DNA-based control of protein activity

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Chapter I

DNA-nanotechnology to control protein-based interactions

Abstract

DNA serves as the blueprint for life, however the highly predictable Watson-Crick base pairing also makes DNA an attractive construction material in nanotechnology. DNA-based nanotechnology is a rapidly evolving field and has already found applications in many different research areas. This chapter provides an overview of DNA-based nanotechnology for biomedical applications. More specifically, the use of DNA templates to control protein activity and the use of DNA as a scaffold for multivalent ligands are discussed. The introduction of biological functionality to DNA often requires robust conjugation strategies. The most common conjugation reactions will be discussed for functionalizing DNA with biomolecules such as peptides and proteins. Since a major part of this thesis describes the application of DNA-based nanotechnology to control antibody activity, an overview is provided concerning the current state of antibody-based therapeutics.
1.1 Introduction

This thesis describes the use of DNA to develop novel biomolecular approaches to control protein-based interactions, with a particular emphasis on developing novel approaches to improve antibody-based therapeutics. The well-understood base pairing properties of DNA combined with the relatively cheap synthesis of DNA make it an excellent construction material for nanostructures. In the past decade, DNA-based nanotechnology has become a rapidly evolving field yielding applications in many different research areas ranging from biomedical applications to single-molecule spectroscopy. This chapter describes the advances in DNA-based nanotechnology with respect to the use of DNA as a construction material, DNA-based computing, its biomedical applications and the use of DNA as a scaffold for multivalent ligands.

1.2 DNA-based nanotechnology

In biology, deoxyribonucleic acid (DNA) serves as the blueprint for RNA and protein synthesis and is thus vital for the function of all living organisms. The double helix structure of DNA is governed by simple base-pairing rules, which is ideally suited for (long-term) information storage and allows for generally applicable mechanisms of information retrieval. The four nucleotide bases used in DNA exhibit a distinct interaction pattern, Watson-Crick base pairing, since adenine (A) pairs with thymine (T) and guanine (G) with cytosine (C). A single strand of DNA can be formed between separate nucleotides by phosphodiester bonds connecting the 5' carbon of the deoxyribose sugar to the 3' carbon of the subsequent sugar (Figure 1.1a and b). Two separate but complementary pieces of single strand DNA can form a double helix. The thermodynamic stability of this double helix is not so much provided by the Watson-Crick base pairing itself, but predominantly relies on the π-π stack interactions between the aromatic nucleobases (purines and pyrimidines) in the double stranded DNA-helix (Figure 1.1c). DNA normally forms a B-type double helix with a diameter of 20 Å (2 nm) and a helical periodicity of 10.5 base pairs per turn (~3.5 nm). A double strand DNA-helix can be considered as a rigid rod with a persistence length of 50 nm, whereas single strand DNA is flexible and can be described as a flexible polymer chain. In addition to the phosphates in the backbone, dsDNA contains two types of grooves that run along the outside of the helix. The major groove is the largest and provides most access to the exterior of the paired bases. Since not all hydrogen bond donor and acceptor groups of the nucleotides are used for the formation of Watson and Crick base pairs, DNA binding proteins or small molecules can form hydrogen bonds with these groups and can bind to specific DNA sequences.
DNA-nanotechnology to control protein based interactions

Figure 1.1: Structural properties of nucleic acids. a) The purine (Adenine and Guanine) and pyrimidine (Thymine and Cytosine) bases used in DNA. Typically, DNA sequences are written from 5' to 3' since that is the direction in which DNA replication and transcription occur. b) Watson-Crick base pairing: two hydrogen bonds are formed between adenine and thymine; three hydrogen bonds are formed between guanine and cytosine. Therefore, DNA-helices with high G-C content are thermodynamically more stable compared to high A-T content helices. c) π-π interactions between aromatic rings of neighbouring nucleobases result in a stable double helix.

In the early 80s Seeman was the first to suggest the use of DNA as an attractive building block for the self-assembly of nanoscale biomolecular structures.[5] Important for the self-assembly of DNA into larger structures was the use of DNA-branched junctions. Four single stranded DNA pieces can form a four-arm junction or Holliday-junction each containing sticky ends. DNA fragments can be linked together by hybridization of overlapping complementary ends followed by ligation to create stable arrays or devices (Figure 1.2a). Many different DNA-structures were assembled by this “bottom-up” approach where the folding of DNA could be predicted by using computer programs. However, internal errors in the assembly process were found to make the construction of large structures using exclusively short single strand oligonucleotides increasingly inefficient.

In 2006, Rothemund’s DNA origami approach boosted the field of structural DNA-nanotechnology.[6] This strategy makes use of one long single strand DNA derived from the M13-phage (scaffold strand, 7249 nucleotides) and multiple short strands (staple strands, ~ 40 nt each) of which the DNA-sequences could be designed by computer algorithms to form the pre-designed shape (Figure 1.2b). This approach allowed the formation of 2D DNA-structures resulting, for example, in 100 nm shaped “smileys” (Figure 1.2c). Although playful at that time, in the years to follow this scientific breakthrough led to extension of the DNA-origami method to form 3D-shaped DNA structures, the assembly of twisted and curved 3D objects and the construction of even larger DNA assemblies by combining individual DNA origami structures.[7] The folding of DNA structures using the origami approach can be carried out in a one-pot reaction.
Figure 1.2: Developments in structural DNA-nanotechnology. 

a) The use of four-arm junctions or Holliday-junctions for the assembly of 2D DNA-arrays. Each of the four double helical arms contains sequence-specific sticky ends: 1 is complementary to 1’ and 2 is complementary to 2’ and the subsequent self-assembly of these DNA-tiles results in a 2D DNA-array. Adapted with permission from [11].

b) Principle of DNA-origami: folding of long 7 kb ssDNA (black) into shapes by multiple (~200) short strands of 40 nt resulting in cross-overs between helices and the formation of pre-designed structures.

c) Examples of DNA-origami structures (top: schematic drawing of the structure, bottom: corresponding AFM or TEM-image), 2D DNA origami smiley face and a 3D DNA origami shaped gear-wheel. Adapted with permission from [11].

d) DNA brick structures which show analogy to LEGO bricks. 32 nt single strand contains 4 domains of 8 nt in length. Every brick or strand has a particular sequence. Each two-brick assembly forms a 90°-angle resulting in a cuboid assembly (2.5 x 2.5 x 2.7 nm³). Shapes can be “carved” by eliminating individual strands from the cube during the assembly process resulting in any pre-designed structure. Adapted with permission from [9].

However, folding of (more complex) DNA nanostructures can be time consuming since all components are mixed at high temperatures and slowly cooled down to allow the single DNA strands to obtain the thermodynamic most favorable (and pre-designed) structure. Depending on the complexity of the structure, folding times have been reported ranging from several hours to more than a week to obtain stable structures at an equilibrium state. [8] Despite the success of DNA-origami to construct arbitrary DNA-structures, drawbacks are the substantial formation of by-products due to entanglement of the large scaffold strand upon folding and the use of the genomic derived M13-phage scaffold strand, of which the sequence may not be the optimal sequence for high-yield folding of DNA-structures. [8] A more modular and robust strategy to create 3D DNA...
structures was recently developed by Yin and coworkers.\[^{9,10}\] Their approach is solely based on the use of single-stranded tiles (SST) or DNA bricks containing four 8-nt domains that allow hybridization to four different neighbor strands. Hundreds of these SSTs result in a molecular 3D canvas of which individual SSTs can be omitted to obtain pre-designed 3D DNA structures in a LEGO-like manner (Figure 1.2d). This method showed that a large number of single strands can self-assemble in desired structures without the formation of byproducts allowing the creation of multiple (100) complex DNA-structures based on a single molecular 3D canvas.

The examples above illustrate the impressive capability of DNA to form predesigned structures (Figure 1.2). More recently the field of structural DNA nanotechnology has started to move from playful science towards potential applications in areas ranging from nanoelectronics to biology and medicine. For many of these applications, site-specific functionalization of the DNA structures with proteins or peptides is crucial, in particular for biomedical applications. Several excellent reviews have recently appeared and many of these applications have been summarized.\[^{7,12}\] Below we provide a few examples to illustrate the potential of structural DNA nanotechnology in therapeutics or in mimicking natural processes.

The advantage of using DNA for biomedical applications, besides the well-understood base pairing, is the natural biocompatibility with the cellular environment. Anderson and coworkers explored the use of DNA structures as a carrier for small interfering siRNAs \textit{in vivo} to suppress the expression of targeted genes, using nude mice bearing tumor cells (Figure 1.3a). Thereto, a DNA tetrahedron was assembled containing single strand DNA pieces that protrude from the structure allowing partial complementary siRNAs to hybridize allowing the functionalization of a maximum of 6 siRNA per structure. In addition, folic acid molecules were used to decorate the DNA structure to specifically target the tumor cells that overexpress the folate receptor enabling intracellular delivery of the siRNA and thereby effective gene silencing. The use of the octahedron as a carrier showed more effective gene silencing compared to siRNA alone, which was attributed to the prolonged blood circulation time (\(t_{1/2}\) of 24 vs 6 minutes) and the targeting capability due to the presence of at least three folic acid moieties per structure.\[^{13}\] DNA nanostructures have also been used as a carrier to deliver doxorubicin, an anti-cancer drug, to cancer cells. For example, Huang and coworkers decorated the surface of a DNA icosahedron with aptamer sequences that specifically recognize MUC1 cell surface receptors, which are overexpressed in epithelial cancer cells. The MUC1-specific aptamers were found to be essential for the selectivity towards MUC1-positive cells and showed increased cytotoxicity effects compared to DNA structures that lacked the specific aptamer sequence.\[^{14}\] Inspired by a natural channel protein, Simmel and coworkers used the origami method to assemble a DNA-structure that forms nanometer transmembrane channels in lipid bilayers.\[^{15}\] Cholesterol-moieties present on one surface of the structure enabled the anchoring of the structure to the lipid bilayer whereas the hollow six-helix bundle protruding from the center of DNA structure
resulted in hollow channels across the membrane with a diameter of 2 nm (Figure 1.3b). Transmission electron microscopy (TEM) showed the docking of the DNA-structure on the exterior of vesicles whereas electrical conductivity measurements, to mimic natural ion-channels, actually proved the formation of membrane pores. The introduction of hollow channels in lipid membrane was suggested to be useful for the intracellular delivery of drugs.

Figure 1.3: DNA-based structures for biomedical applications. a) Targeted siRNA delivery in tumor bearing mice using a tetrahedral DNA structure with 10 nm edges. The siRNA sequence (maximum of 6 per structure) was chemically modified with a phosphorothioate bond to enhance serum stability and hybridized to single stranded DNA handles on the structure, resulting in more effective gene silencing than siRNA alone. Adapted with permission from[13]. b) Synthetic DNA membrane channel constructed from 54 double helical domains packed in a honeycomb lattice resulting in a 47 nm long membrane channel (red). Adhesion of the DNA structure containing cholesterol moieties (orange) pierces the lipid bilayer creating an artificial transmembrane channel. The adhesion to lipid vesicles was visualized using transmission electron microscopy. Adapted with permission from[15]. c) The programmed opening of a DNA-origami based box (42 x 36 x 36 nm$^3$) upon addition of full complementary strands (keys) via toehold-mediated strand displacement reactions. The opening of the lid could be monitored by fluorescence resonance energy transfer (FRET) measurements between Cy3 and Cy5. The decrease in FRET (red curve) proved the disruption of the partial double strand (locks) after the addition of keys. Adapted with permission from[16].

Previous examples represent static DNA structures, however DNA nanostructures can also be equipped with dynamic properties. Dynamic DNA-nanotechnology is based on the use of DNA strand displacement reactions. One of the first examples of (re-)programming active states of DNA structures using strand displacement was designed by
Kjems and coworkers.\cite{16} The lid of a DNA origami based box was closed by oligonucleotides present on the lid as well as on one side of the box (Figure 1.3c). This lock could be opened upon the addition of full complementary oligonucleotides (keys), which initiated toehold-mediated strand displacement resulting in the opening of the lid. Controlling the open and closed states of DNA-based containers allows the selective release of molecular payloads upon external triggers.

Toehold-mediated strand displacement is not only useful to trigger different conformational states in DNA nanostructures, but also provides the basis for DNA-based molecular computing. The key feature of programming straightforward strand displacement reactions is the presence of a toehold sequence. Toehold-mediated strand displacement can be explained by the hybridization of a single strand piece of DNA to a partial double-strand complement (bearing the toehold). The toehold sequence initiates hybridization resulting in branch migration and the release of an “output” strand (Figure 1.4a).\cite{17-19} Branch migration can be described as an enthalpic (hybridization) and entropic (strand release) driven random walk process and is non-directional due to the absence of enzymes like DNA-polymerases that guide the replication in biology. Initiation of toehold binding followed by strand displacement can be kinetically controlled by changing the toehold length. Zhang and Winfree related the thermodynamics of toehold hybridization to strand displacement kinetics of the displacement reaction and observed a difference over 6 orders of magnitude for the rate constant (1 M$^{-1}$ s$^{-1}$ to 6 x 10$^6$ M$^{-1}$ s$^{-1}$) of the strand displacement reaction depending on the toehold length and the content (A-T or G-C).\cite{20,21} Maximal exchange rates were observed for toeholds longer than 7 nt. The reverse reaction is in theory possible, however, the rate of this reverse reaction is 6 orders of magnitude slower than the forward reaction and the toehold-mediated strand displacement reaction is therefore under kinetic control.

The displacement of a shorter strand by a longer strand limits the DNA sequences and lengths for downstream reactions. Therefore, Zhang and Winfree developed the toehold exchange reaction to increase the complexity of DNA-circuits.\cite{20} Toehold exchange is similar to toehold-mediated displacement in that the invading strand binds to the toehold sequence followed by branch migration, but differs in that the total number of base-pairs of the hybrids before and after the displacement is the same (enthalpy) (Figure 1.4b). Toehold exchange allows the construction of much more complex reaction networks in which the reaction is entropically driven by an overall increased release of strands. The revealed toehold-sequence can be used in subsequent exchange reactions and was shown to be particular useful in catalytic circuits in which one input oligonucleotide releases a specific output oligonucleotide and can serve as a catalyst in order to produce more output strands in presence of excess fuel strand. This enzyme-free catalysis showed exponential kinetics and allowed the detection of 10 pM oligonucleotide (catalyst).\cite{22}
Toehold-mediated DNA strand displacement reactions are more commonly used in biological applications than toehold-exchange reactions. For example, Deiters and coworkers developed a DNA-based AND-gate that could respond to endogenous intracellular miRNA sequences specific for certain human diseases. Two consecutive toehold-mediated displacement reactions resulted in the release of a fluorophore from a quencher, indicating the presence of specific miRNAs (miR-21 and miR-122). Their approach can be easily extended to identify other miRNA-sequences. In addition, incorporating photolabile caging groups in DNA-strands allowed the spatial and temporal control over displacement reactions in cells by using light as a trigger. Toehold-mediated displacement reactions have also been used to characterize the cell surface of lymphocytes (Figure 1.5). Subpopulations of lymphocytes are defined by the presence or absence of cell surface markers such as CD45 and CD20. The current method to distinguish the expression (levels) of these cell surface markers is by using fluorescently labeled antibodies and flow cytometry. Rudchenko and coworkers developed an autonomous biomolecular-based computer or logic gate by using oligonucleotides conjugated to anti-CD45 and anti-CD20 antibody. The presence of either one (OR-gate) or both (AND-gate) cell surface markers initiated a strand displacement cascade, liberating or quenching different fluorophores present on the oligonucleotide strand.
1.3 Controlling protein activity using DNA as a scaffold

Besides using displacement reactions in biomolecular computing to control output functions by DNA strand release, DNA can also be used to control protein activity. In these applications proteins are often used for which the activity can be easily read-out such as enzymes and fluorescent proteins. A common approach is to split the protein into two halves and conjugate each to a specific oligonucleotide. Typically, the split-variants of these proteins do not exert high affinity for one another and can only regain their fluorescence\cite{27} or enzymatic activity\cite{28} upon complementation via DNA hybridization.

