Controlling the activity of peptides and proteins with smart nucleic acid–protein hybrids

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Oligonucleotide–peptide conjugates have frequently been used to control the localisation of the conjugate molecule. For example, the oligonucleotide segment has allowed spatially addressed immobilization of peptides and proteins on DNA-arrays *via* hybridisation while the peptide part has most frequently been used to confer transfer of oligonucleotide cargo into live cells. The regulation of functional properties such as the affinity of these bioconjugates for protein targets has rarely been addressed. This review article describes the current developments in the application of smart oligonucleotide–peptide hybrids. The mutual recognition between nucleic acid segments is used to constrain the structure or control the distance between peptide and protein segments. Application of these new type of oligonucleotide–peptide hybrids allowed not only the regulation of binding affinity of peptide ligands but also control of enzymatic and optical activity of proteins.

Introduction

Nucleic acids and proteins evolved to serve distinct purposes. DNA is perfectly adapted to store and transfer information. The expression of a clear-cut nucleobase-coded binding pattern facilitates recognition events required in key biological processes like replication, transcription and translation. By contrast, proteins are chemically and structurally more diverse and lack a simple recognition code. Protein function is determined by the spatial arrangement of the numerous functional groups and the dynamics of conformational rearrangements. The display of functional groups is maintained by the protein framework which serves to scaffold the peptide segments by restricting the conformational freedom. However, the folding of complex protein frameworks is difficult to predict *de novo.* On the contrary, the rules that govern the formation of nucleic acid-based structures are comparatively well understood. Very recently, it has been discovered that nucleic acid-mediated recognition may be used to design constraints that limit the degrees of freedom of a peptide or protein structure. This approach is probably most readily put into practice by means of chimeric molecules or conjugates that harbor both nucleic acid and protein functions.

The option of combining nucleic acid functions with protein functions is rarely put into effect by nature. The most significant example is found in the process of translation, in which the two worlds of biopolymers are transiently merged to allow the transfer of information. However, nucleic acid–protein conjugates have frequently been constructed by man. Peptides and proteins have often been attached to oligonucleotides with the aim to

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modify the properties of the nucleic acid part. Lemaitre and colleagues[1] showed as early as 1987 that cationic peptides such as poly-L-lysine enhanced the cellular delivery rates of an antisense oligonucleotide. Less toxic peptides such as $(Arg\text{-}Ahx\text{-}Arg)_3$-Arg-βAla-Arg have been identified and there is a vast body of literature describing the conjugation of such 'delivery peptides' to *e.g.* antisense or antigene oligonucleotides.[2–6] The use of nucleic acids to control the function of peptides and proteins has less frequently been explored. The main emphasis in the field was placed on attaining control over the spatial arrangement of peptides and proteins. In these approaches the nucleic acid part has been employed to enable the immobilisation of proteins onto DNA-arrays. Several review articles describe the opportunities provided by DNA–DNA hybridisation as a capture tool.[7–10] This article is focused on the still largely unexplored concept of using nucleic acids as regulatory elements of peptide and protein function in conjugate molecules.

Oligonucleotides attached to proteins

A conceptually elegant means of gaining remote control over protein function was introduced by Choi and Zocchi *et al.* (Fig. 1A).[11] They recognized the different persistence length of single stranded DNA (ssDNA) and double stranded DNA (dsDNA) and envisioned double strand formation as a way to exert a mechanical tension on a protein and thereby regulate its activity. A cysteine residue and an oligohistidine tag were introduced by site directed mutagenesis into maltose-binding protein (MBP). These two handles allowed the attachment of a 60 nucleotide long ssDNA. For this purpose the DNA was equipped with N^{α},N^{α}-biscarboxymethyllysine (NTA-Lys) on one terminus and a thiol group at the other terminus to address the oligohistidine tag *via* nickel-based complexation and the cysteine side chain *via* disulfide formation, respectively. In the single stranded state the DNA is flexible and has no effect on the protein conformation. In contrast the addition of complementary DNA and the accompanying formation of a double strand induces a mechanical constraint on the DNA attachment points, which was expected to alter the conformation of the protein. As a result of DNA hybridization the binding affinity of MBP for maltotriose was reduced from $K_a = 5.3\ \mu M^{-1}$ to $K_a = 3.4\ \mu M^{-1}$. The 35% change of binding affinity is a rather small effect. The authors noted that the MBP–DNA conjugate tested still contained unconjugated protein.

