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C-reactive protein (CRP) aptamer binds to monomeric but not pentameric form of CRP

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Abstract

Native C-reactive protein (CRP) is composed of five identical subunits arranged in a pentameric structure (pCRP). Binding of pCRP to damaged cell membranes produces a second isoform, modified CRP, which has similar antigenicity to isolated monomeric subunits of CRP (mCRP). Emerging evidence indicates that modified CRP plays a role in inflammation and atherosclerosis, however, there are very few techniques that can distinguish the different isoforms of CRP. Here we show that an RNA aptamer binds specifically to mCRP and not to pCRP. Using this aptamer, we describe a simple, fast, and sensitive assay to detect nanomolar concentrations of mCRP using fluorescence anisotropy. In addition, we show that this aptamer can be used to detect mCRP in polyacrylamide gels and bound to a surface using total internal reflection fluorescence microscopy. The biological activity of the mCRP we prepared by heating pCRP with 0.1% sodium dodecyl sulfate was confirmed by observing binding to the complement protein, C1q. This probe provides an important tool for CRP research and has the potential to improve clinical diagnostics that predict risk for cardiovascular disease.

Keywords

C-reactive protein; isoform; aptamer; cardiovascular disease; TIRF microscopy; fluorescence anisotropy

Introduction

C-reactive protein (CRP) is an inflammatory marker and serum CRP levels increase as much as 10,000-fold in response to inflammation [1-3]. Cardiovascular disease (CVD) is correlated to chronic inflammation and serum CRP above 3 mg/ml is predictive of increased risk for CVD [1]. Structurally, CRP is composed of five identical 23 kDa subunits (protomers), linked together noncovalently to form pentameric CRP (pCRP) [4,5]. CRP exists in other isoforms, including monomeric CRP (mCRP) with a molecular weight of 23 kDa. mCRP can be produced *in vitro* by subjecting pCRP to urea/ethylenediaminetetraacetic

acid (EDTA) chelation [6,7], acid denaturation [7,8], or heating [9]. pCRP also undergoes conformational rearrangement in the absence of calcium, dissociating into mCRP [10-12]. The modified CRP isoform is generated *in vivo* when pCRP binds to damaged membrane surfaces such as activated platelets [13], apoptotic blebs [14], platelet-derived apoptotic microparticles [15], liposomes containing lysophosphatidylcholine [2,16], and oxidized but not native low-density lipoprotein [17]. Modified CRP expresses neo-epitopes and displays antigenicity that is distinct from pCRP [6,7,18]. Although modified CRP binds to similar antibodies as does mCRP [2], there is no evidence that modified CRP exists as isolated monomers. In fact, modified CRP may exist as aggregates of mCRP on cell membrane surfaces [2]. One critical function of modified CRP is binding to C1q and activation of the immune system's complement cascade [19]. Despite evidence that modified CRP is more strongly associated with inflammation [13,12,20], current CRP diagnostics are unable to distinguish between the common isoforms. It is, therefore, critical to develop assays that can distinguish between these isoforms in order to study which isoform most accurately predicts future CVD risk [21].

Aptamers are single-stranded oligonucleotides (DNA or RNA) selected against a target molecule using systemic evolution of ligands by exponential enrichment (SELEX) [22,23]. Aptamers have affinities and specificities that rival antibodies [24]. Using SELEX, aptamers have been isolated against a variety of diagnostic biomarkers including thrombin [25-27], HIV-tat protein [28,29], and CRP [30-32]. The two aptamers isolated against CRP consist of a 44-base RNA [32] and a 40-base ssDNA [31]. One critical difference to antibodies is that the SELEX process requires the target antigen to be immobilized on a solid support, such as plates or beads [31,33]. It is noteworthy, therefore, that surface immobilization of CRP on enzyme-linked immunosorbent assay (ELISA) plates [7] or SPR chips [34] results in conversion to modified CRP. If CRP aptamers were selected against individual protomers of CRP resulting from immobilization, they could have preference for mCRP over pCRP.

In this work, we determined that the RNA aptamer binds preferentially to the mCRP isoform instead of pCRP. To demonstrate the versatility of this aptamer probe as a research tool and potential clinical diagnostic for mCRP, we validated its use with multiple techniques, including fluorescence anisotropy, dot blot, electrophoretic mobility shift assay (EMSA), and total internal reflection fluorescence (TIRF) microscopy. Furthermore, we confirmed that the mCRP we prepared by heating with a small amount of sodium dodecyl sulfate (SDS) presents a binding epitope for C1q, the first component of the complement cascade. This suggests similarity to modified CRP. The availability of an isoform-specific CRP probe will be a valuable analytical tool for understanding the function of mCRP, its relationship to modified CRP, and its role in CVD.