Ghosh and coworkers developed an RNA-detection system based on the zinc-finger proteins AaRT and E2C, which were fused to split-luciferase domains (Figure 1.6a). The zinc-finger fusions are able to bind to hairpin DNA sequences, which in turn can hybridize to a specific RNA sequence.\cite{29} Templating of both zinc-finger fusions is only possible in the presence of the correct RNA sequence, allowing the reconstitution of the enzyme structure and thereby regaining its activity. Recently, Deiters and coworkers extended the use of this zinc finger fused split-luciferase with toehold-mediated strand displacement reactions to construct a variety of DNA logic gates with luciferase activity as read-out.\cite{30} The careful choice of DNA strands enabled simple and modular gate design resulting in the construction of AND- and OR-gates using only one set of zinc-finger fusions of AaRT and E2C. Different gates could also be connected in series to create a DNA computation circuit with protein function as output. Furthermore a NOR-gate was created by using specific microRNA sequences (miR-122 and miR-21) as input, resulting in the disruption of the enzyme and thereby showing a potential application for the detection of cellular cancer markers (Figure 1.6b).
Figure 1.6: Controlling protein-activity by DNA-templated assembly of zinc-finger proteins AaRT and E2C fused to split-luciferase halves. a) Zinc-finger domain E2C was fused to the C-terminal fragment of Firefly luciferase (ZF1) and AaRT is fused to the N-terminal fragment (ZF2). Zinc-finger fusions bind to dsDNA hairpin which functions as a guide since the adjacent sequence can hybridize to a target RNA-sequence and thereby reconstitute the enzymatic activity of the luciferase resulting in a luminescent signal. Adapted with permission from [29]. b) A NOR-gate was constructed by preincubating AaRT and E2C split-luciferase fusions on dsDNA template containing a toehold sequence (high level of luminescence, output = 1). The presence of one or both microRNAs initiates toehold-mediated strand displacement resulting in the disruption of complemented split-luciferase and the subsequent decrease in luminescence (output = 0). Adapted with permission from [30].

A different approach to control enzyme activity is to apply mechanical stress on an enzyme by DNA hybridization. Zocchi and coworkers showed that the activity of a DNA-enzyme chimera could be controlled by using DNA as a molecular spring turning it into a mechano-sensitive enzyme. Two pieces of DNA were conjugated near the substrate binding pocket of a luciferase, resulting in a "two-arm" DNA-enzyme chimera. The stress exerted on the luciferase caused by the hybridization of matching or mismatched ssDNA resulted in the deformation of the substrate binding site and allowed the enzymatic activity to be tuned (Figure 1.7).
Switching between inactive and active states of an enzyme has also been achieved by DNA-controlled formation of enzyme-inhibitor complexes. Ghadiri and coworkers developed a semi-synthetic enzyme-inhibitor complex by fusing a ssDNA linker between an enzyme and a relatively weak small molecule inhibitor (Figure 1.8a).\textsuperscript{[33,34]} The flexibility of the ssDNA-linker provides a high local concentration of the inhibitor and thereby effectively inhibits the activity of the enzyme. The inhibition could be reversed upon hybridization of a matching DNA sequence resulting in a rigid DNA linker, thereby spatially separating the inhibitor from the enzyme resulting in restoring the enzyme activity and allowing the detection of ssDNA at pM concentrations.

An alternative strategy to switch between active states of an enzyme by intramolecular control was developed by Seitz and coworkers.\textsuperscript{[35]} The intramolecular binding of an SH2-domain to a phosphorylated tyrosine-residue on the Src-kinase leads to inactivation of the kinase. A phosphopeptide-PNA chimera was developed which could switch between active and inactive conformations depending on DNA/RNA inputs and thereby control Src-kinase activity (Figure 1.8b). The chimera in a loop-conformation shows a weak affinity to the SH2-domain. The phosphopeptide switches to a more extended conformation in the presence of a specific RNA sequence, resulting in the binding to the SH2 domain and thereby reactivating 86% of the Src-kinase activity.
Figure 1.8: Controlling intramolecular enzyme-inhibitor complexes by DNA hybridization. **a)** An oligonucleotide strand was fused between Cereus Neural Protease and a small molecule inhibitor resulting in effective intramolecular inhibition of the enzyme. Addition of a complement DNA strand results in a rigid DNA-linker, removing the inhibitor from the active site and activating the enzyme. Adapted with permission from [33]. **b)** The inactive Src-kinase conformation is stabilized by the interaction between pTyr527. The chimera-DNA complex has a weak affinity to the SH2-domain (i). Switching the phosphopeptide conformation by an RNA input to a more extended and linear conformation competes with pTyr527 for binding to SH2 resulting in an active Src-kinase (ii). Adapted with permission from [35].

Another way to control protein activity is the use of multiple protein domains that complement each other’s function. For example, Niemeyer and coworkers used DNA to control the distance between protein domains originating from the native cytochrome P450 enzyme and thereby investigated the effect of distance on enzyme cascade reactions. Native cytochrome P450 is composed of a reductase domain BMR, bearing an FAD and an FMN group, and a hydroxylase domain (BMP) containing a heme moiety (Figure 1.9a). Intact cytochrome P450 transfers electrons from NADPH via FAD and FMN (BMR) to the heme-moiety (BMP) and finally uses oxygen as a substrate for a hydroxylation reaction of a (reporter) substrate. The individual subdomains (BMP and BMR) were tethered with oligonucleotides and templated on a DNA-based stem-loop structure resulting in the maximum hydroxylation of the substrate. The hybridization of short complementary strands (21 or 27 nt) to the stem-loop structure did not result in a decrease of catalytic activity of the BMP-subdomain, whereas complementary strands of 33 nt or more resulted in a 4-fold decrease of catalytic activity. The introduction of a toehold sequence also allowed reversible control over catalytic activity upon the addition of a full complementary strand. DNA-origami based tiles have been used as a more static approach to precisely pattern multiple enzymes for investigating the effect of interenzyme distance on the efficiency of enzyme cascades. Yan and coworkers used glucose oxidase (GOx) and horseradish peroxidase (HRP) as a model system to mimic cellular enzymatic cascades (Figure 1.9b). GOx converts glucose and oxygen into gluconic acid and hydrogen peroxide. Hydrogen peroxide is subsequently used as a substrate by HRP.
to oxidize ABTS yielding a green product for read-out purposes. The increase in enzymatic activity that was observed for distances < 10 nm was explained by the formation of a hydration layer between the enzymes that promotes transfer of H₂O₂ more effectively than diffusion.

Figure 1.9: DNA-control of inter-enzyme distance to modulate enzyme-cascade reactions. a) DNA-based reassembly of two subdomains of the cytochrome P450 BM3 enzyme. DNA-scaffold containing a stem-loop structure (11 bp double strand, 23 nt loop) brings both subdomains (BMR and BMP) in close proximity resulting in effective electron transfer and an increase in catalytic activity of the BMP-subdomain. The stem-loop is destabilized upon hybridization with a complementary strand, resulting in a decrease in enzymatic activity. Adapted with permission from [36]. b) DNA-origami based tile and ODN-protein conjugates controlling the inter-enzyme distance (spacing from 10 to 65 nm) and cascade reaction between GOx and HRP. Adapted with permission from [37].

1.4 DNA as a scaffold for multivalent ligands

Multivalency is a phenomenon that occurs in many biological systems, where multiple low affinity interactions together yield highly specific and strong binding interactions. Multivalency plays a fundamental role in adhesion of virus and bacteria to cell surfaces, in the immune response, in receptor clustering and in mediating protein-protein interactions.[38] The importance of multivalency in nature has inspired the development of multivalent ligands to act as inhibitors or effectors of biological processes. Most semi-synthetic multivalent ligands developed to date employ flexible linkers, allowing them to adapt to the precise orientation of the receptor domains, maximizing the enthalpy of the interaction.[39–42] The nature of the linker used to connect individual ligands is important to obtain a high affinity binding multivalent ligand. Flexible linkers are effective when targeting multivalent targets in which the ligand binding sites are close in space (< 6 nm). However, the amount of possible conformations increases exponentially with increasing linker length and thus the enhanced affinity of a multivalent ligand will decrease drastically for longer distances due to entropic constraints.[43] The use of a more rigid linker reduces the amount of possible conformations of the ligand resulting in a lower entropic penalty, but may strain the ligand-receptor interaction due to a non-matching linker length (too short/long) reducing the enthalpy of the interaction (Figure 1.10).[43]
Figure 1.10: The linker is important for the design of a multivalent ligand. In contrast to rigid linkers, flexible linkers provide more conformational freedom, maximizing the enthalpy of binding but resulting in a higher entropic penalty. A rigid linker avoids entropic losses due to reduced conformational freedom and the possibility to pre-orient the ligands. The highest thermodynamic stability between a multivalent ligand and receptor can be achieved when the linker is rigid and exactly matches the distance of the receptor moieties. Adapted with permission from[43].

Certain features make DNA an attractive scaffold for the construction of multivalent ligands. Single strand DNA is flexible whereas dsDNA provides a rigid linker with a persistence length of 50 nm and thereby the flexibility/rigidity of the multivalent ligand can be tuned. The linker length can be precisely tuned by adjusting the number of bases (3.4 Å per base). In addition, the orientations of ligands can be controlled by the helical nature of DNA. The self-assembling nature of DNA allows rapid screening of multivalent ligands, with different structural properties, towards the target of interest compared to the more laborious synthesis of dendrimers or polymers.[44,45]

Several studies have used DNA as a linker to construct multivalent ligands for protein binding, reporting varying degrees of affinity enhancement. Chaput and coworkers used the yeast regulatory protein Gal80 as a target protein in a proof of concept illustrating the advantages of using DNA as a linker for bivalent ligands.[46] Peptides obtained from a library were shown to bind in the µM-range to non-overlapping regions on the protein. Conjugation of the peptides to dsDNA at different nucleotide positions resulted in a small library of bivalent DNA-peptide hybrids separated by distances of 1-9 nm and different orientations (Figure 1.11a). Screening procedures resulted in a so called synbody with the peptides separated by ~ 4.3 nm and oriented at the same face of the DNA resulting in a 1000-fold increase in affinity (K_d of 5.6 nM) compared to the individual monovalent peptides. In a similar approach, they compared the binding of a peptide-dsDNA based synbody with a commercial antibody towards growth factor receptor bound protein 2 (Grb2). Again, a library of synbody constructs was obtained by displaying individual µM-binding peptides at different distances and orientations. An impressive increase in affinity to a K_d of 7 nM was observed when using a dsDNA-linker of 4.1 nm, resulting in a five- to ten-fold stronger binding to Grb2 compared to commercial antibodies.[47]
Figure 1.11: DNA as a scaffold for bivalent ligand display and subsequent protein binding. a) Combinatorial screening of peptide ligands and linkers to create a synbody binding to the yeast regulatory protein Gal80. Different peptides were attached at different nucleotide positions and to allow screening of different distances between as well as orientation of the peptide ligand with respect to bivalent binding towards the target protein. Adapted with permission from [46]. b) Structural interrogation of tandem proteins (SH2) or protein dimers (estrogen receptor, ER) by using DNA as a linker connecting the cognate ligands. Peptides or small molecules were attached to nucleotides and the flexibility/rigidity of the bivalent ligand can be adjusted by the DNA-hybridization state of the bivalent ligand. A single strand (flexible) bearing two ligands becomes rigid upon hybridization to a full complementary DNA strand and introduces ligand orientation dependency besides spatial separation of the ligands. A ternary complex resulting in a semi-rigid DNA-linker provides more flexibility and reduces the orientation constraint of the ligands and allows structural adaptability towards the target protein. Adapted with permission from [45].

The disadvantage of a rigid DNA-linker is that small spatial mismatches (orientation and length) may result in diminished binding. Seitz and coworkers investigated the influence of flexibility and rigidity of DNA-based bivalent ligands on binding to a target protein. [48] A well characterized protein, tandem SH2-domain of the Syk-kinase, was used as a model to investigate the binding of bivalent ligands containing a flexible, semi-flexible or rigid DNA linker connecting the two SH2-binding phosphopeptides. The single strand DNA linker connecting the two phosphopeptides did not show linker length dependency (2-21 nt) in terms of bivalent binding, whereas the use of a rigid DNA-linker showed helical periodicity with respect to binding. The introduction of a nicked site or the formation of ternary complexes with single strand domains, with a critical maximum of 13 nt (~ 44 Å) in between the phosphopeptides, avoided the orientation limitation caused by the helical turn effect and restored the bivalent binding effect (Figure 1.11b). In a similar approach DNA-based bivalent ligands were assembled to evaluate binding to the
estrogen receptor dimer (35 Å). In both studies relatively minor affinity enhancements were observed, most probably due to the inherent flexibility of the chosen proteins and the short distances, for which the potential benefit of using DNA as a (semi-)rigid linkers is likely to be minor.[48,49]

An excellent example of a bivalent ligand design that capitalizes on the long persistence length as well as the modularity of DNA was shown recently by Bjorkman and coworkers.[50] Their research aimed to develop homo- and heterobivalent antigen binding fragments (Fab) that bind strongly within the HIV-1 envelope spike protein trimer (Env) (Figure 1.12). The architecture of Env spikes and its low density on the cell surface prevents the intra- and inter-spike bivalent binding of anti-HIV-1 antibody. The weaker monovalent binding of natural anti-HIV-1 antibodies leads to mutation of Env spike proteins and antibody evasion. Homobivalent ligands were constructed by connecting two Fab fragments using different length of dsDNA. Up to a 100-fold increase in binding affinity was observed compared to the full anti-HIV-1 antibody for the optimal dsDNA linker length of 60 bp, whereas much less efficient binding was observed using both shorter and longer dsDNA linkers. The 60 bp dsDNA-linker is close to the predicted distance between the Env spike trimer binding sites which supported the optimal intra-spike bivalent binding. Furthermore, heterobivalent Fab-ligands, recognizing different epitopes of the Env spike trimer, were also connected via dsDNA linker. In this case the optimal linker was found to be 40 bp, showing more than a 100-fold increase in affinity compared to the monovalent binding.

