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Altered Binding of a Multimeric Protein by Changing the Self-Assembling Properties of its Substrate

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Abstract: Artificially controlled cell recognition has potentially far-reaching applications in both the understanding and altering of biological function. The event of recognition often involves a multimeric protein binding a cellular membrane. While such an interaction is energetically favorable, it has been surprisingly underexploited in artificial control of recognition. Herein we describe how changing properties of substrate (phosphocholine, PC) self-assembly can affect both binding behavior and substrate affinity to a pentameric recognition protein (C-reactive protein, CRP). PC was modified with a short, self-assembling DNA strand to make the substrate self-assembly sensitive and responsive to ionic environment. A significant shift in CRP binding affinity was observed when substrates were assembled in the presence of Cs^+ rather than K^+. Furthermore, alteration of the linker length tethering PC to DNA showed trends similar to other multivalent systems. In optimizing these linker lengths, positive cooperativity increased and K_d of the substrate assembly to CRP improved roughly 1000-fold. Such experiments both inform our understanding of biological, multivalent interactions in self-assembling systems and present a potential method to exogenously control events in cell recognition.

Introduction

Membrane recognition is a crucial biological function, as it influences proper intercellular communication.[1,2] The mechanism of recognition often involves multimeric protein binding to a cellular membrane. Simultaneous binding of multiple cell surface molecules not only ensures accurate target selectivity but also is predicted to be highly energetically favorable. The close proximity and multiplicity of small molecules is thought to lower the entropy of binding and be the driving force for this type of multivalent association.[3,4] The resultant ΔG for binding of these clustered substrates thus has the potential to be greater than the sum of ΔG's for each individual substrate. Attempts to exploit this phenomenon and study these clusters have almost exclusively involved covalently linking substrates to a core scaffold.[3,5,6] By designing self-assembling substrates, however, the possibility exists both to model natural substrates and to artificially control protein binding affinity and function.[7] Herein, we examine ways to control binding affinity to pentameric C-reactive protein (CRP) by altering the self-assembling properties of its substrate, phosphocholine (PC).

In addition to being synthetically simple and modeling the natural substrate, self-assembled biomolecules offer the advantages of a dynamic scaffold, through which binding affinity can be controlled by addition of an exogenous factor.[8−13] For example, a change in conditions (e.g., pH, temperature, ionic environment) can induce a change in properties of the assembly (e.g., substrate stoichiometry, orientation) that can affect its multivalent binding properties. Herein, we show that by tethering PC to a DNA scaffold, we can significantly alter binding to and aggregation properties of CRP by changing the type of alkali metal cation present. Furthermore, we provide evidence that these self-assembled substrates exhibit multivalent binding properties.[14,15] By designing and incorporating a linker of appropriate length between PC groups within an assembly,

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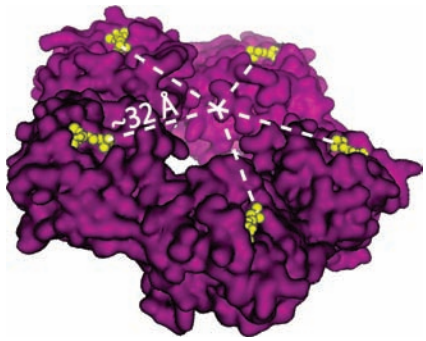

Figure 1. Distance between phosphocholine (PC, yellow) bound to C-reactive protein (CRP, purple) and a coplanar point in the center of the protein. PDB ID=1B09.

binding affinity and behavior can be significantly improved (1000-fold decrease in K_d).

