ACS Combinatorial Science

Research Article

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Magnetic-Assisted Rapid Aptamer Selection (MARAS) for Generating High-Affinity DNA Aptamer Using Rotating Magnetic Fields

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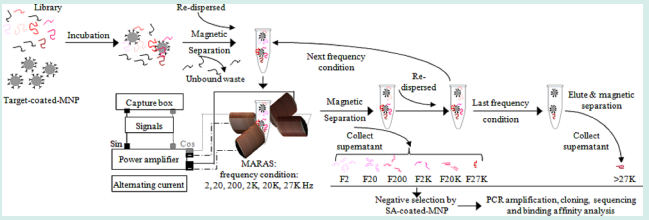
Supporting Information

ABSTRACT: A new SELEX protocol for the development of DNA aptamers has been demonstrated, referred to as magnetic-assisted rapid aptamer selection (MARAS). This method uses magnetic beads and an externally applied rotating magnetic field to provide the competitive mechanism for the selection aptamers with different affinities to the molecular target. The MARAS protocol efficiently generated aptamers with high affinity and specificity for C-reactive protein, a common cardiovascular disease indicator. The binding affinities of the selected aptamers could be varied by changing the frequency of the externally applied rotating magnetic field and optimal cases bound with low-nanomolar dissociation constants.



KEYWORDS: *aptamers, SELEX (systematic evolution of ligands by exponential enrichment), C-reactive protein, magnetic nanoparticle, MARAS*

INTRODUCTION

Systematic evolution of ligands by exponential enrichment (SELEX) is an in vitro selection method for the isolation of DNA or RNA sequences (aptamers) that bind to a target of interest from large combinatorial libraries of random sequences.[1] Once the sequence of an aptamer is identified, the aptamer can be produced entirely by chemical synthesis. Furthermore, aptamers can be easily modified with chemical groups that increase their stability in various biological applications that are otherwise harmful for a nucleic acid. Aptamers are therefore useful in many situations that have traditionally been addressed by antibodies.[2]

The single-strand SELEX (ss-SELEX) protocol was first described in 1990.[3−5] Since then, many modifications have been proposed, but the basic steps of the process (incubation, separation, elution, amplification, and purification) have remained largely unaltered. The procedure is repeated for several rounds, starting with the enriched library obtained in the previous round. Usually, between 5 and 15 SELEX selection rounds must be performed until no further enrichment of the functional nucleic acid species is detectable and dissociation constants (K_d) of low-micromolar to low-nanomolar are achieved. Standard SELEX, therefore, requires a significant investment of resources, time, and biomolecular technology.

Efforts to speed and streamline the process have recently included automation,[6,7] the use of capillary electrophoresis (CE-SELEX),[8] and microfluidics (M-SELEX).[9] CE-SELEX uses the high efficiency of capillary electrophoresis to separate aptamers bound with targets by virtue of an induced shift in electrophoretic mobility. The aptamers that are obtained after a few (i.e., 1−4) CE-SELEX selection rounds have high affinities to target molecules with K_d values at the nanomolar level. However, CE-SELEX is less effective in screening aptamers that bind to small molecules, because small molecules do not induce sufficient shift for separation. M-SELEX consists of a continuous-flow magnetically activated chip-based separation (CMACS) device and magnetic nanoparticle-assisted SELEX. In the best example, an aptamer with K_d of 33 ± 8 nM was identified via a single round of M-SELEX. However, both CE-SELEX and M-SELEX require technology and instruments that are not common in biochemical and medical laboratories. Other modified SELEX procedures that are less commonly used include Photo-SELEX, SPIEGELMER-Technology, and Genomic SELEX.[10−12]

We describe here an efficient aptamer selection platform that uses biofunctionalized magnetic nanoparticles to separate target-bound DNA oligonucleotides from a library, selecting those interactions that survive a disruptive force generated by the movement of the particles in an externally applied rotating magnetic field. We used C-reactive protein (CRP) as a study model, which was coated on the surface of biofunctionalized magnetic nanoparticles. CRP is generally used as a common indicator of inflammation, heart attack, stroke and cardiovascular diseases (CVD).[13−15] This novel technology, called the magnetic-assisted rapid aptamer selections (MARAS) process, produced high-affinity aptamers against CRP in a straightforward and inexpensive manner using equipment common to most biochemistry laboratories. Moreover, we found that the MARAS protocol consumed fewer resources, that the entire procedure was capable of being performed within an hour, and that the process can be automated to provide high throughput aptamer selection using a single machine.