Materials and methods

Purified human CRP was from Sigma (St. Louis, MO), purified human C1q was from Calbiochem (EMD Biosciences, San Diego, CA), and monoclonal anti-CRP antibody (clone C6) was from Meridian Life Sciences, Inc. (Saco, ME). All other chemicals were from Sigma and used as received unless otherwise specified. RNA and DNA aptamers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). The RNA aptamer was end labeled either with Cy3, Cy5, or biotin, and stocks of aptamers were reconstituted with DEPC-treated water (Applied Biosystems/Ambion, Foster City, CA) and stored at -20 °C until use. The RNA and DNA aptamer sequences were:

5'-Cy3-RNA: 5'-Cy3-GCC UGU AAG GUG GUC GGU GUG GCG AGU GUG UUA
GGA GAG AUU GC-3'

3'Cy5-RNA: 5'-GCC UGU AAG GUG GUC GGU GUG GCG AGU GUG UUA GGA GAG AUU GC-**Cy5**-3'

5'Biotin-RNA: 5'-**biotin**-GCC UGU AAG GUG GUC GGU GUG GCG AGU GUG UUA GGA GAG AUU GC-3'

ssDNA: 5'-ACA CGA TGG GGG GGT ATG ATT TGA TGT GGT TGT TGC ATG ATC GTG G-3'

Generation of monomeric CRP

Acid-treated, monomeric CRP (mCRP_{HCl}) was prepared, as described previously [31]. Specifically, pCRP was incubated in an acidic solution (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.1 M HCl, pH 2) for 5 min and neutralized with 0.1M NaOH. CRP treated with heat in SDS (mCRP_{SDS}) was prepared by modification of existing heat-denaturing methods [35]. Specifically, pCRP was heated in an SDS containing buffer (20 mM HEPES, 140 mM NaCl, and 0.1% SDS) for 1h at 85 °C to provide mCRP_{SDS}. Total protein concentration after modification was determined by the bicinchoninic acid method (Pierce BCA Protein Assay Kit, Thermo Scientific) and the purity of mCRP was confirmed by SDS-polyacrylamide gel electrophoresis (PAGE) showing the presence of only one 23 kDa mCRP band with no obvious proteolytic degradation during treatment.

Preparation of CRP–aptamer complex

CRP–aptamer complexes were prepared by incubating either mCRP_{SDS}, mCRP_{HCl}, or pCRP with RNA aptamer in binding buffer (20 mM HEPES, 140 mM NaCl, 50 mM KCl, 0.1 mM DTT, and 5% glycerol at pH 6.5). Incubation was carried out at room temperature for 20 min and analyzed immediately. While calcium is not necessary for aptamer binding, 2 mM CaCl₂ was added to the pCRP sample to retain its folded pentameric structure.

Fluorescence anisotropy

Solution binding studies were performed on a Perkin Elmer LS55 fluorometer in a quartz cuvette with excitation and emission wavelengths of 555 and 570 nm and slit widths of 2.5 and 10 nm, respectively. Baseline anisotropy of the 5'Cy3-RNA was acquired for 5 min. Changes in anisotropy were measured after normalizing to the initial anisotropy of the 5'Cy3-RNA aptamer in buffer. CRP was then added to the cuvette and incubated for 5 min at room temperature before anisotropy measurements were taken. For the displacement assay, titrations of an unlabeled ssDNA aptamer [31] were added to the CRP-5'Cy3-RNA complex and the measurements were taken immediately. Final concentrations of CRP and 5'Cy3-RNA were 9.1 and 0.59 μg/mL, respectively. Tabulated data were reported as mean ± SD from three independent experiments.

Dot blot assay

A 2 μL aliquot of protein was blotted on nitrocellulose membrane, air dried and blocked with 3% BSA + 0.05% Tween-20/PBS overnight at 4 °C. After washing 3 × with 0.005% Tween-20/PBS, the membrane was probed with 170 nM (2.38 μg/mL) 5'biotin-RNA aptamer for 1 h at 25 °C followed by a 30-min incubation with 1:1,000 streptavidin-conjugated IR800 and imaged on an Odyssey imager (Li-COR Biotechnology, Lincoln, NE).

Polyacrylamide gel electrophoresis

CRP and CRP–aptamer complex were resolved by PAGE according to standard Laemmli methods [36]. A 10% (29:1 acrylamide/bis-acrylamide) gel containing 0.005% SDS was

used to visualize both CRP isoforms on the same gel [9]. To preserve the conformation of each CRP isoform, native loading buffer without β -mercaptoethanol or SDS were used and the samples were not heated prior to loading. Electrophoresis was carried out at 80 V for ~2 h at room temperature (BioRad, Hercules, CA). The electrophoretic migration of free 3'Cy5-RNA and bound CRP-3'Cy5-RNA complex were observed using near-infrared (NIR) imaging of the wet gel using the 700 nm channel on an Odyssey imager and CRP protein was visualized by subsequent silver staining of the same gel. The final concentration of CRP was 18 and 11 μ g/mL for the 3'Cy5-RNA aptamer.

Electrophoretic mobility shift assay

The binding affinity of the RNA aptamer to CRP was analyzed using an EMSA. Increasing amounts of 3'Cy5-RNA (0.48-3.8 mM final concentration) were incubated with CRP (fixed at 23 μ g/mL) in binding buffer for 20 min at room temperature before electrophoresis. The CRP-aptamer complex was loaded on a 5% polyacrylamide gel (19:1) in 0.5 $\times$ TBE (45 mM Tris, 45 mM boric acid, and 1 mM EDTA) and electrophoresed at 110 V for 40 min at 25 $^{\circ}$ C.

