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A Stable Bidentate Protein Binder Achieved via DNA Self-Assembly Driven Ligand Migration

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Abstract

Herein we disclose the development of two complementary single stranded DNA-small molecule chimeras (DCs) that by themselves only bind weakly to a protein target (human serum albumin; HSA). However, upon self-assembly, the DC duplex facilitates a ligand migration reaction leading to a covalently fastened high-affinity, bidentate, protein-binder that resides at the terminus of only one of the DC strands. Due to this specific localization, the bidentate projection remains intact—and thus the system continues to strongly bind HSA—even under conditions that denature and degrade the DNA scaffolds.

The development of self-assembled protein binders, that interact with their target proteins via directed and multidentate binding, is an area of significant interest because these systems can be rapidly prepared through predictable supramolecular assembly modes and can lead to significantly enhanced protein-binding affinity and selectivity.¹ In this regard, the use of DNA cores tethered to protein-binding head-groups is particularly attractive since self-assembled duplexes, quadruplexes, and other higher-order structures can be used to achieve multivalent protein-binding.² In particular, DNA duplex-based protein-binders have received primary attention due to the simplicity and programmability of canonical Watson-Crick base-pairing.^{2a,b} Such duplexes have been exploited in DNA encoded chemical library (DEL) technology to select and identify pairs of small molecule binders against a variety of target proteins.³

Alternatively, these duplex based DNA-small molecule chimeras (DCs)^{2a,2b,4,5} can be envisaged to serve directly as the synthetic pharmacophore which has the potential advantage of being conditionally assembled, leading to possible spatio-temporal activation of high affinity protein-binders. For instance, the separate yet selective delivery, via cellular receptor targeting carriers, of two single stranded DCs (that individually are low affinity protein binders) only to disease cells can lead to co-localization of both DCs, resulting in duplex assembly and formation of an activated bidentate protein binder (Scheme 1a).

The abovementioned strategy is attractive since protein binding and inhibition will occur selectively only in cells that express *both* receptors, thereby leading to safer therapeutics.

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However, the first step towards achieving such a goal is to develop *in situ* self-assembling duplexes that stably project a bidentate conformation throughout the lifetime that the protein binding/inhibition is being orchestrated. Indeed, for a double helical system, the longer the duplex DC resides in the cell, the greater the likelihood of off-target binding (that disrupts the duplex leading to inactive single stranded DCs) and/or nuclease digestion. Both of these events may lead to attenuated protein-binding efficacy. Thus there is a need to probe novel mechanisms that enhance the integrity of such bidentate binding systems.

One option to address the abovementioned issue is via judicious oligonucleotide (ODN) modifications (such as using PNA or LNA backbones)⁶ that form stable and nuclease resistant duplexes. Herein we introduce a complementary strategy that could further enhance the integrity of the bivalent projection pattern. Specifically, we reasoned that if the lifetime of the self-assembled duplex can be minimized via a self-assembly driven ligand migration reaction that transfers a protein binding moiety from one strand to the other, and covalent bond formation results; via DNA templated synthesis (DTS)⁷. Then upon completion of the ligand transfer step, a high-affinity bidentate protein binder can be generated at the termini of only one of the DC strands (Scheme 1b and Scheme 2).⁸ Hence, once the DTS step is complete, the off-target induced dis-assembly of the duplex or even nuclease induced cleavage of the single strand should not substantially influence protein-binding effectiveness since the bivalent projection still remains intact in these latter systems (Scheme 1c).

In order to demonstrate proof-of-concept, we focused on DC based binders to human serum albumin (HSA) as it is a well-understood model protein with known bidentate binders.^{2a} In particular, the group of Neri has shown that 4-(*p*-iodophenyl)butyric acid (**ip**) and fluorescein (**fl**) can be tethered to a small molecule scaffold to produce high-affinity binders for HSA that interact with site II on HSA.⁹ Based on these binding head-groups, we were interested in developing self-assembling DCs that can undergo rapid DNA templated ligand transfer to afford a bidentate DC product with both head-groups located on one strand. In particular, we synthesized complementary DCs **1** and **2**, with DC **2** projecting an N-hydroxysuccinimide (NHS) ester-linked **ip** head-group on its 5' terminus whilst DC **1** contains a **fl** moiety on the terminal thymidine residue as well as a 3' primary amine unit (Scheme 2). Hence, when **1** is hybridized with **2**, a duplex forms that brings the primary amine group on DC **1** in proximity to the NHS ester of DC **2** (Scheme 1b i). This duplex facilitates a ligand migration reaction leading to product duplex **1':2'** (Scheme 1b ii) which is expected to be a high-affinity bidentate binder to HSA. Importantly, since the bidentate product HSA binding unit is now projected only on the 3' terminus of DC **1'** (Scheme 2), we reasoned that the integrity of duplex **1':2'** or even single strand **1'** should not critically influence the HSA binding capacity of the system.