Received: December 10, 2013
Revised: April 9, 2014
Published: May 9, 2014

   dx.doi.org/10.1021/co5000272 | ACS Comb. Sci. 2014, 16, 321−327

RESULTS AND DISCUSSION

MARAS Experimental Setup. As shown in Figure 1, the MARAS process used a rotating magnetic field generated by

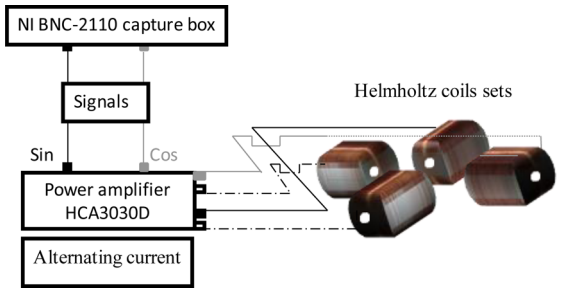


Figure 1. Schematic diagram of the MARAS apparatus.

two sets of Helmholtz coils placed orthogonally. Two signals, $\cos(\omega t)$ and $\sin(\omega t)$, were fed into a 2-channel power amplifier by a LABVIEW program via a NI BNC-2110 capture box. These two signals were amplified equally, driving the coils to produce rotating magnetic fields. The sample was placed at the intersection of the central lines of the two sets of Helmholtz coils, and the field strength was calibrated using a gauss meter.

Parameter-Dependence of MARAS Procedure. A single-stranded oligo-DNA library with a 20-nucleotide randomized region preceded and followed by constant primer regions was purchased from Invitrogen Life Technologies (Invitrogen Life Technologies, Grand Island, NY, USA). The library was dissolved with BD buffer (1 nM). Ten microliters of this library (containing 2.84×10^{11} copies) was heated to 95 °C for 5 min and then snap cooled at 4 °C. The CRP-coated, biofunctionalized magnetic nanoparticles (CRP-MNP), obtained by magnetic separation from 5 μL of CRP reagent, were added to the library and the mixture was incubated for 30 min at room temperature. The magnetic nanoparticles were separated from unbound nucleotides with a magnetic stand, and the bound mixture was washed 3 times with 1 mL of BD buffer. BD buffer (100 μL) was added to resuspend the bound mixture in the microtube, and the tube was placed in the apparatus depicted in Figure 1.

For a frequency-dependent study of the MARAS protocol, the experimental procedure shown in Figure 2 was performed. The resuspended bound mixtures were sequentially subjected to a constant magnetic field strength (14 gauss) and a series of increasing rotating field frequencies (2, 20, 200, 2000, 20K, and 27K Hz, the highest frequency possible at 14 gauss with the power amplifier used), each for 10 min with stirring every 2.5 min by pipetting to avoid agglomeration due to the action of the magnetic field on the magnetic nanoparticles. After each 10 min period, a magnetic separation was performed to collect supernatant, 100 μL of BD buffer was added to resuspend the retained bound mixture, and the mixture was subjected to the next higher rotating field frequency in the series. After the process was completed with the applied magnetic field with the final frequency, a magnetic separation was used to separate the supernatant, and the bound mixture was retained. Finally, 100 μL of BD buffer was added to resuspend the retained nanoparticles and heated to 95 °C for 5 min to elute the bound aptamers from the CRP-MNPs. A magnetic separation was performed to collect the supernatant.

In the standard MARAS protocol, the supernatant obtained after magnetic separation at each successive field frequency was mixed with streptavidin-coated magnetic nanoparticles (SA-MNP), incubated for 30 min at room temperature, and then subjected to magnetic separation to remove aptamers that bind to the SA-MNPs rather than the target CRP. The resulting samples were named for the corresponding frequency as shown in Figure 2 (F2, F20, ..., >27K). All the collected supernatants were precipitated with 1 mL cold ethanol to isolate the DNA. The above negative selection could also be performed before the positive selection step with similar results.

