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Oligonucleotide-Based Systems for Input-Controlled and Non-Covalently Regulated Protein-Binding

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Abstract

Supramolecular chemists continuously take inspiration from complex biological systems to develop functional molecules involved in molecular recognition and self-assembly. In this regard, “smart” synthetic molecules that emulate allosteric proteins are both exciting and challenging, since many allosteric proteins can be considered as molecular switches that bind to other protein targets in a non-covalent fashion, and importantly, are capable of having their output activity controlled by prior binding to input molecules. This review discusses the foundations and passage toward the development of non-covalently operated oligonucleotide-based systems with protein-binding capacity that can be precisely regulated in an input-controlled manner.

Keywords

input-responsive; protein binding; oligonucleotide; supramolecular; structure-switching; multivalency

1. Introduction

The complex systems and functionalities found in nature have been a continual source of inspiration for supramolecular chemists.¹ A particularly interesting example is allosteric proteins, which often interact with other target proteins (13) in a cascade (either activating or inactivating them) in a response to a specific trigger molecule.² These allosteric proteins are able to undergo a structural change in response to trigger binding, which in turn activates or inactivates their ability to bind their target (Figure 1). The use of non-covalent interactions is key in both events in order to maintain the reversibility that allows for living organisms to fluidly respond to changing conditions. Indeed, these features make allosteric proteins salient players in various cellular circuitries, including those that sustain homeostasis, induce differentiation and initiate growth (14, 15). Accordingly, the development of analogous synthetic systems capable of targeted protein binding through activation by a non-covalently interacting stimulus is an important challenge in supramolecular chemistry.

The development of such synthetic systems can serve not only as models from which allosteric proteins can be better understood but also have salient applications, including serving as chemical tools to probe and control protein activity (16), as components in artificial networks that re-wire cellular processes (14), and as stimuli-responsive therapeutic agents. While there has been a great deal of work focused on the development of systems

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¹In addition to being inspired by biological systems, supramolecular chemists have also developed a range of synthetic systems that interface with biology. For further reading, please see the following references (1–12).

²Other functions include covalent modification (such as phosphorylation) of the downstream protein target.

capable of protein binding triggered by covalent transformations (including “caged” systems triggered by photo-induced cleavage of a protecting group (17–19), assembly-promoted synthesis of an active agent by group-transfer reactions (20–25) and systems activated by enzymatic group transfers (26) or cleavage)³, the development of systems capable of responding to non-covalent stimuli remains largely underexplored. Such non-covalent systems benefit from being reversible (e.g., a second stimuli could be used to de-activate the triggered protein-binder). Furthermore, the use of covalent transformations such as activation via enzymatic cleavage require specific substrates and particular reactions, in contrast, using molecular recognition as a trigger theoretically allows for significantly broadening the types of stimuli that can be used. The design and construction of molecules able to undergo specific, sequential molecular recognition events (and able to do so in a competitive aqueous media under biological conditions) is, however, a formidable challenge.

This review focuses on the development of supramolecular systems based on an oligonucleotide (ON) scaffold capable of non-covalently operated stimuli-responsive protein binding.⁴ We commence by discussing the advantages of using ON based scaffolds in terms of developing input-responsive molecules. Next, we provide some examples of foundational work on (a) key structural transitions that ONs can undergo, as well as on (b) ON derived assemblies linked to synthetic ligands for multivalent protein-binding. Last, we illustrate how combining the structure-switching and self-assembly capacities of ONs along with the molecular recognition properties of rationally designed synthetic protein-binding motifs can lead to stimuli-responsive protein-binders that are operated in a purely non-covalent manner.

2. Advantages of ONs as Input Responsive Scaffolds

One possible starting point in the development of non-covalently triggered protein binders is to modify existing allosteric proteins to be responsive to new stimuli. Since many allosteric proteins use structurally segregated, regulatory and effector domains (33) to perform stimuli-responsive function, protein engineers have successfully reprogrammed natural allosteric proteins to impart novel function. For instance, Lim and co-workers (34) have created analogues of the neuronal Wiskott-Aldrich Syndrome Protein (N-WASP) wherein the native inhibitory domains have been replaced by synthetic domains, enabling activation of the protein by non-native stimuli as shown in Figure 1. In addition to this so-called “domain recombination” method, a number of alternative strategies have been devised to modify existing proteins to introduce novel ligand-responsiveness (14, 35, 36).

While the ability to modify existing proteins is a large step forward, complementary approaches are required because it remains difficult to rationally design proteins that are both responsive to a specifically chosen input molecule and can be subsequently activated to bind to a particular downstream protein target. This limitation arises because the folded structure (and hence, function) of a protein cannot be readily known based on its sequence. Furthermore, from a practical standpoint, protein-based systems often have drawbacks including short shelf-life (*i.e.*, are denatured rapidly), tendency for aggregation, and batch-to-batch variability.