Experimental Design

We have previously described the recognition of multimeric proteins by molecular fragments assembled on DNA quadruplexes and pentaplexes.[16−18] In these studies, assembly of substrates was achieved by their conjugation to the termini of deoxyguanosine (G)- or deoxyisoguanosine (iG)-rich oligodeoxynucleotides (ODNs). These ODNs have a propensity to form tetrameric or pentameric complexes, respectively, in the presence of specific counterions. We have most recently reported that PC assembled by an iG pentaplex can target pentameric CRP with ~100 times greater affinity than a nonassembled PC−ODN conjugate.[17] Pentameric ODN−PC assemblies were designed to mirror protein stoichiometry (five PCs to occupy each of CRP's five binding sites) with adequate spacing between PC groups. As depicted in Figure 1, PC groups bound in the pockets of the pentameric CRP are ~32 Å from a central, coplanar point. Thus a 27-atom PEG group of >32 Å straight-chain length was utilized to link PC to each ODN (Figure 2A, **27P**, and Figure S1). The design was intended to result in a 1:1 ratio of CRP complex to pentameric PC assembly.

In this paper we report the structure−activity relationships in PC assemblies that target CRP by altering linker lengths between PC and ODN (Figure 2A) and by changing the stoichiometric ratio of the PC:CRP binding site (Figure 2B). A number of nonassembling, monomeric PC (**M** species), in which the sequence of appended DNA precludes ODN aggregation, were also examined as controls. PC−DNA linkers both longer and shorter than the original 27-atom chain were studied (Figure 2A). Additional experiments examined the effects of monovalent cation type in solution, a condition that alters assembly stoichiometry from five to four PC molecules (Figure 2B).[19,20] Methods to quantify binding included an immobilized-phase binding affinity assay (ELISA) and quantification of solution-phase aggregation (fluorescence light scatter) of PC−ODN and CRP.

Materials and Methods

Synthesis of Conjugates and Formation of Assemblies. PC−ODN conjugates were synthesized and purified by methods outlined in a previous report.[17] Unless otherwise noted, pentameric (**P**) and monomeric species (**M**) were prepared in an aqueous solution of 80 mM CsCl, then diluted 2-fold with an 80 mM CsCl, 100 mM Tris-HCl, 300 mM NaCl, 2 mM $CaCl_2$, pH = 7.4 buffer prior to use in assays. Quadruplex species **27Q** and monomeric **27M** in KCl were prepared in an aqueous solution of 80 mM KCl, then diluted 2-fold with an 80 mM KCl, 100 mM Tris-HCl, 300 mM NaCl, 2 mM $CaCl_2$, pH = 7.4 buffer prior to use in assays. All assemblies were prepared by dissolving in the 80 mM salt solution and heating to 95 °C for 12 min, followed by slow cooling to room temperature and incubation at 4 °C for 48 h prior to dilution in the second buffer. Conjugation and purity of ODNs were confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) spectroscopy. Complete sequences of PC−ODN conjugates and circular dichroism spectra are outlined in the Supporting Information (Figures S1 and S2).

Enzyme-Linked Immunosorbent Assay (ELISA). All physical conditions and instrumentation for the direct-binding ELISA were equivalent to those used in the previous report,[17] with the exception of **27Q** and **27M** in KCl. In these two assays, all buffers that otherwise contained CsCl contained instead an equivalent concentration of KCl. Hyperbolic data analyses were conducted using the one-site saturation ligand binding program in SigmaPlot software to determine the maximum absorbance at saturation of binding by species **36P**, **27P**, and **27Q**. Logarithmic data plotting and fitting were conducted using the sigmoidal dose−response ligand binding program in SigmaPlot software, max=0.46, with the exception of the fit for species **16P** (Origin software with a fixed y maximum=0.46 yielded a similar K_d of 2.74 μM as compared to 4.84 μM for Sigmaplot, with significantly less variance in the curve fit). Data shown are the average of experiments conducted in triplicate. Additional controls by ELISA, including determination of nonspecific binding of DNA−biotin assemblies, were presented in a previous report.[17]

