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Selection Strategy to Generate Aptamer Pairs that Bind to Distinct Sites on Protein Targets

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Abstract

Many analytical techniques benefit greatly from the use of affinity reagent pairs, wherein each reagent recognizes a discrete binding site on a target. For example, antibody pairs have been widely used to dramatically increase the specificity of enzyme linked immunosorbent assays (ELISA). Nucleic acid-based aptamers offer many advantageous features relative to protein-based affinity reagents, including well-established chemical synthesis, thermostability and low production cost. However, the generation of suitable aptamer pairs has posed a significant challenge, and few such pairs have been reported to date. To address this important challenge, we present Multivalent Aptamer Isolation SELEX (MAI-SELEX), a technique designed for the efficient selection of aptamer pairs. In contrast to conventional selection methods, our method utilizes two selection modules to generate separate aptamer pools that recognize distinct binding sites on a single target. Using MAI-SELEX, we have isolated two groups of 2′-fluoro-modified RNA aptamers that specifically recognize the αV or β3 subunits of integrin αVβ3. These aptamers exhibit low nanomolar affinities for their targets, with minimal cross-reactivity to other closely related integrin homologs. Moreover, we show that these aptamer pairs do not interfere with each other's binding, and effectively detect the target even in complex mixtures such as undiluted serum.

Introduction

Compared to monovalent receptor-ligand interactions, multivalent molecular systems often yield dramatically higher affinity and specificity. In nature, microorganisms frequently exploit multivalent binding to their advantage; for example, although individual lectin-glycan interactions exhibit relatively low affinities, the influenza virus achieves dramatic

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affinity enhancement by implementing multivalent binding as a prelude to endocytosis.[1] Many molecular diagnostic and analytical techniques also exploit multivalency, in the form of affinity reagent pairs that each bind to distinct sites of a target protein. For example, the enzyme-linked immunosorbent assay (ELISA), the most widely-used molecular diagnostic platform, employs antibody pairs in a sandwich format that markedly increases specificity and thereby minimizes false diagnoses.[2,3] These advantages have been also shown for aptamer reagents, wherein the use of aptamers pairs that bind to different sites of target proteins, demonstrates significantly improved performance.[4] However, the process of generating aptamer pairs is a significant technical challenge, as evidenced by the small number of pairs reported to date.[5–8] Thus, there is an unmet need for alternative strategies for the effective and unbiased selection of aptamer pairs that bind to distinct sites.

Toward this goal, we report Multivalent Aptamer Isolation SELEX (MAI-SELEX), a novel selection strategy for generating aptamer pairs. MAI-SELEX employs two distinct selection stages: the affinity module and the specificity module. The affinity module enriches for target-binding aptamers without driving the selection to convergence against a single dominant binding site. Then, starting from this enriched pool, the specificity module separates the aptamers into groups based on their binding sites. The specificity module integrates the elements of two classic methods - counter selection[9,10] and toggle selection[11,12] - in a novel combination. This strategy efficiently yields two or more aptamer groups from a single selection with minimal bias.

As a demonstration of MAI-SELEX, we selected 2′-fluoro-modified RNA aptamer pairs that bind to different binding sites of integrin αVβ3. We chose this protein because it is an important biomarker of cancer,[13,14] and because previous selection efforts did not yield aptamer pairs for this target.[15] We obtained two families of aptamers that specifically recognize the αV and β3 subunits, and selected candidates from each pool for further characterization. These aptamers exhibit low nanomolar affinities for their respective subunits, with minimal cross-reactivity to other closely-related integrin homologs. Moreover, these aptamers can effectively bind to their targets in complex mixtures such as undiluted serum, and do not interfere with each other in binding to their respective sites on the same protein.

Experimental Section

Reagents and instruments

Bovine serum albumin (BSA), N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich, Inc. (Saint Louis, MO). Integrin αIIbβ3 was purchased from Enzyme Research Laboratories, Inc. (South Bend, IN). Integrin αVβ3 and Integrin αVβ6 were purchased from R&D Systems (Minneapolis, MN). The single-stranded DNA (ssDNA) library and all PCR primers were synthesized and purified by Integrated DNA Technologies (Coralville, IA). HotStart Master Mix and water for PCR were purchased from Qiagen (Hilden, Germany). The magnetic beads, including Dynabeads MyOne C1 streptavidin-coated beads and M-270 carboxylic acid-coated beads, were purchased from Invitrogen (Carlsbad, CA). Fluorescence measurements were performed in black 96-well microplates (Microfluor 2, Thermo Scientific, Waltham, MA) using a microplate reader (Tecan, Männedorf, Switzerland), and surface plasmon resonance (SPR) measurements were performed on a Biacore 3000 instrument (GE Healthcare, Waukesha, WI). Real-time PCR equipment (IQ5, Bio-Rad, Hercules, CA) was used to measure the DNA concentrations.