In contrast to proteins, the three-dimensional self-folded structure of oligonucleotides (ONs) can be more readily predicted in advance given their sequence. Indeed, the field of DNA

³Nucleic acid based enzymes is also an important area of research that is, however, outside the scope of this review. For recent overviews on the use of enzymatic RNA and DNA systems, please see references (27–29).

⁴A range of supramolecular approaches have been used to bind to and control proteins, from synthetic peptides (2) to metal mediated assemblies (30) and DNA-based molecular springs (31, 32). Several recent reviews highlight some of the approaches not discussed in detail here (7–10, 12).

nanotechnology has emerged as a result of this striking attribute that allows for the *de novo* design of elegant structures with specific geometries and function (27, 37–39). Further, an important feature of ON sequences is the ability to access wide variety of intermolecular assemblies as well as intramolecular self-folded structures *via* rational design strategies. The interplay between these available structures makes it possible to design a range of ON-based molecular switches that respond to the presence of a particular stimulus. We discuss some key examples of such molecular switches in section 3 (*vide infra*). For comprehensive surveys of ON derived molecular switches, the reader is directed to several timely reviews (40–43).

Another advantage of ONs is that their structural transitions have been extensively investigated by a number of analytical tools. These include UV-Visible spectroscopy, circular dichroism, NMR, X-Ray spectroscopy, and gel electrophoresis. As will be evident from the examples highlighted in this review, fluorescence spectroscopy, and FRET in particular, is especially useful. FRET pairs are highly sensitive to both the distance separating the donor and acceptor (either a fluorophore and quencher, or two fluorophores) as well as the relative orientation of the two moieties. There are a number of available fluorophores (e.g., fluorescein, Cy-3, Cy-5, TAMRA) and quenchers (Dabcyl, Iowa Black, BHQ) that can be readily synthesized onto ONs either at the termini, or embedded within the sequence (44).

Finally, a distinct advantage of nucleic acids is that they are convenient to synthesize and manipulate. A range of programmable and automated solid phase oligonucleotide synthesizers are currently available that use mild phosphoramidate chemistry to prepare natural and highly modified ONs (45, 46). Additionally, several post-synthetic modification strategies of ONs are possible (47, 48). Taken together, these properties allow for the attachment of synthetic domains (which can be, for example, small molecules or synthetic peptides) onto a given site on ON sequences (49, 50). As will be discussed in sections 4 and 5, these synthetic domains are particularly useful in serving as protein-binding moieties while the core ON domains are well suited to provide input responsiveness.

3. Input Responsiveness *via* ON-Structure Switching

A prerequisite for developing protein-binding ON-based systems that exhibit input-responsiveness is the identification of distinct and inter-convertible ON conformations. Further, the energy balance between the inter-convertible states is crucial—each must be stable under the desired conditions, yet should also be able to readily switch given the proper trigger. The use of ONs capable of specific intramolecular folds can assist with modulating such transitions due to the increased stability of the folded structure over an unfolded strand. A range of structure switching ON motifs (examples of which are shown in Figure 2), many of which incorporate intramolecular folding, have been identified and implemented into input-driven cycles. These systems are commonly based on transitions between a DNA duplex (double helical) state and a second distinct conformational state such as DNA hairpins (51, 52), cytosine quadruplexes (i-motifs) (53–56), DNA triplexes (57–60), and guanine quadruplexes (61). In addition, structure-switching from a duplex state to a ligand-bound aptamer state has also received much attention (60, 62–69), since aptamers with high affinity binding to a particular target can be readily identified by high throughput selection and enrichment strategies (70, 71). In the following paragraphs, we provide a few examples to highlight how input driven systems can be designed based on either intramolecular quadruplexes or aptamers, as these two motifs have been particularly utilized in the development of stimuli-responsive protein binding systems (cf. Section 5).

Adding to the diversity of available scaffolds, the groups of Mergny and Tan (72, 73) simultaneously introduced intramolecular quadruplex based cycles (with analogous functionality and ON input-responsiveness to canonical duplex hairpin molecular beacons) in the early 2000s. Their systems involved a core quadruplex-forming sequence able to be cyclically opened and closed through the successive addition of two stimulus strands—the first partially complementary to the core quadruplex sequence, and the second fully complementary to the first input (Figure 3A). The original systems had a drawback- the G-rich second input strand (“G-Fuel”, blue) could form a stable intramolecular quadruplex in solution. Such secondary structure formation inhibited hybridization with the toehold region of the first input strand (“C-Fuel”, green) and significantly slowed the “closing” step of the cycle. Based on previous work by Turberfield and colleagues (74), Ong and coworkers (75) found that a short catalytic sequence could be used to inhibit the formation of the G-rich input quadruplex, while being easily displaced upon strand exchange with the extended form of the system.