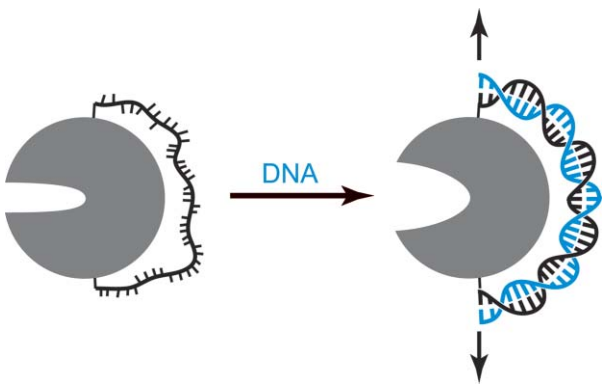

Fig. 1 Regulation of enzyme activity by a DNA molecular spring that is covalently attached to engineered proteins.

Furthermore, the nickel complex was suspected of being unstable in the constrained state.

In subsequent work the authors applied their concept to allosterically control the activity of enzymes through mechanical tension.[12,13] Two cysteines were introduced into guanylate kinase (GK). The conjugation with a DNA strand, which contained thiol reactive linkers, established the stably linked protein–DNA conjugate. In addition, this conjugation chemistry allowed the removal of unreacted protein by thiol affinity chromatography. The activity of the GK–DNA conjugate was assessed by measuring the concentration of adenosine triphosphate (ATP), which is consumed during the GK-catalyzed phosphorylation of guanosine monophosphate (GMP). It was found that the formation of a double strand reduced the enzymatic activity of GK by a factor of 4. Importantly, the hybridization induced activity decrease was reversible as DNase treatment completely restored the enzyme activity. More detailed measurements revealed that mechanical stress affected only the GMP binding site (10-fold reduction of the Michaelis–Menten constant K_M) and not the ATP binding site. Thus, the approach of using DNA-hybridization to exert mechanical stress may be used to explore the "stiffness" of binding sites, which may also allow classifications of ligand-induced changes of conformations that occur upon induced-fit binding.

The preceding two examples described decreases of protein activity upon double strand formation. Interestingly, the concept was also applied by the authors to positively control the activity of protein kinase A (PKA).[14] This enzyme exists as a tetramer comprised of two regulatory and two catalytic subunits. The affinity of the regulatory subunit for the catalytic subunit is normally regulated by cAMP, which upon binding causes a protein conformational change. The altered conformation induces dissociation of the tetrameric complex, thereby releasing and activating the catalytic subunit. Attachment of the DNA molecular spring to the regulatory subunit of PKA rendered the PKA-conjugate responsive to hybridization. Double strand formation was accompanied by a 1.5-fold increase of enzymatic activity, presumably by reducing the affinity of the regulatory for the catalytic subunits. This efficiency is comparable to the activation achieved by the natural cAMP ligand.

Oligonucleotides attached to protein segments

Protein–protein or protein–nucleic acid interactions have frequently been explored by complementation of proteins or protein complexes. One such technique is based on fluorescent protein complexes which may be formed when nonfluorescent protein segments are brought into proximity by means of the biomolecular interactions under scrutiny.[15,16] In seeking a complementation system that allows for rapid development of fluorescence signals, Demidov *et al.* explored reassembly of fluorescent proteins triggered by DNA hybridization.[17] A large and a small fragment of enhanced green fluorescent protein (EGFP) were expressed with extra cysteine residues and biotinylated. Conjugation to streptavidin and subsequent coupling with 5′- or 3′-biotinylated oligonucleotides yielded the desired oligonucleotide conjugates. Each conjugate proved virtually non-fluorescent. However, upon mixing the protein–streptavidin–oligonucleotide conjugates, strong fluorescence occurred (Fig. 2).

