers isolated in R1 for the four target proteins were PCR amplified to synthesize the APs for R2. Since a greater number of APs displaying each aptamer sequence were present in R2, we used the optimal screening conditions described above. Specifically, we set the sort gate at $F_{\text{max}}/3$ and decreased the target concentration such that we collected 0.1–0.2\% of the APs (Figure 2a, rows 2 and 3). Experimental conditions for all protein targets and screening rounds are summarized in Table S1 in the Supporting Information.

After three rounds of screening, the average affinity of the aptamer pool dramatically increased for all four target proteins. This can be readily observed from the fact that relative to the starting library, a far greater percentage of the AP population resides outside of the reference gate in R3, at a considerably lower target concentration. For example, the fraction of sorted APs for thrombin jumped from 0.1\% for the initial pool (Figure 2a) to 14.6\% for the R3 pool at a 50-
fold lower thrombin concentration (Figure 2b). Given this clear increase in binding affinity, we cloned the R3 pools into E. coli (see the Supporting Information) and picked 20 aptamer clones from each pool (Table S2). We then synthesized APs displaying each sequence (Figure S2) and measured their relative fluorescence at a fixed target concentration (see the Supporting Information; Figure S3). From these data, we chose the aptamers with the highest relative fluorescence for each target (Table S3), and subsequently obtained their $K_d$ values using a standard fluorescence-based assay. We present the binding isotherms in Figure 3 and secondary structures as modeled by mfold in Figure S6.

Particle display consistently generated aptamers with exceptional affinities for all four target proteins. For thrombin and ApoE, the aptamers discovered with particle display exhibited considerably higher affinities than those of previously reported aptamers for the same targets. For example, Thrombin-03 exhibited a $K_d$ of 7.04 pM (Figure 3a), approximately two and three orders of magnitude superior to existing thrombin aptamers described by Bock (2.6 nM) and Tasset (5.4 nM), respectively, as measured with the same binding assay (Figure S4). Similarly, our best ApoE aptamer (ApoE-06) exhibited a $K_d$ of 938 pM (Figure 3b), four times better than an aptamer previously isolated by our group using high-stringency microfluidic selection. Importantly, particle display also yielded high-affinity DNA aptamers for PAI-1 and 4-1BB, for which previous attempts to generate natural DNA aptamers by SELEX were unsuccessful. Our PAI-1-01 aptamer exhibits a $K_d$ of 339 pM (Figure 3c) and 4-1BB-07 shows a $K_d$ of 2.32 nM (Figure 3d), both comparable to the $K_d$ values of aptamers previously generated using modified bases. We also obtained similar $K_d$ measurements using aptamers in solution to bind bead-conjugated target proteins, further validating these results (Figure S5).

In this work, we report a novel aptamer discovery technology that yields superior aptamers in fewer rounds in comparison to conventional selection. By transforming solution-phase aptamers into aptamer particles by means of emulsion PCR, we could subsequently use FACS to quantitatively measure the affinity of every displayed aptamer and individually sort them in a high-throughput manner. Our theoretical analysis revealed how the stringency of our screen can be tuned, and we identified two key experimental parameters that enable efficient isolation of the highest affinity aptamers in a given pool. Using these parameters, we generated natural DNA aptamers with excellent affinities for four different proteins in three rounds. Our aptamers for thrombin and ApoE exhibited affinities that were orders of magnitude better than previously reported values. Equally importantly, our method also yielded high-affinity natural DNA aptamers for PAI-1 and 4-1BB, the two targets for which previous attempts to generate such aptamers were unsuccessful. The affinities of our aptamers were comparable to those obtained with these chemically modified aptamers, which suggests that natural DNA aptamers may be able to target a broader swath of the proteome than previously hypothesized, with the aptamer selection strategy representing a critical limiting factor in determining success or failure for a given target.

We have identified a number of opportunities for future expansion of our technique. For example, we believe that the multicolor sorting capabilities of FACS could be exploited by...
labeling closely related protein variants with distinct fluorophores as a means to generate highly selective aptamers that can discriminate subtle variations, such as posttranslational modifications or splice variants, as has been previously demonstrated with yeast display techniques.[26] Additionally, a growing number of nonnatural nucleotides with diverse structures and functional groups are now available, and we anticipate that our particle display technique could be adapted to accommodate these novel nucleotides to both improve aptamer performance and further expand the accessible target space. Finally, our technique offers an opportunity to perform aptamer discovery directly in complex mixtures such as serum, cell lysate, and various patient samples. The resulting aptamers could prove valuable for a host of applications in molecular diagnostics and targeted therapeutics[5,6,28] and is a subject of current investigation.

Given that our particle display strategy employs a pair of technologies that are readily commercially available (i.e., ePCR and FACS), we believe our technique should be broadly accessible to many research laboratories. Particle display should therefore offer a promising mechanism for generating high-quality aptamer reagents in a rapid and economical manner for the accelerated exploration and characterization of the human proteome.

Received: October 25, 2013
Published online: March 18, 2014

Keywords: aptamers · directed evolution · high-throughput screening · SELEX