**Figure 1.12:** Tuning the length of a DNA-linker connecting antigen binding fragments (Fab) allows optimal bivalent and intramolecular binding to the HIV-1 trimer protein. The potency of the homobivalent Fab-ligand was increased when a 60 bp DNA-linker was used to connect the two Fab-fragments, whereas a 40 bp DNA-linker increased the potency of a heterobivalent Fab-ligand recognizing two different epitopes on the trimer protein. Adapted with permission from[50].
Seitz and coworkers conjugated carbohydrates (LacNAc) at internal positions of PNA resulting in the formation of semi-rigid multivalent ligands (bi-, tri-, tetra) assembled on different DNA templates (Figure 1.13a).\cite{51} Although an 800-fold increase in binding was observed for the tetravalent ligand (\(K_d\) of 1 µM) the individual lectin protein has only two binding sites which are opposite of one another (65 Å). The rigidity of the DNA-linker can be adjusted to the properties of the target protein without extensive engineering. Thereto, the one to one binding to the lectin protein was investigated by constructing a bivalent ligand that bends and bridges a distance of 100 Å around the protein to allow simultaneous binding of a bivalent DNA based LacNAc-ligand (Figure 1.13b). The bivalent ligand with a LacNAc separation of 104 Å favored a flexible linker (\(K_d\) of 12 µM) rather than a more rigid linker (\(K_d\) of 21 µM) separating two LacNAc-ligands.

**Figure 1.13:** *Multivalent presentation of glycoligands (LacNAc) to test lectin binding.* a) DNA-programmed assembly of PNA-LacNAc conjugates to create multivalent ligands with different valencies, ligand separation and adjusted flexibility/rigidity of the DNA-linker connecting the ligands (I, II and III). b) Molecular modelling studies of a DNA-based bivalent LacNAc-ligand in presence and absence of more rigid regions of dsDNA and thereby tuning the flexibility of the DNA-scaffold. The lectin binding pockets are opposite of each other and separated by 65 Å, therefore bending or concave presentation of the bivalent LacNAc-ligand is required for simultaneous binding to the lectin protein and bridging a total distance of approximately 100 Å. Adapted with permission from \cite{51}.

Appella and coworkers investigated DNA/PNA-based multivalent ligands by targeting cell-surface receptors.\cite{52,53} Cell-adhesion was studied by targeting integrins (\(\alpha_\beta_3\)), which are overexpressed in certain tumors, using multivalent ligands based on cyclic RGD-PNA conjugates of 12 residues. Four different cRGD-PNA conjugates were synthesized and different lengths of DNA served as a template, resulting in fifty-two individual multivalent constructs with different ligand positions, valencies and ligand densities (Figure 1.14).\cite{52} The ability of the different multivalent cRGD-PNA/DNA assemblies to inhibit metastatic melanoma cells binding to the extracellular matrix was examined. The most potent inhibitor was the hybrid displaying 15 cRGD–ligands (5 times the 3 ligand cRGD-PNA conjugate assembled on ssDNA) showing a 100-fold increase in potency compared to the monovalent ligand. A similar approach was used to investigate the effect of a small molecule antagonist (XAC, µM affinity) binding to a G-protein coupled receptor which is believed to communicate via protein-protein interactions.\cite{53} An exceptional binder was obtained bearing a total of 8 XAC-ligands of which the ligands were spaced by 4 nt
resulting in a $K_d$ of 26 nM whereas a 12-mer ligand with a ligand spacing of 3 nt showed two fold weaker binding ($K_d$ of 54 nM). These results illustrated that, depending on the target, spacing between ligands is important to obtain high affinity binding and that an increase in valency does not automatically result in higher affinities.

Figure 1.14: Generation of a 45-compound library based on cRGD-PNA conjugates for the interrogation of cell-adhesion. cRGD was conjugated at the terminus (A) or at internal positions of a 12 residue PNA using L-lysine γ-substituted PNA displaying 1, 2 or 3 cRGD-peptides (B, C and D). Assembly of PNA-cRGD conjugates on DNA templates yielded different valencies and distances between cRGD-ligands. Multivalent cRGD-PNA/DNA ligands bind to integrins ($\alpha_v\beta_3$) and thereby inhibit melanoma cells (C32) to adhere to a Vitronectin-coated surface. Adapted with permission from [52].

More complex DNA nanostructures have also been used to study multivalent binding to thrombin. Two aptamer sequences were used that bind to distinct sites of thrombin. Yan and coworkers constructed a rigid DNA-tile structure composed of a five-helix tile that was modified with closed-loop thrombin binding aptamer sequences extending from the ends of the helices.[54] The tile could provide distances of 2, 3.5, 5.3 and 6.9 nm between the two aptamers (Figure 1.15a). Tiles containing different inter-aptamer distances were separately incubated with increasing amount of thrombin concentrations and complex formation was observed by gel-shift mobility assays. The inter-aptamer distance of 5.3 nm was determined to be optimal for the bivalent binding to thrombin, resulting in an apparent $K_d$ of 10 nM. The individual aptamers bind with 10-fold lower affinity to thrombin. A nice example of exploiting the distance-dependent bivalent binding to thrombin was by developing a dynamic DNA-tweezer that could switch between bivalent and monovalent binding states.[55] The inter-aptamer distance of the DNA-tweezer in the closed state was 4-6 nm and incubation with thrombin resulted in complex formation. The addition of a fuel strand initiated a toehold-mediated displacement reaction resulting in an increase of the inter-aptamer distance (open) releasing thrombin again (Figure 1.15b). The transition between the open and closed state of the DNA tweezer could be repeated by subsequent addition of anti-fuel and fuel strands. The closed-state of the DNA tweezer showed an apparent $K_d$ of 15 nM, whereas a $K_d$ of 500 nM was found for the open-state.
1.5 DNA-conjugation strategies

Many applications of DNA-based nanotechnology in biomedicine or chemical biology require robust oligonucleotide (ODN) conjugation strategies to decorate DNA-structures with biomolecules like peptides, proteins or sugars. The synthetic accessibility of DNA by solid-phase synthesis enables the introduction of unique chemical functionalities mostly at the 5'- or 3'-end, which facilitates the conjugation to biomolecules. A variety of bioconjugation strategies has been developed for conjugation of oligonucleotides to proteins and peptides, ranging from relatively straightforward approaches, which often lack regioselectivity, to more advanced site-specific conjugation strategies. Here we only discuss the most extensively used approaches that allow coupling of oligonucleotides and peptides or proteins in solution.

A good bioconjugation reaction is defined by (1) a high yield, (2) rapid reaction under biological conditions (aqueous solution, ambient temperature and near physiologic pH) and the (3) selectivity of the reaction. Classic bioconjugation approaches use functional groups that are already present in peptides and proteins such as lysines. The amino group of lysines is a good nucleophile and readily reacts with, for example, activated esters to form a stable amide bond. However, since lysines are abundantly present in proteins, this conjugation strategy often results in heterogeneous mixtures of conjugates.

Cysteines are much less abundant in proteins than lysines, which typically allows for more site-specific conjugation. Often a single cysteine can be introduced via site-directed mutagenesis, which reacts in a subsequent chemical modification to allow site-specific protein conjugation. One of the first examples of an oligonucleotide-modified protein was the spontaneous disulfide bridge formation under oxidizing environments between an alkylthiol-modified oligonucleotide and a lysine-to-cysteine mutated staphylococcal

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**Figure 1.15: DNA nanostructures for multivalent protein binding.**

a) Rigid DNA-tile to study the distance dependence for heterobivalent aptamer binding to thrombin. Adapted with permission from [54].
b) Reversible regulation of bivalent thrombin binding by toehold-mediated DNA strand displacement reactions. Repeated addition of fuel and anti-fuel strands allows switching between bivalent and monovalent binding states. Adapted with permission from [55].
nuclease. Irreversible bioconjugation strategies involving cysteines or thiols are alkylation with bromo- or iodoacetamide and Michael addition reaction with maleimides yielding thioether linkages. In the latter reaction, the thiol acts as a nucleophile resulting in the hydrothiolation of the C=C bond of the maleimide moiety (Figure 1.16a). Maleimide-functionalized oligonucleotides are commercially available or can be obtained relatively straightforward in a one-step reaction. A common approach is to equip an amine-functionalized oligonucleotide via an amine-to-sulfhydryl crosslinker (Sulfo-SMCC) with a maleimide functionality. Subsequently, the maleimide-functionalized oligonucleotide can react with a cysteine introduced on the protein or peptide. Using this strategy, oligonucleotides have been conjugated to for example fluorescent proteins to study FRET mechanisms or split-enzymes which could regain enzymatic activity upon DNA-directed complementation. The bivalent synbodies reported by Chaput and coworkers were also obtained by conjugation of cysteine-containing peptides SMCC-modified oligonucleotides. The coupling-order can also be reversed by reacting a thiol-functionalized oligonucleotide to a maleimide-functionalized streptavidin creating a DNA-streptavidin hybrid for the self-assembly of biotinylated-proteins. Maleimide-functionalized glycans have been coupled to thiolated-PNA nucleotides to create multivalent ligands. Although the thiol-maleimide reaction has been widely used, it also has some limitations. The thiol present in the protein, peptide or oligonucleotide must be maintained in a reduced form by treatment with dithiotreitol (DTT) or tris[2-carboxymethyl]phosphine (TCEP), which can interfere with the presence of native disulfide bridges. In addition, some proteins contain functionally important cysteines which would also be modified by this procedure.

Several alternative coupling strategies have been developed that are orthogonal to the functional groups present in natural proteins. An important example is native chemical ligation (NCL) or expressed protein ligation (EPL), which was originally developed to facilitate the chemical synthesis of proteins by ligation of two or more peptide fragments. NCL requires an N-terminal cysteine and a C-terminal thioester and proceeds through a reversible transthioesterification step followed by an intramolecular S,N-acyl shift resulting in formation of a native peptide bond. The NCL reactions proceeds under aqueous conditions, neutral pH and the efficiency is increased when using a thiol additive as catalyst. Native chemical ligation has been widely used to conjugate peptide and proteins to a broad variety of synthetic molecules and materials, including dendrimers, lipids and biosensor surfaces. The most common strategy to obtain an oligonucleotide-protein/peptide conjugate via native chemical ligation is to introduce an N-terminal cysteine on the oligonucleotide, which can subsequently react with the peptide or recombinant protein bearing a C-terminal thioester. NCL has been used to conjugate oligonucleotides to fluorescent proteins, to Ras-proteins and to maltose-binding protein for DNA-directed immobilization in protein microarray technology. Nagamune and coworkers introduced a thioester on an amine-functionalized oligonucleotide by using a bifunctional reagent bearing an N-hydroxysuccinimide ester and benzyl thioester group. Using this approach they were able
to site-specifically react with a cysteine at the N-terminus of a split-luciferase half, restoring enzyme activity upon DNA-hybridization.[27]

Figure 1.16: Schematic overview of common used chemoselective reactions for peptide/protein conjugation to oligonucleotides. a) Michael addition between thiol and maleimide. b) Native chemical ligation reaction between a thioester and a N-terminal cysteine. c) Cu(I)-catalyzed [3+2] cycloaddition between azide and alkyne (CuAAC). d) Strain-promoted azide-alkyne cycloaddition (SPAAC).

Azides have also proven to be a very useful functional moiety for bioorthogonal reactions. Unlike thiols, azides are totally absent in biological systems and may therefore be considered as a unique functional group.[59] Azides can participate in Huisgen-type [3+2] cycloadditions, such as the Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC).[77] When Cu(I) is used as catalyst, the reaction between an azide and an alkyne results in the exclusive formation of a highly stable 1,4-substituted triazole product (Figure 1.16c). Furthermore, the reaction proceeds in a wide range of solvents including aqueous solutions. Non-natural amino acids can be used to introduce azide-functionality in proteins by using an amber stopcodon binding-tRNA and corresponding aminocyl-tRNA synthetase.[78,79] Both, azides and alkynes can be introduced on oligonucleotides[80] and the CuAAC-reaction has successfully been used in different studies to construct PNA/DNA-peptide[49,81] or -protein conjugates.[82–84] The CuAAC-reaction between peptides and PNA/DNA can be achieved by introducing the alkyne-moiety on the PNA/DNA and the azide-functionality on the peptide, or vice versa.[80,81] Besides the use of non-natural amino acids, one can also use a chemoenzymatic labeling method to site-specifically introduce azide-functionality in proteins. DiStefano and coworkers used the enzyme protein farnesyl transferase (PFTase) to site-specifically conjugate an
oligonucleotide to GFP.\textsuperscript{[82]} PFTase was used to label GFP containing a C-terminal tetrapeptide tag, CVIA, with an azide-modified isoprenoid diphosphate, which was subsequently conjugated to an alkyne-functionalized oligonucleotide using CuAAC-chemistry. Despite the excellent chemoselectivity of the CuAAC-reaction, the use of Cu(I) as a catalyst also has drawbacks. The redox capability of Cu(I) can catalyze the generation of damaging reactive oxygen species, which can lead to protein denaturation,\textsuperscript{[85]} cytotoxicity,\textsuperscript{[86]} and breaks in double strand DNA.\textsuperscript{[87]} To avoid the use of Cu(I), Bertozzi and coworkers developed strain-promoted alkyne-azide cycloaddition (SPAAC) reaction.\textsuperscript{[88]} The SPAAC-reaction has been used to label or conjugate proteins,\textsuperscript{[89]} oligonucleotides\textsuperscript{[56,90–92]} and can even be used in living systems.\textsuperscript{[93–95]} DiStefano and coworkers explored the SPAAC-reaction for the synthesis of ODN-protein conjugates between a dibenzocyclooctyne (DIBO)-functionalized oligonucleotides and azide-functionalized GFP and mCherry (Figure 1.16d).\textsuperscript{[96]} The Cu(I)-free conjugation of ODN-protein were comparable to Cu(I)-catalyzed reactions in yield and kinetics. The laborious synthesis of cyclooctynes and the possible thiol-yne reaction with cysteines yielding non-specific labeling limits the use of the SPAAC-strategy.\textsuperscript{[97]} Recently, the inverse Diels-Alder reaction between electron deficient tetrazines and highly strained trans-cyclooctene (TCO) has become of interest for labeling biomolecules in vivo.\textsuperscript{[98]} So far, no proteins/peptides have been conjugated to oligonucleotides using the tetrazine-TCO reaction.

