High-affinity RNA aptamers to C-reactive protein (CRP): newly developed pre-elution methods for aptamer selection

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Abstract. We have developed a modified SELEX (systematic evolution of ligands by exponential enrichment) method to obtain RNA aptamers with high affinity to C-reactive protein (CRP). CRP is a clinical biomarker present in plasma, the level of which increases in response to infections and noninfectious inflammation. The CRP level is also an important prognostic indicator in patients with several syndromes. At present, CRP content in blood is measured immunochemically using antibodies. To develop a more sensitive method using RNA aptamers, we have attempted to obtain high-affinity RNA aptamers to CRP. We succeeded in obtaining an RNA aptamer with high affinity to CRP using a CRP-immobilized Sepharose column and pre-elution procedure. Pre-elution is a method that removes the weak binding portion from a selected RNA population by washing for a short time with buffer containing CRP. By surface plasmon-resonance (SPR) analysis, the affinity constant of this aptamer for CRP was calculated to be \( K_D = 2.25 \times 10^{-9} \) (M). The secondary structure, contact sites with CRP protein, and application of this aptamer will be described.

Introduction
RNA aptamers are biologically functional RNA molecules with specific binding abilities to target molecules, such as proteins and small biomolecules [1–3]. The aptamers are usually artificially selected from randomized RNA pools (combinatorial library) by several rounds of selective amplification. This selection procedure is called SELEX (systematic evolution of ligands by exponential enrichment) [2]. Many aptamers for different molecules have been reported [2,4,5]. We have also previously
reported an RNA aptamer that specifically inhibits the highly active bacterial protease subtilisin [6]. Aptamers are also of interest as unique tools in diagnostics and therapeutics, because their action is similar to that of antibodies. An RNA aptamer has already been developed as an RNA drug for inhibition of macular degradation by specifically targeting the vascular endothelial growth factor [7].

C-reactive protein (CRP) is a clinical biomarker present in plasma, and the level of CRP increases in response to infections and noninfectious inflammation [8]. Initially, CRP was thought to only be a marker of vascular inflammation, but a recent study indicated that it is also important in atherogenesis [9]. The CRP level is also an important prognostic indicator in patients with acute coronary syndrome. At present, CRP content in blood is measured immunochemically using antibodies [10]. To develop a more sensitive RNA aptamer-based method, we have attempted to obtain a high-affinity RNA aptamer to CRP.

Here, we report the development of an RNA aptamer with high affinity to CRP using a newly developed method involving pre-elution. The RNA aptamer to CRP obtained here showed much higher affinity to CRP than antibodies. The secondary structure of the aptamer and contact sites with CRP protein are also reported.

**Materials and methods**

**Materials**

C-reactive protein (recombinant, CRP) was purchased from Oriental Yeast Co. CNBr-activated Sepharose 4B was purchased from Pharmacia. T7 RNA polymerase and RNasin (human placenta ribonuclease inhibitor) were from Toyobo. RNase-free DNase I (RQ-DNase) was purchased from Promega. All other enzymes and chemicals used in this study were purchased from commercial sources. CRP-immobilized Sepharose 4B was prepared by coupling of CNBr-activated Sepharose 4B with CRP according to the supplier’s instructions. The CRP content in the gel was 1.2 mg/mL of gel. The CRP-immobilized Sepharose 4B gel was washed several times and stored in binding buffer consisting of 20 mM Tris-HCl (pH 7.5), 140 mM NaCl, 2 mM CaCl₂, and 1 mM MgCl₂ at 4°C.

**Synthetic DNAs**

The template DNA used for synthesis of the initial random RNA population was constructed with the following oligonucleotides: Template FR (5’-TCCGCATACGAGCACTGAGT(N₅₀)GTACTACGGATCGAAGTTATAGTGA-
Template SL contained a predetermined stem structure of high G+C content consisting of the sequences 5'-GGCGGGCC-3' and 5'-GGCCCGCC-3' (underlined). This stem structure had a random loop (N\textsubscript{12}), and this stem-loop structure was flanked by two random sequences (N\textsubscript{12}) containing fixed sequences the same as that of template FR. Primer 1 had the whole T7 promoter sequence and a sequence complementary to the 3'-terminus of Template FR or Template SL. Primer 2 had the same sequence as the 5'-terminal fixed region of Template FR or Template SL.