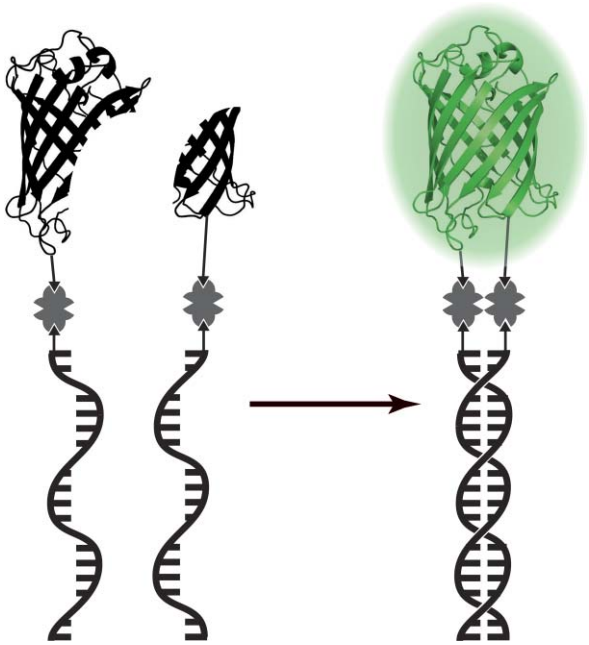

Fig. 2 Concept for fast fluorescence complementation by hybridization. Two essentially nonfluorescent EGFP fragments are conjugated to complementary oligonucleotides by biotin–streptavidin–biotin interaction and upon hybridization, the EGFP fluorescence is restored.

The fluorescence increase was fast, the half maximal increase of fluorescence occurred in less than 1 min. Thus, hybridization-triggered complementation proceeded within the time range required for restoration of fluorescence from denatured EGFP. Nevertheless, the fluorescence spectra suggest that the fluorophore in the restored tripartite complexes is located in a different environment than the fluorophore in intact EGFP. When excess unconjugated oligonucleotide was added, the fluorescence intensity was reduced by about 50%. Thus, the formation of the restored split EGFP was only partially reversible. The authors speculated that the incomplete quenching was caused by an enhanced stability of the tripartite complex (which is maintained by DNA–DNA and protein–protein interactions) and/or by a rearrangement of biotin conjugates at the streptavidin linchpin and suggested covalent conjugation chemistry as a possible solution. Obviously the fast response of the EGFP-based system may provide interesting opportunities for the detection of other pairwise interactions or promoter activities in living cells.

Takeda *et al.* employed a bivalent crosslinker bearing a *N*-hydroxysuccinimide ester and a benzyl thioester group for the covalent attachment of protein fragments to oligonucleotides.[18] These constructs allowed the re-assembly of split luciferase either by DNA–DNA interaction (both protein fragments attached to oligonucleotides) or by DNA–protein interactions (one protein fragment attached to an oligonucleotide and the other attached to a zinc finger protein).

Oligonucleotides attached to peptides

Peptides can tightly interact with proteins. Thus, nucleic acid–peptide conjugates that enable a hybridization-mediated regulation of peptide activity may be used for a remote control of protein function. Recently, Portela *et al.* demonstrated that the affinity of a DNA–peptide conjugate for the transcription factor Jun can be controlled by DNA-hybridization.[19]

The approach was based on the interaction of Jun with yet another transcription factor, Fos and double-stranded DNA. Wild type c-Fos forms a heterodimer with c-Jun by means of a leucine zipper region generating the transcription factor AP-1, which is involved in transformation and progression of cancer. Therefore the down-regulation or inhibition of AP-1 formation should allow the inhibition of cancer growth.

