Supramolecular Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713649759

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To cite this Article Tagore, Debarati M. , Sprinz, K. Ingrid and Hamilton, Andrew D.(2007) 'Duplex DNA as a Self-Assembling Template for the Identification of Bidentate Protein-Binding Agents', Supramolecular Chemistry, 19: 1, 129 — 136
To link to this Article: DOI: 10.1080/10610270600990253
URL: http://dx.doi.org/10.1080/10610270600990253

Supramolecular Chemistry, January–March 2007 Vol. 19 (1–2), pp. 129–136

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Duplex DNA as a Self-Assembling Template for the Identification of Bidentate Protein-Binding Agents

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(Received 14 July 2006; Accepted 29 August 2006)

This paper reviews the versatile self-assembly of bidentate protein-binding agents on a duplex DNA scaffold, as demonstrated using the biotin-streptavidin system. The preferential binding and stabilization of bidentate biotin-containing duplexes is reflected in a 12°C increase in the melting temperature of the duplex in the presence of streptavidin.

Keywords: Duplex DNA; bidentate; self-assembly

INTRODUCTION

In the post-genomic era, it is becoming increasingly evident that there are a vast number of uncharacterized proteins. The identification and characterization of proteins is an arduous task given their functional and structural diversity. It is even harder to characterize proteins that have unknown structure and function and hence most proteomic research is biased towards proteins that have well-defined shapes and activities. In order to effectively assign protein function, different chemical and genetic approaches, like genomic enzymology, proteomics and metabolomics, have to be successfully integrated [1]. In the proteomics approach to assigning protein function, the study of protein recognition by small molecules has typically targeted the active site of a protein for a number of reasons [2–4]. The enzyme's natural substrate can be used as a template for the design of small molecules that inhibit or antagonize the activity of the enzyme. The active site provides clues to small molecule inhibitor design because important functionalities involved in the binding of the substrate are localized. It is also relatively easy to screen libraries if the inhibition involves a loss or gain in the activity of the target.

More recently, there has also been a focus on protein surface inhibitors, which can be targeted towards protein–protein interactions [5]. The main challenge in targeting protein–protein interactions is that there are usually large surface areas involved and the key residues are often unclear or discontinuous and spread across a large domain. Traditional methods of identifying protein surface inhibitors have included the use of antibodies and peptides that mimic the protein-binding partner [6–10]. These methods suffer from poor bioavailability and cellular uptake of the inhibitors. An alternate approach is to design small molecules that mimic of one of the binding partners in a protein–protein interaction [11,12]. Such proteomimetics can potentially compete with the natural partner for binding to the surface and can be used to probe biologically interesting systems [13,14].

ANTIBODY-LIKE RECOGNITION SYSTEMS

Potent inhibitors of protein–protein interactions can be generated either by rational methods whereby the structural information of one of the interacting protein partners is integrated in the design of a mimic [15–18], or through combinatorial approaches [19–21]. A particular, recent combinatorial approach that has been successful, involves fragment-based assemblies, in which designed agents achieve high-affinity by utilizing multiple key interaction points on the protein surface. The underlying concept within this approach involves the identification of

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ISSN 1061-0278 print/ISSN 1029-0478 online © 2007 Taylor & Francis
DOI: 10.1080/10610270600990253

small molecules with low affinity for different regions on the protein surface that are then linked together to generate high-affinity binders [22]. These fragment-based approaches have involved the establishment of structure activity relationships by nuclear magnetic resonance spectroscopy (SAR by NMR) [23], and by mass spectrometry (SAR by MS) [24], covalent tethering [25], privileged scaffold screening by NMR [26] and template-assisted dynamic combinatorial assembly of binding fragments in the presence of the target [27,28].

In the design and synthesis of synthetic molecules that might recognize large regions of a protein surface, Nature provides an elegant example and guidance in the form of antibodies. The key structural component of antibodies is the interaction between the light chain and heavy chain proteins, each of which contains a constant binding domain and a variable binding domain [29]. The convergent association of light and heavy chains through their constant domains allows combinations of their hypervariable domains to provide a wide variety of recognition surfaces, from which specific binding agents are selected. Artificial analogs of this approach, in which the variable domains are brought together through the association of a constant domain, has been demonstrated using a variety of synthetic strategies [30,31].