Preparation of RNA pools

RNAs were synthesized by \textit{in vitro} transcription using T7 RNA polymerase. First, to prepare template double-stranded DNA (dsDNA), PCR-like reaction (filling in of nucleotides into the single-stranded regions) was performed using 50 pmol of Template FR (or 50 pmol of Template SL), 100 pmol of Primer 1, and \textit{Taq} polymerase (Toyobo). Seven cycles of temperature shifts (denaturation, hybridization, and elongation) were performed. This reaction was calculated to yield approximately $3 \times 10^{13}$ different dsDNA molecules. Using ten times the amount of dsDNA ($3 \times 10^{14}$ different dsDNA molecules) from Template FR or SL as a template, the first RNA pool was prepared from the reaction mixture containing T7 Buffer (Toyobo), 0.5 mM each rNTP, 1 units/μL T7 RNA polymerase, and 0.27 units/μL RNasin in a total volume of 300 μL. The mixture was incubated at 37°C for 90 min. The DNA template was removed by RQ-DNase I treatment and the RNA was purified by denaturing 10% PAGE (polyacrylamide gel electrophoresis). After this purification, the RNAs obtained with Template FR and Template SL were mixed and this preparation was used as the first generation of the RNA pool.

In \textit{vitro} selection

The selection of RNA was performed using a CRP-immobilized Sepharose 4B column (0.08 – 0.5 mL gel bed volume = 0.1 – 0.6 mg of total CRP protein immobilized; see Table 1). The column was equilibrated with binding buffer consisting of 20 mM...
Tris-HCl (pH 7.5), 140 mM NaCl, 2 mM CaCl$_2$, and 1 mM MgCl$_2$. The first RNA preparation ($6 \times 10^{14}$ molecules; see Materials and methods) was dissolved in 100 µL of binding buffer and heated at 65°C for 10 min, then incubated for 30 min at room temperature. To remove the Sepharose 4B-binding population, the RNA solution was first poured into the pre-column containing only 0.5 mL of Sepharose 4B gel. After application of the RNA preparation, 3 mL of the binding buffer was then added to the pre-column to obtain the eluate. This pre-column treatment was performed only in the first three selection rounds of SELEX (Table 1). The eluate was then poured into the CRP-immobilized Sepharose 4B column (0.5 mL of gel containing 0.6 mg immobilized CRP; see Table 1). The column was then washed three times with 5 mL of the binding buffer. The total volume of this washing buffer was 30 column volumes. For elution of the CRP-binding population, 1 mL of the elution buffer (binding buffer containing 10 µM CRP) was added to the column and incubated for 30 min at room temperature. This elution was repeated and the RNA preparation was collected by ethanol precipitation. This RNA preparation was reverse transcribed and the cDNA was then amplified by PCR. Reverse transcription of selected RNAs was performed using Primer 2 and a Thermo Script RT kit (Invitrogen). The resulting cDNA was amplified by PCR using Primers 1 and 2.

**Binding assay using disk-filters**

RNA aptamers were first radiolabeled internally by *in vitro* transcription using [$\alpha$-$^{32}$P] UTP as described [11]. The labeled RNA was dissolved in binding buffer. The RNA solution was incubated at 65°C for 10 min and then at room temperature for 30 min to allow proper folding. The RNA was mixed with various concentrations (see Figs. 1 and 3) of CRP protein solution and incubated at 25°C for 30 min. The final concentration of RNA in the binding reaction mixture was 40 nM. The mixture was filtrated *in vacuo* through a disk-filter (MF-membrane filter with 0.45 µm pore size; Millipore), and the filter was washed with binding buffer. After drying, the radioactivity on the filter was measured using a liquid scintillation spectrophotometer (Aloka).