TIRF Microscopy

Individual chambers were created using CoverWells (Life Technologies, Eugene, OR) that were cut and adhered to cleaned coverslips (No. 1 thickness, Ted Pella). Coverslips were coated with monoclonal anti-CRP antibody (C6, 1:1,000 dilution in 30 mM sodium carbonate/70 mM sodium bicarbonate) for 1 h at room temperature, blocked with 3% BSA for 2 h, and rinsed 3 $\times$ with 0.01% Tween-20/PBS buffer, and 1 $\times$ with PBS. CRP (mCRP or pCRP, 6 μ g/mL) was pre-incubated with 0.3 μ g/mL of 5'Cy3-RNA in binding buffer for 20 min at room temperature. The samples were then added to each well, incubated for 10 min, rinsed 3 $\times$ with 20 mM HEPES/140 mM NaCl buffer to remove unbound material, and imaged immediately. Objective-style TIRF microscopy was performed using a Nikon Eclipse Ti-U microscope equipped with a TIRF/epifluorescence illuminator and a 60 $\times$ TIRF oil objective lens (NA = 1.49). Excitation of Cy3 was performed using a fiber launch (Solamere Technology Group, Salt Lake City, UT) equipped with an AOTF to shutter the excitation from a 50 mW, 561 nm diode pumped solid-state laser (Cobalt Jive 50, Cobalt AB, Sweden). Emission was filtered using a 570-nm long pass dichroic (Omega Optical, Brattleboro, VT) and a band pass filter (605/55, Chroma Technology, Bellows Falls, VT). The images were acquired on an Andor iXon EMCCD with an exposure time of 200 ms and an EM gain of 200 using freely available software (μ Manager, <http://www.micro-manager.org/>). The intensity of Cy3 fluorescence was analyzed using ImageJ (<http://rsbweb.nih.gov/ij/>). Each image was filtered using a rolling ball filter (ball radius of 10 pixels) to eliminate background noise and correct for image tilt. The intensity was integrated across the full image to obtain a reading of total fluorescence. TIRF microscopy data are presented as mean $\pm$ SE from at least five independent images. A p value of <0.05 using a Student's *t* test was considered as significant.

Results and discussion

RNA aptamer binds specifically to monomeric CRP

In this study, we demonstrated that an aptamer previously isolated against CRP [34] has a strong affinity for the mCRP isoform and does not associate with pCRP under the same conditions. A solution binding analysis using fluorescence anisotropy was used to identify which isoforms of CRP bind to the aptamer, 5'Cy3-RNA (Fig. 1). Fluorescence anisotropy measures the change in rotational diffusion rate of a fluorescent probe and has been used to measure the interactions of biomolecules [37-39]. Labeled aptamer should freely rotate and have a lower anisotropy than if it is bound to CRP. Addition of mCRP_{SDS} to the 5'Cy3-

RNA aptamer resulted in a significant increase in anisotropy (Table 1), with a Dr of $+0.097 \pm 0.019$. Addition of mCRP_{HCl} to the 5'Cy3-RNA aptamer also resulted in a significant increase in anisotropy (Table 1), with a Dr of $+0.107 \pm 0.016$. In contrast, when the same amount of pCRP was added to the 5'Cy3-RNA aptamer the Dr was $+0.014 \pm 0.007$ showing that the much larger pCRP had negligible affinity for the aptamer. The final concentrations of 5'Cy3-RNA and CRP were 0.59 and 9.1 $\mu\text{g/mL}$, respectively. In an additional control, no binding was observed between the aptamer and BSA (Fig. 1) confirming that the interactions observed were specific.

C1q binding confirms that mCRP_{SDS} retains a functional form

Although the RNA aptamer recognizes both forms of mCRP, the bioactivity of each mCRP form was noticeably different. Since modified CRP has been shown to activate complement *in vivo* [31], binding studies between mCRP and C1q were further evaluated by anisotropy to assess the biological activity of the each mCRP (Fig. 1). The mCRP_{SDS} isoform bound to C1q, resulting in a significant increase in anisotropy, with a Dr of $+0.099 \pm 0.016$ in the presence of 0.01% SDS (Fig. 1). This confirms that mCRP_{SDS} is functional even though it is not in the pentameric form. In contrast, mCRP_{HCl} did not interact with C1q (Dr = $+0.013 \pm 0.009$) (Fig. 1) and adding the same concentration of SDS to mCRP_{HCl} did not restore C1q binding, consistent with acid treatment resulting in complete and irreversible denaturation. The final concentration of C1q in each case was 0.58 $\mu\text{g/mL}$. In addition, ELISA confirmed that mCRP_{SDS} bound C1q better than either pCRP or mCRP_{HCl} (Electronic supplementary materials (ESM)). The binding between C1q and mCRP_{SDS} was twice as strong compared with that of the mCRP_{HCl} by ELISA (ESM). As expected, no binding was observed between pCRP and C1q by ELISA (ESM). The strong binding between C1q and mCRP_{SDS} suggests that CRP heated in a minimal amount of SDS is likely to retain a folded structure as do other proteins at low SDS concentration [40]. On the other hand, the acid treatment (mCRP_{HCl}) rendered the CRP irreversibly denatured [6,8] and without the ability to activate complement, which is in agreement with previous observations [8]. These results demonstrate that the mCRP_{SDS} remains biologically active and expresses the neo-epitopes responsible for C1q binding which occurs through the Asp 112, Tyr 175, and His 38 residues on CRP [41]. This also suggests that the aptamer specifically recognizes a portion of CRP that is blocked when protomers are adjacent to each other, and that the mCRP_{SDS} shows similar antigenicity to the modified CRP found in tissues and human arterial intima [42,19] that are relevant to biological function in complement activation. Because of its high affinity to C1q, we consider the mCRP_{SDS} as similar to the biologically relevant form of modified CRP. Although both mCRP_{SDS} and modified CRP bind to C1q, additional study will be required to fully understand the antigenicity of mCRP_{SDS}. Since mCRP_{SDS} appears to be a better model of modified CRP than does mCRP_{HCl}, and because modified CRP and mCRP_{SDS} both have an intact C1q binding site [2], we therefore sought to further characterize the interactions of the aptamer probe with mCRP_{SDS}.