2′-F RNA MAI-SELEX: target and library preparation

Both M-270 and MyOne C1 beads were used to immobilize integrin proteins. M270 beads were activated with EDC and NHS, after which the proteins were immobilized according to the manufacturer's procedure. Prior to immobilization onto MyOne C1 beads, the protein was first biotinylated using the sulfo-NHS-LC-biotin reagent (Thermo Scientific) and then incubated with MyOne C1 beads, following the manufacturer's procedure. The immobilized proteins were quantified using the LavaPep Quantification Kit (Gel Company, San Francisco, CA). Each element of the ssDNA random library was composed of 50 randomized nucleotides flanked by 5′ and 3′ primer sites (5′-TAATACGACTCACTATAGGGAGGACGATGCGG-[50N]-CAGACGACTCGCCCGA-3′). The ssDNA library was amplified by PCR, and then transcribed to generate 2′-F RNA using a DuraScribe T7 Transcription Kit (EPICENTRE Biotechnologies, Madison, WI). We then digested the DNA template in the reaction using Turbo DNAse I (Ambion, Austin, TX), and purified the RNA library by urea-PAGE gel followed by electro-elution. The eluate was desalted and concentrated by ethanol precipitation, dissolved in PBS and quantified with a NanoDrop spectrophotometer (Wilmington, DE).

MAI-SELEX: affinity module

We used commercially available Dynabeads and a DynaMag magnet (Invitrogen) for MAI-SELEX in order to demonstrate the broad applicability of the method. Protein-coated magnetic beads were washed two times with 500 μl of PBSMT buffer (PBS supplemented with 2.5 mM $MgCl_2$ and 0.01% Tween-20) before each selection. Integrin αVβ3-coated M270 beads were used in the affinity module: 100 nM in the first three rounds, followed by 8 nM for rounds 4 and 5. At the start of SELEX, ~50 pmole random DNA library was transcribed into ~500 pmole of RNA library to be used in the first round. The 2′-F RNA library was folded by denaturing at 95 °C for 5 min and snap-cooling on ice for 5 min. We used an initial library concentration of 5 μM in round 1, and then reduced the concentration to 1 μM in rounds 2 and 3, and to 370 nM in rounds 4 and 5. Integrin αVβ3-coated beads were incubated with the library in 100 μl selection buffer (PBS supplemented with 2.5 mM $MgCl_2$, 0.1% BSA and 100 μM yeast tRNA) for 2 hours at room temperature. After incubation, the beads were magnetically trapped in order to remove the supernatant. The trapped beads were washed three times with 500 μl wash buffer (PBS supplemented with 2.5 mM $MgCl_2$ and 0.1% BSA); the total duration of washing was 15 min for round 1 and 1 hour for subsequent rounds. Target-bound aptamers were eluted from beads by heating at 95 °C for 5 min, and then reverse-transcribed to generate cDNA using the ThermoScript RT-PCR System (Invitrogen). We monitored the progress of the selection by subjecting 1 μl cDNA to quantitative PCR (qPCR). Everything else being equal, the decrease in threshold cycle (Ct) value from one round to the next indicates the increase in the proportion of target-binding sequences. The qPCR result is also valuable in predicting the proper number of PCR cycles to use for full-scale library amplification. After reverse transcription and PCR amplification of the total aptamer eluate, we transcribed the PCR product back to 2′-F-modified RNA and purified the RNA pool for the next round of selection, following the procedure described above.

MAI-SELEX: specificity module

MyOne C1 beads were used in the specificity module, to avoid accumulating sequences that bind to M270 beads. 100 nM aptamer pool RNA was incubated with 5 μM integrin αIIbβ3-coated beads in 30 μl selection buffer for 2 hours, after which the integrin αIIbβ3-coated beads were trapped and the supernatant was transferred to another tube to incubate with 20 nM integrin αVβ3-coated beads in 30 μl selection buffer for 2 hours. The integrin αIIbβ3-coated beads were then washed with wash buffer for 1 hour. Finally, the integrin αVβ3-

coated beads were also trapped and washed with wash buffer for 1 hour. The aptamers were eluted from both sets of beads and amplified following the same procedure as described in the affinity module.

Cloning and sequencing of aptamer pools

The αV and β3 pools were reverse transcribed and amplified by PCR, and then cloned into *E. coli* using the TOPO TA Cloning Kit (Invitrogen). 25 colonies from each pool were randomly picked and sequenced at Genewiz Inc. (South Plainfield, NJ). The sequences were then analyzed and aligned using Geneious v5.1 (Biomatters Ltd, New Zealand). Two representative aptamer sequences from the αV pool and three from the β3 pool were selected for further affinity measurements.

