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Self-assembly of bivalent protein-binding agents based on oligonucleotide-linked organic fragments

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Abstract—A library of bidentate fragments linked through an oligonucleotide duplex was tested for binding to streptavidin. When one fragment was biotin, only biotin-containing duplexes were selected by streptavidin but when heated above the melting temperature, only bidentate biotin ligands were obtained. Thermal denaturation experiments showed that the melting temperature, thus stability, of the monodentate versus bidentate binding ligand increased from 59 to 71 °C in the presence of streptavidin. Substituting biotin with 2-iminobiotin led to the exclusion of all other duplexes by the bidentate iminobiotin duplex in binding streptavidin.

In the identification of effective protein-binding molecules, Nature offers guidance in the form of antibodies. The association of light and heavy chains through their constant domains allows the combinations of their variable domains to provide a wide variety of recognition surfaces, from which specific binding agents are selected (Fig. 1A). We have previously employed this strategy by using metal templation to bring together two constant terpyridyl domains linked to variable binding fragments (Fig. 1B).[1] In this way, self-assembled combinatorial libraries can be prepared that undergo dynamic interconversion and subsequent selection depending on the metal/ ligand exchange rate.[2] However, condition limitations prevented the formation of certain functionalized metal complexes, prompting the search for more aqueous-compatible designs. One attractive possibility is the use of complementary hydrogen bonding groups within the constant domain. Hydrogen bonding between two binding fragments provides stability and exchange kinetics that are governed by the nature of the donor/ acceptor structure, affinity, and kinetics of the pairs (Fig. 1C).

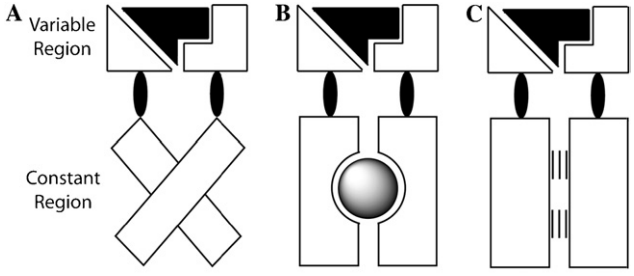

Figure 1. Strategies for combining two recognition fragments based on (A) antibody light and heavy chain association; (B) metal–ligand assembly; (C) hydrogen bond template association.

Particularly suited to this role are single-stranded oligonucleotide sequences that are functionalized on the 3′- or 5′-end and can associate with complementary strands to position two binding fragments across one terminus of the DNA duplex. In a DNA duplex, the phosphate end of the 5′-strand and the hydroxyl end of the 3′-strand are ~20 Å apart and can be coupled to organic fragments. Adding a linker modification at the 5′- or 3′-end of a duplex allows the attachment of a range of organic fragments and also increases their flexibility relative to the duplex. Single-stranded oligonucleotides have previously been linked to organic fragments as coding sequences in solution-based combinatorial synthesis,[3] as templates for controlling biomolecular reactions,[4] or as a constant binding group to select for other binding components.[5] In the present

Keywords: Bidentate; Monodentate; Thermal denaturation; Combinatorial library.
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design, however, the DNA duplex acts as a non-covalent linker between two binding fragments that can undergo exchange with other functionalized oligonucleotide strands to give a combinatorial library of bivalent ligands for screening against protein targets.

We tested this approach against streptavidin (SA), which is known to bind four biotin molecules into surface accessible clefts, with the highest affinity known for a protein–small ligand interaction ($K_d = 10^{-15}$ M).[6,7] Attachment of a biotin fragment to the 3′- and 5′-ends (Fig. 2A) of two complementary oligonucleotide strands should lead to the formation of a duplex displaying two biotin ligands on one end. This should allow, if the geometry of the two biotins were correct, a bidentate contact with the SA surface (as shown in Fig. 2B). In this way, the DNA duplex forms a non-covalent link between two binding fragments, increasing their affinity for the target through multivalency. Protein binding will, in turn, lead to a stabilization of the DNA duplex, which should be reflected as an increase in the melting temperature, T_m, of the duplex.

Conjugation of biotin at the 3′- or 5′-end of two complementary C3 amine-functionalized oligonucleotide strands was achieved by incubating *N*-hydroxysuccinimidobiotin with the oligonucleotide under slightly basic conditions. The complementary oligonucleotides were combined to form a duplex that denatured at 59 °C, as observed from thermal denaturation studies (Fig. 3). Addition of SA led to the formation of a ternary complex, which resulted in a duplex T_m of 71 °C.