CRP-RNA-aptamer complex formation

To provide direct visual evidence of the protein-aptamer complex, we performed a dot blot assay and adapted standard PAGE methods for EMSA to create an in-gel assay using a 3'Cy5-RNA and NIR imaging. The 5'biotin-RNA showed strong affinity for mCRP, with relatively weak binding to pCRP, and no affinity for BSA (Fig. 2a). To further observe the specificity of aptamer for mCRP_{SDS} over pCRP, the proteins were separated by PAGE and visualized using silver stain (Fig. 2b) and NIR imaging (Fig. 2c). When resolved on a 10% polyacrylamide gel containing 0.005% SDS, pCRP showed an apparent M_r of 140 kDa and mCRP_{SDS} had an apparent M_r of 48 kDa (Fig. 2b). This high M_r is caused by the nearly native conditions used and is in agreement with a previous study using the same gel conditions [9]. NIR imaging of the same gel revealed a distinct band at 48 kDa in the

mCRP_{SDS} and 3'Cy5-RNA co-incubated sample (Fig. 2c). This suggests that mCRP_{SDS} forms a complex with 3'Cy5-RNA that has a similar mobility to the protein alone. In contrast, no additional bands were observed in the pCRP + 3'Cy5-RNA lane (Fig. 2c), indicating that 3'Cy5-RNA did not form a persistent complex with pCRP. To further evaluate the complex formation of mCRP_{SDS}-3'Cy5-RNA, EMSA was performed using increasing concentrations of 3'Cy5-RNA into a fixed amount of mCRP_{SDS} and resolved on a 5% 0.5× TBE acrylamide gel (Fig. 3). NIR EMSA gel showed increasing mCRP_{SDS}-3'Cy5-RNA complex formation with increasing 3'Cy5-RNA concentration and a difference in mobilities of the free 3'Cy5-RNA and the mCRP_{SDS}-3'Cy5-RNA complex bands (Fig. 3a), confirming the formation of an mCRP_{SDS}-3'Cy5-RNA complex. Silver staining of the same gel also confirmed that the mCRP_{SDS}-3'Cy5-RNA complex migrated differently than free mCRP_{SDS} (Fig. 3b), and the mCRP_{SDS}-3'Cy5-RNA complex bands from both techniques overlapped with the 3'Cy5-RNA bands observed by NIR imaging (Fig. 3c). In contrast, migration of pCRP did not change in the presence of 3'Cy5-RNA and no complex was observed between pCRP and 3'Cy5-RNA. Both dot blot and EMSA results clearly showed that 3'Cy5-RNA interacts only with mCRP_{SDS} and not pCRP, as indicated by a distinct mCRP_{SDS}-3'Cy5-RNA complex band, visible by NIR imaging (Fig. 3b). The main advantage of this adapted EMSA method is that both the protein and protein–aptamer complex bands can be resolved on the same gel without the need to perform a Northern blot or the use of radioisotopes.

Quantitative binding studies between RNA aptamer and mCRP

Increasing concentrations of mCRP_{SDS} were added to 5'Cy3-RNA to obtain a binding constant for RNA aptamer and mCRP_{SDS} using anisotropy (Fig. 4a). The starting concentration of 5'Cy3-RNA was 0.68 µg/mL and aliquots of mCRP_{SDS} were titrated into the solution. The titration curve showed a saturation binding equilibrium between 5'Cy3-RNA and mCRP_{SDS}, starting with an initial anisotropy value of 0.299 ± 0.009 for the 5'Cy3-RNA probe, followed by a gradual increase in anisotropy, and finally reaching a maximum anisotropy of 0.381 ± 0.007 . The data were fit using nonlinear least squares regression (GraphPad Prism 5). From the fit curve, the CRP concentration at half-maximum was 4.34 µg/mL. This corresponds to a K_d of 187.7 nM (using the M_r for mCRP of 23 kDa), comparable to that of the previously reported K_d for this aptamer [32].

Competitive displacement analysis of the RNA aptamer-mCRP complex

In addition to the RNA aptamer, a second aptamer against CRP has been recently selected [31]. Because the ssDNA aptamer was selected against CRP bound to magnetic beads [31], we expected the ssDNA aptamer could also be mCRP-specific and possibly compete for the same binding site as the RNA aptamer. To determine if the DNA aptamer also binds mCRP, we performed a competitive displacement study using the CRP-specific ssDNA aptamer as a competitor (Fig. 3b). Increasing amounts of ssDNA aptamer were added to the mCRP_{SDS}-5'Cy3-RNA complex until the anisotropy reached the baseline value (Fig. 4B). The initial concentrations of the mCRP_{SDS} and 5'Cy3-RNA were 18 µg/mL and 0.68 µg/mL, respectively. The initial anisotropy of the mCRP_{SDS}-5'Cy3-RNA complex was 0.381 ± 0.006 (the maximum anisotropy value) and no significant changes in anisotropy were detected at lower titrations of ssDNA. After an initial lag phase, a decrease in anisotropy was observed and the drop in anisotropy proceeded to a plateau at a final minimum anisotropy of 0.288 ± 0.01 . The competitive displacement data was fit to a dose-dependent Hill equation and the concentration of ssDNA needed to displace 50% of the 5'Cy3-RNA from the mCRP_{SDS}-5'Cy3-RNA complex (EC_{50}) was 0.83 µg/mL (GraphPad Prism). A complete displacement of the 5'Cy3-RNA was achieved at ssDNA concentrations greater than 1.6 µg/mL, or approximately 2× molar excess of the 5'Cy3-RNA. In an inhibition assay, when ssDNA aptamer was added to mCRP_{SDS} prior to the 5'Cy3 RNA aptamer