Filter-binding assay

RNA aptamers were treated with Antarctic phosphatase (New England Biolabs, Ipswich, MA) and then labeled at the 5′ end with radioactive γ-^{32}P ATP using T4 polynucleotide kinase (New England Biolabs). We then incubated 0, 10, 25, 100, 400, and 500 nM of protein with 1 nM radiolabeled RNA in 20 μl reactions, and separated protein-bound RNA from unbound RNA by passing the solution through a 0.45 μm pore-size Millipore (Billerica, MA) mixed cellulose ester filter membrane. This membrane has strong affinity for protein but not RNA, such that protein and protein-bound RNA will be retained while unbound RNA will flow through. We then quickly washed the membrane twice with 1 ml PBS and measured the amount of protein-bound RNA by measuring remaining scintillation counts on the membrane. For K_d measurement, at least six different concentrations of protein were used in the filter-binding assay, and the scintillation count values were plotted against protein concentrations to generate a binding curve. The curve was then fitted using the GraphPad Prism software (La Jolla, CA).

Surface Plasmon Resonance Measurements

We biotinylated the αV-1 and β3-1 aptamers at the 5′ end by adding a mixture of 5′-biotin-G-monophosphate (Trilink, San Diego, CA) and GTP (molar ratio 3:2) to the *in vitro* transcription reaction. Biotinylated aptamers were then purified as described above, and immobilized by flowing 10 nM aptamer solution at 20 μl/min onto the surface of the Biacore SA chip (GE Healthcare). Next, we obtain a series of SPR sensorgrams using the Kinetics Wizard software: at each cycle of the kinetic measurement, we applied varying concentrations of integrin protein for association (0/25/50/100 nM for αV-1 measurement, where duplicate tests were performed at the 25 nM and 50 nM concentration points; 0/10/50/100/200/500 nM for β3-1 measurement) at 20 μl/min for 2 mins, stopped the protein injection, and allowed 5 mins for dissociation. We then regenerated the sensor surface by injecting 10 mM glycine pH 3.0 at 30 μl/min for 30 s. A flow cell without immobilized aptamers was used as reference.

Enzyme-linked oligonucleotide assay (ELONA)[16]

Individual aptamers were biotinylated at the 5′ end as described above. For ELONA, microtiter plate wells were coated with integrin proteins by adding 50 μl protein solution (at 25 nM unless noted otherwise) and incubating at 4 °C for 37 hours. All subsequent steps were performed at room temperature. After incubation, we washed the plate once with 200 μl PBS supplemented with 0.05% Tween-20 (PBST buffer) and then blocked each well with 100 μl 1% BSA in PBST for 1 hour. We then washed the plate with 200 μl PBST three times, added 100 μl of biotinylated aptamers (at 10 nM unless otherwise noted) dissolved in PBST supplemented with 0.1% BSA (PBSTB buffer), and incubated for 1 hour. Next, we washed the plate three more times with 200 μl PBST, and added streptavidin-conjugated

horseradish peroxidase (HRP) dissolved in 100 μl PBSTB buffer at 1:500 dilution. After 30 min incubation, we washed the plate five times and added the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) substrate. This substrate becomes oxidized by HRP to produce a blue-green color, which we measured with a Tecan plate reader at 405 nm wavelength (OD_{405}). The ELONA test in serum was performed in a similar manner, except that the biotinylated aptamers were dissolved in 100 μl undiluted fetal bovine serum (FBS) instead of PBSTB buffer.

Results and Discussion

Overview of the MAI-SELEX process

MAI-SELEX employs two separate selection modules ('affinity' and 'specificity') to generate aptamer pairs (Fig. 1). The process starts within the affinity module, which enriches aptamers that bind anywhere on the target protein (in this case, integrin αVβ3). We synthesized a library of DNA molecules, each consisting of a random 50-nucleotide region flanked by a pair of constant primer regions.[17] We then amplified the DNA library by PCR, and used it as a template to generate 2′-F RNA by *in vitro* transcription (step 1). Next, we incubated integrin αVβ3-coated magnetic beads with the 2′-F RNA library (step 2) and isolated target-bound aptamers via magnetic separation (step 3). We amplified the isolated RNA sequences by reverse-transcription PCR (RT-PCR) (step 4), and repeated the above process to further improve aptamer affinity. We performed five rounds of selection in this module to obtain an aptamer pool with sufficient affinity (the αVβ3 pool). In order to ensure that the resulting αVβ3 pool had not converged and retained aptamers targeting a wide variety of binding sites, we monitored the affinity of the pool after each selection round and terminated affinity module selection when the average dissociation constant (K_d) reached ~10 nM as measured by filter-binding assay (see Experimental Section).