Another synthetic strategy for site-specific conjugation of oligonucleotides to peptides and proteins involves the use of enzymes or self-labeling protein domains. These strategies typically require small peptide or protein fusions to the protein of interest in order to site-specifically react with (functionalized) oligonucleotides. Microbial transglutaminase (MTG)\textsuperscript{[99]} and Sortase A\textsuperscript{[100,101]} are examples of enzymes that have been used for conjugation between PNA/DNA and proteins. MTG catalyzes an acyl-transfer reaction between the ε-amino group in lysine and the γ-carboxamide in a glutamine. Kamiya and coworkers fused an acyl acceptor tag sequence, MKHKGS, either at the N- or C-terminus of a phosphatase (PfuAP) and used MTG to catalyze the reaction between the lysine-tagged phosphatase and a glutamine labeled (QG) oligonucleotide to create an enzyme-oligonucleotide hybrid.\textsuperscript{[99]} This strategy was used for highly sensitive DNA-detection. Sortases are transpeptidases found in Gram-positive bacteria.\textsuperscript{[102]} The recognition sequence for Sortase A, LPXTG, can be introduced near the C-terminus of the protein or peptide of interest. Sortase A cleaves the amide bond between threonine and glycine and transfers the acyl-fragment to an N-terminal polyglycine-modified oligonucleotide (or PNA) resulting in the formation of an amide bond (Figure 1.17a).\textsuperscript{[102–104]} The Sortase A mediated reaction has been used to conjugate oligonucleotides containing an N-terminal polyglycine sequence to M13-capsid proteins (pIII, PVI and PIX) bearing a LPETG-sortase recognition motif at the C-terminus. The covalent attachment of oligonucleotides to different M13-capsid proteins allowed the DNA-templated assembly of multiphage structures in a controlled manner.\textsuperscript{[101]}
Self-labeling proteins such as SNAP, CLIP or Halo-tag have also been used as site-specific labeling strategies of protein-ODN conjugates. SNAP and CLIP-tags are engineered variants of the human DNA repair protein O6-alkylguanine-DNA alkyltransferase that react rapidly and specifically with O6-benzylguanine (BG) and O6-benzylcytosine (BC), respectively (Figure 1.17b). Specific oligonucleotides bearing either benzylguanine or benzylcytosine have been conjugated to SNAP- and CLIP-fusions (20kDa) of FKBP and FRB proteins, thereby inducing protein-protein interactions by DNA-hybridization at the cell-surface of transfected cells. The Halo-tag has also been used to site-specifically conjugate oligonucleotides to fluorescent proteins (EYFP and mKate) for addressing nanostructures or to protein domains for DNA-mediated assembly of a full protein. The Halo-tag is a modified haloalkane dehalogenase in which the active site aspartate nucleophile reacts with an alkyl chain bearing a chloride at the terminus to form a stable covalent alkyl-enzyme intermediate (Figure 1.17c). The small molecules necessary for the reaction with these protein fusions are often available as NHS-ester and can therefore be introduced easily to amine-functionalized oligonucleotides.

Figure 1.17: Schematic overview of frequently used orthogonal protein coupling to oligonucleotides. a) Sortase A (SrtA) mediated ligation of a protein (POI) bearing the recognition sequence LPXTG at the C-terminus with a polyglycine-functionalized oligonucleotide. b) Covalent coupling between a SNAP-tag fusion protein and O6-benzylguanine (BG) functionalized oligonucleotide. c) Covalent bond formation between Halo-tagged protein and chlorohexane-functionalized oligonucleotide. d) Non-covalent interaction between His6-tagged protein and nitrilotriacetic acid (NTA) functionalized oligonucleotide mediated a nickel ion.
The modification of a protein of interest with a His$_6$-tag facilitates the nickel-mediated and non-covalent interaction between the His-tagged protein and a nitrilotriacetic acid (NTA), which is introduced at the oligonucleotide (Figure 1.17d).\[109,110\] This interaction is rather weak and reversible upon the addition of a chelator (EDTA). Gothelf and coworkers recently used the nickel-mediated interaction between a His-tagged protein and NTA-functionalized oligonucleotides in a template-directed covalent conjugation strategy. A single oligonucleotide was functionalized with a NTA-moiety, whereas the partial complementary strand was functionalized with an NHS-ester. The NTA-oligonucleotide functions as a guide to allow the reaction between the NHS-oligonucleotide and a lysine close to the His-tag of the protein. Finally, the NTA-oligonucleotide can be displaced by a toehold-mediated displacement reaction resulting in a covalent protein-oligonucleotide conjugate.\[111\]

1.6 Antibody-based therapeutics and controlled targeting

Efforts to develop drugs that seek and destroy diseased cells without affecting healthy cells dates back to Paul Ehrlich, who postulated the magic bullet principle at the beginning of the 20th century.\[112\] The intrusion of pathogenic organisms triggers the immune response, resulting in elevated levels of nature’s magic bullets, antibodies. Different classes of antibodies (IgG, IgA, IgE, IgD and IgM) exist, but they all share a common bivalent Y- or T-shaped structural architecture consisting of two identical heavy polypeptide chains that are connected to two identical light chains via disulfide bridges. Antibodies bind specific antigens at antigen binding sites found at the interface of the heavy and light chain variable domains.\[2\] The specificity and high affinity of antibodies for their respective antigens initiated a long quest to produce tailor-made antibodies binding to any target of interest. The hybridoma technology developed by Milstein and Kohler in 1975 resulted in major breakthrough in this research area, yielding access to monoclonal antibodies (mAbs) that bind to one specific antigen.\[113\] However, the high murine content of the first mAbs initiated immune responses upon injection of these mAbs in human patients. The cloning of antibody genes enabled the fusion of murine variable loops with human constant domains creating chimeric (70% human) or humanized (85-90% human) antibodies reducing adverse reactions.\[114\] The availability of these humanized antibodies significantly increased the clinical use of antibodies.

To date, more than 30 monoclonal antibodies (chimeric, humanized or human) have been approved for therapeutic use to treat diseases including cardiovascular, autoimmune and oncologic diseases.\[115\] Several mechanisms exist by which antibodies can eliminate (diseased) cells (Figure 1.18). (1) Antibodies can bind in an agonistic manner to cells and thereby directly activate intracellular signalling cascades resulting in apoptosis (programmed cell death). (2) Once bound to the antigen, the Fc-domain of IgG can initiate an immune response recruiting for example macrophages (or neutrophils) that possess Fc-receptors resulting in phagocytosis. (3) The binding of an antibody to a
receptor can also block the natural ligand to bind to e.g. growth factor receptors, thereby exerting an antagonistic effect.[116] (4) The conjugation of radiolabels or toxins can increase the efficacy of antibodies. For example, a two-step approach of pre-targeted radioimmuno-imaging and therapy strategies uses the long half-life of antibodies itself to accumulate in tumor tissue, whereas the small probe is rapidly cleared reducing the background.[117] Bioorthogonal chemistry is increasingly used for chemoselective conjugation of drugs, toxins or radiolabels to antibodies.

Figure 1.18: Different mechanisms by which antibodies can be used to kill tumour cells. (1) Agonistic-binding activates cell signalling cascades inducing apoptosis. (2) Immune-mediated tumor cell killing. For example, macrophages recognize the Fc-domain of the antibody followed by taking up the tumour cell by phagocytosis and digesting the cell. (3) Antagonistic-binding prevents the binding of natural ligands to the receptor and thereby blocks receptor dimerization resulting in reduced proliferation and apoptosis. (4) Antibody-drug(toxin) conjugates can increase the effect of tumor cell killing. Adapted with permission from[116].

The large size of monoclonal IgGs (150 kDa) has been shown to limit tissue penetration, while their long serum half-life limits their application for radioimmunotherapy and imaging purposes due to irradiation of healthy tissue and substantial high background. Protein engineering has therefore been used to construct smaller monovalent fragments, such as Fab-fragments, single chain fragments (scFv) and single domain antibodies (sdAb, nanobodies) (Figure 1.19).[118] Although these smaller fragments penetrate tumors more efficiently, this effect is sometimes counterbalanced by their weaker antigen binding affinities and shorter serum half-life, which may lead to a decrease in overall uptake. In addition, the smaller antibody fragments lack the Fc-domain, which prevents them from initiating immune-mediated tumor cell killing. Less than 10% of all the antibodies that are FDA-approved for therapeutic use comprise of small antibody fragments.[115]
Current antibody-based therapies are based on binding to antigens that are overexpressed at diseased cells compared to healthy tissue. Examples of specific antigens that have been successfully targeted include epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF) receptor and cytotoxic T-lymphocyte associated antigen 4, CD20, CD 30 and CD52.[116] Binding of antibodies to the aforementioned antigens in healthy tissue can result in side-effects, however, which limits the potential benefit of antibody-based therapy for some patients. Recently, strategies have been proposed to limit off-target effects by using bispecific antibodies that recognize two cell surface antigens or by controlling the binding mode of antibodies using additional biomarkers to increase the specificity of antibody-based therapeutics towards diseased tissue.[119–122] Proteolytic enzymes are known to be highly specific and locally activated in oncological and vascular pathologies.[123,124] Williams and coworkers showed, as a proof of concept, a first example of controlling the binding activity of a scFv fragment by fusing it to its cognate target protein. The scFv fragments of the EGFR-specific antibodies cetuximab and matuzumab were genetically fused to domain III of the EGFR (EGFRdIII). A flexible linker containing a matrix metalloprotease (MMP-9) substrate was introduced between both domains to allow efficient intramolecular masking of the antigen binding
site. Mutations were introduced in EGFRdIII to attenuate the affinity to the antigen binding site to allow dissociation and diffusion after cleavage of the linker. An 8-fold increase in binding to immobilized EGFRdIII was observed after proteolytic cleavage. The linker between the scFv and EGFRdIII was initially designed to yield monomeric masked scFv’s but appeared to predominantly form dimers (Figure 1.20a). In a similar approach, Daugherty and coworkers developed a protease-activatable antibody (pro-antibody) towards atherosclerotic plaques and tumors and tested its specificity in mice. A bacterial display peptide library was used to screen for binders towards anti-VCAM. This antibody targets vascular cell adhesion molecule 1 (VCAM-1), a marker of atherosclerotic plaques. The best peptide binder ($K_d$ of 80 nM) was fused via a MMP-1 substrate and flexible linker (26 amino acids) to an anti-VCAM-derived scFv (Figure 1.20b). This impressive piece of antibody engineering resulted in pro-antibodies that can selectively target MMP producing aortic plaques in vivo and thereby reduce binding to normal tissue. The effective concentration, provided by the flexible linker, was estimated to be 1 mM, allowing even modest affinity binders ($\mu$M) to block the antigen binding site. A similar strategy was applied to the FDA-approved antibody cetuximab. A 21-mer masking peptide was selected from a bacterial peptide display library and genetically fused to the N-termini of cetuximab (Figure 1.20b). A substrate linker was introduced between the masking peptide and cetuximab, which could be cleaved by proteases that are up-regulated in a variety of human carcinomas. The binding activity of the masked pro-antibody to EGFR-expressing cells was reduced by 400-fold compared to cetuximab. Proteolytic activation of the pro-antibody by endogeneous proteases specific for the tumor tissue was demonstrated. The pro-antibody was shown to be as effective as cetuximab at suppressing tumor growth in mice with established tumors, but showed reduced toxicity compared to cetuximab in non-human primates.

**Figure 1.20:** Examples of protease-activatable masked antibody(-fragments). a) Single chain fragment of cetuximab (C225 scFv) fused to domain III of EGFR results in masked antibody-fragments. Dimer formation between scFv-EGFRdIII fusions were observed instead of the postulated monomer and intramolecular masking of the scFv. Adapted with permission from [119]. A substrate peptide-linker between domain III and the scFv allows activation by matrix metalloprotease 9. b) Pro-antibody design for anti-VCAM targeting atherosclerotic plaques and cetuximab targeting specific tumor tissue. A substrate sequence in the linker allows proteolytic activation by endogeneous proteases and thereby restores antibody activity. Adapted with permission from [120,122].
In an impressive engineering approach Church and coworkers used structural DNA nanotechnology to block the binding of scFv’s to human leukocyte antigen (HLA) by shielding the scFv in a DNA-origami based barrel.[121] The barrel (35 x 35 x 45 nm³) consisted of two halves, which were closed by two (different) aptamer sequences that function as locks. The scFv’s only became accessible in the presence of the appropriate key(s) or proteins at the surface of the cell. The modularity of this system allowed the substitution of the payloads and locks (2 similar or 2 different aptamer locks) enabling the targeting of different cell-types in a logic gate type of fashion (Figure 1.21). The presence of characteristic proteins at the cell-surface enabled the opening of the vehicle allowing the antibody to bind to the HLA at the cell-surface.

**Figure 1.21:** A logic-gated DNA nanorobot evaluates the cell surface and reveals its payload only in presence of two specific markers. a) A DNA-origami barrel (35 x 35 x 45 nm³) was loaded with single chain antibody fragments towards human leukocyte antigen. The aptamer-encoded locks consists of a DNA-aptamer (blue) and a partial hybridized complementary strand (orange) and function as an AND-gate responding to molecular inputs (shown in red) expressed by cells allowing the opening of the barrel. b) The DNA-barrel showed specific opening in the presence of two different surface markers. The color match between lock and key indicates the opening of the lock and thus the DNA-barrel followed by the binding of the revealed scFv to the cell. Six different cell lines were specifically targeted by using different combination and cell-type specific locks (protein-aptamer interactions). Adapted with permission from[121].

1.7 Aim and outline of the thesis

The aim of the work described in this thesis was to develop generic approaches to allow DNA-based control of protein activity, in particular to reversible control the activity of enzymes and antibodies.

Chapter 2 describes the DNA-templated reversible assembly of an enzyme-inhibitor complex. TEM1-β-lactamase and its inhibitor protein BLIP were conjugated to different oligonucleotides resulting in an inactive enzyme upon the presence of a DNA template strand. The enzyme-inhibitor complex could be disrupted upon hybridization of a target strand to the template strand, resulting in a rigid dsDNA linker and thereby restoring the enzyme activity of β-lactamase. The non-covalent and modular assembly of the enzyme-inhibitor complex allowed the screening of different lengths as well as sequences of both
template and target strands. Toehold displacement reactions were used to reversibly switch between active and inactive states.

The core of the work described in this thesis involves the development of bivalent peptide-DNA ligands to reversibly block the antigen binding sites of an antibody. In Chapter 3 it is shown that double stranded DNA provides an attractive rigid linker to effectively span the large distance between antigen binding sites. In this proof of concept study we used anti-HIV-1 as a model antibody and investigated the influence of different dsDNA-linker lengths with respect to antibody complex formation. Fluorescence anisotropy binding studies were performed to investigate the difference in affinity between monovalent peptide and bivalent peptide-dsDNA ligand. Using a thermodynamic model the interaction between bivalent peptide-dsDNA ligand and antibody was found to be 500-fold stronger than that of the monovalent peptide, allowing effective blocking of the antigen binding sites in a non-covalent manner. Introduction of a protease cleavage site allowed the antibody blocking to be reversed by treatment with matrix metalloproteinase 2, as monitored using Surface Plasmon Resonance.