addition, no change in anisotropy was observed (data not shown). This competitive displacement assay showed that the ssDNA has strong affinity to mCRP_{SDS}, suggesting that both aptamers may bind to the same epitope on the mCRP_{SDS} consistent with the ssDNA also being specific to mCRP. This further points to a general problem with using SELEX to obtain aptamers for proteins that are prone to aggregation or conformational changes.

Binding studies using TIRF microscopy

The utility of this aptamer as an mCRP-specific probe for fluorescence microscopy was demonstrated using TIRF microscopy [35] with an indirect antibody capturing method that was used to avoid pCRP conversion that occurs in direct capture method [8]. Using TIRF microscopy, the surface is illuminated by an exponentially decaying evanescent wave and only fluorophores within the evanescent field penetration depth (~100 nm) are observed [43]. Use of TIRF permitted the selective excitation of 5'Cy3-RNA attached to the surface while excluding any 5'Cy3-RNA fluorescence from the bulk solution. Therefore, binding events were detected with low background noise by comparing the intensity and distribution of fluorescence spots on the surface. Integrated fluorescence intensities (I) over the area of the entire image of each sample were subjected to rolling ball integration and plotted (Fig. 5). The integrated intensity of the sample with 5'Cy3-RNA but no CRP on the anti-CRP coated coverslip was $19.2 \pm 0.5 (\times 10^6)$. A significant increase in integrated intensity was measured ($\Delta I = + 17.8 \times 10^6$, $p < 0.001$) when mCRP_{SDS}-5'Cy3-RNA was added to the antibody-coated coverslip as evident from the increase in spot density on the surface (Fig. 5b). In contrast, addition of pCRP-5'Cy3-RNA (Fig. 5c) caused an insignificant increase in fluorescence intensity ($\Delta I = + 0.9 \times 10^6$, $p = 0.216$), and the spot density on the surface was comparable to the 5'Cy3-RNA control (Fig. 5a), demonstrating that 5'Cy3-RNA bound to mCRP but not pCRP. This demonstrates the utility of the aptamer as an mCRP-specific probe to identify CRP isoforms in microscopy applications.

Conclusions

CRP immunoassays are used as a clinical test to predict future CVD risk [11,3]. While CRP immunoassays are capable of detecting low levels of CRP, the antibodies used in current assays cannot distinguish between the CRP isoforms. A commercially available monoclonal antibody (clone 8, C8) has been reported to bind preferentially to mCRP that is directly immobilized on ELISA plates [34,44]. However, its specificity for mCRP remains in dispute [45] and a number of studies have shown that C8 interacts with both mCRP and pCRP [13,46]. The fact that binding of pCRP to ELISA plates can result in isoform conversion [34] likely contributes to the confusion. Because each CRP isoform has distinctly different bioactivity, and substantial evidence has implicated modified CRP with pro-inflammatory and atherogenic effects [13,11,12], it has become increasingly important to distinguish mCRP from pCRP, to accurately study CVD.

In this study, we showed that the aptamers selected by surface immobilization of CRP [32,31] are specific for mCRP. Although the aptamers were originally selected using pCRP, dissociation is likely to have occurred during the SELEX process since CRP was directly immobilized onto ELISA plates [7]. Direct immobilization of pCRP on ELISA plates has been previously reported to cause isoform conversion of pCRP resulting in neo-epitope exposure [7]. This led to our hypothesis that the CRP epitope recognized by the aptamer is inaccessible in the pentameric structure, most likely existing at contact points between adjacent protomers.

Together, our results suggest that caution should be used in performing SELEX on proteins prone to conformational changes. Consequently, prior reports of aptamer binding to pCRP [30] could be explained by the facile isoform conversion of pCRP on a variety of surfaces

[34,7], over long-term storage [47], or when calcium is not present [13]. Such binding studies performed with pCRP need to be re-examined in consideration of the fact of pCRP dissociation.