In order to determine the effect of stem and loop regions on switching ability of intramolecular quadruplexes, Mergny and colleagues (76) probed stems and loops of different lengths. Using a Fluorescein/Dabcyl FRET pair to detect hybridization (Figure 3B), the researchers showed that the central loop could be expanded to contain up to 21 bases while still retaining a stable, closed quadruplex structure. Further, the quadruplex was able to efficiently open when exposed to an input sequence complementary to the central loop region. This finding is important because having relatively large loop regions expands the specific biological ON sequences that can be recognized and potentially used as inputs.

The aforementioned examples are driven by ON inputs. However, the incorporation of aptamers allows for the development of systems responsive to non-ON stimuli (27). Accordingly, aptamers have been well investigated as input-responsive systems. Two aptamers in particular have received much attention through the years, due in part to their early discovery and well understood structures and binding mechanisms- the thrombin binding aptamer (TBA) (77) and the adenosine triphosphate (ATP) binding aptamer (78). Taken together, these two sequences exemplify the versatility of ON-based aptamers since the former sequence is able to strongly bind (and inhibit) a large protein, and the latter sequence is able to specifically bind a small molecule.

An early example of incorporating an aptamer into an input-responsive system comes from Li and coworkers (63), who designed a FRET-based tripartite biosensor. Their system is composed of a “core” ON sequence derived from an aptamer (Figure 4A, blue) to which two complementary sequences are hybridized, FDNA (red) and QDNA (green), carrying the terminally conjugated fluorophore and quencher of a FRET pair, respectively. In the absence of the cognate ligand, QDNA and FDNA are both hybridized to the core sequence in a tripartite DNA duplex such that the emission from fluorescein is quenched by the proximity of the dabcyl quencher. In the presence of the target molecule, however, the portion of the core ON complementary to QDNA preferentially forms an aptamer-target complex, releasing the QDNA sequence with a corresponding increase in fluorescence. The modularity of this system was highlighted by the ability to facilitate detect either ATP or thrombin, or more recently, theophylline (79), by simply changing the aptamer sequence, and modifying the complementary QDNA sequence accordingly.

Another example of aptamer-based biosensors comes from Ho and coworkers (68), who developed hairpin-based systems responsive to ATP and thrombin. Differing from Li *et al* (63), however, these researchers chose a “signal off” sensing arrangement wherein the presence of the input causes a decrease rather than an increase in the fluorescence of the system. Specifically, Ho and colleagues designed a duplex-based system, with a “molecular

beacon” strand capable of forming an intramolecular hairpin (Figure 4B, green strand) and a “sensing” strand (blue) able to form an aptameric complex with the target molecule. In the absence of the target, the two sequences form a duplex, separating the terminally conjugated 6-carboxyfluorescein/Iowa Black FQ pair on the molecular beacon strand. When the input is introduced to the system, the sensing strand preferentially forms an aptamer-target complex, releasing the molecular beacon strand which subsequently folds into an intramolecular hairpin, bringing the FRET pair together and quenching the fluorescence signal.

4. Multivalent Protein Binding *via* ON Self Assembly

A critical component in developing stimuli-responsive protein-binders is to ensure that the different ON conformational states possess differential protein-binding abilities. As will be evident from section V, toggling between monovalent and multivalent protein-binding conformers is one potential pathway to achieve differential protein-binding states. Since, in this regard, achieving multivalency is important, here we provide examples of how ON self-assembly (e.g., into motifs such as duplexes, quadruplexes, pentaplexes, and those created by DNA origami (80)) has been utilized to project multivalent protein binders. For more extensive reviews on multivalency in protein-binding and in other biological systems, the reader is directed to the following articles (49, 81).