Four aptamers obtained from the DNA pool isolated at each step of the MARAS selection were obtained by PCR amplification, cloning into *Escherichia coli*, and sequencing of randomly selected colonies. The detail sequence results are shown in Table 1. The predicted secondary structure was analyzed by using Mfold program.[16] The representative secondary structures of selected aptamer, which screened from each step of MARAS, are shown in Supporting Information Figure 1S. The result illustrated structural similarities between the sequenced clones, which are consistent with aptamer recognition of the target conformation. There are two possible conserved motifs that might interact with CRP protein in the experiment. One of the motif is a central circle connected with several stem loops (F2−1, F20−1-A, F200−3-

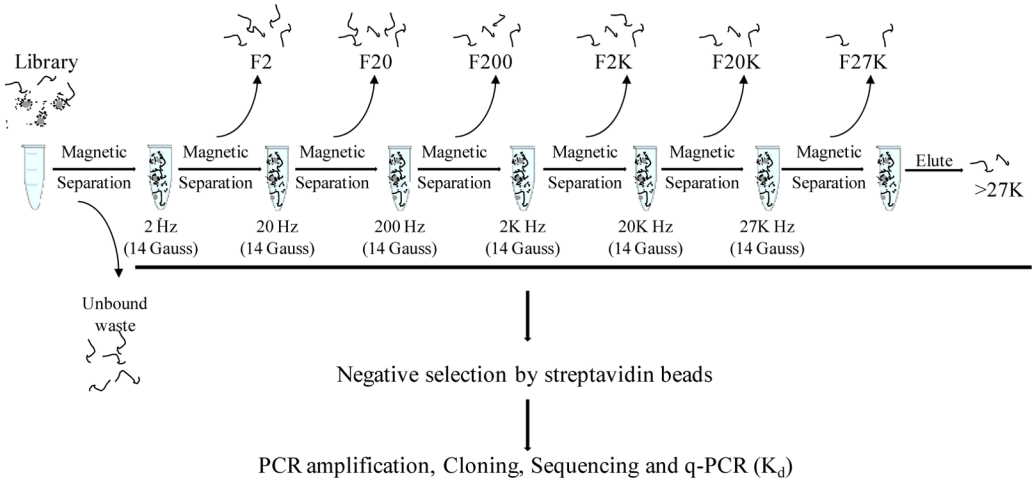


Figure 2. Schematic diagram of a frequency-dependent analysis by the MARAS process.

dx.doi.org/10.1021/co5000272 | *ACS Comb. Sci.* 2014, 16, 321−327

Table 1. Sequences and Dissociation Constants (K_d) of 20N Region in the Aptamers Selected by Using MARAS Protocol

clone name	5′-sequence-3′	K_d mean ± SD (nM)
20N RO F2-1	AGACCTTTGAAGGCCCCAAG	121.26 ± 6.23
20N RO F2-2	TTGGTTTGTAACATTGATGG	125.73 ± 7.67
20N RO F2-2-B	TAGTTTATCATTTTCCAGTG	119.02 ± 6.09
20N RO F2-4-C	GGAGTTGTATTCGCACTAGG	124.38 ± 4.28
20N RO F20-4	AGTTAGTTTCAGTGGGTCCC	85.88 ± 3.38
20N RO F20-5	ACCGTGTTGTCTGAGGTCTC	91.92 ± 2.78
20N RO F20-1-A	GACCCGTCAGCTCCTAATGG	90.74 ± 2.12
20N RO F20-3-B	TCGGGTAAAACACTGTTGAT	88.37 ± 1.00
20N RO F200-1	TCGGTGTGCCACTTTCTCAA	81.06 ± 2.68
20N RO F200-3	TTTTAGTTGTGCCTGGTGCG	78.78 ± 1.08
20N RO F200-1-A	CTAATCGAGGCCACATGAGT	79.32 ± 2.15
20N RO F200-3-B	GTTTCGCCTTCCGTAGTGAT	75.45 ± 2.09
20N RO F2K-1	GGTGTGCGTTATTCTGTGGC	54.04 ± 1.47
20N RO F2K-4	CTCTTGGACAACTGGATTAG	57.89 ± 3.41
20N RO F2K-1-A	AAATCTACTGGAGTGTCGAG	58.36 ± 1.44
20N RO F2K-3-C	GCCAAGGCTCCTTCCCCTAT	51.00 ± 1.37
20N RO F20K-1	ACCCTACGTACGTTCTCATG	24.59 ± 1.15
20N RO F20K-2	ATATAATTTTGCTCTTTATT	25.76 ± 1.84
20N RO F20K-1-A	GCCGCGTTCATCTTCGATGA	25.90 ± 0.71
20N RO F20K-3-B	ACTCGTAGTGGCTGTGCGGA	28.80 ± 2.05
20N RO F27K-4	GCCCCCGAAGTTGCTTAGTC	22.71 ± 0.46
20N RO F27K-6	CACATTAGGTGTGATAAGGC	22.76 ± 0.88
20N RO F27K-2-A	AAGCGAAGTTGGGAATCAAG	19.88 ± 0.99
20N RO F27K-3-B	GGGACTCTGCTCTCCTTAAC	21.42 ± 0.89
20N RO > 27K-1	TCTGTAATTTATAGTTCCAT	7.65 ± 2.62
20N RO > 27K-5	TTTTTCGCTGGTGTTCGACC	12.48 ± 1.74
20N RO > 27K-2-A	GTATCACAGTGGAGAGTGCT	11.06 ± 1.22
20N RO > 27K-4-B	AGCGCATTGTGGAGACAGTG	11.02 ± 0.54