We have previously replicated the antibody strategy by using metal templation to bring a library of binding fragments in proximity (Fig. 1) [32]. Strong metal binding terpyridine ligands were chosen as the constant domains, and these were further functionalized with variable binding regions that associate in the presence of the metal ion to generate a synthetic receptor. The receptor can then be screened to identify the best binding substrate. A library of fifteen receptors was generated using terpyridine derivatives linked to various thiourea, crown ethers and hydroxyl groups as the variable domains. Ruthenium ion was used to form different octahedral complexes projecting the two variable domains in a fixed orientation (Fig. 2). The library was then screened for binding to bis(tetrabutylammonium) pimelate, a dicarboxylate substrate. Microcalorimetry was used to estimate the affinities of the

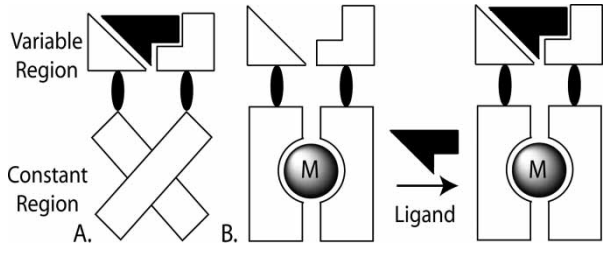

FIGURE 1 Mimicking the association of antibodies to generate recognition motifs. (A) Antibody displaying constant and variable region; (B) Metal-templation to direct ligand assembly.

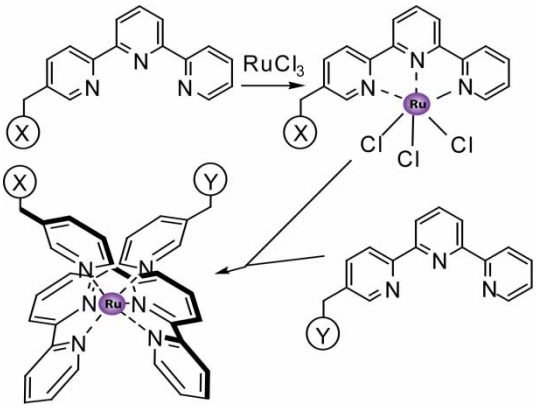

FIGURE 2 Formation of metal-templated libraries projecting a variable substrate-binding domain (X and Y) on a constant metal-binding domain.

receptor to the substrate, with the best receptor showing a $K_a > 10^4\,M^{-1}$.

Combinatorial libraries on an assembled metal template can undergo dynamic exchange and amplification dictated by the exchange rates of the metal/ligand complex [33]. However, there are limitations of this approach in water where the metal/ligand interaction might be weak and incompatible with an aqueous environment. One alternative and potentially attractive design involves the use of complementary H-bonding pairs to mimic the constant region of antibodies. H-bonding driven self-assembly has been used extensively to construct and direct the formation of supramolecular motifs, where the stability of the H-bond can be modulated through judicious choice of the donor/acceptor pair and the kinetics of the association (Fig. 3) [34–36].

DNA AS A SUPRAMOLECULAR SCAFFOLD

Nature has demonstrated the power of non-covalent H-bonded self-assembly in the association of single-stranded oligonucleotides into duplex, triplex and quadruplex structures. Sequence-specific recognition of complementary deoxyribonucleic acid (DNA) sequences is of supreme importance in the storage and transfer of genetic information. The self-assembly is mediated by H-bonding between the two bases, stacking interactions and van der Waals forces. This genetic code has been exploited in the

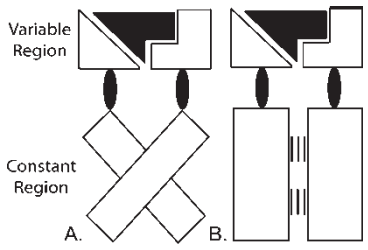

FIGURE 3 H-bonded template association of two recognition motifs.

development of supramolecular assemblies that utilize the exquisite selectivity in the recognition of the H-bonding partners [37–39]. These supramolecular structures are generated with high fidelity and provide spatial control for the generation of unique architectures in a predetermined fashion [40–43]. Indeed, oligonucleotide-assembly has inspired the development of highly sensitive probes for immunoassays as well as the design of biochip and sensors with widespread applications in material science and nanobiotechnology [44–48]. The cooperativity between multiple individual H-bonding interactions has also been exploited in the design and synthesis of alternate H-bonding motifs that mimic the recognition properties of a DNA duplex [49–52].