**Molecular biological methods**

DNA manipulation, cloning techniques, and nucleotide sequencing were performed as described by Sambrook and Russell [12]. Terminal labeling with [$^{32}$P], secondary structure analyses, and gel electrophoresis of RNAs were performed as described by Kikuchi *et al.* [13, 14].
Results and discussion

Effects of pre-elution by target ligand on SELEX
The selection of RNA was performed using a CRP-immobilized Sepharose 4B column. After the tenth round of selection without pre-elution as described in Materials and methods, we found that the RNA preparation did not have any significant binding ability to CRP in the binding assay using the disk-filter method (Fig. 1). This indicated that the binding ability of the selected RNA preparation to the target may be too weak to detect by this disk-filter method, even if the RNA preparation was trapped by the affinity column during the selection rounds.

To remove the weak binding portion from the RNA population, on and after the sixth selective round (Table 1), we introduced the pre-elution step just before the true elution step. In the pre-elution step, 1 mL of the elution buffer (10 μM CRP-containing binding buffer) was added to the column and incubated for a short time (0.5 – 15 min). These pre-elution steps were repeated several times in some selective rounds (see Table 1). The composition of the pre-elution buffer was the same as the elution buffer. A total of 19 rounds of selective amplification were performed (Table 1). The pre-elution steps were very effective to obtain the strong binding fraction (see below).

Figure 1 shows the results from the disk-filter assay of RNA populations from some selective rounds. The selected RNA populations were labeled internally by in vitro transcription of the dsDNA preparations from the tenth, fourteenth, and nineteenth rounds of selection using [α-32P] UTP as described in Materials and methods. The RNA preparation (10000 – 100000 cpm) was dissolved in 100 μL of binding buffer, heated at 65°C for 10 min, and incubated for 30 min at room temperature to allow appropriate folding. The RNA preparation was then mixed with the CRP solution of various concentrations as shown in Fig. 1. Values of binding are expressed as % of total input RNA. The RNAs from the tenth, fourteenth and nineteenth rounds of selection with pre-elution step showed dose-dependent binding. First, the RNA preparation from tenth round containing pre-elution steps at 6th to 10th rounds (see Table 1) clearly showed the binding abilities, although none of the RNA preparation from tenth round without pre-elution step showed the ability (compare open squares with closed squares in Fig. 1). The RNA preparation from the nineteenth round of selection showed extremely high binding ability. Fig. 1 shows that the binding ability increased especially after the fourteenth round. During the fourteenth and seventeenth rounds, we adopted four pre-elution steps for 10 min (Table 1). The incubation time of the pre-elution of the last two rounds (the 18th and 19th) was extended to 15 min. Pre-elution was performed under
very stringent selection conditions indicating that this was a very effective means of obtaining RNA aptamers with high binding ability.

**Table 1. Selection conditions of RNA populations.** All selection rounds (19 rounds) using CRP protein-immobilized Sepharose column are shown. **Pre-column,** +: in first 3 rounds, RNA populations were poured into free Sepharose column (no CRP) to remove Sepharose-binding portions. **CRP in column:** total amount of CRP immobilized on Sepharose gel used. **Wash:** volume of washing buffer and frequency are shown. **Pre-elution:** frequency and time (min, in parentheses) of pre-elution using 1 mL of elution buffer are shown. For example, “2 (5)” in 8th and 9th rounds indicates that two 5-min incubations with elution buffer were performed. See text for full description.