Fluorescence Light Scattering. All light scattering experiments were conducted with a Hitachi F-4500 fluorescence spectrometer (scan speed 240 nm/min, excitation wavelength 468 nm) and a 45 μL quartz fluorescence cuvette, light path 3 mm × 3 mm (Hellma). Samples of 50 μL were prepared and incubated at room temperature. Spectra of the samples containing CRP (2.0 μM) with varied concentrations of assembled or monomeric species were collected after 120 min. Final buffer conditions for incubation and data collection of protein/ODN samples: 80 mM KCl, 50 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, pH = 7.4 for the monomeric species **27M** in KCl and the tetrameric species **27Q**, and 80 mM CsCl, 50 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, pH = 7.4 for all other monomeric **M** species, pentameric **P** species, and pure, nonconjugated PC. Beer's law was used to determine concentration of all PC−ODN conjugates, and the concentration of nonconjugated PC was determined by obtaining the mass of the compound prior to its dissolution in buffer. Additional controls for light scatter of PC/CRP have been assessed in the previous study.[17]

Results and Discussion

Affinity of Pentameric Assemblies with Varied Linker Lengths. We[17,21] and others[3,5,6] have previously employed ELISA as a means of determining binding affinity of multimeric substrates

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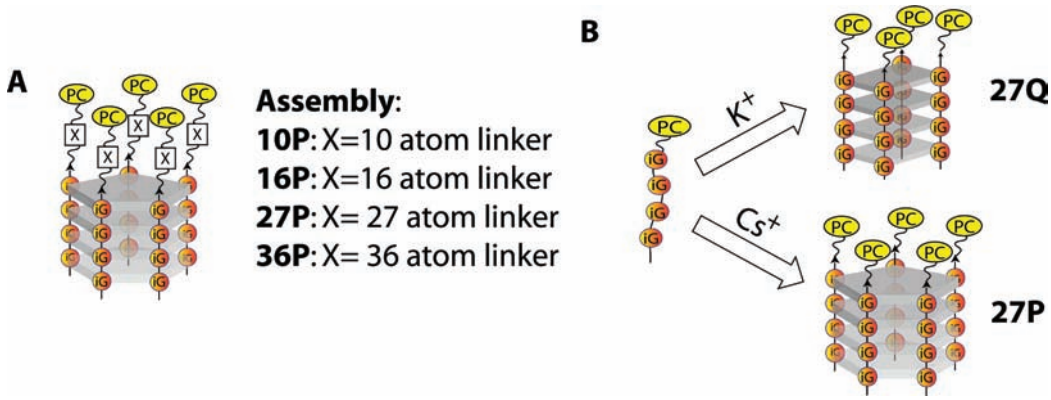
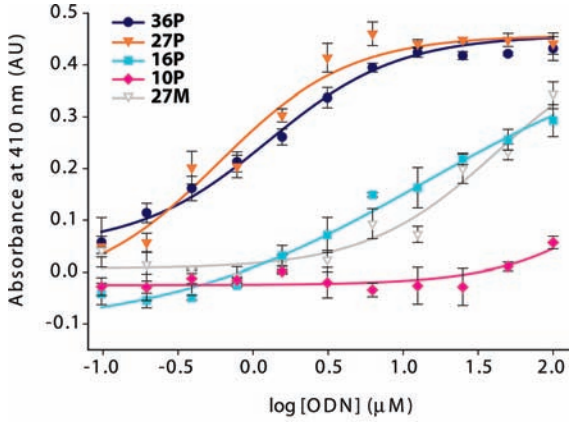


Figure 2. (A) PC assembled on a DNA, iG (deoxyisoguanosine) pentaplex scaffold with varying linker lengths. (B) PC assembled on iG scaffolds, with stoichiometry (tetrameric, pentameric) determined by the presence of K^+ or Cs^+.



Species	K_d (μM)	K_d per PC (μM)
36P	0.251	1.26
27P	0.117	0.585
16P	2.74	13.7
10P	116	580
36M*	50.5	50.5
27M	42.1	42.1
16M*	64.0	64.0
10M*	188	188