Next, we utilized the specificity module to separate aptamers that bind to the αV subunit from those that recognize the β3 subunit. This module employs a "decoy" protein, which may be a homologous protein that shares a common subunit with the target or a subdomain of the target protein itself. In this work, we used magnetic beads coated with integrin αIIbβ3 as a decoy, which shares the β3 subunit with our target. We incubated the decoy proteins with the αVβ3 pool from the affinity module (step 5), in order to capture the aptamers that bind to this shared subunit. During this process, it is critical to add sufficient decoy protein to capture the majority of β3-binding aptamers. We then eluted these from the beads to obtain a β3-specific aptamer pool (the β3 pool) (step 6a). With those aptamers that did not bind to αIIbβ3 (Step 6b), we performed one additional round of magnetic selection with αVβ3 (step 7) to obtain a pool of aptamers that specifically recognize the αV subunit (the αV pool) (step 8).

Selection of aptamers that specifically target αV or β3 subunits

We observed a continuous increase in the fraction of RNA bound to αVβ3 during the five rounds of affinity module selection. To quantify the fraction of target-bound aptamers after each round, we labeled the 5′ end of the aptamers with radioactive γ-^{32}P-ATP and performed a series of filter-binding assays[18] (see Experimental Section). We found that the percentage of target-binding RNA monotonically increased from 0.96% (unselected library) to 2.26% after three rounds of selection, 2.95% after four rounds and finally 8.77% after five rounds (Fig. 2A). We used this filter-binding assay to estimate the K_d of the αVβ3 pool, incubating 1 nM ^{32}P-labeled round 5 αVβ3 pool RNA with varying concentrations of integrin αVβ3 and then measuring the scintillation counts for each sample (Fig. 2B). Using commercial software (GraphPad Prism) and assuming 1:1 Langmuir binding, we calculated the K_d value of the αVβ3 pool to be 3.8 nM.

We subsequently performed a single round of selection with the round 5 αVβ3 pool in the specificity module. The resulting αV pool and β3 pools were amplified by RT-PCR, and cloned into *E. coli*. We then randomly picked colonies for sequencing (Table S1). We found that most sequences only existed in one pool, with the exception of one sequence, (αVβ3-1), which appeared in both pools with multiple copies. We selected two unique sequences from the αV pool (αV-1 and αV-2), three from the β3 pool (β3-1, β3-2 and β3-3), as well as αVβ3-1, for further characterization.

Aptamer pairs with high affinity to distinct binding sites

Using ELONA, we show that the unique sequences from the αV and β3 pools possess desired subunit specificity. Both αV-1 and αV-2 aptamers exhibited significant affinity for integrin αVβ3, but not for integrin αIIbβ3; in contrast, the β3-1, β3-2 and β3-3 aptamers showed significant binding to both integrin αVβ3 and integrin αIIbβ3 (Fig. S1A). When tested with integrin αVβ6, both αV-1 and αV-2 showed significant binding, while β3-1, β3-2 and β3-3 showed minimal binding (Fig. S1A). Taken together, these results indicate that both aptamers from the αV pool (αV-1 and αV-2) bind exclusively to the αV subunit, while all three aptamers from the β3 pool (β3-1, β3-2 and β3-3) only bind to the β3 subunit. Interestingly, the αVβ3-1 sequence, which was present in both pools at high copy numbers, showed minimal binding to integrin αVβ3 (Fig. S1B). We suspect this sequence may have originated from synthesis bias.[19]

We selected αV-1 and β3-1 sequences for further characterization, because they were the most abundant sequences within their respective families. We used the mfold software to model the secondary structure of αV-1 and β3-1 (Fig. S1C & S1D).[20] Interestingly, the aptamers αV-1 and αVβ3-1 only differed by a single base in the primary sequence, yet exhibit dramatically different binding properties. Such dramatic differences arising from a single base difference have been previously reported in literature.[19,21]

We first measured the equilibrium dissociation constants (K_d) of the two aptamers using two separate methods: ELONA and surface plasmon resonance (SPR). For ELONA, we coated plate wells with 0.5 nM integrin αVβ3 and then added varying concentrations of αV-1 or β3-1 into each well and plotted the OD_{405} value against the aptamer concentration for each sample. Using GraphPad Prism, we calculated the K_d values of αV-1 and β3-1 to be 2.7 nM and 6.5 nM, respectively (Fig. 3A & 3B). The K_d value of β3-1 is comparable to that of previously reported molecules.[15]