| Round | Pre-column | CRP in column [mg] | Wash       | Pre-elution (min) |
|-------|------------|--------------------|------------|------------------|
| 1     | +          | 0.6                | 5 mL × 3   | -                |
| 2     | +          | 0.6                | 5 mL × 3   | -                |
| 3     | +          | 0.6                | 5 mL × 3   | -                |
| 4     | -          | 0.3                | 5 mL × 3   | -                |
| 5     | -          | 0.3                | 5 mL × 10  | -                |
| 6     | -          | 0.3                | 5 mL × 10  | 1 (0.5)          |
| 7     | -          | 0.3                | 5 mL × 10  | 1 (1)            |
| 8     | -          | 0.2                | 5 mL × 10  | 2 (5)            |
| 9     | -          | 0.2                | 5 mL × 10  | 2 (5)            |
| 10    | -          | 0.1                | 5 mL × 10  | 3 (10)           |
| 11    | -          | 0.3                | 5 mL × 10  | 3 (10)           |
| 12    | -          | 0.3                | 5 mL × 10  | 3 (10)           |
| 13    | -          | 0.3                | 5 mL × 10  | 3 (10)           |
| 14    | -          | 0.3                | 5 mL × 10  | 4 (10)           |
| 15    | -          | 0.3                | 5 mL × 10  | 4 (10)           |
| 16    | -          | 0.3                | 5 mL × 10  | 4 (10)           |
| 17    | -          | 0.3                | 5 mL × 10  | 4 (10)           |
| 18    | -          | 0.3                | 5 mL × 10  | 2 (15)           |
| 19    | -          | 0.3                | 5 mL × 10  | 2 (15)           |
Figure 1. Binding assay of selected RNA populations using disk-filter. Binding abilities of RNA populations of several rounds of selection were assayed using increasing concentrations of CRP. Closed circles, RNA from 19th round; open circles, from 14th round; closed squares, from 10th round; open squares, from round 0, tRNA, and from 10th round without the pre-elution. See text for full description.

CRP1-1, an RNA aptamer with high affinity to CRP

The selected RNAs from the nineteenth round were reverse transcribed, and resulting cDNAs were amplified by PCR and subcloned into the plasmid vector pGEM-3Z (Promega). We arbitrarily chose 110 clones and sequenced the inserts of these plasmids. All clones sequenced originated from Template FR, although the first populations contained RNAs originating from equal amounts of Templates FR and SL (see Materials and methods). Template SL was designed to take predetermined stem-loop structures in the population. Although the stem-loop structures were thought to provide an advantage for binding ability selection, there were no such structures in the clones sequenced. This suggested that the stem-loop structures, even if they are dominant in the selection steps, were disadvantageous in the RT-PCR or transcription steps, because the stem with high G+C content may cause elongation pauses during reactions with reverse transcriptase or T7 RNA polymerase. If we try another SELEX starting with only Template SL, it is possible that another aptamer containing this stem-loop may be obtained, although we have yet to perform such experiments.

Of 110 clones sequenced, 87 (79%) had the same sequence shown in Fig. 2A, which we call CRP1-1. Thirteen of the 110 clones (12%) had the sequence shown in Fig. 2B as CRP1-2. Other clones had completely different sequences and lower binding
capabilities compared to CRP1-1 and CRP1-2 (data not shown). We have confirmed the binding abilities of the aptamers CRP1-1 and CRP1-2 using disk-filter assay. As shown in Fig. 3, CRP1-1 had higher binding ability to C-reactive protein than CRP1-2. Therefore, we examined CRP1-1 in more detail.

Figure 2. Nucleotide sequences and secondary structures of the RNA aptamers CRP1-1 (A) and CRP1-2 (B). The secondary structures were generated by computer. Colored regions (red and green letters) originated from random region of starting template and the black regions are constant regions used as primer binding sites for amplification.

Figure 3. Binding of the RNA aptamers CRP1-1 and CRP1-2. Binding abilities of the aptamers CRP1-1 (open circles) and CRP1-2 (closed circles) to increasing concentrations of CRP protein were assayed.
**SPR analysis of CRP1-1**