One of the most recognizable and well-studied DNA structures is the double helix composed of two complementary single strands. As such, it is not surprising that duplex DNA has been utilized to project bivalent binding moieties (82). An early use of self-assembled duplexes for protein-binding comes from the “encoded self-assembling chemical” (ESAC) libraries approach pioneered by the groups of Neri and Liu (83–85). Utilizing DNA strands as structural assembly units and coded “tags”, two sub-libraries of small-molecule binders were terminally conjugated to sets of complementary ONs (Figure 5), which were allowed to hybridize to form a combinatorial library of bidentate binders. By exposing this library to an immobilized protein target and washing off weak- and non-binding members, the remaining strong-binders could be analyzed using PCR amplification of their DNA “tags” to determine which pairs of inhibitors showed the strongest activity. Once pairs of protein-binding head-groups are identified by the DNA assembly method, then the binding moieties can be incorporated into short covalent scaffolds. This strategy has been successfully used to identify strong-binding bidentate ligands for a variety of proteins, including streptavidin (86), matrix metalloproteinase-3 (87), trypsin (88), human serum albumin and carbonic anhydrase-II (89), and a variety of kinases including VEGFR2 (90).

It is also possible to use the long helical axis of the DNA, in conjunction with internal modifications, to display protein-binding units with discrete distances and angles between them. Chaput and coworkers (91) used this approach via standard amine coupling reactions to couple pairs of short peptide sequences (previously identified through binding studies to the target protein) to amine modified nucleobases on each of two complementary strands. In a similar fashion to ESAC libraries, these researchers attached one binding peptide (Figure 6A, red) to the 5'-terminus of one strand and a second peptide (Figure 6A, purple) to a range of internal positions along the complementary strand. Upon hybridization the researchers could then generate a library of synthetic antibodies, or “synbodies”, with defined inter-peptide distances from 3–9 nm. Utilizing this technique, termed “combinatorial examination of ligands and linkers” (CELL), high affinity binders for Gal80 ($K_d = 5$ nM) and human transferrin ($K_d = 68$ nM) were developed. The power of using bivalency is exemplified by the fact that for both target proteins, Chaput et al were able to take weakly binding peptides, and through conjugation with a precise control over distances improve protein-binding affinity by greater than 1000-fold. More recently, these researchers have extended this method to a wider range of protein targets (92).

In addition to using DNA scaffolds to generate combinatorial libraries of bivalent assemblies, bivalent ligands can be displayed along the helical axis of duplex DNA to serve as a “molecular ruler” to interrogate structural properties of target proteins. For example, the Seitz group (93) has explored bivalent molecular rulers of varying flexibility. In particular, protein binding was investigated using pairs of phosphopeptides projected from (a) a highly flexible single-stranded system, (b) a rigid duplex scaffold, or (c) a ternary complex (wherein two short ONs are hybridized to the same longer strand) with intermediate rigidity. With these differentially rigid scaffolds, the researchers then introduced distance variation between binding groups (as shown in Figure 6B) in order to probe the distance dependence of the binding interaction to a pair of homologous pockets on the tandem SH2 domain of Syk kinase as a function of the flexibility of the linker. The single-stranded system was found to bind consistently, without any distance dependence due to the complete lack of rigidity, whereas the rigid duplex was found to have highest binding when the ligands were placed 37 Å apart, which conforms closely to the 34 Å separation between the binding sites found by crystallography. The ternary complexes displayed a wider range of available stable binding distances, due to increased rotational freedom, as well as decreased rigidity. The same group has also applied the use of bivalent display of ligands *via* nicked duplexes to the binding of the estrogen receptor (94). In a related effort, the Merck group (26) has taken advantage of the strong distance dependence of bivalent binding in a rigid duplex system (Figure 6C) to develop a method of inactivating antibodies in the presence of a duplex displaying two terminally conjugated antigens.

Using the helical axis of duplex DNA to display binding groups also opens up the potential to expand to higher valencies. For example, Kobayashi and coworkers (95) have developed a multivalent lectin-binding system through the ordered display of varying numbers of galactose domains from a central nicked duplex scaffold shown in Figure 6D. Through the use of a repeating system (in which galactose-displaying strands were assembled through the use of “half-sliding cDNA” with two halves, complementary to the “head” (Figure 6D, red) of one strand and “tail” (Figure 6D, blue) of the next strand, respectively) multivalent scaffolds with a consistent, periodic distance between the binding units were developed. These systems were shown to bind to their target, RCA120 lectin via fluorescence quenching of a FITC labelled lectin ($K_{a,app} = 5.5 \times 10^4 \text{ M}^{-1}$), and showed a significantly increased binding over monovalent systems. Further, Hill plots suggest cooperative binding.⁵ A different method for ON organized multivalent binding of RCA120 lectin has been recently shown by Seitz and coworkers (97) with similar results.

A remarkable ability of DNA is their ability to form higher-order assemblies composed of more than two ON strands. Hamilton and coworkers (98) have pioneered the use of higher-order assemblies as a means of projecting multivalent protein binders. For instance, by exploiting the ability of guanine-rich sequences to assemble into a parallel quadruplex (in the presence of templating potassium ions), a self-assembled tetravalent scaffold was formed that can bind and denature cytochrome c (Figure 7A). More recently, Hamilton and colleagues (99) have extended this strategy to bind and inhibit α -chymotrypsin using a quadruplex that projects cyclic hexapeptide binding groups (Figure 7B).