B, F2K-3-C, F20K-1, F27K-4, and >27K-1), another is a linear form with circle at both end of the structure (F2−4-C, F20−3-B, F200−1, F2K-1-A, F20K-3-B, F27K-2-A, and >27K-5). Furthermore, binding affinities of selected aptamers to the target protein, given as dissociation constants K_d, were measured by q-PCR[17] and a nonlinear fitting curve as described in the Experimental Procedures. The representative nonlinear fitting curve results are shown in Supporting Information Figure 2S. The detail K_d values of the 20N aptamers are shown in Table 1 and were plotted against the field frequency as shown in Figure 3. In the figure, K_d was found to be inversely proportional to the rotational field frequency, suggesting that faster oscillatory fields produced greater selection pressure for tight binders. The lowest K_d value observed was 7.65 ± 2.62 nM for aptamer >27K − 1. This suggests that an aptamer having a desired range of affinity for the target can be selected by properly choosing a lower cutoff and an upper cutoff for the frequency of the applied magnetic fields in the MARAS method. We therefore refer to this procedure as a "window-type" MARAS operation.

As the sample is subjected to a rotating magnetic field, the bound aptamer−nanoparticle mixtures are subjected to a driving torque (produced by a phase lag between the dipole moment of magnetic nanoparticles and the magnetic field direction) producing rotational motion or angular oscillation of the nanoparticles (depends on the applied field frequency), and a dissipative torque (because of the angular velocity of the nanoparticle). These two counter torques exert a stretching force on the binding interaction between oligonucleotide sequences and CRP-MNPs that increases with increasing field rotational frequency. The oligonucleotide sequences remain

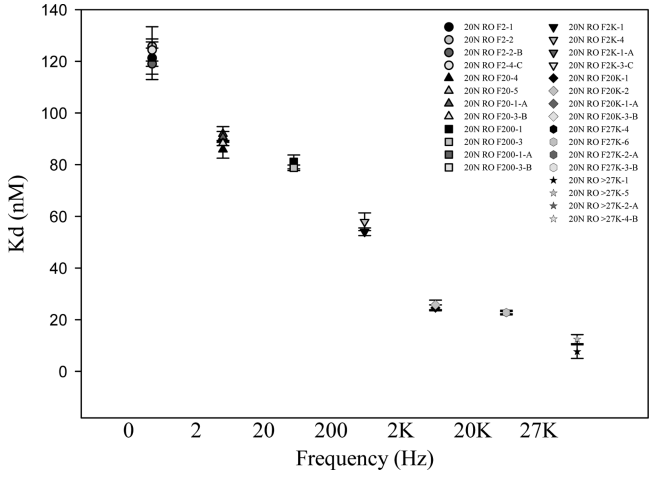


Figure 3. K_d vs magnetic field rotation frequency for the 20N aptamers produced by the MARAS protocol. The dot and error bar of each data point indicate the mean value and the standard deviation of the dissociation constant for three separate experiments.

bound to the nanoparticles until this stretching force counteracts the binding strength of the oligonucleotide toward the CRP-MNPs. The stretching force could also be increased by raising the field strength of the rotating magnetic field or the size of the magnetic particles, but rotational frequency is easiest to control.

One-Shot MARAS Procedure. To further demonstrate the ease and the viability of the MARAS protocol for the selection of high-affinity aptamers, a one-shot MARAS (OS-MARAS) was performed using the setup shown in Figure 1. A CRP-

dx.doi.org/10.1021/co5000272 | ACS Comb. Sci. 2014, 16, 321−327

MNP-bound mixture from the same naïve 20N library used above was subjected directly to a high rotating magnetic field frequency of 20K Hz (at 14 gauss) for 10 min, with stirring every 2.5 min by pipetting. After a magnetic separation, the supernatant was removed and the retained bound mixture was resuspended with 100 μL of BD buffer. The remaining steps (elution from the nanoparticles at 95 °C for 5 min, magnetic separation to collect the supernatant, negative selection to remove potential SA-MNP bound aptamers, precipitation, PCR, cloning and sequencing of two randomly selected aptamers) were performed as above. The 20N aptamers thus obtained from this one-step procedure were designated OS-RO-20N > 20K-3 and OS-RO-20N > 20K-6, and were found to have similar binding affinities to the best aptamers isolated from the stepwise MARAS procedure (11.05 ± 0.93 and 13.89 ± 3.20 nM, respectively; see Table 2 for detailed sequences).