For SPR measurements, we biotinylated the αV-1 and β3-1 aptamers and immobilized them onto the surface of a Biacore SA chip (GE Healthcare). Then, we applied varying concentrations of integrin αVβ3 (for αV-1) or αIIbβ3 (for β3-1) protein to obtain a series of SPR sensorgrams and analyzed the data using the BIA evaluation software (Fig. 3C & 3D). For αV-1, we calculated the association constant (K_{on}) to be 2.77×10^5 $M^{-1}s^{-1}$ and the dissociation constant (K_{off}) to be 2.47×10^{-3} s^{-1}, yielding K_d = 8.9 nM. For the β3-1 aptamer, we measured $K_{on} = 5.33\times10^4$ $M^{-1}s^{-1}$, $K_{off} = 5.57\times10^{-4}$ s^{-1} and K_d = 10.5 nM. The higher K_d values (*i.e.* lower affinity) obtained via SPR are presumably due to the surface immobilization of the aptamer, as it is well known that immobilization conditions and linker design can affect apparent affinity on surfaces.[22]

αV-1 and β3-1 aptamers effectively distinguish the target from homologs

As there is significant homology among the different subunits in the integrin protein family,[14,23] it is critical that selected aptamers are highly specific and can effectively distinguish among homologous proteins. To test the specificity of the αV-1 and β3-1 aptamers for their target subunits, we coated plate wells with various concentrations of

integrin αVβ3, integrin αIIbβ3 or integrin αVβ6 and then added biotinylated αV-1 or β3-1 for ELONA. The results showed that while αV-1 effectively recognizes both αV-containing integrins at 100 pM concentration, it exhibits negligible affinity for the homologous αIIb subunit even at 25 nM. This demonstrates the remarkable specificity of αV-1 for its αV target, in that it fails to bind even a 250-fold higher concentration of a homologous protein (Fig. 4A). Similarly, while β3-1 effectively recognizes both β3-containing integrins at 100 pM concentration, its affinity for the homologous β6 subunit is negligible even at 25 nM (Fig. 4B).

αV-1 and β3-1 aptamers do not interfere with each other in target binding

Since any affinity reagent pair used in a sandwich assay must efficiently bind to the same target, we tested whether αV-1 and β3-1 interfere with each other in binding to integrin αVβ3 using ELONA. We measured the affinity of biotinylated αV-1 for integrin αVβ3 in the presence of an excess of unlabeled β3-1 or αV-1 (as a positive control). We observe that, while unlabeled αV-1 greatly reduced the interaction between biotinylated αV-1 and integrin αVβ3 by competing for binding sites, the presence of unlabeled β3-1 did not result in signal reduction (Fig. 4C). From this result, we can conclude that β3-1 does not interfere with the binding of αV-1 to integrin αVβ3. Conversely, we also found that αV-1 does not interfere with the binding of β3-1 to integrin αVβ3 (Fig. 4D).

Next, we tested whether these aptamers can recognize their targets in complex biological mixtures, such as undiluted fetal bovine serum (FBS). To do so, we coated microtiter plate wells with 0, 10 or 50 nM integrin αVβ3 and added undiluted FBS containing 50 nM αV-1 or β3-1 into each well for ELONA. The signal from the 0 nM integrin αVβ3 sample was used as the baseline, and subtracted from that of other samples. Despite the extremely high concentration of non-target protein present in the serum (~1 mM), both aptamers were able to bind to their binding sites and successfully detect integrin αVβ3 (Fig. 4E).

Finally, we verified whether αV-1 and β3-1 aptamers indeed function as a reagent pair in a sandwich ELONA assay. As the capture reagent, we immobilized 400 nM biotinylated αV-1 aptamer on microtiter wells pre-coated with streptavidin, and blocked the remaining streptavidin sites with free biotin. Then, we added either 0, 50, or 250 nM integrin αVβ 3 in undiluted serum, as well as 100 nM biotinylated β3-1 pre-incubated with streptavidin HRP. HRP-bound β3-1 need to form a sandwich complex with integrin αVβ3 and immobilized αV-1 in order to elicit signal. Indeed, the aptamers functioned as a reagent pair and successfully detected integrin αVβ3 (Fig. 4F). We note that the ELONA signal can be further improved by optimizing the process of aptamer immobilization, incubation time, reagent concentrations and other related factors.[24]