A structural isomer of guanine, isoguanine (iG), has received attention recently as ONs containing repetitive iG bases can form five-stranded pentaplexes in the presence of cesium ions (100, 101). Hamilton and coworkers (102) were able to target the pentameric human C-reactive protein (CRP) using the cesium induced self-assembly of DNA pentaplexes projecting phosphocholine (PC, a cognate ligand of CRP) headgroups (Figure 8). The

⁵While not a system working *via* multivalent protein binding, *per se*, the groups of Muruyama and Goto (96) have used a similar multi-stranded hybridization method to organize Thrombin using sequentially hybridized TBAs.

researchers showed strong CRP binding ($K_d = 141$ nM) for the pentaplex (*via* a sandwich ELISA assay that used streptavidin-linked Horseradish Peroxidase as the reporter group) relative to much weaker binding for the non-templated single stranded ON ($K_d = 50$ μ M). In more recent work, Hamilton *et al* (103) showed that a single ON sequence when potassium-templated leads to a tetradentate quadruplex structure while the same sequence when templated with cesium displays a pentadentate structure. Furthermore, the quadruplex form displays weaker affinity for CRP ($K_d = 450$ nM) relative to the pentaplex form ($K_d = 117$ nM). Taken together, this work highlights the importance of the ability to precisely control the valency in the development of multivalent scaffolds.

5. Input-Controlled Protein-Binding *via* Multivalent ON Switches

The previous two sections have highlighted how ONs can be designed to undergo stimulus induced structural transitions (section 3) and how ON derived assemblies can be linked to synthetic protein-binding ligands to achieve multivalency (section 4). In this section, we illustrate how the concepts of ON structure-switching and self-assembly (in combination with multivalency) has led to the development of non-covalently driven and input-controlled protein-binders.

A conceptually simple, yet attractive, method to envision a trigger controlled protein-binder based on nucleic acids is to use an aptamer/complementary strand pair. Here, the conformation of the aptamer (and thus its protein-binding activity) can be modulated by addition of a complementary sequence. Such systems are particularly attractive as potential drug/antidote pairs where rapid neutralization of the drug is an important property. For instance, a major risk in the use of anticoagulation agents is bleeding. To tackle this problem, Rusconi *et al* (62) have used *in vitro* selection to identify an RNA aptamer that tightly binds ($K_d = 0.6$ nM) to coagulation factor IXa (fIXa) and also inhibits its activity. Furthermore, using an activated partial thromboplastin time (APTT) assay that measures the plasma clotting time, the researchers demonstrated that the anticoagulant activity (using human plasma) of the PEG-modified anti-fIXa aptamer could be neutralized upon addition of complementary “antidote” ONs (Figure 9A). Additionally, these authors have shown that the aptamer/antidote system is also effective in animal models (64).

A related antidote-responsive system has been more recently developed by the Tan group (104), who used dual Thrombin Binding Aptamers (TBAs) connected by an internal single stranded DNA sequence. With no hybridization, the flexible single strand allows the two aptamers to bind to different sites on the Thrombin surface in a bi-dentate fashion. In the presence of a complementary strand, however, the rigid duplex formation prevents both from binding. Using this inhibitor-antidote system, Tan and coworkers were able to show successive inhibition and recovery of thrombin activity.

Simmel and coworkers (65) have designed an input-driven aptamer-based protein-binder that not only can be neutralized upon addition of an antidote strand but can also be subsequently reactivated by a second ON stimulus. Building upon earlier work on quadruplex forming systems (see section 3), these researchers have modified the TBA sequence such that it can bind and release thrombin in a cyclic fashion (Figure 9B). In their design, Simmel and colleagues attached a 12-base toehold sequence to the 5' terminus of TBA and the 3' thymidine residue was functionalized with Oregon Green 488 (to serve as the fluorophore for FRET assays). In the absence of any stimulus sequence, the intramolecular quadruplex binds to thrombin with nanomolar affinity, but on the addition of an “opening” input sequence (Q: containing a 5' TAMRA quencher) that is complementary to the toehold region (and a portion of the aptamer sequence) the quadruplex unfolds to form the more stable duplex structure, releasing thrombin. Importantly, strand Q is not fully

hybridized to the modified TBA sequence since it contains an 8-base un-paired toehold on the 3' terminus. This allows for the addition of a "removal" strand, R (that is fully complementary to Q), to displace the TBA sequence, allowing it to fold back into a quadruplex thereby once again able to sequester thrombin. Using both FRET and gel shift assays, the authors were able to demonstrate both efficient switching and repeatable thrombin binding and release. In addition to targeting thrombin, the same group (67) has extended the concept of input controlled aptamer-based ONs to inhibit and re-activate *Taq* polymerase *via* the competing addition of two ON inputs. This latter example is particularly noteworthy since it shows how stimuli regulated ONs can control the important biological function of DNA polymerization.