Table 2. Sequences and Dissociation Constants (K_d) of the 20N Region in the Aptamers from a One-Shot MARAS Processes, with Negative Selection after or before (Rev) the Positive Selection

clone name	5′-sequence-3′	K_d
OS-RO-20N > 20K-3	TACAGTTTAGTGAGTGCAAG	11.05 ± 0.93
OS-RO-20N > 20K-6	CTATGTGAGTATTTCGCCAC	13.89 ± 3.20
Rev-OS-RO-20N > 20K-2	CAGCTGGTCTGGGGGGCGTT	13.05 ± 3.88
Rev-OS-RO-20N > 20K-6	CTCTTACTTTTGCTCGTCTC	12.59 ± 0.69

Supporting Information Figure 3Sa shows the predicted secondary structures and dissociation constant results of the OS-RO-20N > 20K-3 and OS-RO-20N > 20K-6 aptamers.

Performing the negative selection first (designated as reversed MARAS) worked well in the one-shot protocol as well. Thus, 10 μL of the randomized 20N library was subjected to a negative selection by incubation with SA-MNPs followed by magnetic separation. The resulting supernatant was used as a reduced library in the same one-shot MARAS procedure described above (14 gauss and 20 kHz, for 10 min with stirring every 2.5 min). The detail sequence results are shown in Table 2 and the predicted secondary structures and dissociation constant data are shown in Figure 3Sb, for two resulting aptamers, designated as Rev-OS-RO-20N > 20K-2 and Rev-OS-RO-20N > 20K-6, showing very similar results as for the initial one-shot procedure in which negative selection was done second.

Direct Observation of Affinity Selectivity to Target of Aptamers Selected by MARAS. Two of the highest affinity aptamers described above (F27K-4 and >27K-1, K_d = 22.71 ± 0.46 and 7.65 ± 2.62 nM, respectively) were labeled with FITC dye as described in the Experimental Procedures. These fluorescent aptamers were added to CRP-MNPs followed by magnetic separation and washing. The resulting particles were shown to be bound by both aptamers by fluorescence microscopy (Figure 4 a, c). These bound particles were then subjected to a rotating magnetic field (14 gauss, 27K Hz) and washed. Fluorescence imaging (Figure 4 b, d) showed detachment of only the aptamer derived from selection at that frequency (F27K-4), but not the higher-affinity aptamer isolated from higher-frequency challenge (F > 27K-1). [In a control experiment, the FITC-F27K-4 aptamer was not observed by fluorescence microscopy to bind to streptavidin-coated nanoparticles lacking C-reactive protein; see Supporting Information, Figure 4S.] These results show that by altering the condition of the externally applied magnetic field in the MARAS process, aptamers with different binding affinities to the target can be differentiated.

CONCLUSION

According to our experimental results, aptamers isolated through the MARAS protocol can interact with targets. Also, the dissociation constants selected through the MARAS protocol are magnetic field-dependent, of which the value decreases as the field frequency increases. In addition, the dissociation constants of single strand aptamers binding with targets could easily reach a single digital nanomolar concentration by applying a rotating magnetic field with a proper frequency and strength for MARAS protocol. In addition, an aptamer to a target molecule with a desired range for the dissociation constant can be selected by properly choosing a lower cutoff and an upper cutoff for the frequency of the applied magnetic fields via the MARAS protocol. Moreover, the MARAS protocol did not require repeated selection cycles. The procedure was streamlined to involve just 2 steps: (1) the application of a proper rotating magnetic field to the sample and (2) the execution of a negative selection, if the material preparation and the post analyses, such as PCR amplification, cloning, and dissociation constant calculation, were excluded which are also needed for other SELEX protocol. Since the procedure is relatively simple, the MARAS protocol proposed here may be performed in any common biochemistry laboratory and would enable the end user to rapidly screen the suitable aptamer for the desired application. As compared to the traditional ss-SELEX process, the MARAS protocol described in this study consumes fewer supplies and can be completed in less than an hour, as compared to several weeks to several months for the ss-SELEX protocol. Furthermore, due to the simplicity of the procedure, future automation using a single machine or miniaturization in a microchip may be feasible.