Guided by the X-ray structure of the AP-1/Fos-Jun complex, Portela *et al.* constructed a ssDNA–peptide conjugate comprising a 5′-thiol terminated oligonucleotide and a maleimide modified 35mer peptide from c-Fos. Subsequent gel shift binding experiments revealed that the DNA–peptide conjugate is unable to bind c-Jun in the ssDNA state but is activated for binding (K_d = 59 nM) when annealed to a complementary DNA (Fig. 3). Control experiments showed that the trapping depends on the presence of both dsDNA and the 35mer c-Fos peptide. However, a match with the cognate DNA sequence recognized by Jun was not required, which suggests that complex formation was driven by recognition of helically arranged phosphodiester groups.

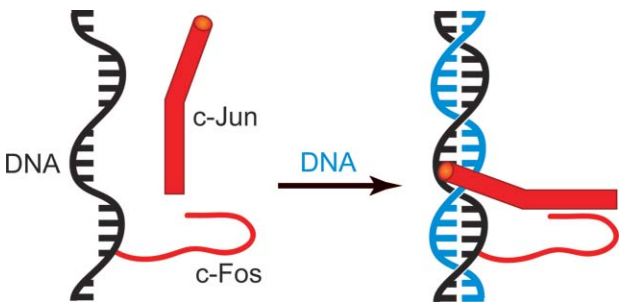

Fig. 3 Targeting the c-Jun transcription factor with oligonucleotide–peptide-conjugates. A dsDNA–peptide chimera is able to interact with both the DNA binding part and the leucine zipper region of c-Jun.

These constructs might be valuable tools for the exploration of biomolecular recognition surfaces as well as for the development of new probes for the capturing of specific transcription factor components. Furthermore, the fact that the activity of the DNA–peptide conjugates can be switched by DNA hybridization offers new perspectives for exerting control over protein function.

We have proposed a potentially generic method for controlling the function of proteins that usually do not interact with nucleic acids.[20] The approach is based on hybrid molecules in which a peptide is equipped with DNA-analogous peptide nucleic acid (PNA) arm segments.

The single stranded PNA–peptide chimeras **1** were envisioned to adopt a random coil-like conformation. However, PNA is known to self-aggregate and it is thus conceivable that ssPNA molecules have the features of a collapsed structure in which exposure of the hydrophobic nucleobases to water is minimized. In spite of this uncertainty, it was considered that binding of complementary DNA to the PNA-arms will have an effect on the structure of the peptide segment. Depending on the DNA used, various peptide structures may be accessible (Fig. 4). For example, it is conceivable that seamless base-pairing in duplex **1**·**3** enforces a loop-like peptide conformation. Alternatively, binding to only one PNA arm (**1**·$\mathbf{2}_a$) or simultaneous hybridization of both PNA arms

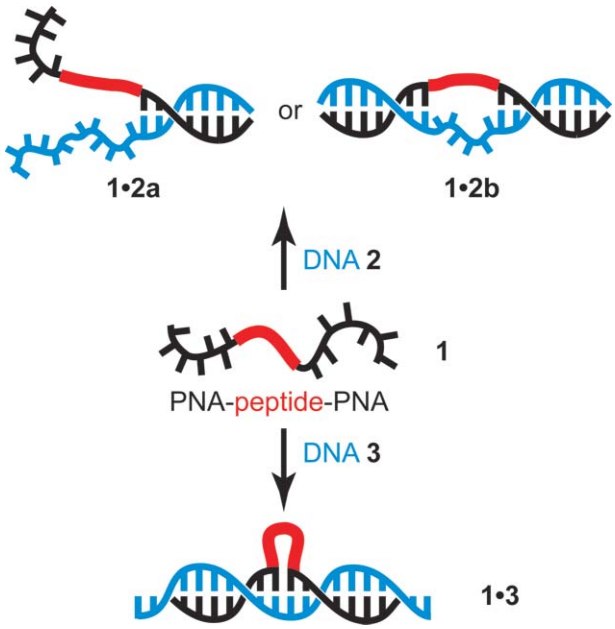

Fig. 4 Concept for the hybridization directed control of peptide conformation. The single-stranded PNA–peptide–PNA–chimera (**1**, gcgtata-Gln-pTyr-Glu-Glu-Ile-ccaatag) is hybridized with complementary oligonucleotides (**2a** = **2b**, TATTGGTATACG; **3**, TGCTATTG-GAGTCAGTATACGCGA). The peptide in the resulting duplexes $\mathbf{1}\cdot\mathbf{2}_a$, $\mathbf{1}\cdot\mathbf{2}_b$ or **1**·**3** experiences different conformational constraints. The cartoon representations illustrate possible architectures rather than defined structures.