The use of DNA as a linker also provides an excellent opportunity to control antibody activity by DNA-based logic operations. In Chapter 4 the introduction of a toehold sequence on the bivalent peptide-dsDNA lock is described to enable antibody activation by toehold-mediated DNA strand displacement reactions. The anti-HA antibody that was used in this study confirmed the generic applicability of peptide-DNA locks to control antibody activity. Anti-HA antibody activation could be quantitatively monitored by flow-cytometric analysis of yeast displaying the HA-epitope at its surface. The introduction of two different toehold sequences on the peptide-dsDNA lock rendered antibody activation conditional on the presence of two oligonucleotides yielding OR- and AND-gates. The kinetics of the toehold-mediated strand displacement reaction was not affected when the peptide-dsDNA lock was bound to the antibody. The range of molecular inputs could be further extended to protein-based triggers by using protein-binding aptamers.

In Chapter 5, the use of bivalent peptide-dsDNA locks was applied to control the activity of the therapeutic antibody cetuximab. Two different approaches were explored based on non-covalent peptide-dsDNA locks. A homobivalent peptide-dsDNA lock was constructed that blocked cetuximab by binding to the antigen binding sites, whereas a heterobivalent peptide-dsDNA lock was developed that controlled the activity of cetuximab at the level of the Fab-fragment. The heterobivalent peptide-dsDNA lock contained two different cyclic peptides, one binding to the antigen binding site of cetuximab and the other targeting a unique pocket within the Fab-framework of cetuximab. A triple mutant of the Fab-fragment binding peptide showed increased affinity and the resulting heterobivalent peptide-dsDNA lock blocked cetuximab more efficiently compared to the wild-type containing peptide-dsDNA lock. Flow cytometric analysis revealed efficient blocking of cetuxumab binding to EGFR-overexpressing cells for both
type of ligands. Moreover, blockage of cetuximab could be reversed by both protease cleavage and toehold-mediated strand displacement reactions.

**Chapter 6** describes the use of Pyrrole-Imidazole (PI) polyamides as an alternative approach to functionalize DNA structures with peptides and proteins in a non-covalent manner. These PI-polyamides are attractive small molecule DNA binders that bind to the minor groove in a sequence-specific manner with nanomolar affinity. Native chemical ligation was explored as a generic approach to couple thioester proteins and peptides to PI-polyamides containing an N-terminal cysteine. The synthesis of the PI-polyamide conjugated to peptide and proteins was successful and the effect on DNA binding was investigated by Surface Plasmon Resonance measurements. Peptide-PI-polyamide conjugates showed a 20-fold attenuation in DNA binding strength compared to non-functionalized PI-polyamide, whereas a further attenuation in binding affinity was observed for protein-PI-polyamide. The practicality of using PI-polyamides to address proteins on DNA in a non-covalent manner therefore remains to be established.

**Chapter 7** contains a general discussion of the obtained results and elaborates on possible applications and suggestions for further research. Furthermore, the possible risks and societal embedding of our novel approach for antibody-based therapeutics will be discussed as part of the Risk Analysis and Technology Assessment (RATA) program within the NanoNextNL consortium.

1.8 References

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Chapter 2

DNA-DIRECTED CONTROL OF ENZYME-INHIBITOR COMPLEX FORMATION: A MODULAR APPROACH TO REVERSIBLY SWITCH ENZYME ACTIVITY

Abstract

DNA-templated reversible assembly of an enzyme-inhibitor complex is presented as a new and highly modular approach to control enzyme activity. TEM1-β-lactamase and its inhibitor protein BLIP were conjugated to different oligonucleotides, resulting in enzyme inhibition in the presence of template strand. Formation of a rigid dsDNA linker upon addition of a complementary target strand disrupts the enzyme-inhibitor complex and results in the restoration of enzyme activity, enabling detection of as little as 2 fmol DNA. The non-covalent assembly of the complex allows easy tuning of target and template strands without changing the oligonucleotide-functionalized enzyme and inhibitor domains. Using a panel of 8 different template sequences, restoration of enzyme activity was only observed in the presence of the target viral DNA sequence. The use of stable, well-characterized protein domains and the intrinsic modularity of our system should allow easy integration with DNA/RNA-based logic circuits for applications in biomedicine and molecular diagnostics.

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2.1 Introduction

The development of robust design strategies for the construction of biomolecular switches is one of the key challenges in bionanotechnology, providing essential tools for molecular imaging, synthetic biology, molecular diagnostics and biomolecular computing.\(^1\) Inspired by the modular architecture of many natural signaling networks, researchers have started to explore similar principles in semi-synthetic systems.\(^1,4,5\) Oligonucleotide-based circuits are attractive in this respect because their highly modular and predictable nature allows the construction of complex circuits using a limited set of principle logic gates and building blocks.\(^1,6,7\) However, the application of these DNA/RNA-based logic circuits in biomedicine and molecular diagnostics is limited by a lack of generic approaches to interface them with protein activity.\(^8\) Most previously reported approaches to control protein activity by DNA are based on the templated assembly of two protein (fragments), either via semi-synthetic DNA-hybrids or zinc-finger fusions recognizing certain double stranded DNA-stretches. Several examples of DNA-directed complementation of split proteins have been reported, including the use of split fluorescent proteins and split luciferase,\(^12,14\) split β-Lactamase\(^18\) and split murine dihydrofolate reductase.\(^19\) Although split reporter enzymes have the advantage of providing low background signal, split enzymes tend to be thermodynamically instable and protein complementation is often not reversible.\(^20\)

An alternative approach is the use of multiple protein domains that complement each other’s function. Examples include the assembly of two enzyme domains that catalyze two consecutive reactions (cascade catalysis) such as glucose oxidase (GOx) and horseradish peroxidase (HRP). Although assembly of enzyme cascades on hierarchical self-assembled DNA-structures has been shown to enhance the efficiency of the overall catalytic process, these systems often show a substantial background activity in the absence of DNA-template due to diffusion processes.\(^10,21–25\) A related strategy is to use split multidomain enzymatic systems. An example is the work of Niemeijer and coworkers who separated the reductase and hydroxylase domains of cytochrome P450 BM3 and conjugated each domain to different 20-mer single stranded oligodeoxynucleotides.\(^26\) Hydroxylase activity was only observed upon hybridization with a complementary template strand and could be further modulated by tuning the length of the dsDNA linker in between the two domains. An advantage of this design is that it does not depend on diffusion of intermediate substrates, but the approach is not easily extended to more common reporter enzymes.

A more generic approach is to control the interaction between an enzyme and an inhibitor, an approach that was used by Ghadiri and coworkers to modulate the activity of cereus neutral protease (CNP).\(^27,28\) A small molecule phosphoramidite-inhibitor was tethered either directly to this enzyme via an oligonucleotide linker or an inhibitor-oligonucleotide conjugate was connected to the oligonucleotide-functionalized CNP via hybridization. This system allowed the detection of pM concentrations of ssDNA and
enabled the construction of logic-gate architectures. However, strategies such as these that rely on direct conjugation of a target binding oligonucleotide to a reporter enzyme, require synthesis and purification of new protein-oligonucleotide conjugates for each new target sequence, hampering high throughput applications and making system optimization time and labor intensive.

Here we report a new approach to control the activity of a reporter enzyme that is based on the reversible assembly and disassembly of a complex between an enzyme and inhibiting protein domain. The well-studied reporter enzyme TEM1-\(\beta\)-Lactamase and its inhibitor domain BLIP were each conjugated to an oligonucleotide strand to allow their interaction in the presence of a template strand, rendering the enzyme inactive (Figure 2.1). Subsequent formation of a rigid dsDNA complex between the template and target strand disrupts this enzyme-inhibitor complex and results in enzyme activation. The non-covalent assembly of the inhibited complex allows easy tuning of the optimal linker/target recognition sequence in the template strand, using only a single pair of oligonucleotide-functionalized enzyme and inhibitor domains.

![Figure 2.1: Concept of DNA-mediated assembly and disassembly of a complex between oligonucleotide-functionalized \(\beta\)-Lactamase and its inhibitor protein BLIP. Formation of the enzyme-inhibitor domains requires the presence of a template strand consisting of complementary sequences and a flexible ssDNA linker. Subsequent hybridization of a complementary strand to the single stranded loop in between the proteins results in a rigid helix, which disrupts the enzyme-inhibitor complex and restores the enzymatic activity.](image)

### 2.2 Results and Discussion

#### 2.2.1 Synthesis of ODN-protein conjugates

TEM1 \(\beta\)-Lactamase is a well-established reporter enzyme for which a broad range of colorimetric and fluorescent substrates is available. The interaction between TEM1 \(\beta\)-Lactamase and its inhibitor protein BLIP has been structurally characterized using X-ray crystallography and has served as a model system for understanding and tuning protein-
protein interactions.\cite{30} Ideally, the interaction between TEM1 β-Lactamase and BLIP should be strong enough to ensure complete intramolecular complex formation when both are linked by the template oligonucleotide strand, but weak enough such that the interaction is easily disrupted upon hybridization with the target DNA sequence.\cite{31,32} Although the thermodynamic driving force provided by DNA hybridization is more than sufficient to disrupt the interaction between wild type TEM1 β-Lactamase and BLIP ($K_i = 0.5$ nM), the use of such a strong enzyme-inhibitor interaction may also promote intermolecular enzyme-inhibitor interactions, which would suppress enzyme activation. We therefore used the E104D mutant of TEM1 β-Lactamase, as this mutant shows the same enzymatic activity as wt TEM1 β-Lactamase, but binds BLIP with a 1000-fold weaker affinity ($K_i = 1500$ nM). A similar attenuation of affinity was found to be optimal in a related approach recently reported by our group in which antibody-induced disruption of an intramolecular interaction between TEM1 β-Lactamase and its inhibitor protein BLIP was used to construct switchable antibody reporter enzymes.\cite{32}

![Figure 2.2: a) Crystal structure of wild type β-Lactamase (red) in complex with BLIP (green). The distance between the N-terminus of BLIP and the C-terminus of β-Lactamase was determined using PyMol (PDB:3C7V). b) Synthetic strategy for site-specific oligonucleotide (ODN) conjugation. The hetero-bifunctional linker Sulfo-SMCC was reacted with a 5'- or 3'-end amine-functionalized ODN. The maleimide moiety reacts with a cysteine introduced at the termini of β-Lactamase and BLIP. c, d) Semi-native 12% SDS-PAGE analysis of the conjugation of the maleimide-activated ODN and β-LactamaseE104D containing a C-terminal cysteine (c) and BLIP containing a N-terminal cysteine (d). Lane 1 shows the protein before the reaction and lane 2 after conjugation to the maleimide-activated ODN. e, f) SEC-traces (absorbance monitored at 280 nm) of ODN, protein and purified protein-ODN conjugates for ODN1-β-LactamaseE104D (e) and BLIP-ODN2 (f).](image)

To prevent the use of bulky tags or fusion proteins between the oligonucleotides and the proteins, thiol-maleimide chemistry was chosen to allow site-specific conjugation of 21-mer oligonucleotides to each protein. A cysteine was introduced at the C-terminus of β-Lactamase and near the N-terminus of BLIP as shown in Figure 2.2a. These positions were chosen because conjugation at the flexible ends of the proteins is unlikely to perturb protein folding or interfere with complex formation. The distance between these
positions in the complex is ~ 42 Å. Both proteins were expressed in *E. coli* with a periplasmic leader sequence to allow proper formation of intramolecular disulfide bonds and an N-terminal His-tag for Ni-affinity purification. To allow conjugation of β-Lactamase to the 3’-end of ODN1 and BLIP to the 5’-end of ODN2 (Table 2.1), these 5’- or 3’-end amine-functionalized 21-mer oligonucleotides were first reacted with a 20-fold excess of the bifunctional cross-linker Sulfo-SMCC. After removal of unreacted Sulfo-SMCC, the maleimide-functionalized ODNs were reacted in a 3-fold molar excess to the cysteine-functionalized proteins (Figure 2.2b). To prevent the formation of disulfide-bridged protein dimers, the proteins were stored in the presence of TCEP, which was removed by buffer exchange prior to the addition of the ODNs. Typical conversions of the reaction ranged between 50 and 95% and were monitored using semi-native SDS-PAGE gels (Figure 2.2c and d). The ODN-protein conjugates were purified by Ni²⁺-affinity chromatography to remove excess ODN. An additional purification step, anion-exchange chromatography, was necessary for BLIP-ODN2 in order to remove unreacted protein. Both conjugates were obtained with an overall yield of ~35% and determined to be > 90% pure based on semi-native SDS-PAGE and analytical size exclusion chromatography (SEC) (Figure 2.2e and f).

### 2.2.2 Enzyme kinetics characterization of ODN-protein conjugates

To ascertain that both proteins remained functional after ODN-conjugation, their activities were first compared to their non-conjugated counterparts. $V_{\text{max}}$ and $K_M$ values were obtained by using the Michaelis-Menten equation (equation 2.1) to fit the hydrolysis rate as a function of nitrocefin concentration. Michaelis-Menten plots showed similar $K_M$ and $k_{\text{cat}}$ values for β-LactamaseE104D ($K_M = 75 \pm 9$ μM, $k_{\text{cat}} = 504 \pm 18$ s⁻¹) and ODN1-β-lactamaseE104D conjugate ($K_M = 88 \pm 13$ μM, $k_{\text{cat}} = 602 \pm 24$ s⁻¹) using nitrocefin as the colorimetric substrate (Figure 2.3). These values are also similar to those reported in literature,[34] and show that neither substrate binding nor substrate turnover are affected by conjugation. To assess whether ODN-functionalization of BLIP affected its interaction with β-Lactamase $K_i$ values were obtained by measuring enzymatic activity at a fixed substrate concentration (50 μM nitrocefin) as a function of inhibitor (BLIP or BLIP-ODN2) concentration. Equation 2.2 was used to fit the fractional hydrolysis rates.[33] Similar $K_i$-values were obtained for BLIP and BLIP-ODN2 ($K_i$ of $1.41 \pm 0.14$ nM and a $K_i$ of $1.44 \pm 0.06$ nM, respectively), which are also consistent with previously reported values indicating that ODN-functionalization of BLIP also does not affect the interaction with β-Lactamase.[35]

\[
v = \frac{k_{\text{cat}} \times [E] \times [S]}{K_M + [S]} \tag{2.1}
\]
\begin{equation}
\frac{v_i}{v_0} = \frac{K_i \times (1 + \frac{[S]}{K_M})}{[I] + K_i \times (1 + \frac{[S]}{K_M})}
\end{equation}

(2.2)

Figure 2.3: Enzyme kinetics measurements investigating the effect of ODN conjugation on protein activity. a) \(\beta\)-Lactamase activity as a function of Nitrocefin concentration at an enzyme concentration of 300 pM. Absorption was measured in a quartz cuvet with a pathlength of 0.1 cm. b) Relative enzyme activity of wild-type \(\beta\)-Lactamase (600 pM) as a function of the concentration of BLIP or BLIP-ODN2. The steady-state Michaelis-Menten equation (equation 2.2) was used to fit the data. Activity assays were performed in 50 mM phosphate, 100 mM NaCl and 1 mg mL\(^{-1}\) BSA at pH 7.0 at 28 °C.