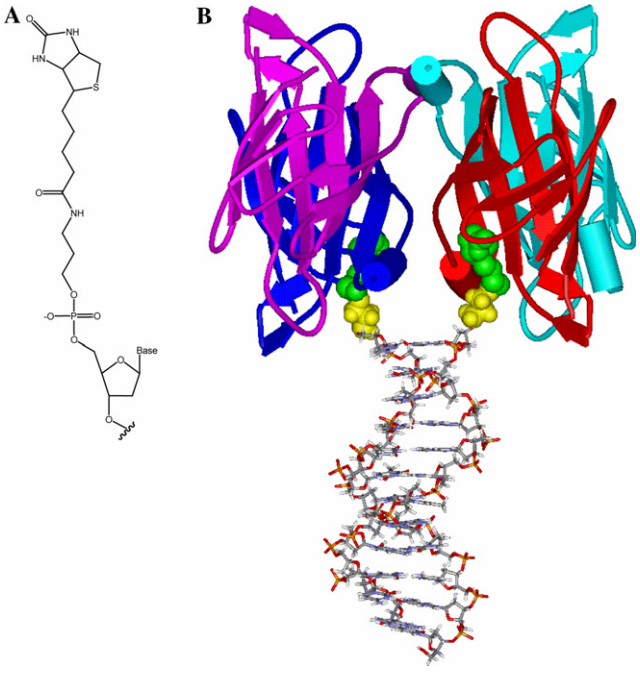

Figure 2. (A) Chemical structure of biotin conjugated to an oligonucleotide through the C3 linker. (B) Schematic of a bidentate biotin ligand binding two adjacent biotin sites on the tetrameric SA. Subunits (red, cyan, blue, and pink) binding biotin (green) through an amine-modified (yellow) DNA duplex. PDB code: streptavidin (1SWG). NDB code: duplex DNA (BDL084).

This increase of T_m by 12 °C clearly points to an increased stabilization of the complex through a pseudo-intramolecular chelate effect arising from a combination of bidentate binding to the protein and duplex formation. Control experiments using a biotin-linked oligonucleotide duplexed with a complementary unfunctionalized strand showed no change in T_m on addition of SA (Fig. 3).

One advantage of this strategy is that the DNA duplexes act not only as scaffolds to display the protein-binding groups but can also be induced to dissociate under denaturing conditions and exchange with other complementary functionalized strands. In this way, mixtures of different oligonucleotide-linked fragments can form a library of x^2 DNA duplexes (where x is the number of library members in each set) displaying different combinations of two binding fragments at one end (Fig. 4A). The library can then be screened to identify which fragment combinations bind the surface or active site of an immobilized protein target (Figs. 4C, D). A further advantage of the DNA scaffold is that, with appropriate choice of sequence, the identity of the binding groups can be detected by polymerase chain reaction (PCR) (Fig. 4F). Hence, dynamic exchange by heating and further selection can lead to an enrichment of the binding combinations and thus to an amplification of the PCR signal.

As a proof of principle of this approach, a small library of organic fragments on oligonucleotide scaffolds was generated and screened for binding to SA. The oligonucleotides used in this approach are designed to have two conserved sequences bordering an identifying region (Fig. 4B). This identifying region is a unique sequence that encodes for the organic fragment attached to the end of the oligonucleotide. PCR conditions were optimized to accommodate only one primer that is complementary only to the identifying region of the oligonucleotide. In traditional PCR, a reverse primer would have to anneal to the conserved region of the oligonucleotide which, in this case, would lead to products synthesized from any and all oligonucleotides in the fraction collected.

To test this approach against SA, a library of six species was generated, consisting of two sets (a, b) of complementary oligonucleotides: one set containing C3 amine modifications at the 5′-end (Z_a) and the other set modified at the 3′-end (Z_b) where Z denotes the oligonucleotide. The two sets of oligonucleotides contain the same three organic fragments: biotin (B_a and B_b), methyl adipate (M_a and M_b), and an unfunctionalized primary amine (X_a and X_b). When the two sets are pooled and allowed to anneal, the duplexes formed will display two organic fragments at one end of the duplex. The sequences of the oligonucleotides were generated so that there was no possibility of self-complementarity, hairpin or loop formation.[8] The organic fragment-conjugated oligonucleotides were tested individually for their ability to bind tetrameric SA and also to form duplexes with every member of the complementary set of oligonucleotides (data not shown). Only the biotin-con-