The synthesis and characterization of the DC sequences are provided in the SI. First, we explored the feasibility of the DNA-templated ligand migration reaction by HPLC and MALDI-TOF. Figure 1 shows the HPLC profile for the reaction as followed conveniently by the absorption of the **fl** protein-binding head-group located initially on reactant DC **1**. In these profiles, only the reactant and product DCs containing this **fl** moiety are observed. Figure 1a is a control HPLC trace of unreacted DC **1**, which displays a retention time of 13.5 min. Figure 1b shows the HPLC profile for the DNA templated reaction at 25 °C, where 1.2

equiv. of DC **2** was added to an aqueous solution (50 mM MOPS and 10 mM Mg(OAc)₂, pH 7.5) of 1 μ M DC **1** and was reacted at room temperature for 30 min and then heated to 80 °C for 5 min. Here, a new peak with a slower retention time of 20 min is observed with a concomitant decrease of the DC **1** peak. MALDI-TOF analysis (Figure 1b; inset) confirmed that the peak at 20 min corresponds to DC **1'** (found MW = 5038.3 Da, calcd. MW = 5041.9 Da) and a 90% yield was estimated based on HPLC integration. In marked contrast to the templated reaction, when a negative control (DC **2m**; a poly T sequence that contains the NHS ester-linked **ip** head-group but is not complementary to DC **1**) was reacted with DC **1**, no ligand transfer is observed (Figure 1c), instead unreacted DC **1** was isolated (found MW = 4768.4 Da, calcd. MW = 4769.9 Da).

With information in hand that the templated ligand migration proceeds smoothly, we next investigated the differential HSA binding ability of the reactant DC **1** and purified product DC **1'** single strands via PAGE. As can be observed from the inset in Figure 2, when HSA is added to a solution of DC **1'**, the fast migrating fluorescent band (from the **fl** unit) of DC **1'** disappears and a slow migrating fluorescent band—asccribed to the HSA:DC**1'** complex—is observed. On the other hand, when HSA is introduced into a solution containing reactant DC **1** no appreciable difference in the migration of DC **1** is observed. These results are consistent with the notion that the bidentate product DC **1'** is a strong binder to HSA compared to DC **1**. Further, quantitative affinity information of the DCs with HSA, were obtained from fluorescence anisotropy (FA) titrations (again using the **fl** handle on the DCs) on purified DCs **1** and **1'** (100 nM) in the presence of increasing concentrations of HSA. Figure 2 shows that for DC **1** no appreciable HSA binding was detected even at 0.1 mM [HSA].¹⁰ However, DC **1'** displays saturation behaviour and exhibits a robust K_d of $1.9 \pm 0.2 \times 10^{-6}$ M.

In addition to analysing the binding of the HPLC purified product DC **1'** to HSA, we also probed the binding of HSA directly with the, non-purified, reaction product solution of DC **1** and DC **2** (i.e., after the formation of duplex **1':2'**). Non-linear regression analysis, using a 1:1 binding mode resulted in a K_d of $2.4 \pm 0.5 \times 10^{-6}$ M, which is close to the K_d for DC **1'** and HSA (*vide supra*). Importantly, when the FA study was conducted with the reaction solution using the negative control DC **2m**, no appreciable HSA binding was detected under the HSA concentrations tested. Taken together, these results are consistent with the notion that the product duplex DC **1':2'** is an activated bidentate binder of HSA while the non-templated reaction does not facilitate formation of the bidentate head-group.

Subsequent to establishing that the *in situ* generated product DC duplex **1':2'** is a strong binder to HSA, we investigated whether the denaturation of duplex **1':2'**, through disassembly, would retain the high affinity binding (see Scheme 1c i). For duplex DC **1':2'** dis-assembly, 10 equiv. of ODN **3** (5' TGC GGAGGAAGGT 3', that has the same core sequence as DC **1'**) was incubated with duplex **1':2'** at 90 °C for 2 min and allowed to cool to room temperature. FA titrations were performed by addition of HSA to the dis-assembled solution. As expected, duplex displacement does not appreciably affect the binding affinity of the system to HSA (Table 1), since the bidentate head group resides only on DC **1'** and thus is not influenced by the assembly state of DC **1'**.