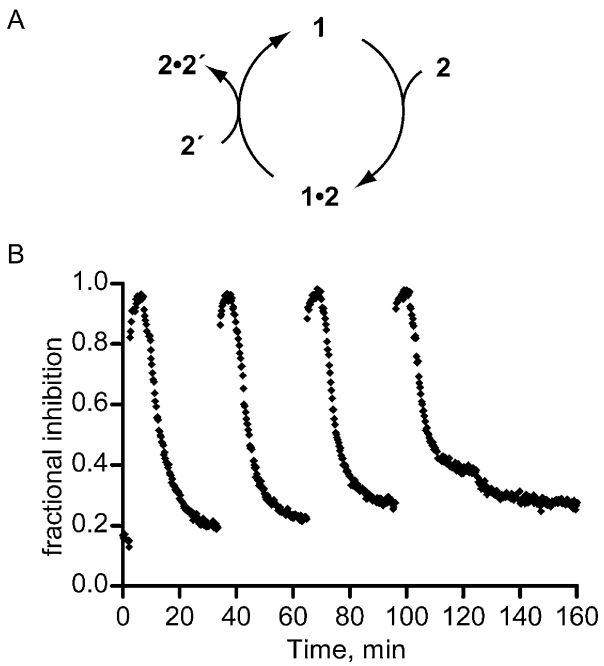

Fig. 5 Reversible switching of the affinity of PNA–peptide conjugate **1** for the Src-SH2 protein by nucleic acid hybridization. (A) Concept: alternating addition of DNA strand **2** and the fully complementary strand **2′** allows increases and decreases of the affinity for Src-SH2. (B) Result of iterative activation (addition of **2** indicated by solid arrows) and deactivation (addition of **2′** indicated by dashed arrows) measured by displacement of a fluorescence labeled reference peptide from the peptide–SH2 complex.

with DNA that contains unpaired spacer nucleotides between the two cognate sequences ($\mathbf{1}\cdot\mathbf{2}_b$) may increase the tendency to adopt extended conformations.

In a paradigm study, we analyzed the interaction of PNA–peptide chimeras with the SH2-domain of Src, a tyrosine kinase involved in the Ras signal transduction pathway. This SH2 domain binds phosphopeptides that contain the consensus motif pTyr-Glu-Glu-Ile in an extended conformation. The single stranded PNA–peptide–PNA chimera **1** inhibited the binding of a reference peptide (FAM-Gly-pTyr-Glu-Glu-Ile-Ala-NH_2, K_d = 0.24 μM) to Src-SH2 with an IC_{50} = 3.4 μM. Addition of DNA **2** that provided both complementarity to at least one PNA arm and unpaired nucleobases yielded duplexes (such as $\mathbf{1}\cdot\mathbf{2}_a$ or $\mathbf{1}\cdot\mathbf{2}_b$) with increased affinity (IC_{50} = 0.5 μM) for the Src-SH2 protein. By contrast, contiguous base-pairing of the DNA bases in **3** (compare **1**·**3**) conferred decreases of binding activity as evidenced by the increased IC_{50} = 6.9 μM. The differences in binding affinities were shown to be sufficient to switch from 0% to 96% fractional inhibition of reference peptide–SH2 binding. The reversibility of DNA-induced affinity switching allowed repeated switch processes. This was shown by applying iterative treatments of chimera **1** with activating DNA **2** (forms duplexes such as $\mathbf{1}\cdot\mathbf{2}_a$ or $\mathbf{1}\cdot\mathbf{2}_b$) and capture DNA **2′**, which was fully complementary to the activating DNA (Fig. 5). The addition of activating DNA **2** was accompanied by a sharp increase in the inhibitory activity of the resulting duplex. The inhibitory power was reduced when capture DNA **2′** was added to induce the formation of duplex **2**·**2′**. We demonstrated that hybridization can be used to regulate the enzymatic activity of Src.[21] Src kinase exists in an autoinhibited state, among others, which is maintained by intramolecular binding of the SH2 domain to an internal phosphopeptide motif. Competition against this internal binding leads to activation of kinase activity. A RNA strand was designed in analogy to activating DNA **2** and it was assumed that the resulting RNA·PNA–peptide–PNA complex would adopt structures such as $\mathbf{1}\cdot\mathbf{2}_a$ or $\mathbf{1}\cdot\mathbf{2}_b$ (Fig. 4). Indeed, the addition of such an RNA to deactivated DNA·PNA–peptide–PNA complex **1**·**3** restored the enzymatic activity of autoinhibited Src. We reckon that it may be feasible to use this approach to reassign the function of cell-endogenous RNA. For example, this RNA may activate a PNA–peptide chimera such as **1** (or deactivated complex **1**·**3**) for interference with select protein–protein interactions.