Liu *et al.* have elegantly utilized the base complementarity of two strands of DNA to direct the multistep synthesis of small molecules through sequence-controlled provision of different reactants. This DNA-templated synthesis occurs with high precision since complementary and reactant functionalized strands generate a duplex which in turn leads to an increase in the local concentration of the reacting groups. Liu has demonstrated the versatility of DNA-programmed synthesis in the generation of polymers, complex natural products, macrocyles, and in the identification of new transition metal catalysts for C—C bond forming reactions [53–58].

FUNCTIONALIZED DUPLEX DNA AS A SOURCE OF PROTEIN-BINDING AGENTS

As part of our work on protein surface recognition, we have sought to extend the use of self-complementarity within DNA for the detection of bivalent protein-binding agents [59]. Functionalization of single-stranded oligonucleotides with organic fragments followed by annealing the single strands to form duplexes will lead to the generation of a H-bonded scaffold projecting two functionalities on one end of the duplex. Our H-bonding strategy is based on a library of binding fragments linked to either the 3′-or 5′- end of an oligonucleotide strand that contains complementary constant sequences and a variable coding sequence that identifies the fragment (Fig. 4a). The identifying (coding) region is unique to each oligonucleotide as it encodes the organic fragment that is attached at the end of the oligonucleotide (Fig. 4b). Due to the natural ability of DNA to anneal, mixing fragment-linked oligonucleotides from the 3′-set with the 5′- set will lead to 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. 4c). Since the identifying sequence is unique to each compound, the duplex formed is a bulged duplex (Fig. 4d). The library can then be screened to identify which fragment combinations bind to the surface or active site of an immobilized protein target. Incubation of the library members with the protein of interest can give rise to a monodentate and a bidentate binding combination (4e, 4f). Following removal of weak/non binders, the binding components can then be detected by a non-traditional polymerase chain reaction (PCR) using a single primer specific for the identifying region of the template (Fig. 4g).

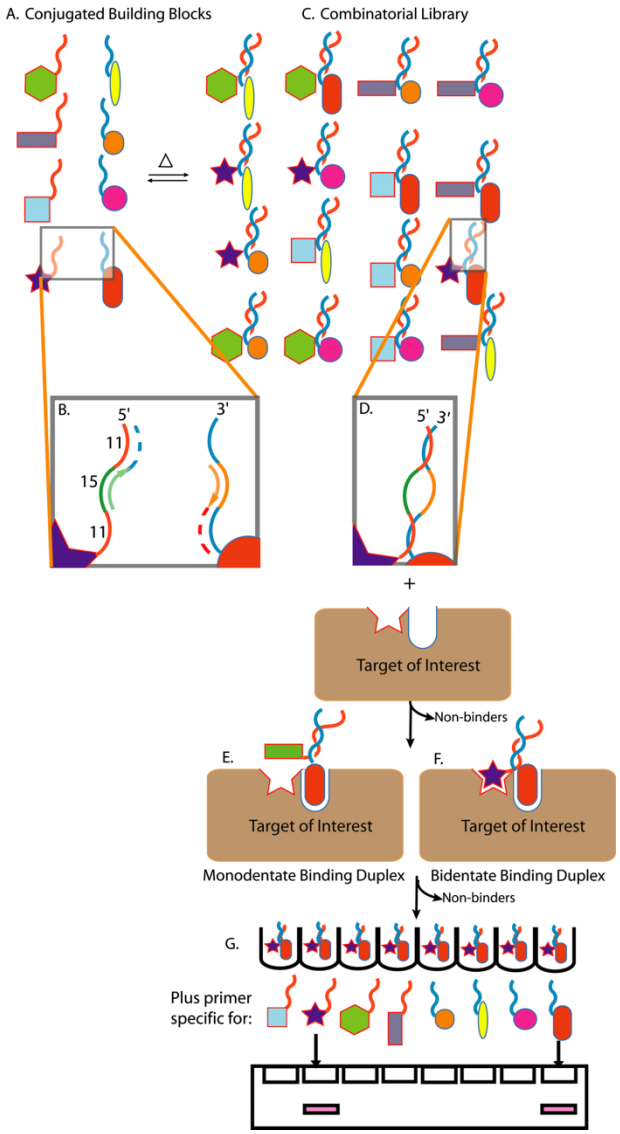

FIGURE 4 Schematic of the design of a library of organic-fragment conjugated oligonucleotides for protein recognition and subsequent detection.