In an interesting manuscript that illustrates input responsive bivalent binding using a peptide sequence as the binding group, Mascarenas and coworkers (105) generated a double-stranded ON-peptide conjugate targeting the oncoprotein and transcription factor cJun. Here, the ON duplex contains two parts: (a) a DNA sequence that is typically recognized by the dimeric Fos/cJun transcription factor and (b) a 35 residue peptide derived from Fos (which can directly interact with cJun through a leucine zipper region). Taken together, the bivalent interactions (Figure 10, right side) lead to a high affinity ($K_d = 50$ nM) binder of cJun while the ssDNA-peptide conjugate alone (Figure 10, left side) showed much weaker binding. Based on the weaker binding of the ssDNA-Fos conjugate relative to the duplex system, the researchers used gel-shift assays to show that binding could be "activated" in the presence of a complementary strand by the formation of the stronger dsDNA-Fos conjugate. Additionally, the use of an overhang sequence on the complementary strand allowed de-activation through a second, longer input sequence complementary to the full length of the first.

The Seitz group (106) has also developed an input-responsive system wherein the protein-binding is achieved *via* peptide sequences. Specifically, central peptide sequences were flanked on both termini with peptide nucleic acid (PNA) sequences. PNA domains were chosen not only due to their Watson-Crick base-pairing ability but also because of their stability in biological medium and ready incorporation in solid-phase peptide synthesis. In this system (Figure 11A), DNA inputs modulate the conformational projection of a central phosphopeptide domain (Gln-pTyr-Glu-Glu-Ile), thereby controlling its binding to the SH2 domain of tyrosine kinase Src. Crystal structures of pTyr containing peptides bound to Src-SH2 had shown that the peptides bind in an extended, linear fashion. Thus, the authors hypothesized that PNA-DNA duplex formation that stabilizes the extended peptide form would lead to stronger binding to the protein. Focusing on a phosphopeptide flanked with two non-complementary 8-mer PNA sequences (Figure 11A, Center), the authors found through a fluorescence polarization assay that this base construct exhibited an IC_{50} of 3.4 μ M. When hybridized to a short DNA input that paired directly to the flanking PNA segments, but contained no central bases opposite the peptide region, the central peptide region was forced into a loop conformation, resulting in an increase in the IC_{50} to 6.9 μ M (Figure 11A, Bottom). In contrast, the use of a longer DNA input containing unpaired bases opposite the peptide region resulted in a significantly decreased IC_{50} value of 0.5 μ M (Figure 11A, Top). This reduction in IC_{50} is thought to be caused by the peptide adopting a more optimal extended conformation.

Seitz and colleagues (107) have also explored the inhibition/activation capacity of PNA-peptide conjugates using an RNA stimulus. As shown in Figure 11B, a previously inactive constrained central peptide loop (as a result of DNA induced DNA-PNA duplex formation), is switched, upon addition of an RNA input, to a more stable RNA-PNA duplex (due to the formation of additional base-pairs with the RNA input sequence) that accesses an active extended peptide conformation. The net effect is an 86% reactivation of Src kinase activity,

as measured using an enzyme-linked UV assay. These results are particularly interesting because a number of unique disease states are promoted by specific RNA sequences and so harnessing RNA inputs to trigger protein-binding has therapeutic implications.

While the abovementioned examples have highlighted ON induced modulation of protein-binding activity through the presentation of nucleic acid or peptide sequences, a complementary strategy towards stimuli induced protein-binders can be achieved by controlling the presentation and valency of synthetic small molecules. A marked advantage of this latter approach is that it can harness the large chemical space of already discovered small molecule based protein antagonists, and thus has the potential to be readily applied to numerous protein targets. In a proof-of-concept study, the Jayawickramarajah group (108) has generated a DNA-small molecule chimera (DC) that can switch between monodentate (low-affinity) and bidentate (high-affinity) protein-binding conformations using a pair of ON triggers (Figure 12). Targeting the serine protease trypsin, an 18-mer core ON domain derived from the TBA sequence was tethered on the 5' and 3' termini with benzamidine (trypsin catalytic site binder) and iodophenylthiourea (trypsin exosite binder) units, respectively. Using a combination of circular dichroism and FRET experiments, it was shown that in the presence of potassium ions the DC folds into an intramolecular quadruplex conformation whilst addition of an ON stimulus that is partially complementary to the DC leads to duplex formation. Importantly, the quadruplex form is a strong trypsin binder (a K_d of 0.8 μM was determined *via* a fluorescence anisotropy assay) as a result of accessing chelate interactions through the coordinated projection of both trypsin-binding arms. In contrast, upon addition of the first input ON (Figure 12, green strand), and formation of a DNA duplex, the two binding arms are presented on opposite sides. This conformational change significantly decreased (*ca.* 20 times) the affinity of the DC to trypsin. A further quality of this system is that the switch between strong and weak trypsin-binding modes is reversible *via* addition of a second input ON (Figure 12, blue strand) that is perfectly complementary to the first, quadruplex-opening, stimulus ON.