PNA–peptide hybrid molecules have been fashioned into probes that allow homogenous protein detection. So-called "hairpin peptide beacons" (HPBs) have been designed in analogy to DNA molecular beacons.[22] These probes feature a peptide segment, which is recognized by the target protein, and self-complementary PNA arm segments which preorganize the PNA–peptide chimera to adopt a stem-loop structure (Fig. 6).[23] In the closed form, a fluorophore and a fluorescence quencher, each appended to

Fig. 6 Concept of the hairpin peptide beacons. A PNA–peptide–PNA chimera featuring a fluorophore and a quencher at the termini is opened upon binding to the target protein.

different termini, are in close proximity and fluorescence is effectively quenched. It was reasoned that the hairpin structure would be opened, and thus fluorescence restored, if binding of the protein to the peptide segment occurred.

The proof-of-principle was demonstrated in two examples. In one, the presence of a Src-SH2 domain was signaled by more than 10-fold increases of pyrene emission. In the other example, the protease renin was used as a target. The renin-specific HPB comprised a near-infrared dye (NIR664) at one terminus, a dabcyl quencher at the other terminus and a statine residue within the recognized peptide segment to prevent proteolysis. This HPB delivered up to 8-fold increase of the near infrared emission upon binding to renin.

It was shown that the conformational constraint induced by the self-complementary PNA arm-segments increases the specificity for the protein target. The authors explained that the restoring force provided by intramolecular hybridization would hinder opening of the hairpin structure by non-specific, low-affinity protein targets. This interesting behavior, improvement of target specificity, has previously been observed for DNA-targeted molecular beacons.[24,25] HPB probes may provide interesting opportunities in protein detection. For example, HPBs may enable the continuous monitoring of both increases and decreases of protease expression, which is feasible because HPBs can respond in real-time without being subject to cleavage. In addition, the technique may be applied to the detection of proteins involved in protein–protein interaction networks, which often lack an enzyme-activity. Such proteins are difficult to detect with the increasingly used activity-based probes.[26,27]

Oligonucleotides attached to saccharides and small molecules

Many proteins recognize and bind more than one ligand. For example, lectins compensate the relatively modest affinity for individual glyco ligands by offering multiple binding pockets for simultaneous binding of multivalent glyco assemblies.[28–31] It has been shown that high binding affinities can only be obtained when the spatial arrangement of the carbohydrate ligands perfectly fit the display of binding pockets of the lectin. Kobayashi and co-workers introduced the use of DNA as a conformationally rigid scaffold of glyco-cluster models and showed that the affinity of lectins for carbohydrate–oligonucleotide conjugates can be regulated by hybridization.[32,33] Galactosyl residues were attached to a central thymidine nucleotide of 18-, 20- and 22-mer oligonucleotides. The sequences were designed to allow oligomerization *via* overlapping hybridization (Fig. 7).