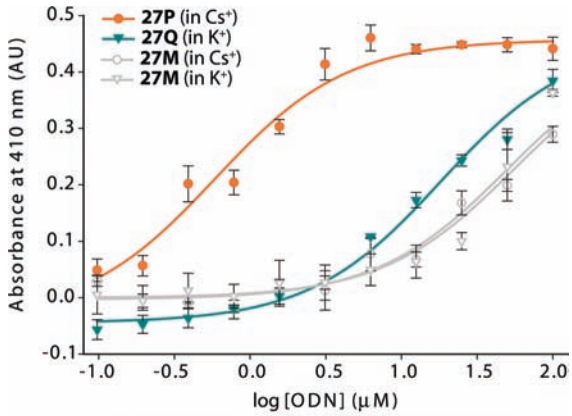
Figure 3. Direct binding analyses of pentameric assemblies (**P**) and monomers (**M**) of PC. *For other monomeric fits see Figure S3 in the Supporting Information.

to protein targets. In this study, a direct binding assay wherein CRP is immobilized to the assay plate by an antibody was used to determine K_d of all **P**, **Q**, and **M** species. ODNs in all assemblies and monomers were dually functionalized, with PC at the 5′ terminus and biotin at the 3′ terminus (Figure S1). The K_d's of species with varied linker lengths (**10P**, **16P**, **27P**, and **36P**) are depicted in Figure 3, and a direct correlation between the protein binding affinity of the species and its linker length is apparent. In pentaplexes **36P** and **27P** (Figure 3, dark blue and orange, respectively), PC is predicted to be tethered at a significantly greater distance than 32 Å from the center of the deoxyisoguanosine assembly, when linkers are in a straight-chain conformation. Thus PC in these assemblies should be more likely than in **16P** and **10P** to simultaneously reach the five binding sites of a pentameric CRP. The respective K_d values of 0.251 and 0.117 μM for **36P** and **27P** reflect binding much greater than that observed for equivalent monomeric species of 50.5 and 42.1 μM.

By contrast, species **16P** (Figure 3, light blue), in which the PC−ODN linker is ∼32 Å in length, does not fully saturate CRP at the maximum ODN concentration used in ELISA. The assembly evinces a markedly lower K_d, 2.74 μM, demonstrating a loss of greater than one order of magnitude affinity compared to species with longer linkers. The species with the shortest linker (**10P**, Figure 3, magenta) shows an even greater change in K_d and has as poor or worse affinity to CRP than single-stranded species (**27M**, Figure 3, gray, and other **M** species, Figure S3), particularly when examining the binding affinity per PC. In this case, the K_d of 580 μM for **10P** is at least three times greater than the highest K_d value for a monomeric **M** species, reflecting a loss in affinity upon substrate assembly.

Protein Binding Affinity of Pentameric vs Tetrameric Assemblies. As noted previously, in all of the deoxyisoguanosine-assembled species, the type of monovalent cation present during assembly plays a crucial role in the resulting stoichiometry of the complex. In species with varied linker lengths (**P** species), all assemblies were pentameric, a result of being formed in the presence of Cs^+. To examine the effect of ion-controlled stoichiometry on substrate binding affinity, tetrameric assemblies formed in K^+ (**27Q**) were assessed by ELISA. As can be seen in Figure 4, altering substrate stoichiometry from pentameric (orange) to tetrameric (blue-green) results in a 15-fold loss in binding affinity. The 4.56 μM K_d of **27Q** is still slightly higher than the monomeric control. To determine whether this difference arose from the ion alone and not ODN stoichiometry, monomeric species were analyzed in the presence of either Cs^+ or K^+. These controls yielded results that were nearly identical (K_d = 42.1 and 50.0 μM, respectively), precluding the role of the ion in affecting factors beyond ODN assembly.

CRP:ODN Assembly Binding Ratio through Maximum Absorbance. The maximum absorbance (MA) observed for different species in ELISA can indicate their relative stoichiometry of binding, as MA is determined by the number of ODNs that bind to a single CRP at saturation. If x equivalents of a PC−ODN assembly bind to a CRP pentamer, the expected MA at 410 nm would be lower than if $>x$ equivalents bind. There is a possibility that the lower relative K_d observed for **27Q** is due to binding fewer equivalents of PC per CRP, and thus fewer signaling molecules (biotin/HRP-streptavidin), resulting in lower MA. To negate this possibility, and to determine potential differences in maxima for binding analyses, selected data were fit as hyperbolae. Three species, **36P**, **27P**, and **27Q**, were shown to clearly approach an MA at 410 nm in the concentration range studied and are compared in Figure 5.



