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Protein-Binding Molecular Switches via Host–Guest Stabilized DNA Hairpins

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Supporting Information

ABSTRACT: Molecular switches, with target protein-binding activity controlled by prior binding to specific input stimuli, are ubiquitously used in Nature. However, the emulation of such responsive systems, especially in a *de novo* fashion, remains a significant challenge. Herein, we disclose a strategy that harnesses an intramolecular β-CD/adamantane host–guest interaction to generate a stabilized DNA hairpin (ΔT_m = 17 °C) that undergoes an input oligonucleotide (ODN)-selective structural transformation from a stem-loop conformation to a duplex. This ODN-induced conformational switch allows for the transition from an inactive state (wherein the adamantane protein-binding headgroup is encapsulated) to an activated protein-binding complex, with a freely accessible adamantane moiety. Given that hairpin domains can be readily modulated to be responsive to alternative ODN triggering sequences and that encapsulating macrocycles, such as β-CD, are good hosts for a number of protein-binding small molecules, this strategy may furnish a general method to develop ODN-responsive protein-binders.

Allosteric proteins are routinely utilized by Nature to exert precise control over cellular events. These complex agents behave as molecular switches, where binding to specific triggers (such as small-molecule or protein inputs) regulates their output (e.g., binding to downstream proteins).[1] Non-natural macromolecules with protein-binding activity that is controlled by initial binding to an input have a number of salient applications, including as tools to control/probe protein activity,[2] components in artificial networks that rewire cellular processes,[3] and as potential stimuli-responsive prodrugs.[4] However, the realization of such molecules remains a formidable challenge and has largely focused on proteinaceous systems.[5] Although elegant, these systems are limited in the choice of input triggers because of their basis on natural proteins. In this regard, protein-binders that are inducible by oligonucleotide (ODN) stimuli are particularly attractive,[6] because of the potential for high-fidelity activation by specific ODN sequences. Furthermore, the ODN inputs could be exogenously introduced (i.e., synthetic ODN sequences) or endogenously present (e.g., mRNA or miRNA).

We have previously shown that ODN triggers can be used to toggle a DNA–small molecule chimera (DC) between bidentate and monodentate protein-binding conformations, with the bivalent[7] state displaying significantly enhanced protein-binding affinity.[8] However, a limitation of this system is that two proximate sites on a protein need to be targeted simultaneously. In this communication, we describe a novel strategy toward ODN-inducible protein-binders, based on supramolecular chemistry[9] in water. In particular, this strategy involves hairpin-forming DCs that harness intramolecular host–guest interactions[10] between a protein-binding headgroup and a macrocyclic host, leading to an inactive protein-binding conformation (Scheme 1A). In the presence of a cognate ODN trigger, however, the hairpin undergoes a conformational change to a duplex state, resulting in an exposed protein-binding headgroup, corresponding to an activated DC conformation. This strategy is particularly attractive because it targets only one site on a protein surface and allows for high selectivity in terms of input ODN sequence (with even single- and double-base mismatches leading to significantly attenuated responsiveness).

The structures of the DCs used in this study are illustrated in Scheme 1B. These DCs (**1** through **1″**) contain a 19-mer core ODN domain that forms a stem-loop conformation composed of a five base pair stem and an unhybridized eight nucleotide loop region.[11] Additionally, the 5′ end features a non-base-paired thymidine residue that serves as an external toehold.[12] The hairpin-forming ODN domain is attached, via spacers, to adamantane and β-CD headgroups at the 5′ and 3′ termini, respectively (see Supporting Information for details on the synthesis of the DCs). The adamantane headgroup was specifically chosen because it serves as a prototypical hydrophobic protein-binder and is also a well-established guest for β-CD (the intermolecular adamantane/β-CD couple is characterized by a dissociation constant, K_d, of 2.0×10^{-5} M).[13] Further, we hypothesized that when the DCs access the stem-loop conformation a more robust adamantane/β-CD inclusion complex should form, as a result of the two binding partners being placed in proximity (this is because intramolecular binding is often more favorable than intermolecular binding as a result, *inter alia*, of entropic considerations).[14] Thus the hairpin conformation is envisioned to result in an inactive protein-binding state. In contrast, addition of a complementary ODN sequence is expected to lead to an activated duplex with an unsheathed adamantane headgroup. β-Lactoglobulin (β-lac) was chosen as the target protein because of its capability to bind promiscuously to a variety of hydrophobic molecules through a central β-barrel calyx.[15]