The library can be induced to exchange by simply heating above the melting temperature, T_m, of the duplex formed by the complementary constant regions. Hence the DNA acts not only as a non-covalent scaffold which increases the affinity for the target through multivalency, but also has the added advantage of being capable of effecting the dynamic interconversion of library members.

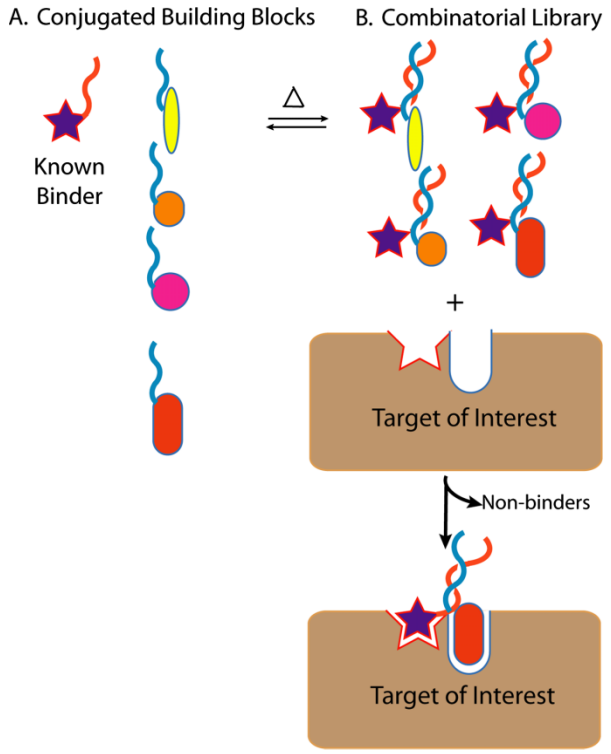

FIGURE 5 Self-assembling library of organic fragments on oligonucleotide scaffold to probe for proximal binding sites using a known binder (ref [49–52]).

A variant of this approach has been demonstrated by Neri *et al.* in the generation of a bidentate library of organic fragments on an oligonucleotide scaffold (Fig. 5) [60,61]. Using a biased library of oligonucleotides, with one strand functionalized with a known binder of the target protein and varying the compound on the other strand, a duplex library is generated to detect fragments that bind in proximity to the lead compound.

The binding components are detected using a combination of affinity capture and detection on a microarray with a fluorescent oligonucleotide. Using linkers of varying lengths to ligate the two fragments yielded bidentate ligands for human serum albumin and carbonic anhydrase with affinities in the low micromolar and low nanomolar range, respectively. Since both binding fragments in our approach are encoded by identifying sequences, we can potentially target any protein of interest without the requirement for structural information.

Streptavidin (SA), a tetrameric protein, was chosen to test the validity of our self-assembly approach since the interaction of SA and biotin is one of the strongest non-covalent interactions known in Nature ($K_d = 10^{-15}$ M) [62–67]. SA binds to four molecules of biotin in surface accessible clefts. The strong affinity of biotin for SA arises from an extensive number of H-bonding and van der Waals interactions as well as the structural reorganization of the binding loop in response to complexation. The distance between two adjacent biotin-binding sites on SA is ~20 Å, which corresponds to the distance between the 3′-end and the 5′-end of a DNA duplex. Incorporation of biotin at the ends of two complementary oligonucleotides results in a duplex displaying bidentate biotin, which can potentially bind to adjacent biotin-binding sites on SA (Fig. 6).