These researchers (109) have also developed an alternative design strategy that hinges on the structure-switching of a DNA hairpin as well as intramolecular host-guest interactions. Specifically, in the inactive hairpin state the adamantane-guest unit (which is also a protein-binder) of the DC is not accessible to the protein as a result of a host-guest interaction with an appended β -cyclodextrin (β -CD) moiety (Figure 13). However, upon addition of a trigger ON, an activated duplex is formed with an exposed adamantane head-group. Fluorescence quenching assays (using a fluorescent pyrrolo-dC nucleobase reporter group) were used to probe the ON induced transition from a stem-loop state to an activated duplex. These studies showed that while a fully complementary input ON could facilitate a hairpin to duplex conformational switch, even a single base-mismatch was substantially less effective. Choosing β -Lactoglobulin (β -lac) as a model protein (as it can promiscuously bind to a variety of hydrophobic molecules), fluorescence anisotropy studies demonstrated that the input-activated duplex state binds β -lac with a K_d of 1.3×10^{-4} M. In contrast, the inactive hairpin conformation of the DC only interacted in a non-specific manner with β -lac (K_d 3.0×10^{-4} M). The use of a hairpin-based structure-switching scheme is especially attractive since it has the advantage that the DC can be designed—due to the variable loop region—to be selectively activated by specific ON input sequences.

In an interesting concept that also uses a central hairpin domain for input responsiveness, Liu *et al* (110) have recently shown that ON-responsive protein-binders can also be developed by modulating the projection of a pair of DNA aptamers. Specifically, these researchers have harnessed a DNA-tile based (80) molecular tweezer that can bind and release thrombin in response to specific ON inputs (Figure 14) as a result of accessing bidentate or monodentate protein-binding interactions based on aptamer ligands. Their

system consisted of a pair of DNA double crossover (DX) motifs (that serve as the arms of the tweezer) connected together through a central Holliday junction (111). The tweezers are opened through the hybridization of a fuel strand with a central intramolecular hairpin motif causing an expansion and hence opening of the tweezers. In this open state, a pair of TBA sequences (incorporated at the termini of the DX motifs) are held apart, and thus can only bind to thrombin *via* monodentate binding ($K_d \sim 500$ nM). Upon the addition of an antifuel strand complementary to the fuel strand, the fuel strand is displaced from the central hairpin through a toehold exchange mechanism, causing the tweezers to close, thereby placing the two TBA sequences 4–6 nm apart. This distance matches the separation between the two aptamer binding sites on thrombin and leads to a high-affinity complex ($K_d \sim 15$ nM) facilitated through bidentate binding. It was shown, using a combination of FRET and PAGE analyses, that the opening and closing of the tweezers was efficient and repeatable.

6. Conclusions and Outlook

Since its inception, the field of supramolecular chemistry has been designing Nature-inspired synthetic systems that have increasingly complex functionality. In this regard, the generation of structure-switching molecules that emulate allosteric proteins-whose downstream protein-binding activity is precisely regulated by upstream biological inputs-is a salient challenge. Researchers have recently begun to address this challenge by focusing on ON based structure-switching molecules.

As the work described in this review illustrates, the genetic molecular recognition capacity of nucleic acids (*e.g.*, *via* base-pairing) can be readily utilized to generate protein-binders that are responsive to ON inputs. Exploiting DNA strands as triggers is important, as it allows expansion into applications where exogenous sequences could be used as orthogonal activators to probe protein activity *in vitro*, or rewire cellular signal transduction pathways *in vivo*. The same base pairing strategies can be extended to harness responsiveness to disease associated RNA biomarkers, potentially enabling the development of prodrugs responsive to non-covalent endogenous stimuli.