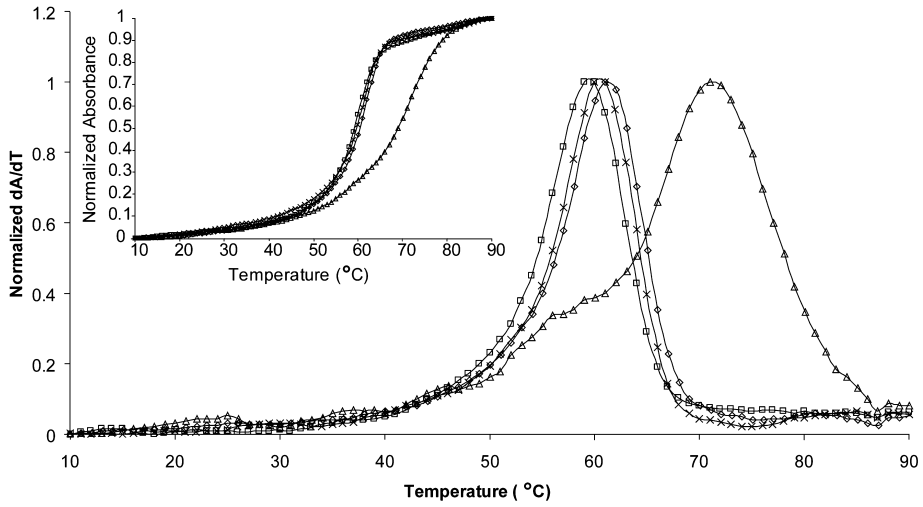

Figure 3. Thermal denaturation studies were performed with 1 μM of either monodentate or bidentate duplex. The denaturation studies were performed both in the absence and presence of SA. B_aB_b (□), B_bX_a (◊), B_aB_b + SA (△), and B_bX_a + SA (×). Derivative and sigmoidal data were smoothed (n = 5) and normalized.

jugated oligonucleotides from each set were found to bind SA. The six oligonucleotides were pooled and allowed to form the nine possible duplex combinations. One of the duplex species that forms will have at one end of the duplex, two biotin molecules (B_aB_b) that could potentially bind two adjacent sites on the tetrameric SA protein (as in Fig. 2B). The other combinations containing one biotin oligonucleotide and another oligonucleotide either containing methyl adipate (M_aB_b or B_aM_b) or just the primary amine modification (X_aB_b or B_aX_b) would bind SA in a monodentate fashion (as in Fig. 4D) through B_a or B_b, since M and X were found not to bind SA individually.

In a typical experiment, the library mixture was incubated with SA immobilized on magnetic beads for a period of 1 h at room temperature. After removal of the flow-through fraction (F), the beads were washed to remove weak/non-specific binders. The binding fragments were then eluted (E) under protein denaturing conditions. The F and E fractions were then aliquoted into PCR tubes, each containing one primer complementary to the identifying sequence, thus encoding one compound. Primers were designed to complement the identifying region and synthesize a new template in one direction. The PCR was performed with different mismatches to ensure that the PCR was specific for each template:primer combination.

When the pool was incubated with SA, the monodentate and bidentate ligands containing biotin (Figs. 4C, D) were found as binding components of the library by PCR, whereas the duplexes without biotin-conjugated oligonucleotides showed no affinity for SA (data not shown). Heating the various SA/duplex complexes above the T_m of the duplexes should lead to the removal of those strands engaged in duplex formation but without making any strong contact with SA. In other words, a heteroduplex that contains a biotin-conjugated oligonucleotide will denature, leading to the removal of non-binding oligonucleotides (e.g., Fig. 4E). The single-stranded biotin oligonucleotide should remain bound to SA until the protein is denatured. Heating the SA/duplex library at 65 °C for 5 min resulted in a flow-through (F65) in which no biotin-linked oligonucleotides were observed, while all four non-binding oligonucleotides were detected (Fig. 5, F65). The absence