The sequence design of the oligonucleotides ensures no self-complementarity or the potential for secondary structure formation between the different members of the library [68]. Since the detection of the library members is through PCR, the sequences were designed so that the resulting PCR product would be distinguishable in size from both the template and primer. Product formation was optimized with one single primer complementary to

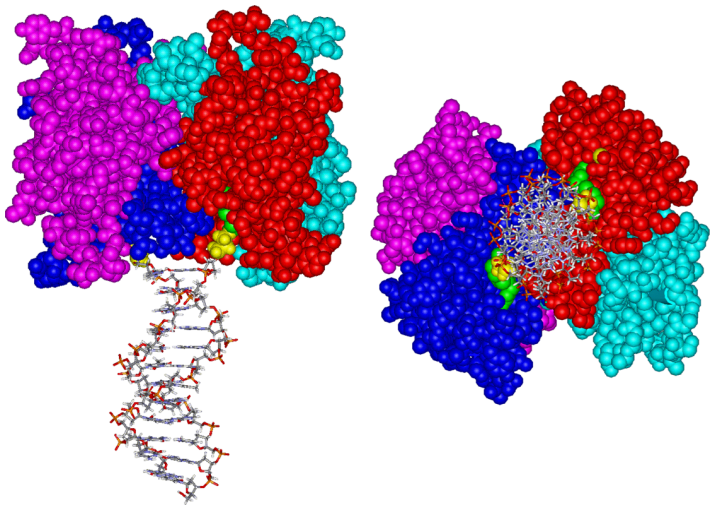

FIGURE 6 Schematic representation of bidentate biotin duplex binding to adjacent epitopes on SA (Left: side view, Right: top view). The biotin molecules are shown in green, the linker in yellow, the protein subunits are shown as ribbons and the DNA duplex depicted as sticks.

the identifying region of the oligonucleotide. This is unlike traditional PCR where there is a forward and reverse primer for the amplification of the template. In our library design, a reverse primer would anneal to the conserved sequence and result in product formation from all the oligonucleotides in the fraction collected.

The organic fragments were coupled under standard amide bond forming conditions between the N-hydroxysuccinimide activated carboxylic acid on the organic fragments and amine modified oligonucleotides. The conjugation was verified by Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry followed by reverse phase HPLC purification. A small library of six organic fragments was generated with two sets of complementary oligonucleotides (a, b); one set functionalized at the 5′-end (Qa) and the other at the 3′- end (Qb), where Q denotes the oligonucleotide. The members of each complementary set were conjugated to the same organic fragments, namely, biotin (Ba and Bb), methyl adipate (Ma and Mb) and a primary amine terminated oligonucleotide (Xa and Xb) (Fig. 7).

All the conjugated oligonucleotides were tested individually for their binding to SA, their ability to form detectable PCR product, the specificity of each primer for the designed template and formation of bulged duplexes with each member of the complementary set of oligonucleotides (data not shown). PCR analysis showed that only the biotin-conjugated oligonucleotides from each set bound to SA with methyl adipate and the amine-modified oligonucleotides not displaying any detectable affinity for SA (data not shown).

Under annealing conditions, the library members should form nine possible duplex combinations. Incubation of the duplex library with SA, immobilized on magnetic beads, should result in the detection of all duplexes containing biotin molecules on their end. Following incubation, the flow -through (F) fraction was collected and the beads washed to remove weak/non-specific binding species. The binding fractions were then eluted (E) under denaturing conditions. Of the nine possible combinations of duplexes, there is only one bidentate biotin-containing duplex. However, there are monodentate biotin duplexes (BaXb, BaMb, BbXa, BbMa) that are also detected due to the high affinity of SA for biotin (Fig. 8).

The melting temperature of the duplexes in the library is ~59 °C as evidenced from thermal denaturation studies. Hence, heating the duplexes above 59 °C and in the presence of SA should disengage the non-binding counterpart of the monodentate biotin duplex. This would ensure the detection of only 'true' binding members of the