Furthermore, we were keen to explore whether the integrity of the core ODN sequence of DC 1' is required to maintain high affinity HSA binding (Scheme 1c ii). Thus single stranded DC 1' was incubated with DNase I for 4 hours (a PAGE study confirmed the nuclease induced cleavage of DC 1', see SI-8). When FA titrations were performed (by addition of HSA to the nuclease degraded DC 1' solution), the system still maintains strong binding to HSA. DNase I nonspecifically cleaves DNA to furnish 5'-phosphorylated di-, tri-, and ODN products.¹¹ Thus, this finding indicates that the fl containing bi-products are still effective in binding to HSA, leading us to conclude that the bidentate projection is maintained in the cleaved biproducts.

Conclusions

In summary, we have disclosed a mechanism to generate *in situ* activated protein-binders wherein two single stranded DCs that are weak HSA binders self-assemble into a duplex that initiates ligand migration from one strand to the other. This DTS step was achieved via an intramolecular NHS-ester amine coupling reaction resulting in a covalently fastened high-affinity bidentate DC duplex. In addition to dramatically enhanced HSA binding, the bivalent head-group is localized on the terminus of only one DC sequence and thus this activated head-group remains effective even upon denaturation of the DC duplex and degradation of the single-stranded DC. We expect that this novel approach to *in situ* activated DCs that display robust protein binding capacity even in harsh conditions could be of general applicability in the design of "smart" protein binders and inhibitors, because (a) a diversity of bivalent synthetic head groups can be identified to target various proteins,¹² and (b) a number of bio-compatible ligand migration chemistries can be utilized.^{7a}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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10. FA studies were also conducted with two control DCs (**4** and **5**). DC **4** has the same core sequence as DC **2**, but has a nonlabile **ip** group on the 5' end and a **fl** unit on the 3' end (these binding units were separated to attenuate any possible bidentate binding, yet still containing a **fl** unit as a spectroscopic handle). The HSA binding studies (Figure SI-15) show that DC **4** is not a strong binder for HSA and therefore neither monodentate head group when separated by a large distance is able to bind effectively to HSA. Control DC **5** has the same structure as DC **4** (including the non-labile **ip** group on the 5' end) but lacks the **fl** unit on the 3' end). DC **5** can hybridize with **fl** containing DC **1** (to form duplex **1:5**) but can not transfer its **ip** group upon duplex formation. The HSA binding data (Figure SI-16) shows that duplex **1:5** is less effective at binding HSA when compared to duplex **1':2'**. Thus, the exact positioning of the **ip** and **fl** units is important to achieve strong bidentate binding
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Scheme 1.

(a) Two single stranded DCs (with low protein binding affinity) are separately transported into target disease cells by two carrier particles each containing a unique targeting ligand. After entrance, the DC strands can self-assemble forming a high-affinity bivalent protein binder. (b) Low affinity DCs **1** and **2** (i) self-assemble into a duplex, (ii) which results in ligand migration and formation of an activated high affinity bivalent human serum albumin binder (i.e., DC duplex **1':2'**). (c) The HSA binding activity of the bivalent head-group located on DC **1'** within duplex **1':2'** is not significantly affected by (i) the presence of excess competitive single strands or (ii) nuclease induced cleavage.

Scheme 2.

The reactants (top) and products (bottom) of a duplex DNA templated ligand migration reaction (*via* intra-molecular NHS ester-amine coupling).

Figure 1.

HPLC analysis of (a) DC **1** only, (b) the products from the reaction of DC **1** and full complementary DC **2**, (inset: MALDI-TOF of peak at 20 min), and (c) products from the reaction of DC **1** and non-complementary DC **2m** (inset: MALDI-TOF of peak at 13.5 min). Note: the HPLC detector wavelength was set to 490 nm.

Figure 2.

FA titrations of purified DC **1** (blue triangles) and purified DC **1'** (brown triangles) with increasing concentrations of HSA. Also shown is the corresponding titration of the crude reaction mixture of DC **1** with 1.2 equivalent of DC **2** (black squares), and DC **1** with 1.2 equivalent of DC **2m** (red circles) with increasing concentrations of HSA. All fluorescently labeled DCs are 100 nM. Inset: PAGE of DC **1'** and DC **1** in the absence and presence of HSA. Both FA and PAGE studies were followed using the fluorescein reporter.

Table 1

Dissociation constant data, based on FA titrations, of HSA binding to DC **1':2'** and its dis-assembled and degraded products.

DC Sequence	DC 1'2'	DC 1'2' +10 eq of ODN 3	DC 1' + DNase I ^a
$K_d(M)$	$2.4 \pm 0.5 \times 10^{-6}$	$3.6 \pm 0.5 \times 10^{-6}$	$3.3 \pm 0.2 \times 10^{-6}$

^aSince DNase I action on DC **1'** produces **fl** containing bi-products, the dissociation constant is an estimated average K_d for all the **fl** containing hydrolyzed products with HSA.