Species	K_d (μM)	K_d per PC (μM)
27P in Cs^+	0.117	0.585
27Q in K^+	4.56	18.2
27M in Cs^+	42.1	42.1
27M in K^+	50.0	50.0

Figure 4. Direct binding analyses of pentameric, tetrameric, and monomeric PC with a 27-atom linker.

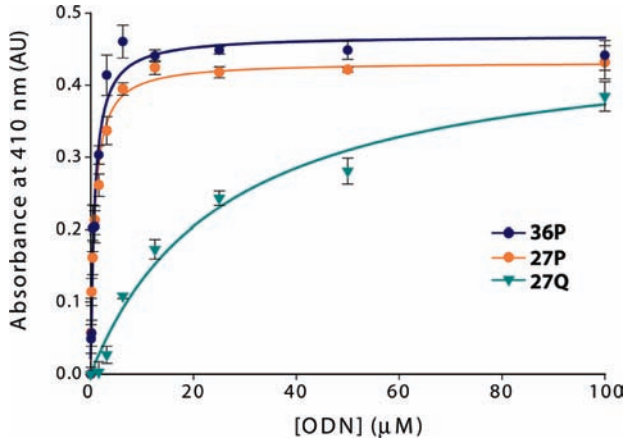


Figure 5. Analysis of species **36P**, **27P**, and **27Q** ELISA as hyperbolae to determine maxima, equal to 0.43, 0.47, and 0.47, respectively.

Absorbance at 410 nm for **36P**, **27P**, and **27Q** begins to approach a common maximum, indicating equivalent PC−ODN: CRP binding ratios for each species. The average of the respective three maxima, 0.43, 0.47, and 0.47, was equal to 0.46. This value was thus defined as the maximum in all sigmoidal dose−response curve fitting and analyses. These results appear to indicate that, by ELISA, changing stoichiometry or linker length in assembled PC does not likely alter the number of assemblies that bind to a single pentameric protein. All species appear to approach the same maximum without significant error or variance of points from the fitted curve.

Solution-Phase Aggregation of CRP and PC Assemblies. While several methods exist to quantify protein binding data of small-molecule species, the multimeric nature of self-assembled species presents a challenge.[13] Branched, multimeric binding species can induce aggregation of their protein target in solution,[22−24] precluding certain methods of fluorescence titration, anisotropy, or calorimetry, for example, to obtain quantitative binding information. Furthermore, the role of CRP in nature is to aggregate damaged cells, as supported by cell-based turbidity assays and clinical quantification of CRP by precipitation.[25] Thus, quantitative information can be garnered from aggregation of the protein itself. In vitro nephelometry has been previously adapted by our group to study precipitation of a multimeric protein by DNA-assembled small molecules.[17] An established technique to quantify precipitation of multimeric complexes (e.g., antibody−antigen complexes), fluorescence light scatter (I_F) of 468 nm light showed significantly greater precipitation by DNA-assembled substrates as compared to monomeric species (both quantitatively and to the naked eye). As can be seen in Figure 6A, a trend similar to that of K_d is observed in light scattering behavior of pentameric PC with varied linker length. Lower concentrations of the long-linker species studied are necessary for aggregation to occur. Both species **36P** and **27P** induce maximum aggregation at relatively lower concentrations, while **16P** does not reach a maximum in the range of ODN concentration shown. **10P**, like single-stranded species, does not induce observable aggregation at the concentrations studied. Neither PC linked to nonassembling