2.2.3 Assembly of protein-DNA complexes

Having established that ODN conjugation did not affect the enzymatic activity of the reporter enzyme or interfere with the interaction between \(\beta\)-Lactamase and BLIP, we next tested the DNA-mediated assembly of the system by monitoring the interactions of various components using semi-native PAGE (Figure 2.4). Addition of a template strand with a 30-mer linker sequence between the two complementary sequences (Templ-30), resulted in a clear shift to higher molecular weight complexes for both ODN1-\(\beta\)-LactamaseE104D (lanes 1 and 2) and BLIP-ODN2 (lanes 3 and 4).

Complete complex formation was observed upon addition of a slight excess (1.2 equivalents) of the template strand. As expected, no complex formation was observed between ODN1-\(\beta\)-LactamaseE104D and BLIP-ODN2 in the absence of a complementary template strand (lane 5). Subsequent addition of Templ-30 to ODN1-\(\beta\)-LactamaseE104D and BLIP-ODN2 resulted in the clean formation of the ternary ODN1-\(\beta\)-LactamaseE104D/Templ-30/BLIP-ODN2 complex (lane 6). To assure complete inhibition of the enzyme activity in the presence of template and reduce background enzymatic activity a 1:1:2:2 ratio of ODN1-\(\beta\)-LactamaseE104D/Templ-30/BLIP-ODN2 was chosen for all activity assays. Using a slight excess of BLIP-ODN2 is not a problem, since the
concentration of non-complexed BLIP-ODN2 is too low to inhibit ODN1-β-LactamaseE104D. Importantly, under these conditions, no bands corresponding to ODN1-β-LactamaseE104D or ODN1-β-LactamaseE104D/Templ-30 were present, showing that all reporter enzyme was bound to its inhibitor protein. A further gel-shift was observed upon addition of the 30-mer complementary target oligonucleotide, consistent with the formation of a 4-component complex in which β-lactamase and BLIP are separated by a rigid 30 bp dsDNA linker. The length and composition of the flexible oligonucleotide linker can be easily tuned in our approach by using a different template strand (Table 2.1), without having to synthesize new protein-ODN conjugates. Lanes 7-12 show that the length of the flexible linker can be varied between 0 and 50 bases without compromising complex formation. A consistent trend of smaller complexes is observed with decreasing linker length, except for Templ-0. The latter shows a slight increase in apparent hydrodynamic radius compared to Templ-10, suggesting the formation of a different, less compact complex.

Figure 2.4: Monitoring the assembly of protein-DNA complexes using semi-native 12% SDS-PAGE stained with Coomassie Blue. Components were sequentially mixed at low µM-concentrations and at a ratio of 1:1.2:2 of ODN1-β-LactamaseE104D/Template/BLIP-ODN2. The complex formed in lane 7 was also incubated with 10 equivalents (with respect to Template) of 30 bases Target strand. Lanes 6, 8, 9, 10, 11 and 12 show the hybridization with both conjugates and different lengths of template strand (30, 50, 40, 20, 10 and 0 bases, respectively, Table 2.1). Sequential hybridization steps were performed for 30 minutes at room temperature.
2.2.4 Varying the template and target lengths and restoration of enzyme activity

The effect of template strand addition and subsequent addition of target ODN on the enzymatic activity was monitored by varying both the length of the linker in the template strand and the length of the complementary target strand between 0, 10, 20, 30, 40, and 50 bases (Table 2.1). ODN1-β-LactamaseE104D and BLIP-ODN2 were first incubated with the template strand at low µM concentration for 30 minutes to ensure complete formation of the inhibited complex. Subsequently, the complexes were diluted to a final concentration of 1.2 nM, followed by the addition of a 10-fold excess (12 nM) of target strand. After 1 hour incubation at room temperature, enzymatic activity was measured by monitoring the hydrolysis of the colorimetric substrate nitrocefin (Figure 2.5). As expected, the enzymatic activity of ODN1-β-LactamaseE104D was not affected by the addition of BLIP-ODN2 in the absence of template strand, confirming the absence of complex formation. Enzymatic activity was strongly inhibited upon the addition of any of the template strands, with the strongest apparent inhibition observed for the longer template loop lengths. At first it may seem surprising that inhibition is also observed for the template with no oligonucleotide linker separating the enzyme and inhibitor domains (Templ-0), since the distance between the two attachment points in the X-ray structure of the β-Lactamase-BLIP complex is ~42 Å. The observed inhibition suggests that this distance can be bridged by the combination of the hexyl spacer on the DNA, the SMCC-linker and the flexible residues at the termini of both proteins and/or that complex formation between the template strand and the ODN1 and ODN2 is somewhat strained. Such strain would be released when increasing the length of the oligonucleotide linker, which explains the more complete inhibition for the longer template sequences.

The effect of target sequence length on the restoration of enzymatic activity depends on the length of the template strand. No restoration of enzymatic activity is observed for complexes containing 0, 10, or 20 base template strands. For 0 and 10 base template strands this can be simply explained by the absence of stable duplex formation between template and target under these conditions. The 20 bases template and its complementary 20 bases target sequence would be expected to result in formation of a stable double helix. Apparently, the length of this rigid linker alone (20 bp ≈ 68 Å) is not sufficient to disrupt the complex between β-LactamaseE104D and BLIP which we again attribute to the inherent flexibility contributed by the linkers of the protein domains and the conjugation-site of the DNA. Consistent with this hypothesis, restoration is observed upon further increasing the lengths of the template strand and target strands. Partial restoration of enzymatic activity was observed for the 30-mer template strand in combination with 30-, 40-, or 50-mer target strands. The observation that activity is only partially restored, suggests that the 10 nm 30-mer linker can still be partially accommodated by fully stretching the linkers between the protein cores and the dsDNA, and possibly by partial destabilization of the DNA duplex at its ends. Complete release of enzyme inhibition and full restoration of enzyme activity was only observed for template
strands with 40- or 50-mer template strands with their full complementary target sequences. While in these experiments enzymatic activity was monitored after 1 hour incubation with target strand, the response time of the system is actually much faster, as the same enzymatic activity was observed when the enzymatic assay was started 1 minute after target addition.

**Figure 2.5:** Influence of template-linker length and target length on sensor performance. Template strands containing a flexible loop of 0, 10, 20, 30, 40 or 50 bases were each combined with complementary target strands varying in length between 0, 10, 20, 30, 40 or 50 bases. ODN1-β-LacE104D, BLIP-ODN2 and different templates were pre-incubated (at low µM-concentrations, similar to Figure 2.4) and diluted to final concentrations of 1 nM ODN1-β-LacE104D, 2 nM BLIP-ODN2 and 1.2 nM Template (1:2:1.2). 10 equivalents of target (12 nM) was added and incubated for 1 hour at room temperature prior to the addition of substrate. Assays were performed using 50 µM Nitrocefin in 50 mM phosphate, 100 mM NaCl and 1 mg mL⁻¹ BSA, pH 7.0 at 28 °C. ODN-sequences used in this assay for template and target strands are displayed in Table 2.1.

An alternative explanation for the increase in enzyme activation with increasing length of template strand is an enhanced thermodynamic driving force. The hybridization of the target strand to the template strand competes with the interaction between enzyme and
inhibitor, which will decrease the overall affinity of the template for the target strand. To provide insight into the relative contribution of these competing interactions, a thermodynamic model was derived that describes the binding of target oligonucleotide to the inhibited complex in 2 steps (Figure 2.6).

![Thermodynamic model](image)

**Figure 2.6:** Thermodynamic model describing the binding of a target oligonucleotide to a complex between ODN1-β-lactamaseE104D, BLIP-ODN2 and template strand in 2 steps. $K_{d1}$ = intermolecular dissociation constant for the equilibrium between closed and open state of complex between ODN1-β-lactamaseE104D, BLIP-ODN2 and template strand. $K_a$ = association constant for hybridization of template and target strand. $K_a^*$ = association constant for binding of target strand to the inhibited complex of ODN1-β-lactamase-E104D, BLIP-ODN2 and template strand.

The first step is the equilibrium between the closed and open state of the complex between ODN1-β-lactamase-E104D, BLIP-ODN2 and template strand. The dissociation constant $K_{d1}$ for this equilibrium is equal to the inhibition constant between β-lactamase-E104D and BLIP ($K_i = 1500$ nM), divided by the effective concentration ($C_{eff}$) of BLIP relative to the enzyme domain because of their linkage by the template strand (equation 2.3). The value of $C_{eff}$ is not exactly known but depends on the linker length, the stiffness of the linker, and the distance that the linker bridges in the complex form. [36,37] In this case $C_{eff}$ is at least 10 μM (otherwise the enzyme would not be inhibited). The second step is the hybridization of the template to the target strand ($K_a$). Combining both steps results in equation 2.4 describing the binding of the target oligonucleotide to the inhibited complex. Translating these equilibrium constants in terms of Gibbs free energy results in equation 2.5 and can be compared to the theoretical Gibbs free energy for hybridization of two DNA strands. In our case, Δ$G^0$ for hybridization decreases by RT ln($K_i/C_{eff}$). Even if $C_{eff} = 15$ mM, and thus $K_i/C_{eff} = 0.0001$, this corresponds to a decrease in
$\Delta G^0$ of 5.4 kCal/mole. This is a relatively small effect compared to the theoretical Gibbs free energy for hybridization, which for a 30-mer is -39 kCal/mole. Based on these calculations, the hybridization of a 30-mer provides more than enough driving force to completely disrupt the interaction between $\beta$-lactamase-E104D and BLIP.

$$K_{d1} = \frac{K_c}{C_{eff}}$$  \hspace{1cm} (2.3)

$$K^* = K_{d1} \times K_a = (\frac{K_c}{C_{eff}}) \times K_a$$  \hspace{1cm} (2.4)

$$\Delta G^{0*} = -RT \ln K^* = -RT \ln(\frac{K_c}{C_{eff}}) \times K_a = \Delta G^0 - RT \ln\left(\frac{K_c}{C_{eff}}\right)$$  \hspace{1cm} (2.5)

### 2.2.5 Sensitivity of DNA-detection

Figure 2.5 shows that the optimal system, consisting of ODN1-$\beta$-LactamaseE104D, BLIP-ODN2 and 40-mer template and targets strands, displays an 8-fold increase in enzymatic activity in the presence of the target strand. To challenge the analytical performance of the system further, we decreased the concentration of the reporter enzyme to 100 pM and used the more sensitive fluorescent substrate CCF2-FA. CCF2-FA is a FRET probe in which the coumarin donor is released upon hydrolysis by $\beta$-Lactamase resulting in an increase in fluorescence at 447 nm. The increased sensitivity of CCF2-FA compared to nitrocefin allows it to be used at a concentration of only 2 μM, which is far below its $K_M$. At this concentration the substrate will compete less with BLIP for binding to the $\beta$-Lactamase active site, which should further suppress enzymatic activity in the absence of template. In addition, unlike nitrocefin, CCF2-FA also shows no background hydrolysis in the absence of the protein complex.

**Figure 2.7:** Hydrolysis of CCF2-FA monitored in time as a function of Targ-40 concentration. a) Fluorescence of CCF2-FA substrate recorded in time (Ex. 409 nm; Em. 447 nm). The assay was performed in duplo at 28 °C using 100 pM ODN1-$\beta$-LacE104D, 200 pM BLIP-ODN2, 120 pM Templ-40, 2 μM CCF2-FA, 50 mM phosphate, 100 mM NaCl and 1 mg mL$^{-1}$ BSA, pH 7.0. b) Hydrolysis rate as a function of Targ-40 concentration.
Hydrolysis of CCF2-FA was monitored in time at different target concentrations and is shown in Figure 2.7a. Reliably measuring the very low hydrolysis rates required pre-equilibration of all the components including the substrate for 30-45 minutes. The hydrolysis rates were obtained after the apparent equilibration of the system after 2000 seconds. A near linear increase in enzymatic turnover was observed up to the addition of 1 equivalent of template strand, when the enzyme is fully activated. Even 10 pM concentrations of template, corresponding to 2 fmol of target DNA, can be distinguished from background activity under these conditions.

2.2.6 Modular design allows the screening for different DNA sequences

The modular, self-assembling properties of the system not only allow straightforward optimization of its architecture, but enable the system to be easily adapted to different target sequences. To illustrate this property, we designed template sequences complementary to 8 different 40-mer viral DNA sequences, flanked by the constant 21-base regions required to hybridize to ODN1-β-LactamaseE104D and BLIP-ODN2. The DNA target sequences were selected in the protein region of interest of Hepatitis B (HBV), Hepatitis C (HCV), Avian influenza (H5N1), Smallpox, Human Immunodeficiency Virus (HIV), Measles, Ebola and Rotavirus C (Table 2.2) without consideration of possible secondary structure formation in template and target strand. Complexes assembled using these 8 different template strands were incubated with all 8 target sequences or no target. The heat-map shown in Figure 2.8 shows that enzyme activation was only observed in the presence of the complementary target sequence.

**Figure 2.8:** Modular sensor architecture allows straightforward tuning of target specificity. Components were pre-incubated at low µM-concentrations and diluted to final concentrations of 1 nM ODN1-β-LacE104D, 2 nM BLIP-ODN2 and 1.2 nM Template. 10 equivalents of Target (12 nM) was added and incubated for 1 hour at room temperature prior to the addition of substrate. The respective assays were performed in duplo, 50 µM Nitrocefin in 50 mM phosphate, 100 mM NaCl and 1 mg mL⁻¹ BSA, pH 7.0. Template and Target sequences are displayed in Table 2.2.
2.2.7 Reversible switching between active and inactive states using toehold-mediated strand displacement reactions

Finally, we chose one of these target sequences (HCV, 40-mer) to show that enzyme activity can be reversible switched on and off by repeated addition of a toehold-containing target strand and a fully complementary displacer strand. To allow reversible switching the target sequence was flanked with a 10 base toehold (Toehold-Target, TT), single strand piece of DNA. The fully complementary strand of the Toehold-Target is 50 bases and referred to as Displacer (D) (Figure 2.9a, Table 2.1). In each step a 2.5 molar excess of TT or D was added compared to the previous addition.

Nearly complete switching was observed between enzymatically active and inactive states for 3 cycles of target and displacer strand addition, respectively, demonstrating that DNA-directed control of enzyme activity is dynamic and fully reversible (Figure 2.9b).