The availability of an mCRP-specific aptamer provides a new molecular probe for CRP research. Since modified CRP has been reported to localize on inflamed sites and in the intima of the blood vessel [42,48], the probe will be useful for monitoring the interaction and subsequent conformational rearrangement of CRP on activated membrane surfaces including atherosclerotic plaques [13], apoptotic blebs [14], and activated platelets [13]. We have shown using TIRF microscopy that the probe is suitable and can be used in microscopy applications to monitor such conformation changes of pCRP *in situ*. Additionally, we have described a simple, fast, and sensitive assay to quantify the concentration of mCRP in solution using fluorescence anisotropy. The concentrations used are within the range of clinically relevant CRP concentrations, suggesting that this technique could be used to assess whether mCRP is predictive of future risk for CVD, for example on circulating microparticles. The orthogonality of the C1q and aptamer binding sites makes this probe especially useful in studies where complement activation is being examined. This solution-based assay shows potential for detecting nanomolar concentrations of mCRP with high specificity and which could lead to clinical diagnostics that may better predict future CVD risk.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Black S, Kushner I, Samols D. C-reactive protein. *J Biol Chem*. 2004; 279(47):48487–48490. [PubMed: 15337754]
2. Ji SR, Wu Y, Zhu L, Potempa LA, Sheng FL, Lu W, Zhao J. Cell membranes and liposomes dissociate C-reactive protein (CRP) to form a new, biologically active structural intermediate: mCRP(m). *FASEB J*. 2007; 21(1):284–294. [PubMed: 17116742]
3. Pepys MB, Hirschfield GM. C-reactive protein: a critical update. *J Clin Invest*. 2003; 111(12):1805–1812. [PubMed: 12813013]
4. Thompson D, Pepys MB, Wood SP. The physiological structure of human C-reactive protein and its complex with phosphocholine. *Struct Fold Des*. 1999; 7(2):169–177.
5. Volanakis JE. Human C-reactive protein: expression, structure, and function. *Mol Immunol*. 2001; 38(2-3):189–197. [PubMed: 11532280]
6. Potempa LA, Maldonado BA, Laurent P, Zemel ES, Gewurz H. Antigenic, electrophoretic and binding alterations of human C-reactive protein modified selectively in the absence of calcium. *Mol Immunol*. 1983; 20(11):1165–1175. [PubMed: 6656768]
7. Potempa LA, Siegel JN, Fiedel BA, Potempa RT, Gewurz H. Expression, detection and assay of a neoantigen (Neo-Crp) associated with a free, human C-reactive protein subunit. *Mol Immunol*. 1987; 24(5):531–541. [PubMed: 2443837]
8. Potempa LA, Zeller JM, Fiedel BA, Kinoshita CM, Gewurz H. Stimulation of human-neutrophils, monocytes, and platelets by modified C-reactive protein (CRP) expressing a neoantigenic specificity. *Inflammation*. 1988; 12(4):391–405. [PubMed: 2459061]

9. Taylor KE, van den Berg CW. Structural and functional comparison of native pentameric, denatured monomeric and biotinylated C-reactive protein. *Immunology*. 2007; 120(3):404–411. [PubMed: 17163961]
10. Singh SK, Suresh MV, Hammond DJ, Rusinol AE, Potempa LA, Agrawal A. Binding of the monomeric form of C-reactive protein to enzymatically-modified low-density lipoprotein: effects of phosphoethanolamine. *Clinica Chimica Acta*. 2009; 406(1-2):151–155.
11. Eisenhardt SU, Thiele JR, Bannasch H, Stark GB, Peter K. C-reactive protein: how conformational changes influence inflammatory properties. *Cell Cycle*. 2009; 8(23):3885–3892. [PubMed: 19887916]
12. Khreiss T, Jozsef L, Potempa LA, Filep JG. Conformational rearrangement in C-reactive protein is required for proinflammatory actions on human endothelial cells. *Circulation*. 2004; 109(16): 2016–2022. [PubMed: 15051635]
13. Eisenhardt SU, Habersberger J, Murphy A, Chen YC, Woollard KJ, Bassler N, Qian HW, von zur Muhlen C, Hagemeyer CE, Ahrens I, Chin-Dusting J, Bobik A, Peter K. Dissociation of pentameric to monomeric C-reactive protein on activated platelets localizes inflammation to atherosclerotic plaques. *Circ Res*. 2009; 105(2):128–U155. [PubMed: 19520972]
14. Gershov D, Kim S, Brot N, Elkon KB. C-reactive protein binds to apoptotic cells, protects the cells from assembly of the terminal complement components, and sustains an antiinflammatory innate immune response: implications for systemic autoimmunity. *J Exp Med*. 2000; 192(9):1353–1363. [PubMed: 11067883]
15. van der Zee PM, Biro E, Trouw LA, Ko Y, de Winter RJ, Hack CE, Sturk A, Nieuwland R. C-reactive protein in myocardial infarction binds to circulating microparticles but is not associated with complement activation. *Clin Immunol*. 2010; 135(3):490–495. [PubMed: 20138585]
16. Volanakis JE, Narkates AJ. Interaction of C-reactive protein with artificial phosphatidylcholine bilayers and complement. *J Immunol*. 1981; 126(5):1820–1825. [PubMed: 7217669]
17. Chang MK, Binder CJ, Torzewski M, Witztum JL. C-reactive protein binds to both oxidized LDL and apoptotic cells through recognition of a common ligand: phosphorylcholine of oxidized phospholipids. *Proc Natl Acad Sci USA*. 2002; 99(20):13043–13048. [PubMed: 12244213]
18. Ying SC, Gewurz H, Kinoshita CM, Potempa LA, Siegel JN. Identification and partial characterization of multiple native and neoantigenic epitopes of human C-reactive protein by using monoclonal antibodies. *Journal of Immunology*. 1989; 143(1):221–228.
19. Ji SR, Wu Y, Potempa LA, Liang YH, Zhao J. Effect of modified C-reactive protein on complement activation-A possible complement regulatory role of modified or monomeric C-reactive protein in atherosclerotic lesions. *Arterioscl Throm Vas*. 2006; 26(4):935–941.
20. Zouki C, Haas B, Chan JSD, Potempa LA, Filep JG. Loss of pentameric symmetry of C-reactive protein is associated with promotion of neutrophil-endothelial cell adhesion. *Journal of Immunology*. 2001; 167(9):5355–5361.
21. Ridker PM, Rifai N, Rose L, Buring JE, Cook NR. Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. *New Engl J Med*. 2002; 347(20):1557–1565. [PubMed: 12432042]
22. Ellington AD, Szostak JW. In vitro selection of RNA molecules that bind specific ligands. *Nature*. 1990; 346(6287):818–822. [PubMed: 1697402]
23. Tuerk C, Gold L. Systematic evolution of ligands by exponential enrichment - RNA ligands to bacteriophage-T4 DNA-polymerase. *Science*. 1990; 249(4968):505–510. [PubMed: 2200121]
24. Tombelli S, Minunni A, Mascini A. Analytical applications of aptamers. *Biosens Bioelectron*. 2005; 20(12):2424–2434. [PubMed: 15854817]
25. Pavlov V, Xiao Y, Shlyahovsky B, Willner I. Aptamer-functionalized Au nanoparticles for the amplified optical detection of thrombin. *J Am Chem Soc*. 2004; 126(38):11768–11769. [PubMed: 15382892]
26. Xiao Y, Lubin AA, Heeger AJ, Plaxco KW. Label-free electronic detection of thrombin in blood serum by using an aptamer-based sensor. *Angew Chem Int Edit*. 2005; 44(34):5456–5459.
27. Xiao Y, Piorek BD, Plaxco KW, Heeger AJ. A reagentless signal-on architecture for electronic, aptamer-based sensors via target-induced strand displacement. *J Am Chem Soc*. 2005; 127(51): 17990–17991. [PubMed: 16366535]