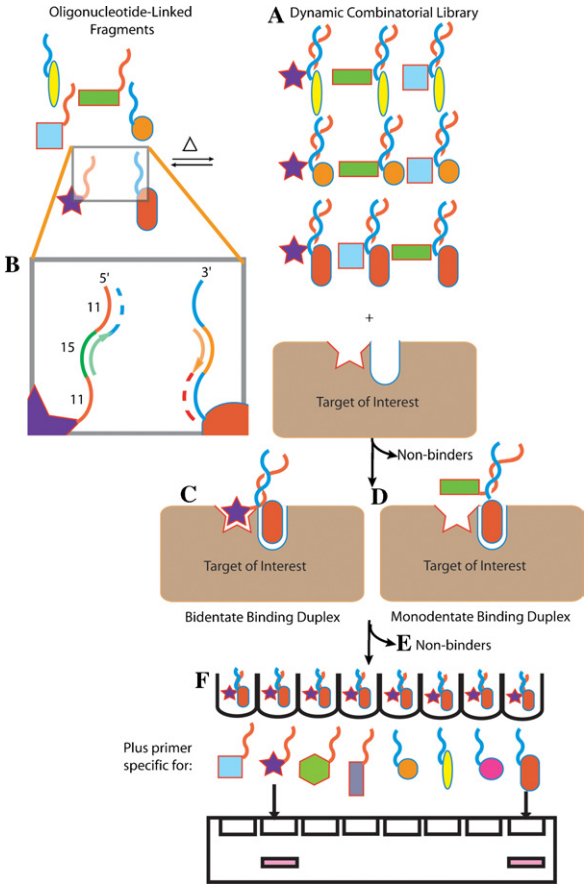

Figure 4. Strategy for protein recognition by oligonucleotide-linked organic fragments.

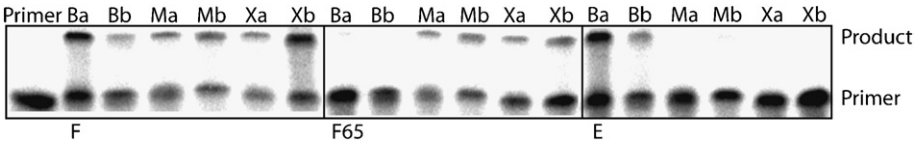

Figure 5. Biotin pool enrichment. After subjecting the duplex pool (2 μM) to SA immobilized beads and collecting the excess oligonucleotides in the F fraction, the pool was heated to 65 °C to remove non-binding fragments from monodentate biotin binding duplexes. The binding components were eluted with 6 M guanidinium hydrochloride at 95 °C for 15 min. PCR was then performed on the flow-through (F), flow-through at 65 °C (F65), and elution (E) with ^{33}P-labeled primers specific for each fragment.

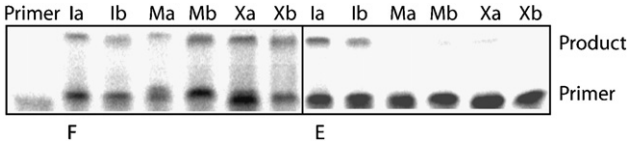

Figure 6. The iminobiotin pool (4 μM) was subjected to SA-linked beads overnight and the F fraction was collected, followed by washing and eluting the binding fragments. The binding components were eluted with 6 M guanidinium hydrochloride at 95 °C for 15 min. PCR on the flow-through (F) and elution (E) was performed with ^{33}P-labeled primers specific for each fragment.

of B_a or B_b in the F65 indicates that the B_aB_b duplex binds in a bidentate fashion. Furthermore, the B_a and B_b from the monodentate duplexes can either form a SA-templated bidentate B_aB_b duplex or remain bound to SA as single-stranded monodentate oligonucleotides.

On account of an extremely slow off-rate of the biotin–SA interaction, testing the competition between monodentate and bidentate biotin-containing duplexes was not realistic. Hence, a further demonstration of the approach was carried out by replacing biotin in the pool of fragment-linked oligonucleotides with 2-iminobiotin (I_a, I_b), a reversible binder of SA.[9] By incubating this new duplex library with SA where the concentration of each duplex was sufficient to saturate SA, only bidentate I_aI_b was detected as the binding species (Fig. 6). The absence of other oligonucleotides in the E fraction suggests that the iminobiotin monodentate duplexes are excluded from the protein, through competition, with a stronger bidentate binding of the I_aI_b duplex.

These results show that the self-assembly of complementary oligonucleotide scaffolds can be used to display two organic fragments at one end of the duplex. The use of an identifying region in each strand allows identification of strong binding fragments through PCR. Dynamic exchange of the library members, as well as removal of non-binding fragments, can be achieved by heating above the T_m of the duplex. This design holds promise for the generation of larger and more diverse libraries to target a range of protein surfaces. Our anticipation is that once optimal bidentate binding duplexes have been identified, highly potent ligands can be synthesized by covalently linking the organic fragments.

Acknowledgments

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Supplementary data

Supporting information available: Oligonucleotide sequences, conjugation procedure, SA assay, PCR conditions and detection, and thermal denaturation experimental conditions. Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bmcl.2005.05.094.

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