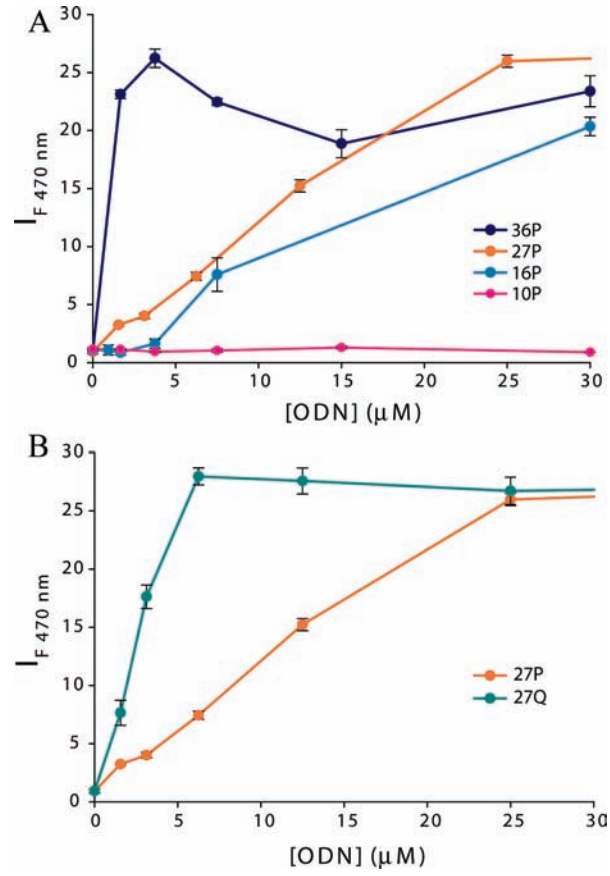


Figure 6. (A) Light scatter at 90 degree angle, 470 nm for pentaplex-assembled PC with varied linker lengths and (B) for pentameric versus tetrameric assemblies.

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Table 1. Free Energy of Dissociation per PC Molecule in Various Assemblies and Monomer **27M**

species	ΔG (in kJ/mol)
36P	3.37
27P	3.56
16P	2.77
10P	1.85
27M	2.50
27Q	2.70

DNA nor PC free in solution (Figure S4) appeared to result in significant precipitation within this concentration range.

Light scattering of tetrameric species assembled in the presence of K^+ (**27Q**) was compared to that of pentameric species assembled in Cs^+ (**27P**). As shown in Figure 6B, a distinct difference is observed between the two deoxyisoguanosine assemblies. The monomers in K^+ and Cs^+ yield no observable precipitation. Interestingly, species **27Q** induces precipitation at lower concentrations than does **27P**. This may indicate that, unlike in an immobilized phase (ELISA), cross-linking may be playing a unique role in solution-phase protein aggregation. A species that does not saturate binding pockets in a 1:1 assembly:multimeric protein ratio can allow for a mechanism of cross-linking-induced aggregation, perhaps explaining aggregation induced by the quadruplex species at lower relative concentrations of ODN.

Behavior in Solution. As seen in Figure 6A, light scatter for pentameric **P** species indicates that longer linker lengths induce aggregation at lower concentrations. This trend is similar to the results from ELISA, wherein longer linkers yield a lower K_d, and binding appears to be nearly abolished for shorter linkers (i.e., assembly **10P**). It is important to note the differences between these two types of assays, particularly the phase in which they are conducted. While CRP is immobilized in ELISA, light scattering reflects solution-based binding of protein and ODN. In the latter method, aggregation can be induced by cross-linking of ODN species and protein or by an inherent aggregating activity of CRP.[24] It is possible, particularly for the species **36P**, that both may be contributing factors. The peak at lower ODN concentrations might be due to greater cross-linking, as long linkers in **36P** allow for less steric hindrance in binding to multiple proteins. The second peak, at a slightly higher concentration, follows the trend of the ELISAs, wherein the affinity of **36P** for CRP falls between that of **27P** and **16P**. This may be accounted for by the natural function of CRP aggregation, or a different mode of cross-linking, wherein there is more than one ODN assembly per CRP pentamer. Indeed, in this concentration range of ODN, the ratio of ODN monomers to CRP monomers is >1. As cell recognition often occurs by multiple multimeric surface proteins, cross-linking is likely a contributing factor in the binding event in vivo.