28. Minunni M, Tombelli S, Gullotto A, Luzi E, Mascini M. Development of biosensors with aptamers as bio-recognition element: the case of HIV-1 Tat protein. *Biosens Bioelectron.* 2004; 20(6):1149–1156. [PubMed: 15556361]
29. Tombelli S, Minunni A, Luzi E, Mascini M. Aptamer-based biosensors for the detection of HIV-1 Tat protein. *Bioelectrochemistry.* 2005; 67(2):135–141. [PubMed: 16027048]
30. Bini A, Centi S, Tombelli S, Minunni M, Mascini M. Development of an optical RNA-based aptasensor for C-reactive protein. *Anal Bioanal Chem.* 2008; 390(4):1077–1086. [PubMed: 18066708]
31. Huang CJ, Lin HI, Shiesh SC, Lee GB. Integrated microfluidic system for rapid screening of CRP aptamers utilizing systematic evolution of ligands by exponential enrichment (SELEX). *Biosens Bioelectron.* 2010; 25(7):1761–1766. [PubMed: 20061133]
32. Kim, SD.; Ryu, JS.; Yi, HK.; Kim, SC.; Zhang, BT. Construction of C-reactive protein-binding aptamer as a module of the DNA computing system for diagnosing cardiovascular diseases. Preliminary proceedings of the tenth international meeting on DNA computing (DNA10); 2004. p. 334–343.
33. Stoltenburg R, Reinemann C, Strehlitz B. FluMag-SELEX as an advantageous method for DNA aptamer selection. *Anal Bioanal Chem.* 2005; 383(1):83–91. [PubMed: 16052344]
34. Biro A, Rovo Z, Papp D, Cervenak L, Varga L, Fust G, Thielens NM, Arlaud GJ, Prohaszka Z. Studies on the interactions between C-reactive protein and complement proteins. *Immunology.* 2007; 121(1):40–50. [PubMed: 17244159]
35. Toomre D, Manstein DJ. Lighting up the cell surface with evanescent wave microscopy. *Trends Cell Biol.* 2001; 11(7):298–303. [PubMed: 11413041]
36. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970; 227:680–685. [PubMed: 5432063]
37. Li W, Wang KM, Tan WH, Ma CB, Yang XH. Aptamer-based analysis of angiogenin by fluorescence anisotropy. *Analyst.* 2007; 132(2):107–113. [PubMed: 17260069]
38. Mackiewicz MR, Hodges HL, Reed SM. C-reactive protein induced rearrangement of phosphatidylcholine on nanoparticle mimics of lipoprotein particles. *J Phys Chem B.* 2010; 114(16):5556–5562. [PubMed: 20364851]
39. McCauley TG, Hamaguchi N, Stanton M. Aptamer-based biosensor arrays for detection and quantification of biological macromolecules. *Anal Biochem.* 2003; 319(2):244–250. [PubMed: 12871718]
40. D'Auria S, Barone R, Rossi M, Nucci R, Barone G, Fessas D, Bertoli E, Tanfani F. Effects of temperature and SDS on the structure of beta-glycosidase from the thermophilic archaeon *Sulfolobus solfataricus*. *Biochem J.* 1997; 323:833–840. [PubMed: 9169619]
41. Agrawal A, Shrive AK, Greenhough TJ, Volanakis JE. Topology and structure of the C1q-binding site on C-reactive protein. *Journal of Immunology.* 2001; 166(6):3998–4004.
42. Diehl EE, Haines GK 3rd, Radosevich JA, Potempa LA. Immunohistochemical localization of modified C-reactive protein antigen in normal vascular tissue. *Am J Med Sci.* 2000; 319(2):79–83. [PubMed: 10698090]
43. Wagner M, Weber P, Bruns T, Strauss WSL, Wittig R, Schneckenburger H. Light dose is a limiting factor to maintain cell viability in fluorescence microscopy and single molecule detection. *Int J Mol Sci.* 2010; 11(3):956–966. [PubMed: 20479994]
44. Schwedler SB, Guderian F, Dammrich J, Potempa LA, Wanner C. Tubular staining of modified C-reactive protein in diabetic chronic kidney disease. *Nephrol Dial Transpl.* 2003; 18(11):2300–2307.
45. Vaith P, Potempa LA. Deposition of modified or native C-reactive protein in atherosclerotic arteries? *Arterioscl Throm Vas.* 2000; 20(4):1173–1174.
46. Hu WP, Hsu HY, Chiou A, Tseng KY, Lin HY, Chang GL, Chen SJ. Immunodetection of pentamer and modified C-reactive protein using surface plasmon resonance biosensing. *Biosens Bioelectron.* 2006; 21(8):1631–1637. [PubMed: 16343891]
47. Singh SK, Suresh MV, Hammond DJ Jr, Rusinol AE, Potempa LA, Agrawal A. Binding of the monomeric form of C-reactive protein to enzymatically-modified low-density lipoprotein: effects of phosphoethanolamine. *Clin Chim Acta.* 2009; 406(1-2):151–155. [PubMed: 19545552]