The resulting macromolecular double helical DNA constructs displayed the galactose residues in 63 Å, 68 Å and 75 Å distances. Strong binding of *Ricinus communis* agglutinin ($K_{af} = 5.5 \times 10^{-4}$ M^{-1}) was observed for complexes which were comprised of 20-mer oligonucleotides (68 Å between galactose units). The apparent affinity constant K_{af} was reduced by 65% when the galactosyl residues were presented at 63 Å distance *via* complexes of oligonucleotide 18-mers, while 22-mer complexes showed minimal binding. The concept of self-organized glyco-clusters was recently extended to spherical DNA-assemblies[34] and applied to the multivalent display of mannose residues.[35]

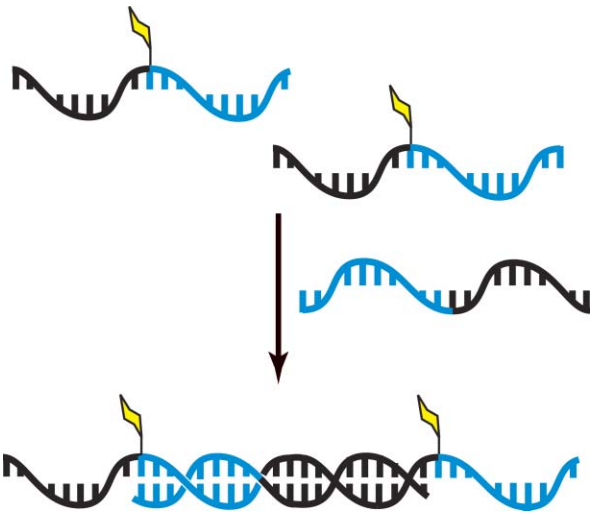

Fig. 7 Hybridization of galactose–oligonucleotide conjugates with a complementary "glue" strand (segments marked in colour are complementary) allows the multivalent display of galactose (yellow) for increased binding to lectins.

The programmed self-assembly of oligonucleotides has been used to organize the bivalent display of pharmacophores.[36,37] In this concept, a known binder with modest affinity for a protein of interest is attached to the 3′-end of one oligonucleotide and allowed to anneal with a library of 5′-modified complementary oligonucleotides. Each member of the library features a different pharmacophore and a unique sequence tag. The resulting duplexes may exhibit enhanced affinities for the target protein provided that both pharmacophores are recognized by the target. For example, in the pursuit of high affinity inhibitors of trypsin, the weak trypsin inhibitor benzamidine was conjugated to the 3′-end of an amino-modified 24-mer oligonucleotide (Fig. 8).[36]

This conjugate was allowed to anneal to a library of 620 DNA-encoded compounds. High affinity binders to trypsin were isolated by performing affinity-capture assays on immobilized trypsin. PCR amplification and microarray-based decoding of the sequence tags allowed the identification of preferred binders such as the phenylthiourea derivative **4**. The bidentate pharmacophores that conferred the highest binding affinities were connected by non-DNA tethers leading to binders such as **5** with dissociation constants in the nanomolar range. The technique may thus be used to facilitate the fragment-based drug discovery. The identification of high affinity binders of bovine serum albumin and carbonic anhydrase was demonstrated.[36] Recently, the assembly of organic biscarboxylic acid fragments on a DNA quadruplex scaffold has been demonstrated.[38] It was shown that quadruplex-scaffolded fragments can recognize cytochrome c and promote trypsin-induced proteolysis, presumably by stabilizing the unfolded state of the protein.

In the above discussed examples, hybridization was used to align protein binders by means of non-covalent interactions. Interesting opportunities for drug discovery may arise when DNA hybridization is used to trigger the synthesis of pharmacophores.[39,40] Here, DNA hybridization is used to control the effective molarity of mutually reactive functional groups and to accelerate bond forming reactions that would proceed less effectively in the absence of the DNA-template. In a library format, the DNA-encoded