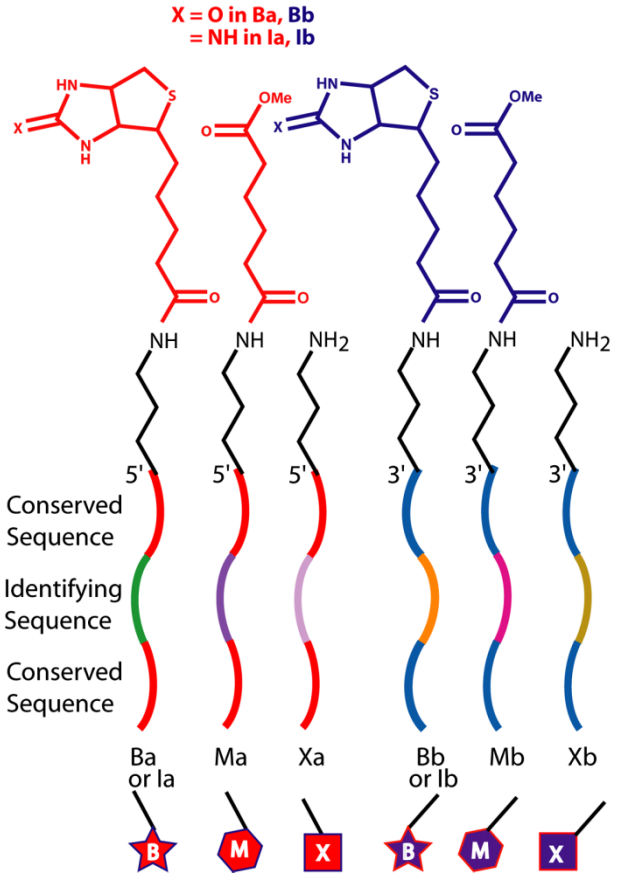

FIGURE 7 The design of the streptavidin library. Sequences shown in red and blue are complementary, with the identifying sequences shown in different colors. One oligonucleotide from each set will have a biotin conjugated at the 5′-end (red star, Ba) and 3′-end (blue star, Bb). Similarly methyl adipate and amine modified oligonucleotide from the 5′-set are denoted by the red hexagon (Ma) and red box (Xa) respectively whereas those from the 3′-modified set are denoted by the blue hexagon (Ma) and blue square (Xa). The biotin conjugated oligonucleotides have also been replaced by 2-iminobiotin conjugated oligonucleotides to generate another library in later experiments.

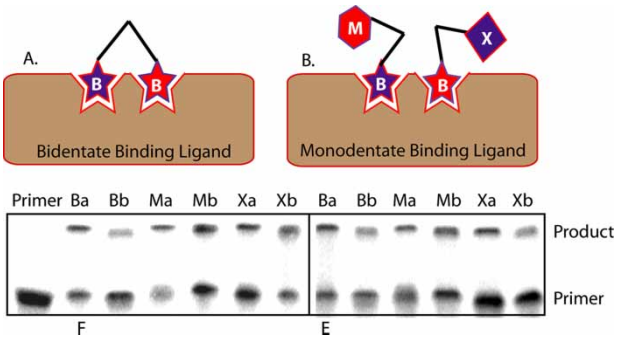

FIGURE 8 (A, B) The two possible binding scenarios of biotin-containing duplexes with SA. (Bottom) 15% TBE-urea denaturing gel showing the PCR products obtained after incubation of library duplexes (2 μM) with SA. The binding members were eluted with 6 M guanidinium hydrochloride at 95°C for 15 min. PCR was performed on each fraction using ^{33}P-labeled primers specific for each identifying sequence. Both monodentate and bidentate ligands containing biotin are amplified by PCR. The presence of oligonucleotides in the F fraction is due to the saturating concentration of library members used for the assay.

library and the removal of strands that form heteroduplexes with biotin-containing oligonucleotides but do not interact with SA. The association of single-stranded biotin oligonucleotide with SA should remain unaffected due to the high-affinity of SA for biotin. The library members were heated at 65 °C in the presence of SA, followed by collection of the flow-through fraction at 65 °C (F65) and elution of the bound fragments.

The absence of biotin-containing oligonucleotides in the F65 fraction shows that the biotin does remain bound to SA and the BaBb duplex binds to SA in a bidentate manner (Fig. 9). The monodentate biotin-containing single-stranded oligonucleotides can either remain as single strands (if a Ba oligonucleotide is adjacent to a Ba oligonucleotide, duplex formation is not possible) or form a SA-templated bidentate duplex (if a Ba oligonucleotide is adjacent to a Bb oligonucleotide).