EXPERIMENTAL PROCEDURES

Oligonucleotide Library and Primers. 20N randomized oligonucleotide sequence was used as the library, which consisted of a randomized 20-mer midsection (5′-AGCAGC-ACAGAGGTCAGATG-N20-CCTATGCGTGCTACCGTG-AA-3′), synthesized at 1 mM scale and PAGE purified (Invitrogen Life Technologies, Grand Island, NY, USA). One set of primers, (Lab-forward 5′-AGCAGCACAGAGGTCAGA-TG-3′ and the Lab-reverse 5′-TTCACGGTAGCACGCATA-GG-3′), was used to anneal the 5′ and 3′ degenerating region of the library during the PCR amplification. 5′-biotin-labeled primer, Lab-biotin-R, with the same sequence as was described above, was used to isolate the forward single strand from the double strand PCR product. A set of primers, Lab-FITC-F and Lab-biotin-R, with the same sequence as described above, was used to generate FITC-labeled, single strand aptamers. The universal T7 primer was used to sequence the nucleotide of the selected aptamer (T7:5′-TAATACGACTCACTATAGGG-3′).

CRP-Coated Magnetic Particles. The streptavidin-coated magnetic reagent (SA reagent) used in this study was purchased from Magqu (Magqu, Taipei, Taiwan). The average hydro-dynamic diameter of the streptavidin-coated magnetic nano-

dx.doi.org/10.1021/co5000272 | ACS Comb. Sci. 2014, 16, 321−327

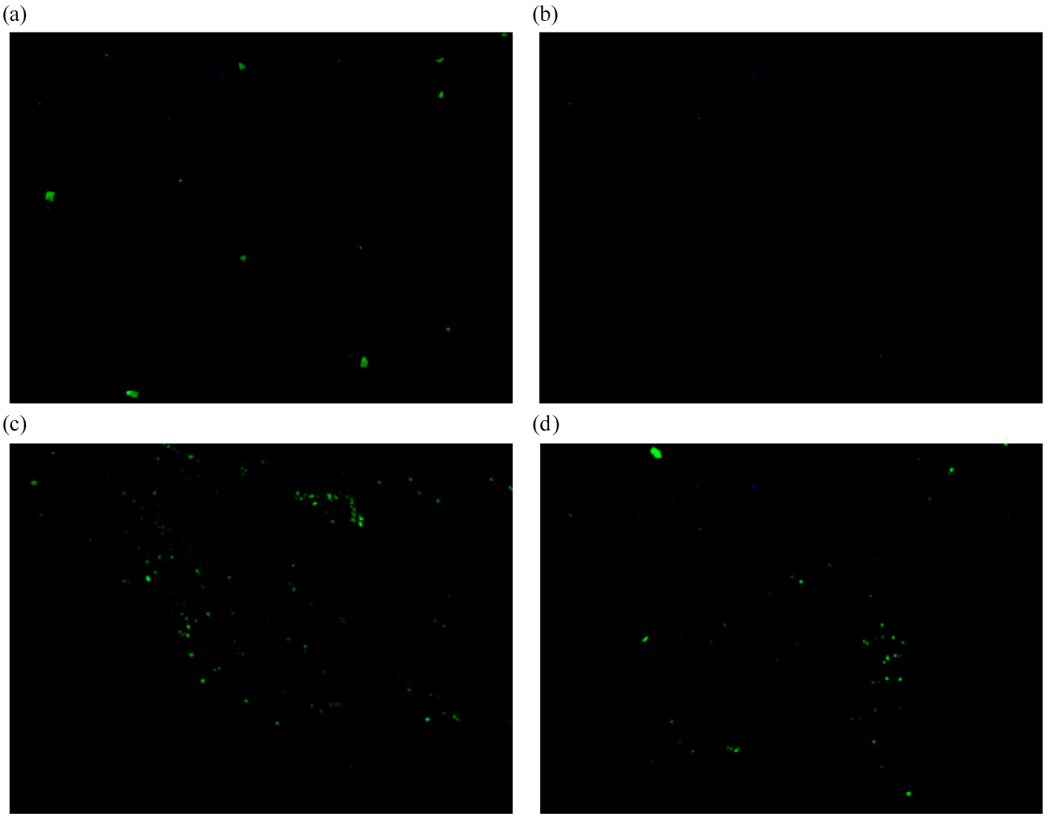


Figure 4. Representative fluorescence microscopy images of FITC-labeled aptamers binding with CRP-derivatized nanoparticles. (a) FITC-F27K-4 aptamer + CRP-MNPs. (b) FITC-F27K-4 aptamer + CRP-MNPs after having been subjected to a rotating magnetic field (14 gauss, 27K Hz, 10 min). (c) FITC->27K-1 aptamer + CRP-MNPs. (d) FITC->27K-1 + CRP-MNPs after having been subjected to a rotating magnetic field (14 gauss, 27K Hz, 10 min).