***K_d*, Binding Energy, and Cooperativity.** The energetics of binding are significantly more favorable for the assemblies with adequate linker length and pentameric stoichiometry assessed in this study. In an optimal multivalent system, ΔG of binding of the pentameric PC would equal 5 times ΔG of binding a monomeric PC, when K_d is expressed in terms of individual PC−ODN (column 3, Figures 3 and 4). With no added energetic benefit from substrate assembly, the ΔG per PC in both pentamer and monomer would be equal. ΔG's of dissociation calculated from K_d per PC are outlined in Table 1.

The experimental values of ΔG for **36P** and **27P** exhibit a degree of positive cooperativity in multivalent binding as compared to the monomer. **16P** and **27Q** show little to no multivalent effect, and **10P** appears to exhibit negative cooperativity, with a ΔG of dissociation 0.65 kJ/mol lower than that of the monomer. While species **36P** and **27P** allow all five PC to simultaneously bind the five sites of CRP, the binding event restricts degrees of freedom of the highly flexible linkers. **36P** is expected to induce more significant entropic loss upon binding as compared to **27P**. The loss of affinity between **36P** and **27P** is relatively small, 0.19 kJ/mol, in comparison to 0.79 and 1.71 kJ/mol for **16P** and **10P**, respectively. This suggests that increasing linker lengths beyond what is sufficient to span all binding sites has relatively little impact on overall free energy.[17]

Insufficient linker length, however, results in a much greater deleterious effect on binding. For species **10P**, for example, unfavorable conformations in either or both CRP and the PC−ODN assembly are likely adopted to accommodate binding of the closely clustered PC.[4] **10P** is the only species to exhibit binding energy per PC lower than that of the monomer. The relative effect on ΔG from both increasing and decreasing linker length in this study aligns well with previous reports on multivalent protein−ligand systems.[3,4]

Biological Event Controlled by Monovalent Alkali Metal Ion. Perhaps the most dramatic shift in binding behavior, both of solution-based aggregation of CRP and in K_d, is between pentameric **27P** and tetrameric **27Q**. Given that the variable is type of group I metal cation, the effect is surprisingly significant. As shown in Table 1, free energy of binding per PC shifts from multivalent in the pentameric assembly (3.56 kJ/mol) to roughly equal to the monomer in the tetrameric assembly (2.70 kJ/mol).

Few types of biological systems distinguish between alkali metal cations.[26,27] With the PC−d(TiG_4T_4) conjugate, the alteration of Cs^+ to K^+ coincides with a change in affinity of 2 orders of magnitude. The change may arise from either or both a decrease in the number of PC (from five to four) and the distance between substrates (PC in **27Q** spans less area in a two-dimensional plane than in **27P**). Both assemblies are robust, and the ion is not expected to significantly alter the stability of the assembly at room temperature. Circular dichroism spectra of **27P** and **27Q** (Figure S2) indicate assemblies have formed, and the DNA quadruplex and pentaplex scaffolds have been extensively outlined in reports by Seela,[20] Switzer,[19] and our group[17] in the conditions used.

Conclusion

A self-assembling system in which response of a protein target is contingent on the type of monovalent cation present and that demonstrates linker-length-based multivalency has been presented. With rational changes in linker length and alteration of assembling conditions, as great as 1000-fold changes in binding affinity are observed. Several possible directions for future applications of the systems described here exist. Families of multimeric proteins that are implicated in both human health and pathology (e.g., cholera toxin, serum amyloid P, and other pentraxins) are potential targets. In addition to targeting proteins, protein substrate−ODN

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conjugates can have potential application in methods of assembly-based protein detection (i.e., as a beacon). Further exploration in detection and induction of cell signaling based on the assembly of conjugated DNA is currently underway.

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Supporting Information Available: (1) Complete sequences of conjugates used, (2) circular dichroism of assemblies studied, (3) graphical fits of monomeric species (ELISA), (4) graphical data for monomeric species (light scatter), (5) complete ref 24. This material is available free of charge via the Internet at http://pubs.acs.org.

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