In order to probe the presence of a stabilizing intramolecular host–guest interaction, thermal denaturation experiments were conducted on parent DC **1** (followed via UV–vis spectroscopy).[16] As shown in Figure 1, a melting temperature (T_m) of 72 °C is observed. Furthermore, this T_m value does not change with

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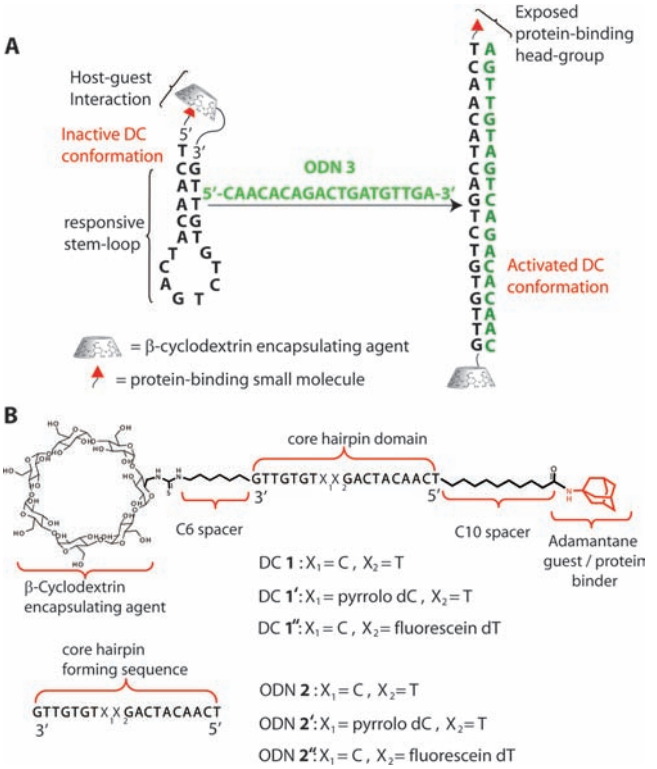
Scheme 1[a]



[a] (A) ODN-induced structure-switching of a DNA−small molecule chimera (DC) between inactive (host−guest interaction stabilized hairpin conformation) and active (duplex) protein-binding states. (B) Design of DC **1**, control ODN **2**, and their respective derivatives.

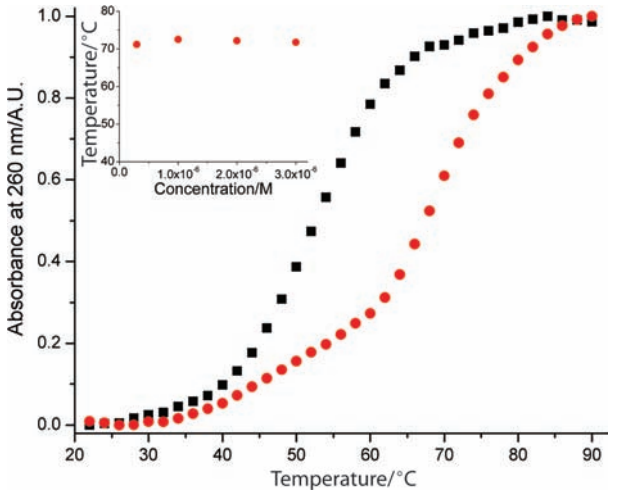


Figure 1. Normalized UV thermal denaturation of DC **1** at 0.3 μM (red dots) and ODN **2** at 1 μM (black squares). Inset: Concentration dependence on the melting temperature (T_m) of DC **1**. Experiments were conducted in 0.1 M NaCl, 0.1 M Tris, at pH = 7.1.

increasing concentration of DC **1** (Figure 1 inset), indicating the presence of a unimolecular hairpin that unfolds upon heating. Importantly, control ODN **2** that contains the core hairpin-forming ODN domain but lacks both synthetic headgroups unfolds at a substantially lower temperature (T_m = 55 °C). This