![Figure 2.9: a) Scheme showing reversible switching of enzyme-inhibitor complex formation by repeated addition of a toehold-containing target strand (TT) and a full complementary displacer strand (D). b) Enzymatic activity of complexes formed upon repeated addition of template and displacer strand. Complex formation was performed at 1 µM concentration of the ODN 1-β-LactamaseE104D. (-) shows the initial state were no TT or D was added. A total of 3 cycles were performed, each subsequent addition of TT or D contained a 2.5 fold higher concentration compared to the previous step. Every addition was followed by a 30 minute incubation step at room temperature. The enzymatic activity of the complexes formed at the various steps was determined by diluting the samples to a concentration of 1 nM ODN1-β-LactamaseE104D, 2 nM BLIP-ODN2 and 1.2 nM Templ_HCV. Assays were performed in triplo using 50 µM Nitrocefin in 50 mM phosphate, 100 mM NaCl and 1 mg mL⁻¹ BSA at pH 7.0 and 28 ºC.]

2.3 Conclusions

In this study we introduced a new concept to control the activity of a reporter enzyme by DNA. Our system does not rely on DNA-templated assembly of a split enzyme system, but is based on the reversible complex formation between a reporter
enzyme and an inhibiting protein domain. By using an enzyme-inhibitor pair with an interaction strength in the low µM range, effective formation of an enzyme-inhibitor complex is only observed in the presence of a template strand. The non-covalent assembly of the inhibited complex allowed easy tuning of the optimal linker/target recognition sequence in the template strand, using a single pair of oligonucleotide-functionalized enzyme and inhibitor domains. Efficient disruption of the enzyme-inhibitor interaction and full restoration of enzyme activity was observed upon formation of a rigid double strand DNA helix of 40 bp or more. The present system consisting of TEM1 β-LactamaseE104D and BLIP allowed detection of as little as 2 fmol of ssDNA using a simple fluorescence assay and was shown to be easily adapted to any target sequence of interest. The use of stable, well-characterized protein components and the intrinsic modularity of the system makes it an attractive system to use in conjunction with DNA-based computing and extend the range of input targets beyond ssDNA/RNA, e.g. using aptamers as ligand binding intermediates. Finally, the concept of reversibly controlling the interaction between two proteins could be easily extended to other output functions, such as self-assembling fluorescent protein domains or other enzyme-inhibitor pairs.

2.4 Experimental section

**Mutagenesis.** DNA encoding for β-Lactamase and BLIP were available in pET29a expression vectors (Genscript).[31] Single point mutations in β-Lactamase and BLIP to introduce a cysteine for ligation purposes were achieved using the QuickChange site-directed mutagenesis (Stratagene) according to the manufacturer’s protocol. The primers used for the glycine to cysteine mutation at position 302 of β-LactamaseE104D were CATTGGGAATTCGGCTGCTGGAGCCATCCGCAG and CTGCGGATGGCTCCAGCAGCCGAATTCCCAATG. The primers used for the introduction at the C-terminus of BLIP were CAGTGGGATCTGGTTTGCTAAGAATTCGGCGGT and ACCGCCGAATTCTTACGAAACCAGATCCACTG. All mutagenesis results were confirmed by DNA sequencing (StarSEQ).

**Protein expression and purification.** β-Lactamase and BLIP were expressed and purified as previously described.[31] In short, E. coli BL21(DE3) cells were transformed with the appropriate pET29a vector. Bacteria containing the plasmid DNA were grown in LB-media (2L) containing kanamycin at 37 °C and induced with IPTG (0.1 mM) at an approximate OD₆₀₀ of 0.7. Induced cells were grown for 20 hours at 16 °C and pelleted by centrifugation for 10 minutes at 10,000 g. The periplasmic fraction (containing the protein) was extracted by an osmotic shock procedure. The bacterial pellet was resuspended in 300 mL osmotic shock solution (30 mM Tris-HCl, 1 mM EDTA and 20% sucrose at pH 8.0) and stirred for 20 minutes at room temperature. Subsequently, a centrifugation step (10,000 g for 20 minutes) was performed followed by resuspending the pellet in 300 mL ice cold 5 mM MgSO₄. The suspension was continuously stirred and incubated for 10 minutes at 4 °C followed by centrifugation of 40 minutes at 40,000 g. The supernatant, containing the periplasmic protein fraction, was adjusted with 200 mM Tris-HCl, pH 7.4 to a final concentration of 20 mM Tris-HCl. The supernatant was loaded onto a prepacked His-bind resin column (Novagen) and native proteins were washed away (20 mM Tris-HCl, 500 mM NaCl, 60 mM imidazole, pH 7.9).
The desired protein was eluted with 20 mM Tris-HCl, 500 mM NaCl, 400 mM imidazole, 2 mM TCEP at pH 7.9. Fractions containing pure protein were pooled and the buffer was exchanged to a storage buffer (20 mM Tris-HCl, 150 mM NaCl, 2 mM TCEP pH 7.9) using a PD-10 desalting column (GE-Healthcare). Protein aliquots were stored at -80 °C. SDS-PAGE analysis and mass spectrometry (Xevo G2 QToF mass spectrometer, Waters) was performed to confirm the purity and correct molecular weight of the proteins (β-LactamaseE104D: \( MW_{\text{calc}} = 31,842 \text{ Da} \), \( MW_{\text{obs}} = 31,843 \text{ Da} \); BLIP: \( MW_{\text{calc}} = 19,470 \text{ Da} \), \( MW_{\text{obs}} = 19,469 \text{ Da} \)). Protein concentrations were determined by measuring the absorbance at 280 nm using extinction coefficients calculated with Vector NTI (TEM1 β-Lactamase: \( \varepsilon_{280\text{nm}} = 33,585 \text{ M}^{-1} \cdot \text{cm}^{-1} \) and BLIP: \( \varepsilon_{280\text{nm}} = 28,675 \text{ M}^{-1} \cdot \text{cm}^{-1} \)).

**ODN-protein conjugation and purification.** Oligonucleotides functionalized with a primary amine via a C6 linker at their 5'-end (ODN1) or 3'-end (ODN2) were purchased from Bioneer (Table 2.1). The oligonucleotides were dissolved in PBS (100 mM NaPi, 150 mM NaCl, pH 7.2) to a final concentration of 1 mM and 20 equivalents of Sulfo-SMCC (Thermo Scientific) were added. Following incubation for 2 hours at room temperature with continuous shaking (850 rpm), excess sulfo-SMCC was removed by (repetitive) ethanol precipitation of the conjugate. The precipitated SMCC-oligonucleotides were pelleted by centrifugation (14,000 rpm for 15 minutes) and dried to air overnight at room temperature.

Prior to ODN ligation to the proteins, the storage buffer was exchanged to ligation buffer (100 mM NaPi at pH 7.0) using a PD-10 desalting column to remove TCEP. Subsequently, a 3-fold molar excess of dried ODN-SMCC was added to 20-60 µM of protein and incubated for 2 hours at room temperature under continuous shaking (850 rpm). The reaction mixture was loaded on a pre-packed His-bind resin column and the column was thoroughly washed to remove unreacted oligonucleotide. Subsequently, the protein and protein-ODN conjugates were eluted using several column volumes of elution buffer (20 mM Tris-HCl, 500 mM NaCl, 400 mM imidazole, pH 7.9). An additional purification step was necessary for BLIP-ODN2 in order to remove non-functionalized protein, the (ODN) protein mixture was loaded on a Strong Anion-exchange Spin Column (Thermo Scientific) and washed with 15 column volumes of a low ionic strength buffer (20 mM Tris-HCl, 50 mM NaCl, pH 8.0) by centrifugation. The ODN-protein conjugate was eluted in 200 µL fractions of high ionic strength buffer (20 mM Tris-HCl, 1 M NaCl, pH 8.0). The reaction yield was determined using extinction coefficients of \( \varepsilon_{260\text{nm}} = 203,900 \text{ M}^{-1} \cdot \text{cm}^{-1} \) for ODN1 and \( \varepsilon_{260\text{nm}} = 225,400 \text{ M}^{-1} \cdot \text{cm}^{-1} \) for ODN2 (UV spectrum calculator of Integrated DNA Technologies, IDT). ODN-protein conjugation and purification was preferably done on the same day to avoid repeated freeze and thaw cycles in order to maintain the enzymatic activity of β-Lactamase. The purity of the ODN-protein conjugates was assessed by SDS-PAGE and by analytical size exclusion chromatography on a Superdex 200 column (GE), using a flow of 0.1 mL·min⁻¹ (20 mM NaPi, 150 mM NaCl, 1 mM EDTA at pH 7.0).

**Hybridization assays.** Preferably, hybridization of DNA-templated Enzyme-Inhibitor complexes was performed on the same day prior to the activity assays. Hybridization was executed in consecutive steps and performed at µM concentrations, involving 30 minute incubation time at room temperature. At first, 1.2 equivalent of template strand was incubated with 1 equivalent ODN1-βLactamaseE104D followed by the addition of 2 equivalents of ODN2-BLIP. Coomassie and Silver-staining of 12% SDS-PAGE gels was performed to analyze the hybridized complexes. Target strands
were added to the already pre-hybridized Enzyme-Inhibitor complex on template and incubated for 1 hour room temperature prior to the start of the activity assay. All target and template strands were purchased from Eurofins MWG Operon.

**Activity assays.** Activity assays were performed in a phosphate buffer at pH 7.0 (50 mM NaPi, 100 mM NaCl and 1 mg mL⁻¹ BSA) in 96-well plates at 28 °C. All target and template strands were HPLC-purified and purchased from Eurofins MWG Operon and used as received (Table 2.1 and 2.2). Hydrolysis of nitrocefin (VWR) was monitored by measuring the increase in absorbance at 486 nm, whereas the hydrolysis of CCF2-FA (Invitrogen) was studied by measuring the increase in coumarine fluorescence (excitation = 409 nm; emission = 447 nm). Both absorbance and fluorescence were recorded on a Safire2 spectrofluorimeter (Tecan). Hydrolysis rates were obtained by calculating the slope of absorbance or fluorescence intensities in time (linear regime) and corrected for background hydrolysis of substrate in the absence of enzyme.
Table 2.1. ODN1, ODN2, template and target sequences used in Figure 2.4, 2.5 and 2.7.
Table 2.2. Oligonucleotide (ODN) sequences derived from Genbank data used as target sequences and ODN-sequences used as template sequences for the experiment depicted in Figure 2.8 and 2.9.

| Virus                  | Target sequence (5’→3’)                                                                 | Nt position | Genbank no. | Bases |
|------------------------|----------------------------------------------------------------------------------------|-------------|-------------|-------|
| **Hepatitis C (HCV)**  | AGCCGAGTAGGCGTTGGTTGCAGAAGGCTCTGTGTTACTG                                               | 251-290     | AB04763     | 40    |
| **Avian Influenza A (H5N1)** | AGACCCAAAGTGAAAGGGCAAGTTGGAAGATGAGTTCT                                                | 646-685     | AF036356    | 40    |
| **Variola major virus** | GAAGGTGATTTTCATGTGAAGTTACAGGATCTAATTTGTGA                                               | 181-220     | L22579      | 40    |
| **Human Immunodeficiency Virus (HIV)** | ATCACAGGTGCTAGGCTACAAAGAGCACTATAGGCTATGCTG                                            | 5751-5790   | EF514704    | 40    |
| **Ebola virus**         | AAGGTGGCTATTTGGAAGCTGTGAAAGGCTCGGCT                                                | 6321-6360   | AF086833    | 40    |
| **Measles**             | TAGTGGAATTACACTCTCTGGACTGAAATTTAATTTGACTCCGCGC                                      | 7611-7650   | NC_001498   | 40    |
| **Ebola**               | AACATCGAGTTTCTAGGACCCCTGCTGTTACAGGCG                                                | 1561-1600   | AF363961    | 40    |
| **Hepatitis B (HBV)**   | TCAAGGCTTTATATCTCTGAGAATAGCAGCTTTCTGAGTTAGTCAGTTCACGTTTCG                            | 436-475     | AJ549087    | 40    |

| Viruses and Abbreviations | Template sequence (5’→3’)                                                                 | |
|---------------------------|----------------------------------------------------------------------------------------|---|
| **Targ_HCV**              | TTCTCTACCTACATACCCATACCTACCTGAGTTGGCGTTGGTCAGAAGGACCTCTGTGTTACTG                      | 82 |
| **Targ_H5N1**             | TTCTCTCCATCACTACATACCTACCTGAGTTGGCGTTGGTCAGAAGGACCTCTGTGTTACTG                      | 82 |
| **Targ_Smallpox**         | TTCTCTACCTACATACCTACCTGAGTTGGCGTTGGTCAGAAGGACCTCTGTGTTACTG                      | 82 |
| **Targ_HIV**              | TTCTCTACCTACATACCTACCTGAGTTGGCGTTGGTCAGAAGGACCTCTGTGTTACTG                      | 82 |
| **Targ_Ebola**            | TTCTCTACCTACATACCTGAGTTGGCGTTGGTCAGAAGGACCTCTGTGTTACTG                      | 82 |
| **Targ_Measles**          | TTCTCTACCTACATACCTGAGTTGGCGTTGGTCAGAAGGACCTCTGTGTTACTG                      | 82 |
| **Targ_HBV**              | TTCTCTACCTACATACCTGAGTTGGCGTTGGTCAGAAGGACCTCTGTGTTACTG                      | 82 |
| **Targ_RotavirusC**       | TTCTCTACCTACATACCTGAGTTGGCGTTGGTCAGAAGGACCTCTGTGTTACTG                      | 82 |
2.5 Acknowledgements

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2.6 References

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Chapter 3

Reversible blocking of antibodies using bivalent peptide-DNA conjugates allows protease-activatable targeting

Abstract

Antibody-based molecular recognition plays a dominant role in the life sciences ranging from applications in diagnostics and molecular imaging to targeted drug delivery and therapy. Here we report a generic approach to introduce protease sensitivity into antibody-based targeting by taking advantage of the intrinsic ability of antibodies to engage in multivalent interactions. Bivalent peptide ligands with dsDNA as a rigid linker were shown to effectively bridge the relatively large distance between the two antigen binding sites within the same antibody, yielding exclusively the cyclic 1:1 antibody-ligand complex. Size exclusion chromatography and small angle X-scattering were used to study the types of complexes formed between a model antibody and peptide-dsDNA conjugates displaying 1 or 2 peptide ligands and different linker lengths. Competitive binding assays using fluorescence anisotropy revealed that the interaction between bivalent peptide-dsDNA conjugate and antibody is 500-fold stronger than that of the monovalent peptide, allowing effective blocking of the antigen binding sites in a non-covalent manner. Cleavage of the linker between the peptide epitope and the DNA by matrix metalloprotease 2 disables this strong bivalent interaction and was shown to effectively restore the binding activity of the antibody in an in vitro binding assay. The approach presented here is broadly applicable, because it takes advantage of the Y-shaped multivalent presentation of antigen binding sites common to all antibodies and could be extended to control antibody activity by other input signals.