Conclusion

In this report, we describe a selection strategy for isolating aptamer pairs that bind to distinct epitopes of a target protein. Using integrin αVβ3, an important cancer biomarker as a model, we have identified two families of aptamers that specifically recognize the αV and β3 subunits. We found that two of the isolated aptamers from these families (αV-1 and β3-1) exhibit low nanomolar affinities for their respective targets, with minimal cross-reactivity to other, closely-related integrin homologs. Moreover, we confirmed that these nuclease-resistant, 2′-F-modified aptamer pairs do not interfere with each other's binding, and can be effectively used as reagents for ELONA assays in buffer as well as complex mixtures such as undiluted serum. An important advantage of method is that, unlike previous approaches,[8] our method does not require a pre-existing aptamer that blocks a particular binding site on the target. Furthermore, our method requires significantly less number of selection rounds compared to performing direct parallel selection/counter

selections. Importantly, because our method starts with a single initial library, it enables identification of sequences that become enriched through PCR and other selection biases. For example, as described above, we found αVβ3-1 to exist in both αV and β3 pools, but showed low affinity to both subunits. If selections were to be carried out using two different initial libraries for the two targets, such identification may be challenging.

An important prerequisite of the MAI-SELEX process is the availability of a decoy for use in the specificity module. This decoy can be a homologous protein that shares a common subunit with the target, or a subdomain of the target protein itself. Although this requirement somewhat limits the generality of our method, considering that homologous proteins from different species are often available, and the fact that ~65% of the human proteome are multi-subunit proteins,[25] we believe that our method would be useful for a wide range of protein targets. Furthermore, even for monomeric protein targets that lack suitable homologues, we may consider the use of fusion proteins (e.g. GST fusions) for the display of independent, properly-folded subdomains of an individual target protein as decoys.

Although beyond the scope of the work presented here, it is interesting to consider how the concept of affinity/specificity modules might be extended to generate aptamers with desired properties. For example, one could consider using two decoy proteins, each representing a different region of the target, as a means to separately capture different aptamer groups and further improve the efficiency of the specificity module as depicted in Fig. S2. Alternately, one may be able to generate a “broad spectrum” affinity reagent that has affinity towards multiple proteins (*i.e.* high-cross reactivity) by using a mixture of decoy proteins. Such reagents could be useful for a number of applications, including as promiscuous kinase inhibitors that recognize several therapeutic targets[26] or antagonists that target both wild type and mutant forms of a viral protein.[9] Finally, it is interesting to consider the use of next-generation sequencing with MAI-SELEX to identify multivalent aptamer reagents through bioinformatic sequence analysis.[19] We believe such extensions of our selection method could help unlock the full potential of aptamer reagents as a tool for molecular diagnostics and targeted therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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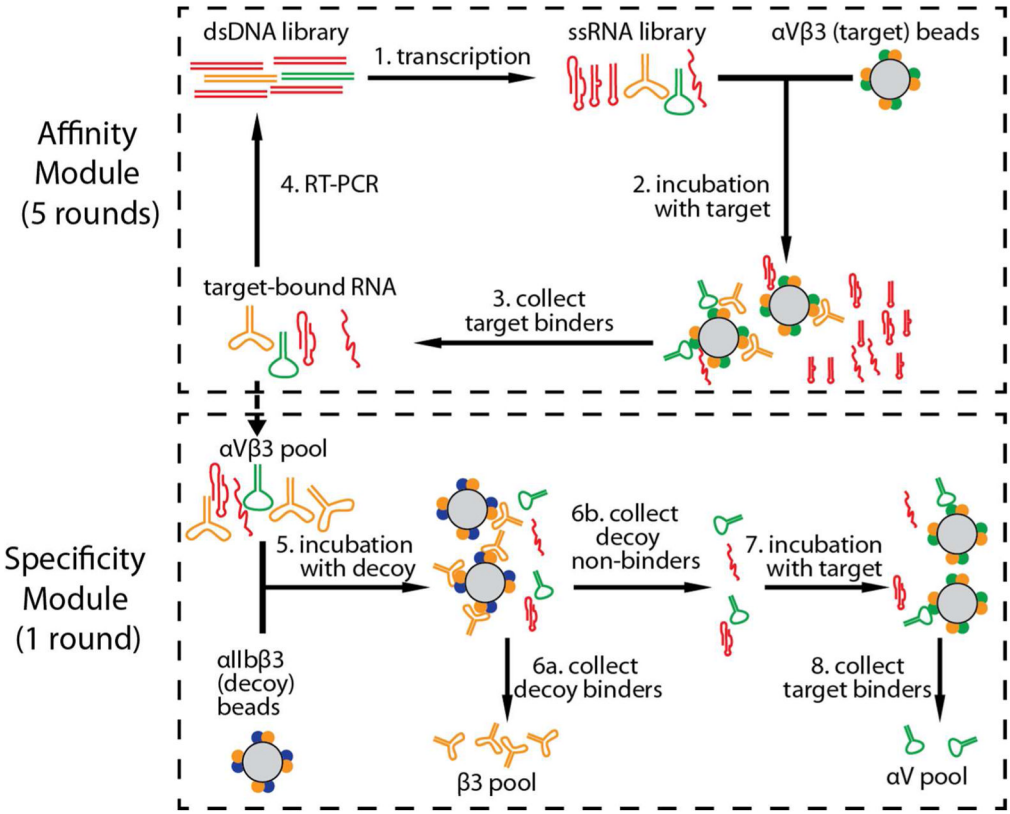