Surface plasmon-resonance (SPR) analysis using Biacore biosensor was performed. Biacore Model X was used throughout this study. First, the 3'-end of CRP1-1 was biotinylated and immobilized on the surface of a streptavidin-immobilized chip. For kinetic analysis, CRP protein solutions of several concentrations (0.25 – 4 μg/mL) were loaded onto the CRP1-1 immobilized surface of a sensor chip at a flow rate of 20 μL/min for 5 min in the association phase. Then, the running buffer was injected at the same flow rate for 5 min in the dissociation phase. Figure 4 shows the sensorgrams of CRP protein binding. The $k_{on}$, $k_{off}$, and $K_D$ values were calculated to be 1.6×$10^5$ (M$^{-1}$s$^{-1}$), 3.6×$10^{-4}$ (s$^{-1}$), and 2.25×$10^{-9}$ (M), respectively. The CRP RNA aptamer reported previously had a binding constant of 125 nM [15]. The binding constants of standard anti-CRP antibodies were reported to be 440 – 550 nM [16]. The binding constant of our CRP1-1 (2.25 nM) has almost two orders of magnitude lower (higher affinity) than those of the reported RNA aptamer and antibodies. CRP1-1 constructed here exceeds the binding constant of standard anti-CRP antibodies.

![Sensorgrams of CRP protein binding](image)

**Figure 4.** Binding kinetics of CRP protein against the RNA aptamer CRP1-1. Sensogram showed the affinity of CRP protein to the CRP1-1. CRP protein at each indicated concentration (0.25 – 4 μg/mL) was loaded onto CRP1-1-immobilized surface of the sensor chip. Biacore X (GE Healthcare) was used.
This experiment indicated that CaCl\(_2\) in the buffer is indispensable for binding. Usually, the binding buffer contained 2 mM CaCl\(_2\). When CaCl\(_2\) was removed from the binding buffer, no binding was detected by SPR analysis. These observations indicated that CRP1-1 recognizes the pentamer of CRP protein, because CaCl\(_2\) was required to form pentamers of CRP [17]. Alternatively, CRP1-1 aptamer may require CaCl\(_2\) for binding itself, irrespective of pentamer formation.

**Secondary structure of CRP1-1**

The secondary structure of CRP1-1 was first generated by computer using GENETYX-MAC 8.0 software (Fig. 2). This secondary structure was confirmed enzymatically using single-strand-specific nuclease S\(_1\) and double-strand-specific ribonuclease (RNase) V\(_1\). RNAs radioactively labeled at the 5'- or 3'-end under native conditions were used as the substrates for these enzymes. The enzymatic reaction products were analyzed by denaturing PAGE (Fig. 5). Based on the results of this experiment, the cleaved positions (the bands in Fig. 5) were marked in Fig. 6. There was good agreement between these experimental results (Fig. 5) and the computer-generated secondary structure (Figs. 2A and 6), indicating that the computer-generated secondary structure was mostly correct.

Figure 5. Analysis of the secondary structure of CRP1-1. Samples of the native form of CRP1-1 (5'-or 3'-end radiolabeled) were partially digested by nuclease S\(_1\) (single-strand-specific nuclease), ribonuclease V\(_1\) (RNase V\(_1\), double-strand-specific RNase), and RNase T\(_1\) (for G-mapping). The products were analyzed by denaturing PAGE. Autoradiograms are shown. S\(_1\), nuclease S\(_1\); V\(_1\), RNase V\(_1\); T\(_1\), RNase T\(_1\); TU, RNase T\(_1\) in the presence of 4 M urea; OH, products of alkaline digestion; C, no enzyme control. Positions of some nucleotides in the CRP1-1 sequence are shown on the right sides of autoradiograms.
Figure 6. Secondary structure of CRP1-1 and the region that makes contact with CRP protein. Sites sensitive to nucleases derived from the results of Figure 5 are indicated by arrowheads. Closed arrowhead, the site cleaved by nuclease S; open arrowhead, the site cleaved by RNase V. Square shown by dotted lines indicates the putative region of contact with CRP protein. See text for full description.

The area in CRP1-1 in direct contacted with CRP protein

Using the footprinting technique, we searched for the area in the CRP1-1 sequence that makes contact with CRP protein. An experiment similar to that shown in Figure 5 was performed but in the presence of CRP protein for nuclease reactions. The intensity of the cleavage signals (bands) from nucleotide number 31 to 65 decreased significantly in the presence of CRP protein in the nuclease reaction mixture (data not shown). This suggested that the region from nucleotide 31 to 65 may make direct contact with CRP protein. This region is indicated by a square (enclosed by dotted lines) in Fig. 6.