particles (SA-MNPs) in the reagent was 50 nm. The reagent had a concentration of SA-MNPs of 0.3 emu/g. The magnetic nanoparticles were biofunctionalized by coating streptavidin on the outermost surface of nanoparticles and were dispersed in PBS (pH = 7.4). The pureness of the human CRP used in this study was greater than 99% and was purchased from MYBIOSOURCE (MYBIOSOURCE, San Diego USA). The biotinylation kit (Biotin Labeling Kit-NH_2) was purchased from Abnova (Abnova, Taipei, Taiwan). In this study, a total volume of 600 μg of pure CRP protein was used. The biotinylated CRP protein was prepared according to the manufacturer's instructions. Afterward, a total of 250 μg biotinylated CRP protein was mixed with 50 μL of SA reagent and incubated overnight at 4 °C. The high affinity binding between the streptavidin and biotin ensured the conjugation between the magnetic nanoparticles and the biotinylated CRP protein. Then, the incubated solution was subjected to magnetic separation to remove the unbound biotinylated CRP. The CRP-MNPs were resuspended in 50 μL PBS-T (50 mM of K_2HPO_4, pH 7.5, 150 mM NaCl, 0.05% Tween-20) to form CRP labeled reagent (CRP reagent) and stored at 4 °C. The final concentration of biotinylated CRP in CRP reagent was 188.47 ± 1.30 ng/μL, as determined by Bradford assay (Bio-Rad, Hercules, CA, USA). Before use, the CRP reagent was washed 3 times with binding buffer (BD: 50 mM of NaH_2PO_4, pH 8.0, 150 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 0.005% (v/v) Tween-20). During each washing step, a magnetic stand (Magqu) was used to collect the CRP-MNPs. Similarly, if the SA-MNPs were needed, then the SA reagent was washed 3 times with BD buffer and the SA-MNPs were collected by using a magnetic stand.

Cloning, Sequencing, and Motif Analysis of Selected Aptamers. The supernatants that were collected from each experimental step were precipitated with 1 mL of 100% cold alcohol and diluted by 100 μL of ddH_2O for subsequent PCR amplification. The collected supernatants were subsequently amplified by PCR, with Lab-F and Lab-R primers. The PCR reaction, which contained 1.25 U of DNA polymerase (Invitrogen), 0.1 mM of dNTPs, 0.5 mM of $MgSO_4$, and 0.5 nM primers, was performed under the following conditions: 5 min at 95 °C; 35 cycles of 40 s at 95 °C; 40 s at 60 °C; 40 s at 72 °C; and 10 min at 72 °C. The PCR product was purified by using a DNA Extraction Miniprep System (Viogene, Taipei, Taiwan). The purified product was subcloned into a pGEM-T Easy vector (Promega, Madison, WI, USA). The cloning procedure was performed according to the manufacturer's instructions. The plasmids of picked-up colonies were purified by using a High-Speed Plasmid Mini Kit (Geneaid, Taipei, Taiwan). The plasmids were sequenced by using an Applied Biosystems PRISM 3730 DNA automatic sequencer and a Big Dye terminator cycle sequencing kit (Foster City, CA, USA). The secondary structures of the aptamers were predicted by using Mfold.[16]

Estimation of Aptamer Binding Affinity by Real-Time Quantitative PCR. The affinity of the aptamers for the CRP target was described by the equilibrium dissociation constant (K_d), which was measured by a real-time PCR.[17] For each plasmid picked from the cloning experiments, 10 ng of aptamer clone plasmid was used as a PCR template to generate double