Fig. 1. MAI-SELEX scheme

In the affinity module (top), we synthesized the RNA aptamer library (step 1), and selected aptamers that recognize either subunit of integrin αVβ3 using magnetic separation (step 2). We collected the aptamers bound to αVβ3 (step 3), and amplified them via RT-PCR (step 4). We repeated these steps until the affinity of the αVβ3 pool reached ~ 10 nM. Then, we performed the specificity module (bottom), wherein we separated aptamers that bind to αV from those that bind β3. We incubated the αVβ3 pool with an excess of integrin αIIbβ3-coated micromagnetic beads, which served as the decoy (step 5). We obtained the β3 pool by performing selections with the decoy protein (step 6a). In parallel, we also collected the supernatant, which contained sequences that did not bind to the decoy (step 6b). Finally, we obtained the αV pool by performing a selection again with αVβ3 (step 7 & 8).

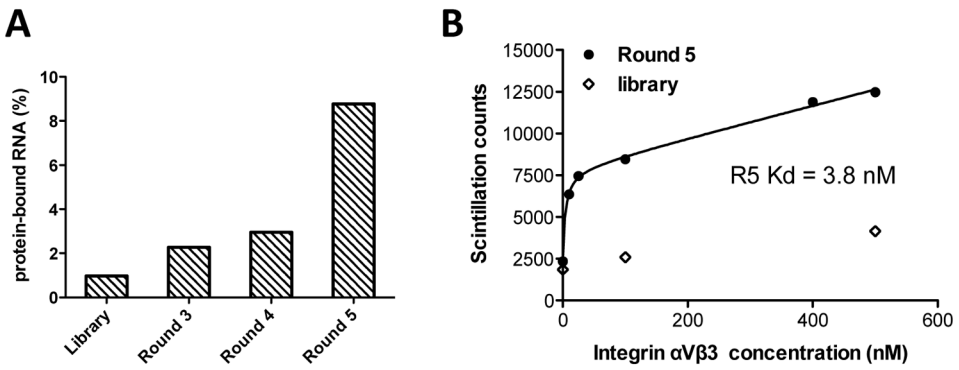

Fig. 2. Aptamer affinity enrichment over five rounds of affinity module selection

(A) We incubated 100 nM integrin αVβ3 with 1 nM radiolabeled RNA from the initial library and affinity module rounds 3, 4 and 5, and measured the percentage of protein-bound RNA in each sample by filter-binding assay. (B) After incubating 1 nM radiolabeled RNA from the round 5 (R5) pool with different concentrations of target protein, we plotted the scintillation counts from the protein-bound RNA. The bulk K_d of the round 5 pool was 3.8 nM. In comparison, the binding signal from library slowly increased with higher protein concentrations, but even at 500 nM concentration the signal was still lower than the R5 binding signal at 10 nM concentration.