Based on this result, we examined whether this region alone is sufficient for binding ability to CRP protein. We synthesized a small RNA containing only this region from nucleotide 31 to 65, but it showed no binding ability to CRP protein. These observations suggested that the region of contact in the CRP1-1 aptamer to CRP protein is indeed from nucleotide 31 to 65, but that other regions are also important for binding or for retaining some higher order structure necessary for binding.

Conclusions

We obtained an RNA aptamer, CRP1-1, with high affinity to CRP protein by the newly
developed pre-elution method. The dissociation constant of CRP1-1 to CRP protein was calculated to be $K_D = 2.25 \times 10^{-9}$ (M) by SPR analysis using a Biacore biosensor. This value is almost two orders of magnitude lower (higher affinity) than that of the previously reported CRP RNA aptamer. The value was derived from the $k_{on}$ value of $1.6 \times 10^5$ (M$^{-1}$s$^{-1}$) and the $k_{off}$ value of $3.6 \times 10^{-4}$ (s$^{-1}$). Until the tenth round of selection, we used gradually decreasing concentrations of CRP protein in the CRP-immobilized column (Table 1). This may have contributed to obtaining a high $k_{on}$ value of selected populations. The pre-elution method described here may contribute to obtaining a favorable $k_{off}$ value of CRP1-1 by removing a population easily released from the CRP-immobilized column.

Various methods for detection of CRP using RNA aptamers with low affinity have been reported [15, 18, 19]. These methods may be improved by use of an RNA aptamer with high affinity.

References

[1] Ellington AD and Szostak JW 1990 Nature 346, 818
[2] Tuerk C and Gold L 1990 Science 249, 505
[3] Gold L, Polisky B, Uhlenbeck O and Yarus M 1995 Ann. Rev. Biochem. 64, 763
[4] Jenison RD, Gill SC, Pardi A and Polisky B 1994 Science 263, 1425
[5] Bock LC, Griffin LC, Latham, JA, Vermaas EH and Toole JJ 1992 Nature 355, 564
[6] Takeno H, Yamamoto S, Tanaka T, Sakano Y and Kikuchi Y 1999 J. Biochem. 125, 1115
[7] Ng EWM, Shima DT, Calias P, Cunningham ET Jr, Guyer DR and Adamis AP 2006 Nature Rev. Drug Discov. 5, 123
[8] Oliveira EB, Gotschlich EC and Liu TY 1977 Proc. Natl. Acad. Sci. USA 74, 3148
[9] Clearfield MB 2005 J. Am. Osteopath. Assoc. 105, 409
[10] Wu TL, Tsao KC, Chang CPY, Li CN, Sun CF and Wu JT 2002 Clin. Chim. Acta 322, 163
[11] Kikuchi Y and Sasaki N 1991 Nucleic Acids Res. 19, 6751
[12] Sambrook J and Russell DW 2001 Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
[13] Kikuchi Y, Ando Y and Shiba T 1986 Nature 323, 824
[14] Kikuchi Y, Sasaki, N and Ando-Yamagami Y 1990 Proc. Natl. Acad. Sci. USA 87, 8105
[15] Bini A, Centi S, Tombelli S, Minunni M and Mascini M 2008 Anal. Bioanal. Chem.
[16] Lin S, Lee CK, Lin YH, Lee SY, Sheu BC, Tsai JC and Hsu SM 2006 *Biosens. Bioelectron.* **22**, 715

[17] Pepys MB and Hirschfield GM 2003 *J. Clin. Invest.* **111**, 1805

[18] Pultar J, Sauer U, Domnanich P and Preininger C 2009 *Biosens. Bioelectron.* **24**, 1456

[19] Centi S, Sanmartin LB, Tombelli S, Palchetti I and Mascini M 2009 *Electroanalysis* **21**, 1309