Further evidence for preferential bidentate binding was provided by replacing biotin in the library with 2-iminobiotin (Ia, Ib), a reversible binder of SA with a $K_d \sim 10^{-11}$ M [62]. The affinity of iminobiotin for SA is strongly pH dependent, with a decrease in affinity at lower pH. The library was generated by replacing biotin oligonucleotides with 2-iminobiotin conjugated oligonucleotides and keeping the other fragments the same. Incubation of the new iminobiotin library with SA followed by PCR results in the detection of bidentate Ia-Ib duplex as the only binding component of the library, when the concentration of each of the duplexes is enough to saturate the protein (Fig. 10).

The DNA duplex acts as a non-covalent linker of two biotin or iminobiotin fragments, thereby increasing their affinity for SA through multivalency. The multivalency is governed by self-assembly, and is

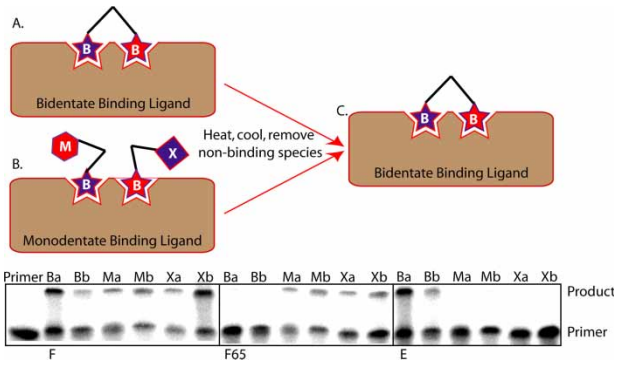

FIGURE 9 (A, B) Two different binding modes of biotin-containing duplexes. (C) Enrichment of 'true' binders through dynamic exchange of the library members to eliminate the non-binders. (Bottom) 15% TBE-urea denaturing gel showing the PCR products obtained after incubation of library duplexes (2 μM) with SA, followed by removal of non-binding fragments by heating the library at 65 °C and collecting the F65 fraction. The binding members were eluted with 6 M guanidinium hydrochloride at 95 °C for 15 min. PCR was performed on each fraction using ^{33}P-labeled primers specific for each identifying sequence.

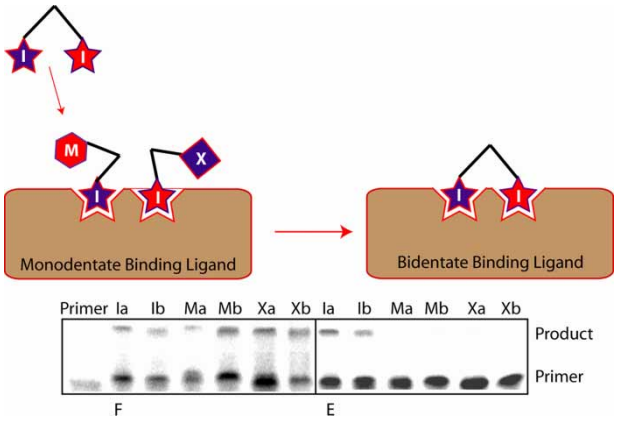

FIGURE 10 (Top) Exclusion of monodentate iminobiotin duplexes by competition with the bidentate iminobiotin duplex. (Bottom) 15% TBE-urea denaturing gel showing the PCR products obtained after incubation of library duplexes (4 μM) with SA, followed by washing and elution of bound fragments. PCR was performed on each fraction using ^{33}P-labeled primers specific for each identifying sequence.

reflected in the increased stability and hence increased affinity when compared to a monodentate ligand. This stability of the protein–ligand interaction should be reflected in an increase in the T_m of the duplex due to a pseudo-intramolecular chelate effect. Thermal denaturation studies indicated that both the monodentate and bidentate biotin-containing duplexes have a melting temperature of ~59 °C. In the presence of SA, there is a 12 °C increase in the melting temperature of the bidentate duplex whereas the corresponding monodentate duplex with SA shows no change in the melting temperature (Fig. 11). This provides clear evidence for the bidentate mode of binding of the duplex to the protein [69,70]. The nature of this non-covalent pseudo-intramolecular stabilization could be seen when the same sequences of Ba and Bb were 'covalently' joined through the addition of an 8mer $d(GA)_4$ turn sequence to obtain a hairpin loop sequence $5'$-B_a-$(GA)_4$-B_b-$3'$. The thermal denaturation of the unmodified hairpin duplex gave a T_m of ~70 °C, which is very similar to that of the ternary complex of bidentate biotin duplex and SA.