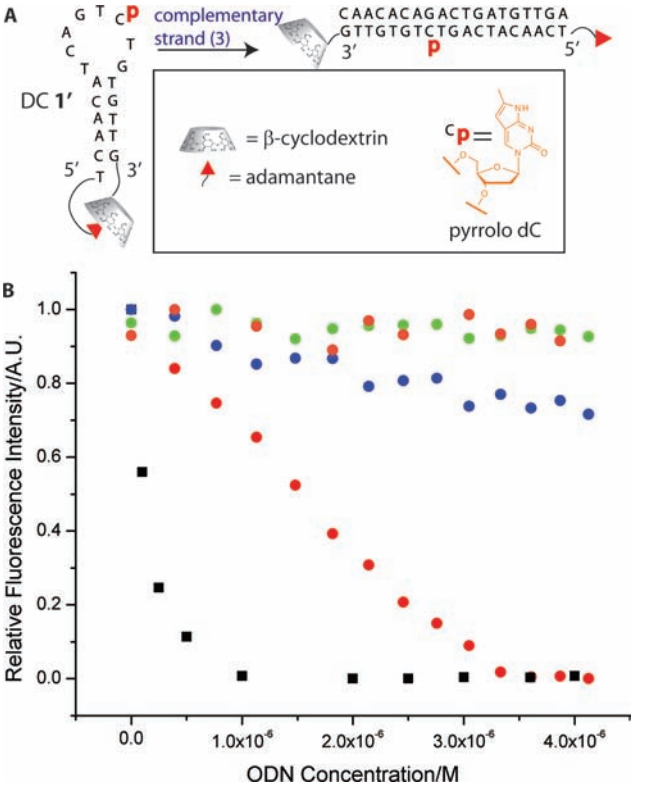


Figure 2. (A) Schematic depicting the hairpin-to-duplex transition of DC **1**′ with fully complementary stimulus ODN **3** (5′-CAACACA-GACTGATGTTGA-3′). (B) Fluorescence quenching of DC **1**′ (1 μM) upon binding to ODN **3** (red dots), single-base mismatch ODN **4** (5′-CAACACAGCCTGATGTTGA-3′) (blue dots), double-base mismatch ODN **5** (5′-CAACACAGCCCGATGTTGA-3′) (orange dots), and scrambled sequence ODN **6** (5′-ATGATCGATAGCAGTCACA-3′) (green dots). Fluorescence quenching of control ODN **2**′ (1 μM) with full complement ODN **3** (black squares). Notes: mismatches are underlined; excitation λ = 345 nm and emission λ = 450 nm; all experiments were conducted at 37 °C in 0.2 M NaCl, 0.1 M Tris, pH = 7.4.

change in T_m of 17 °C corresponds to a stabilization of 0.55 kcal/mol, as determined by van't Hoff analysis.[17]

With evidence that the intramolecular host−guest interaction can stabilize the hairpin conformation of DC **1**, we next investigated the ODN-induced structure-switching ability of the DC. Specifically, we synthesized DC **1**′, a congener of DC **1** where a cytidine in the loop region is replaced with pyrrolo-deoxycytidine (pyrrolo dC; see Figure 2A). Pyrrolo dC is a cytidine derivative that when unhybridized is fluorescent, but when base-paired with guanine (via three-point Watson−Crick H-bonding interactions) in a duplex shows dramatic quenching of its fluorescence emission.[18] As a control, the core hairpin-forming sequence, ODN **2**′, incorporating the pyrrolo dC modification, was also obtained.

Initial titration experiments of DC **1**′ (1 μM) and ODN **2**′ (1 μM), with a fully complementary sequence (ODN **3**), further verified that a stabilizing adamantane/β-CD interaction was present. In particular, whereas 1 equiv of ODN **3** was able to complete the hairpin-to-duplex transition for control ODN **2**′, approximately 3 equiv of ODN **3** is required to facilitate the same transition for DC **1**′ (Figure 2B). Given that complementary ODN trigger **3** was less effective at converting DC **1**′ to a duplex state, we investigated whether DC **1**′ could effectively discriminate

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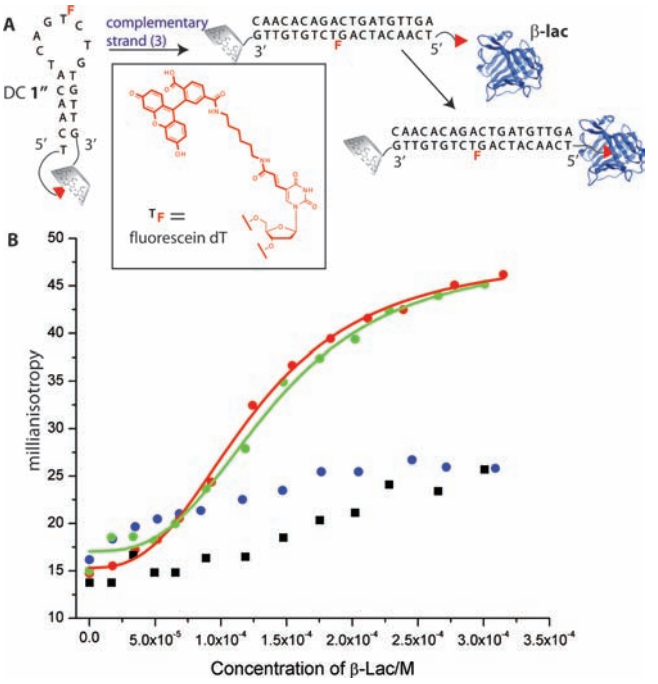