48. Khreiss T, Jozsef L, Hossain S, Chan JSD, Potempa LA, Filep JG. Loss of pentameric symmetry of C-reactive protein is associated with delayed apoptosis of human neutrophils. *Journal of Biological Chemistry*. 2002; 277(43):40775–40781. [PubMed: 12198121]

Abbreviations

CRP	C-reactive protein
CVD	Cardiovascular disease
TIRF	Total internal reflection fluorescence
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
SELEX	Systemic evolution of ligands by exponential enrichment
NIR	Near infrared
DEPC	Diethylpyrocarbonate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
SDS	Sodium dodecyl sulfate
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
DTT	Dithioreitol
PBS	Phosphate buffered saline
SPR	Surface plasmon resonance
BSA	Bovine serum albumin
PAGE	Polyacrylamide gel electrophoresis
EMSA	Electrophoretic mobility shift assay

Fig. 1.

Solution binding analyses between 5' Cy3-RNA, CRP, and C1q using fluorescence anisotropy. Baseline anisotropy readings of 5' Cy3-RNA (0.59 $\mu\text{g/mL}$) were acquired prior to the addition of (circles) mCRP_{SDS}, (squares) mCRP_{HCl}, (triangles) pCRP or (diamonds) BSA (each at 9.1 $\mu\text{g/mL}$) at the first indicated time point. At the second indicated time point, an aliquot of C1q (5.8 $\mu\text{g/mL}$) was added to the CRP-aptamer complex and evaluated for complement activity.

Fig. 2. CRP-RNA complex formation. **a** Dot blot assay was carried out by blotting CRP and BSA onto nitrocellulose and detected using a biotinylated aptamer (177 nM) followed by streptavidin-conjugated-IR 800 (1:1,000) and imaged on an Odyssey NIR imager. CRP was pre-incubated with 3'Cy5-RNA before being resolved on a 10% polyacrylamide gel containing 0.005% SDS. Protein bands were visualized by **b** silver staining and the gel was imaged for 3'Cy5-RNA and CRP-3'Cy5-RNA complex using **c** NIR imaging.

Fig. 3. EMSA of mCRP and pCRP using 3'Cy5-RNA probe. **a** In-gel visualization of 3'Cy5-RNA and 3'Cy5-RNA-mCRP complex using NIR imaging and **b** visualization of CRP bands by silver staining of the same gel. **c** Superimposed image of the NIR image and silver stain gel, highlighting the overlapping areas of the 3'Cy5-RNA-mCRP complex.

Fig. 4.

Quantitative binding studies between RNA aptamer and mCRP_{SDS}. (a) Saturation binding curve of 5'Cy3-RNA aptamer (0.68 μg/mL) with increasing mCRP_{SDS} concentrations. (b) Competitive displacement assay with increasing concentration of ssDNA. Titrations of ssDNA were added to a solution containing mCRP_{SDS}-5'Cy3-RNA complex (18 μg/mL mCRP_{SDS} and 0.68 μg/mL 5'Cy3-RNA) Data represents mean ± SE, $n=3$.

Fig. 5. Single molecule binding analysis between 5'Cy3-RNA and CRP assessed by TIRF microscopy. Representative TIRF images of **a** 5'Cy3-RNA, **b** 5'Cy3-RNA + mCRP_{SDS}, and **c** 5'Cy3-RNA + pCRP. Scale bar = 40 μ m. **d** Average integrated intensity of the 5'Cy3-RNA and 5'Cy3-RNA-CRP complex. Data represents mean $\pm$ SE, $n = 5$, ** $p < 0.01$ compared with the 5'Cy3-RNA control; # $p < 0.05$, compared with pCRP.

Table 1

Degree of binding between 5' Cy3-RNA and CRP (Δr_1) and between CRP and C1q (Δr_2) determined by the change in anisotropy.

Sample	Δr_1 (mean $\pm$ SD)	Δr_2 (mean $\pm$ SD)
mCRP _{SDS}	0.097 [*] $\pm$ 0.019	0.099 [*] $\pm$ 0.015
mCRP _{HCl}	0.107 [*] $\pm$ 0.016	0.013 $\pm$ 0.009
pCRP	0.014 $\pm$ 0.007	0.013 $\pm$ 0.008
BSA	0.018 $\pm$ 0.005	0.009 $\pm$ 0.005

n = 3

^{*} P < 0.001 compared with BSA control.