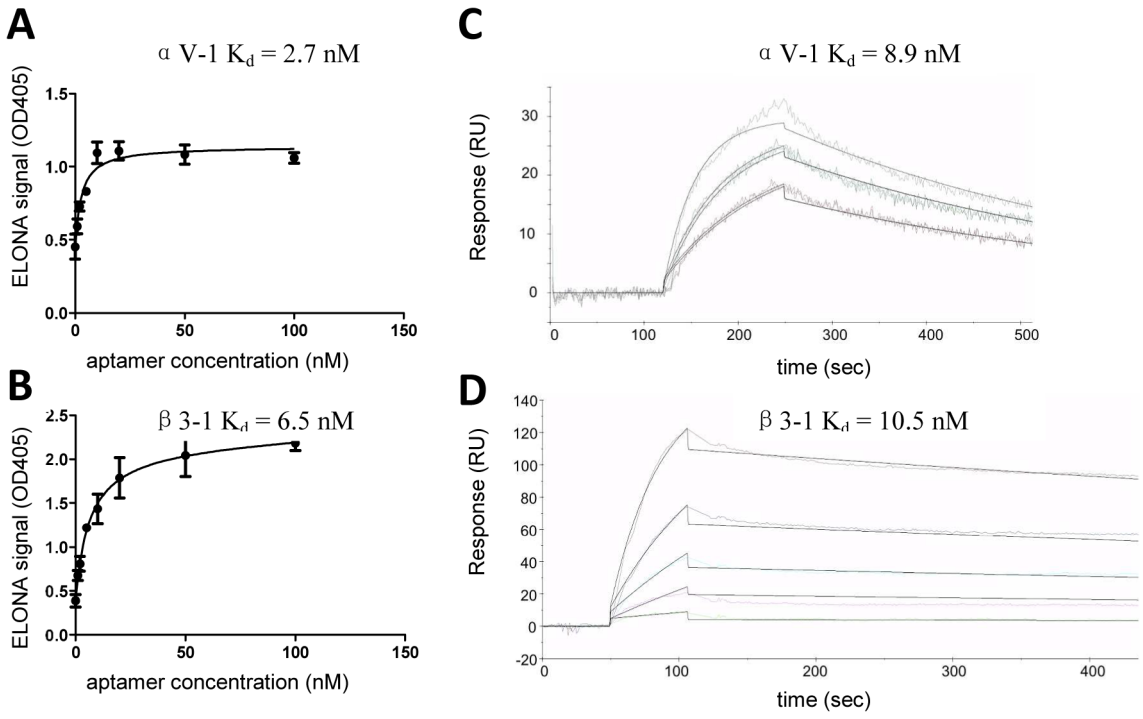

Fig. 3. Affinity measurement of aptamers αV-1 and β3-1

(A) & (B) We measured aptamer affinity using ELONA. We coated plates with 0.5 nM integrin αVβ3, and then added varying concentrations of αV-1 or β3-1 into each well to generate a binding curve. We calculated K_d values for αV-1 and β3-1 to be 2.7 nM and 6.5 nM, respectively. (C) & (D) Biotinylated αV-1 and β3-1 were immobilized on a Biacore SA chip. We then injected different concentrations of integrin protein (αVβ3 for αV-1, αIIbβ3 for β3-1) and recorded the binding activity in a series of sensorgrams, and calculated the K_d value using the BIA evaluation software. Based on these measurements, we calculated K_d values for αV-1 and β3-1 to be 8.9 nM and 10.5 nM, respectively.

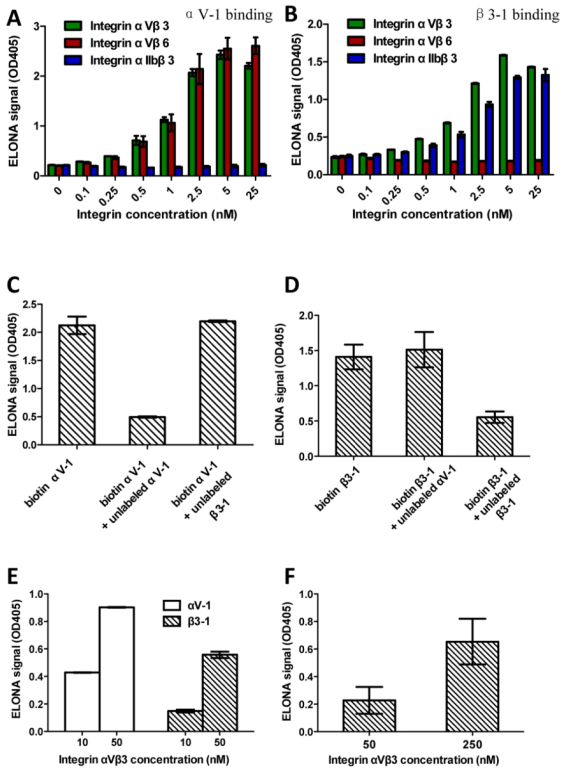

Fig. 4. Specificity measurement of aptamers αV-1 and β3-1

(A) & (B) We measured the specificity of αV-1 and β3-1 using ELONA. We coated a plate with various concentrations of integrin αVβ3, integrin αIIbβ3 or integrin αVβ6 and then added biotinylated αV-1 or β3-1 for ELONA. Both αV-1 and β3-1 detected their respective targets (αV and β3 subunits) at pM concentrations, and exhibited negligible affinity for homologous subunits (αIIb and β6 subunits). (C) & (D) We measured whether αV-1 and β3-1 competed with each other in binding to αVβ3, by incubating excess amount of unlabeled αV-1 or β3-1 together with biotinylated αV-1 or β3-1 in ELONA. The unlabeled β3-1 does not affect the binding signal of αV-1, and unlabeled αV-1 does not affect the binding signal of β3-1, indicating that the aptamers do not interfere with each other in binding to αVβ3. (E) The binding performance of αV-1 and β3-1 was measured in serum. Both aptamers were able to detect integrin αVβ3 at 10 and 50 nM concentrations in serum. (F) The αV-1 and β3-1 aptamers effectively function as a reagent pair in a sandwich ELONA to detect integrin αVβ3.