Figure 3. (A) Schematic illustrating the hairpin-to-duplex transition followed by β-lac binding for DC **1**″. (B) Fluorescence anisotropy titrations of β-lac in the presence of the hairpin (blue dots) or duplex **1**″:**3** (red dots) conformations of DC **1**″. Controls: duplex **2**″:**3** (black squares) that contains the core ODN **2**″ without any headgroups, and positive control ODN **7** (green dots) (5′-adamantane-TCAACAT-CAGT$_F$CTGTGTTG-3′) that contains only an adamantane moiety. Note: fluorescence anisotropy experiments were performed at 37 °C; excitation λ = 495 nm and emission λ = 525 nm; all experiments were done in 0.2 M NaCl, 0.1 M Tris, pH = 7.4. The concentrations of fluorescein-tagged ODNs were 2 nM. The line shows the curve fit to a 1:1 binding model.

between slight variations in input sequence. Specifically, we probed input sequences containing one (ODN **4**) or two (ODN **5**) base-pair mismatches, as well as a scrambled version of **3** (ODN **6**). As can be gauged by inspection of Figure 2B, the fluorescence of DC **1**′ does not show appreciable change upon addition of the double-mismatch sequence or the scrambled sequence, while the single-base mismatch shows only modest fluorescence quenching, with no saturation behavior, under the concentrations tested. When taken together, these studies lead us to suggest that the synthetic host–guest interaction shows high fidelity in terms of being responsive to the complementary input sequence.

Having shown that the adamantane/β-CD stabilized hairpin is selective toward its fully complementary input sequence, we were keen to determine if there is a difference in β-lac binding ability between the hairpin and ODN **3**-induced duplex conformations of the DC. In an effort to explore protein-binding, DC **1**″ was synthesized (Figure 3A) that contains a fluorescein-labeled thymidine residue as a handle for fluorescence anisotropy-based binding studies. Additionally, control ODN **2**″ (containing only the core hairpin domain) and ODN **7** (that projects the 5′-adamantane arm but lacks the β-CD host) were also prepared. Figure 3B shows the change in anisotropy of the fluorescein-labeled ODNs with increasing concentrations of β-lac (followed at 525 nm; excitation at 495 nm). The hairpin conformation of DC **1**″ and control duplex **2**″:**3** both displayed only small increases in anisotropies at the concentrations tested, attributed to nonspecific binding to β-lac ($K_d \gg 3.0 \times 10^{-4}$ M). In contrast, the ODN **3**-induced duplex state of DC **1**″ (i.e., DC **1**″:**3**) showed a clear increase in anisotropy with saturation behavior and is characterized by a K_d of $(1.26 \pm 0.04) \times 10^{-4}$ M. Importantly, this value is close to the K_d of $(1.41 \pm 0.08) \times 10^{-4}$ M for positive control ODN **7** that contains a freely accessible adamantane headgroup. Taken together, these studies clearly show that the β-lac binding ability of DC **1**″ can be induced by stimulus ODN **3**.

In conclusion, we have demonstrated a proof-of-concept strategy that harnesses host–guest chemistry in water and the structure-switching ability of nucleic acid hairpins to develop DCs with protein-binding ability inducible by selected nucleic acid inputs. We envision that this strategy will be of broad utility because the DC design is highly modular: for instance, the hairpin domain can be interchanged to engender responsiveness to other input sequences (i.e., in a manner similar to that popularized by research on molecular beacons).[19] Furthermore, β-CD and its analogues are established hosts for a plethora of drug and drug-like molecules;[20] thus, the protein-binding DC headgroup can also be "dialed in" to sequester alternative protein targets. We are currently exploring these avenues.

■ ASSOCIATED CONTENT

Supporting Information. Synthetic procedures, MALDI-TOF, and fluorescence titrations. This material is available free of charge via the Internet at http://pubs.acs.org.

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