CONCLUSIONS

Using duplex DNA as a supramolecular scaffold, we have demonstrated the utility of oligonucleotides as a non-covalent 'linker' in the identification of bidentate protein-binding agents. The versatility of the DNA scaffold and the ease of manipulation make it very attractive for the identification of molecules that bind in proximity on a protein. This approach holds promise for the rapid identification of lead fragments that can then be synthetically ligated to provide a route for the design of rational analogs. The combinatorial library design can be readily

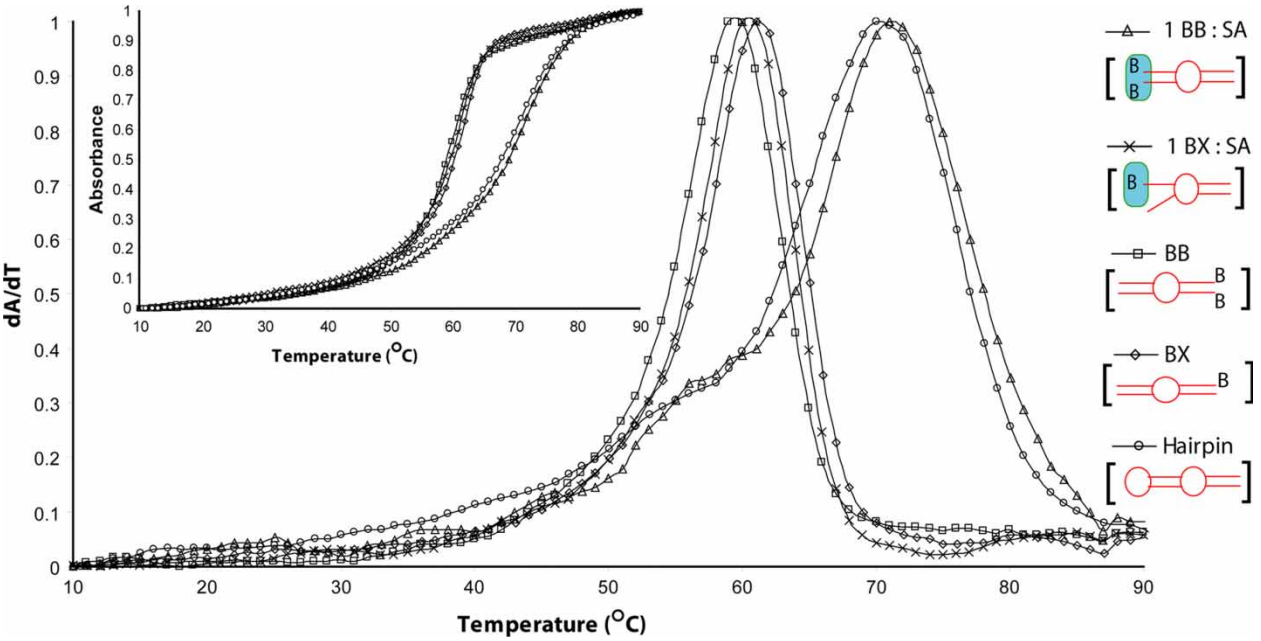

FIGURE 11 Thermal denaturation studies of monodentate and bidentate biotin duplexes (1 μM) in the presence of SA (shown in blue). The denaturation studies were performed both in the absence and presence of SA. B_aB_b (□), B_bX_a (◇), B_aB_b + SA (Δ), B_bX_a + SA (x) and hairpin (○). Derivative and sigmoidal data was smoothed (n = 5) and normalized.

expanded to include a repertoire of diverse fragments that encompass the most commonly found functionalities in drugs. It also holds tremendous potential for the development of inhibitors of protein-protein interactions.

Acknowledgements

We thank the National Science Foundation and the National Institutes of Health (GM 35208) for financial support of this work.

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