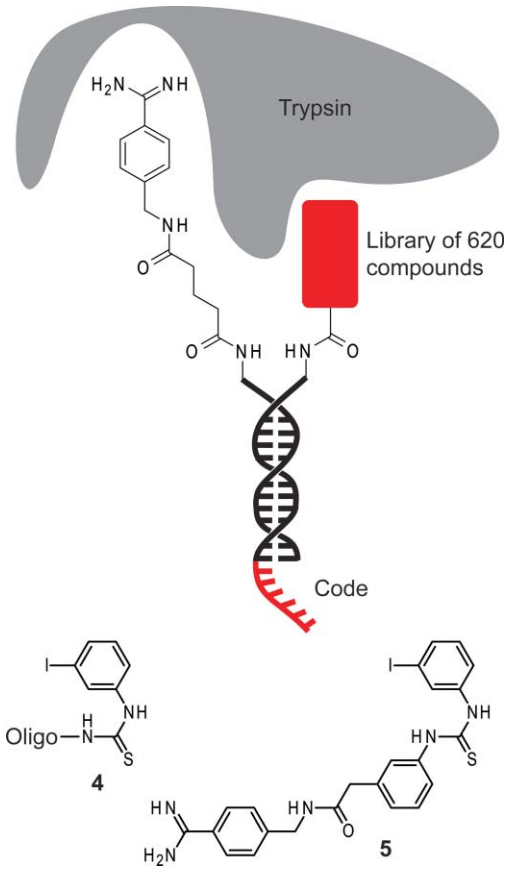

Fig. 8 Bivalent display of pharmacophores attached to nucleic acid segments.

information that instructed the formation of a specific reaction product remains attached to the putative protein binder. It is thus possible to subject the reaction products of DNA-templated synthesis to an *in vitro* evolution procedure. Molecules that bind to the protein target can be enriched by affinity selection and subsequent PCR amplification of the information that coded for the instructed synthesis of the protein binder. The pool of compounds can be passaged to another iteration of the selection. The identity of the selected protein binders can be inferred through the sequence of the amplified DNA that survived the selection procedure. The feasibility of this approach was demonstrated in the DNA-templated synthesis and selection of a binder for carbonic anhydrase.

Conclusions and outlook

Although the first synthesis of an oligonucleotide peptide conjugate has already been described in 1987, the majority of the described applications were aimed, until recently, at altering properties of the nucleic acid part. However, several laboratories pursue a new perspective, in which nucleic acids and the mutual recognition of nucleic acids are used to regulate the activity of peptide and proteins. The range of applications is diverse and encompasses regulation of ligand binding as well the control of enzymatic activity and optical properties. This has been achieved by the construction of new types of biohybrid fusion molecules comprising a nucleic acid part as well as a protein, peptide, saccharide or small molecule. By employing these constructs, nucleic acid hybridization can be used in different ways. One approach draws upon the hybridization based regulation of protein or peptide conformation. This approach allows the direct as well as the indirect (by controlling the conformation of a regulating ligand) regulation of protein activity. Another approach uses DNA–peptide conjugates in which both the nucleic acid and the peptide part target recognition sites of a transcription factor. This approach uses the different affinities of the transcription factor to the single stranded and the double stranded conjugate to achieve a hybridisation-controlled targeting of the transcription factor. Oligonucleotides attached to saccharides and small molecules have been used to target multivalent recognition sites in a hybridization controlled fashion. Furthermore, duplex formation allowed the bivalent display of different pharmacophores.

Regardless of the methods used, investigations into this area will open new avenues to novel enabling technologies in biosciences because hybridization can be employed as a regulatory element under biocompatible conditions. Significant challenges remain. For example, conjugation chemistry has to be further optimized to avoid the problems described for the regulation of enzyme activity and the fluorescence complementation. Furthermore, in order to be useful for *in vitro* studies, switching needs to extend to binding regimes in the low nanomolar range. This issue will probably be solved when nucleic acid researchers team up with protein and peptide scientists. Another challenging task is the introduction of the described oligonucleotide conjugates into living cells. However, cell delivery is an intensively studied issue in antisense and RNA interference projects and one may expect that the current efforts[41] towards achieving small molecule-, protein-, lipid- or nanotransporter-based delivery systems will help to overcome these limitations.

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