While the above strategies show initial promise, much work is still necessary to bring these approaches to *in vivo* systems; including (a) increasing nuclease resistance through scaffold modifications and (b) developing appropriate delivery systems. Although noteworthy challenges, these two areas are already being intensely investigated in terms of other ON derived therapeutic strategies (such as traditional antisense ONs for mRNA degradation, therapeutic ribozymes, and siRNA-based target degradation). In addition, a particular advantage of the designs illustrated in this review is that the selective activation of protein-binders can come from endogenous ON inputs and thus incorporation of such protein-binding molecules within site-selective delivery systems (*e.g.*, tissue specific vectors), could lead to dual selectivity (*i.e.*, activated only in disease cells within a particular tissue). A related strategy, in which the ONs act as the delivery vectors for activatable, site-selective delivery has been recently shown by Church *et al* (112).

Another attractive direction for future work is the development of ON-based systems responsive to non-ON stimuli. As the work in section 3 shows, small molecules and proteins can be used as inputs to drive DNA devices (*i.e.*, *via* structure switching aptameric scaffolds). A useful extension of that work will be to translate such strategies to activate protein binders in response to therapeutically useful small molecule or protein stimuli.

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Figure 1. Re-engineered N-WASP that imparts novel input-responsiveness. Intramolecular binding between a non-native PDZ domain and its peptide partner leads to a conformationally deactivated protein that is subsequently activated in the presence of excess free peptide.

Figure 2.
Schematic showing the range of inter- and intra-molecular ON motifs discussed in the following section.

Figure 3. Schematic of two input responsive quadruplex-based systems designed by the Tan (A) and Mergny (B) groups, respectively. In both cases a FRET system was used to detect the structure switching. The fluorophore (F) is represented by a yellow star, and the quencher (Q) by a purple circle.

Figure 4.

Schematic of two aptamer-based systems triggered upon input recognition. A “signal on” device (A) prepared by Li *et al*, and a “signal off” device (B) designed by Ho and colleagues resulting in increased and decreased fluorescence signals, respectively. The fluorophore (F) is represented by a yellow star, the quencher (Q) is shown as a purple circle and the input (i.e., target) as a large orange star.

Figure 5.

Strategy for bivalent protein recognition based on ESAC libraries. Hybridization of two sublibraries leads to a self-assembled large library wherein each member represents a potential bidentate binder against the target protein. After biopanning, washing and removal of unbound molecules, the remaining binders are PCR-amplified, decoded and conjugated covalently to yield potential pharmacophores.

Figure 6.

Representations of (A) a DNA-peptide bio-conjugate "Synbody" utilized for bivalent binding, developed by Chaput *et al*, as well as multivalent protein binding systems using the helical axis of DNA designed by (B) Seitz and coworkers, (C) Merckx and colleagues, and (D) Kobayashi *et al*.

Figure 7. Potassium cation induced self-assembly of parallel G-quadruplexes functionalized with protein-binding head-groups. (A) Iminodiacetate ligand used for the recognition and denaturation of cytochrome c. (B) Cyclic peptides harnessed to inhibit α -chymotrypsin. Note: the light blue squares represent guanine quartets.

Figure 8.

Sandwich ELISA assay featuring a bi-facial DNA pentaplex. Microwells are first coated with CRP antibody. Subsequently, CRP is exposed to the wells and then DNA-pentaplexes capable of binding both CRP and streptavidin are added. The captured CRP is detected by the addition of streptavidin-linked HRP which provides a colorimetric signal.

Figure 9.

Schematic designs for a set of aptamer-based controllable protein binding agents. (A) An anticoagulant capable of deactivation through use of an “antidote”, designed by Rusconi *et al* (B) A toehold-exchange driven machine capable of binding and releasing thrombin *via* addition of two ON inputs.

Figure 10. Stimuli-responsive transcription factor binding system developed by Mascarenas *et al.* (A) A schematic showing the necessity of ON hybridization to cJun binding. On the right, following hybridization exposure to cJun leads to strong binding, while on the left, the ssDNA/peptide conjugate alone shows much weaker binding. (B) shows the structure of the bound system.

Figure 11. Schematic design of peptide-PNA conjugates developed by Seitz *et al* for protein binding controlled by (A) DNA or (B) RNA inputs.

Figure 12. A stimuli-responsive DNA chimera, designed by Jayawickramarajah *et al*, that can switch between monodentate and bidentate trypsin binding conformations.

Figure 13. Stimuli-responsive DNA chimera utilizing an intramolecular host-guest system on a DNA hairpin scaffold for modulating its protein binding ability.

Figure 14.

Input-driven DNA-based molecular tweezer that controls the projection of a pair of thrombin binding aptamers for selective protein binding.

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