dx.doi.org/10.1021/co5000272 | ACS Comb. Sci. 2014, 16, 321−327

strand DNA (dsDNA) with Lab-biotin-R and Lab-F primers. The PCR condition and procedure were as described above. After the completion of PCR amplification, the PCR product was mixed with SA-MNPs, obtained by magnetic separation from 5 μL of SA reagent. Forward single strand aptamers (nonbiotinylated strand) were separated from the immobilized complementary strand, by being incubated with 0.15 N of fresh NaOH for 5 min. The bound SA-MNPs were removed with a magnetic stand. An equal amount of 0.15 N of HCl was added to the collected supernatant to adjust the final pH to 7.0, after which the forward ssDNA was precipitated with 1 mL of 100% ice-cold alcohol. The concentration of the single strand aptamers was determined with a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). A series of progressively diluted aptamers (200−1.5625 nM) in 20 μL of BD buffer were heated to 95 °C for 5 min and cooled at 4 °C for the formation of secondary structures. Partial diluted aptamers were retained as an input control (input). CRP-MNPs, obtained by magnetic separation from 5 μL of CRP reagent, were added into each microtube containing diluted aptamers and incubated for 30 min at room temperature. The bound CRP-MNPs were washed twice with 100 μL of BD buffer. The bound aptamers were eluted from the CRP-MNPs by heating the aptamers at 94 °C for 10 min in 20 μL of ddH_2O. The CRP-MNPs in the solution were removed with a magnetic stand, and the supernatants were collected. Both the input control and eluted aptamers were precipitated with 1 mL of 100% ice-cold alcohol. The input control and eluted aptamers were individually dissolved in test tubes filled with 100 μL of ddH_2O. The quantities of the aptamers in each test tube, including input control tube and eluted aptamer tubes, were calculated by real-time quantitative PCR (q-PCR). q-PCR was performed with MicroAmp optical 96-well reaction plates, and the threshold cycle (ct) value was calculated automatically using the maximum correlation coefficient approach with StepOnePlus Real-Time PCR Systems software, version 2.0 (Applied Biosystems). The mixture for each q-PCR run was 10 μL containing 2.5 μL of SYBR Green PCR master mix (Applied Biosystems) and 0.5 nM of primer Lab forward and Lab reverse. The reaction condition was as follows: 95 °C for 3 min; 40 cycles at 94 °C for 30 s; 60 °C for 30 s; and 72 °C for 30 s. The concentrations of the aptamers in the input control and of the eluted aptamers were calculated, using a 200 nM concentration of aptamers as indicative of maximum binding. The K_d value of the selected aptamer was then determined by fitting a saturation binding curve based on the experimental data via a curve fitting program, CurveExpert1.3 (curveexpert.webhop.net). The K_d value of the selected aptamer was performed in duplicate for each q-PCR run and were expressed as the mean ± standard deviation from three separate experiments performed.

FITC-Labeling Aptamer Preparation, Selectivity, and Specificity Analyses of Selected Aptamers. The plasmids of the aptamers were used as templates and a set of primers, Lab-FITC-F and Lab-biotin-R, was used for PCR amplification. The PCR condition and purification procedure of the FITC-labeled, single strand aptamer procedures were as described above. For the selectivity analysis of the aptamers via MARAS, 10 nM FITC-labeled, single-strand aptamers were heated to 95 °C for 5 min to disrupt the formation of secondary structures, snap cooled at 4 °C for 10 min, and incubated with 5 μL of CRP reagent for 30 min at room temperature. The bound compounds were washed 3 times with BD buffer to remove unbound aptamers by magnetic separation. The bound mixtures of the FITC-labeled aptamers with CRP reagent were redispersed in 20 μL of BD buffer and were observed by a fluorescence microscopy (DP72; Olympus, Center Valley, PA). Sequentially, the redispersed bound mixtures were subjected to a rotating magnetic field (14 gauss/27K Hz) for 10 min, and stirred every 2.5 min by pipetting, via the setup of Figure 1. The bound compounds were washed 3 times with BD buffer to remove detached aptamers by magnetic separation. The resultant bound mixture was redispersed in 20 μL of BD buffer and was analyzed with a fluorescence microscopy. For the specificity analysis of aptamers, 10 nM of fluorescence-labeled aptamers were heated to 95 °C for 5 min to disrupt the formation of secondary structures, snap cooled at 4 °C for 10 min, and incubated with CRP reagent and SA reagent for 30 min at room temperature, separately. The bound compounds were washed 3 times with BD buffer to remove unbound aptamers by magnetic separation. After it was washed, the CRP reagent and SA reagent bound with/without aptamers were redispersed with 20 μL of BD buffer and examined by using a fluorescence microscopy. The fluorescent images shown in the figure can be pulled by using a magnet that assures these green spots containing the target-bound magnetic nanoparticles binding with the FITC-labeled aptamers

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Representative predicted secondary structures of selected aptamer sequences from each step of MARAS using MFold analysis, representative non-linear fitting curve results of aptamer from each step of MARAS, representative secondary structures and non-linear fitting curve results of the 20N region in the aptamers from a one-shot MARAS processes, and representative images of fluorescence of the aptamer binding specificity with CRP reagent. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the National Science Council of Taiwan under grant number 102-2221-E-005-054.

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