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3.1 Introduction

Antibodies play a central role in biomedicine with applications ranging from diagnostic assays and molecular imaging to targeted drug delivery and therapy. While the intrinsic affinity and specificity of antibodies for their target can be very high, the ability of antibody-based targeting to distinguish diseased cells from healthy cells is often limited by the presence of a substantial background of target receptors in healthy tissues.[1] Dual-specific targeting strategies that require the presence of specific proteases in addition to the overexpression of a receptor would be attractive for monitoring disease processes such as atherosclerosis, angiogenesis, and tumor metastasis that are characterized by overexpression of matrix metalloproteases (MMPs).[2–4] Protease-responsive ligands have recently been reported for cell penetrating peptides[5,6] and proteins targeting macrophages[7] and collagen.[8,9] In addition, Williams and coworkers developed a protease-activatable single chain variable fragment (scFv) by fusing a scFv to its cognate protein domain via a protease-cleavable linker.[10] However, these strategies require substantial engineering for every new target and cannot be easily combined with existing targeting strategies that still mostly depend on full length monoclonal antibodies. Here we explore a non-covalent strategy to block the antigen binding sites of native monoclonal antibodies in a protease-reversible manner by exploiting the intrinsic ability of full length antibodies to engage in multivalent interactions. Our approach is based on bivalent peptide ligands that effectively bridge the two antigen binding sites present in full-size monoclonal antibodies and in this way block the binding of the antibody to its target (Figure 3.1). Provided that the difference in affinity between bivalent and monovalent interactions is sufficiently large, this blockage can be reversed by introducing a protease recognition sequence between the peptide epitopes and the linker.

Figure 3.1: Concept of bivalent peptide-dsDNA conjugates to act as protease-activatable ligands for antibody (IgG,) blocking. The bivalent peptide ligand blocks the antigen binding sites of the IgG, by forming a stable and inactive, cyclic 1:1 complex. Once the linker between the epitope and dsDNA is cleaved by MMP-2, the monovalent epitopes dissociate allowing the antibody to bind to its intended target.
The interaction between multivalent ligands and antibodies has been an area of active research for some time, but typically these interactions result in higher order aggregates. For example, a variety of well-defined oligomeric complexes have been reported to form between anti-DNP (dinitrophenyl) IgG and bivalent and trivalent DNP ligands, which has been applied to suppress background binding to surfaces with a low density of binding sites and to develop a non-chromatographic method for IgG purification.[11–13] The main challenge in the development of effective bivalent ligands for antibodies is the large distance between the antigen binding sites. Flexible linkers are only effective in multivalent ligands that target binding sites that are close in space (< 6 nm).[14–20] The amount of possible linker conformations increases exponentially over distance and the enhanced affinity of a multivalent ligand will therefore decrease drastically for longer distances due to entropic constraints.[21] For example, calculations based on the wormlike chain model show that for a flexible 80 amino acid linker (GlyGlySer repeat),[22,23] the effective concentration to span the distance observed between 2 antigen binding sites in an antibody (120 Å) is 1000-fold lower than its effective concentration to span half of this distance, 60 Å (Figure 3.2).

**Figure 3.2:** The effective concentration ($C_{eff}$) for a flexible peptide linker depends on the number of the amino acids in the linker and the distance that the linker needs to bridge.

The structural rigidity of double stranded DNA (dsDNA) in combination with the possibility to rationally and precisely tune its dimensions make dsDNA an attractive scaffold for multivalent ligand design.[24–37] Recent studies have reported varying degrees of affinity enhancement for DNA-based multivalent ligands. Chaput and coworkers constructed synthetic antibody mimics, or synbodies, showing a 1000-fold increase in affinity towards the target protein, compared to the monovalent counterpart.[38,39] In a similar approach, Seitz and coworkers observed modest affinity enhancements towards their target when screening a library of bivalent ligands consisting of different linker lengths and display orientations.[40,41] In this work we show that bivalent peptide ligands with dsDNA as an easily accessible rigid linker effectively block the antigen binding sites of antibodies by almost exclusive formation of the cyclic 1:1 complex between antibody
and bivalent peptide-dsDNA ligand. Size exclusion chromatography and small angle X-scattering were used to study the types of complexes formed between a model antibody and peptide-dsDNA conjugates displaying 1 or 2 peptide ligands and different linker lengths. Competitive binding assays using fluorescence anisotropy allowed quantitative determination of the avidity of the bivalent interaction ($K_d^{\text{avidity}}$). Finally, we investigated the reversibility of antibody blocking by MMP-induced cleavage of the antigen peptides from the DNA linker. The generic approach presented here endowing protease sensitivity enables dual-specific targeting by native monoclonal antibodies (Figure 3.1).

### 3.2 Results and Discussion

In this study we used anti-HIV1-p17 (IgG$_1$), a murine monoclonal IgG$_1$-b12 antibody targeted against the p17 protein of HIV1. This antibody is commercially available and its binding to linear peptide epitope sequences has been well characterized in a number of independent studies.$^{[42-44]}$ Recent work on FRET-based sensor proteins for antibody detection suggested that flexible peptide linkers cannot efficiently bridge the distance between the two antigen binding sites in this antibody.$^{[44]}$ The X-ray structure of a related murine antibody of the same subclass shows a Y-shaped structure with a separation of $120$ Å between the two antigen binding sites$^{[45]}$, but in solution the two Fab arms might experience substantial flexibility with respect to the Fc part and each other.$^{[46]}$ To assess the adaptability of the IgG$_1$ structure we synthesized mono- and bivalent peptide dsDNA conjugates with DNA linkers consisting of 20, 35 and 50 bp, representing dsDNA linker lengths of 68, 119 and 170 Å, respectively. The length of the 35 bp dsDNA linker should thus optimally span the distance between the antigen binding sites according to the X-ray structure (PDB: 1HZ).

#### 3.2.1 Synthesis of peptide-ODN conjugates

Protease Cleavable Antigen Peptides (PCAP) consisting of an MMP2-recognition sequence (PLG|LAG) fused to an epitope sequence specific for anti-HIV p17 (ELDRWEKIRLRP) were prepared using standard Fmoc-mediated solid phase peptide synthesis and functionalized at their N-terminus with 3-maleimidoproprionic acid. For every linker length two complementary single strand pieces of DNA (ssDNA) with a thiol at the 5'-end were obtained and reduced prior to conjugation with the maleimide-functionalized peptide.$^{[35,47,48]}$ Analysis of the reaction mixture using a native 15% polyacrylamide gel revealed that this reaction proceeded to near completion for all oligonucleotides (Figure 3.3a and c). The peptide-ssDNA conjugates were subsequently purified to homogeneity by using size exclusion chromatography (SEC) and reverse phase HPLC followed by Q-ToF-MS characterization (Figure 3.3d). Monovalent ligand ($n$DS1x, with $n = 20$, 35 or 50 bp) and bivalent ligand ($n$DS2x) were prepared by hybridization of the (peptide-functionalized) complementary strands. Analysis using native 15%
polyacrylamide gel electrophoresis and SEC (Figure 3.3e and f) confirmed the formation of the expected nDS1x and nDS2x conjugates in high purity (> 95%).

**Figure 3.3:** Synthesis and characterization of mono- and bivalent peptide-dsDNA conjugates. a) Structure of maleimide functionalized PCAP-peptide, containing the protease cleavable (PLG|LAG) MMP-2 linker and the ELDRWEKIRLPR binding epitope to anti-HIV p17 (IgG1), ligated to 5'-end thiol functionalized oligonucleotide (n nucleotides). b) Scheme of bivalent PCAP-dsDNA conjugates (nDS2x, with n = 20, 35 or 50 bp). c) 15% native polyacrylamide gel was used to determine the conversion of peptide-DNA conjugation reactions for 20-, 35-, and 50-mer oligonucleotides. Lane 1, 5, 8: untreated thiol-functionalized ssDNA. Lane 2: TCEP-resin treated ssDNA (20 nt). Lane 3, 6, 9: reaction mixture leading strand. Lane 4, 7, 10: reaction mixture complementary strand. d) Pure ODN-peptide conjugates were analyzed by electrospray ionization in negative mode and direct injection. Representative Q-ToF mass spectrum of 20 nt oligonucleotide-PCAP (20-pep) conjugate (MW_{calc} = 8679.9 Da, MW_{obs} = 8680.4 Da). e) Native 15% polyacrylamide gel showing the hybridization of (functionalized) oligonucleotides of different lengths (nt) resulting in non-, mono- and bivalent ligands (nDS0x, nDS1x and nDS2x). f) Representative SEC-chromatogram of 35DS2x.
3.2.2 Structural characterization of antibody complexes

Complexes of different stoichiometry formed between antibodies and bi-and trivalent ligands can be readily distinguished using size exclusion chromatography (SEC) as was shown by Whitesides and coworkers.\cite{11,12} The complexes that can form between an antibody and a ligand depend on the length and the nature of the linker used in the bivalent ligand. We hypothesized that only the rigid linker which exactly and effectively spans the distance (35 bp, 120 Å) between the antigen binding sites of the antibody will yield a cyclic 1:1 closed complex. This compact complex can be distinguished from dimers or oligomer complexes which could be a result of incubating antibodies with ligands containing too long or too short linkers (20 and 50 bp, respectively).

SEC requires relatively high concentrations (µM) of IgG1 in order to analyze the complexes that are formed between the antibody and different lengths of bivalent ligands. Initially, the commercially available IgG1 and the bi- or monovalent ligands were injected separately using 1 µM concentration. This concentration is well above the monovalent dissociation constant of the epitope and was expected to result in stable complex formation. IgG1 and mono- or bivalent ligands were incubated for at least 15 minutes at room temperature prior to injection. Figure 3.4 shows SEC-traces for the complexes formed between IgG1 and the mono- or bivalent ligands of different linker lengths. For all linker lengths, incubation of 1 µM IgG1 with equimolar amounts of the bivalent nDS2x resulted in the disappearance of free IgG1 and nDS2x, with n being the amount of basepairs, and the formation of a new species with a retention time that is shorter compared to the free IgG1. In contrast, incubation of equimolar amounts of the antibody with monovalent nDS1x ligands resulted in the formation of a broader peak, which we assigned to a combination of 1:1 and 1:2 IgG1:nDS1x complexes. Importantly, the retention times of the IgG1:nDS1x complexes are shorter than the IgG1:nDS2x complexes, providing strong evidence that the bivalent ligand forms a more compact structure in which the ligand bridges the two antigen binding sites in a single antibody.

Additional evidence for this assignment was obtained by monitoring the absorbance at 260 nm and 280 nm for each peak. The absorbance at 260 nm is indicative for DNA and increases when the IgG1 is in complex with either the mono- or bivalent ligand (nDS1x or nDS2x, respectively). In order to assign the formed complexes more accurately we compared the calculated and the observed absorbance 260/280-ratios. Equation 3.1 was used to calculate the theoretical 260/280 nm ratios based on the stoichiometric ratio of binding (s), the known extinction coefficients (ε) of both IgG1 and the DNA-based ligands at 280 nm. The absorbance weight factors (w) of IgG1 and the DNA-based ligand were derived from their individual SEC-traces.

\[
\rho = \frac{W_{f(IgG)} \times \varepsilon_{280(IgG)} + s(W_{f(DNA)} \times \varepsilon_{280(DNA)})}{(\varepsilon_{280(IgG)} + s\varepsilon_{280(DNA)})} 
\]

(3.1)
Figure 3.4: SEC-analysis of the various complexes in mixtures containing monovalent or bivalent ligands (nDS1x or nDS2x, with n = 20, 35 or 50 bp) and IgG1. a, b, c) SEC-traces of bivalent ligands (black), IgG1 (magenta) and equimolar mixture (1 µM) of IgG1 and nDS2x (red) or nDS1x (blue) for linker lengths of 20 bp (a), 35 bp (b) and 50 bp (c). d) Comparison of SEC-traces for IgG1 in the absence (magenta) and presence of 20DS2x (black), 35DS2x (red) and 50DS2x (blue) to show differences in retention times.

The observed 260/280-ratio was consistent with the formation of a 1:1 IgG1:nDS2x complex, whereas the ratio observed for the complexes with the monovalent ligands indicated an IgG1:nDS1x stoichiometry that is closer to a 1:2 (Table 3.1). Almost exclusive formation of the cyclic 1:1 complex is observed for the 20 and 35 bp linkers (20DS2x and 35DS2x), whereas an additional peak with a lower retention time is observed for 50DS2x, indicating the formation of an additional higher molecular weight complex. We tentatively assign this peak to cyclic 2:2 complex, as the absorbance spectrum of this peak is consistent with the presence of equimolar amounts of IgG1 and 50DS2x (Figure 3.4 and Table 3.1).
Table 3.1: Overview of the retention times and the 260/280 nm ratios (calculated and observed) of the complexes formed between IgG1 and mono- or bivalent ligands of different DNA-linker lengths (nDS2x or nDS1x, with n = 20, 35 or 50 bp).

| Complex          | RT (min.) | Calculated | Observed |
|------------------|-----------|------------|----------|
| IgG1:20DS2x (1:1)| 8.53      | 1.06       | 1.06     |
| IgG1:20DS1x (1:1)| 8.27      | 1.12       | 1.14     |
| IgG1:20DS1x (1:2)| 8.10      | 1.33       | 1.21     |
| IgG1:35DS2x (1:1)| 8.30      | 1.27       | 1.24     |
| IgG1:35DS1x (1:1)| 8.06      | 1.29       | 1.31     |
| IgG1:35DS2x (1:2)| 7.78      | 1.48       | 1.41     |
| IgG1:50DS2x (1:1)| 8.04      | 1.39       | 1.36     |
| IgG1:50DS2x (2:2)| 7.16      | 1.39       | 1.40     |
| IgG1:50DS1x (1:1)| 7.85      | 1.40       | 1.45     |
| IgG1:50DS1x (1:2)| 7.57      | 1.56       | 1.52     |

Surprisingly, the SEC experiments show that bivalent peptide ligands with 20 and 35 bp linkers exclusively form cyclic 1:1 closed complexes and even the ligand with a 50 bp dsDNA linker still predominantly formed the cyclic 1:1 IgG1:50DS2x. This remarkable adaptability probably reflects the inherent flexibility present in the hinge region separating the Fab arms and the Fc part of the IgG1. To study this structural plasticity we performed small angle X-ray scattering (SAXS) experiments on the free antibody and the antibody in complex with 20DS2x and 35DS2x. The SAXS profiles show an increase in the slope and forward scattering intensity in the presence of both ligands (Figure 3.5a), signifying an increase in molecular weight and size. In addition, the globular structure of the protein transforms to a more stretched structure after addition of either 20DS2x or 35DS2x. For the free IgG1 a radius of gyration, \( R_g = 5.1 \pm 0.1 \) nm was obtained from a Guinier approximation, which is very similar to the radius of gyration previously reported for IgG1 b12 (\( R_g = 5.3 \pm 0.1 \) nm).\(^{[49]}\) Binding of dsDNA to the antibody resulted in a significant increase in the radius of gyration from \( R_g = 5.3 \pm 0.1 \) nm to \( R_g = 6.7 \pm 0.2 \) nm and \( R_g = 6.5 \pm 0.2 \) nm for the cyclic IgG1:20DS2x and IgG1:35DS2x, respectively. More information on the flexibility and solution structure of the free IgG1 and its cyclic 1:1 complex with 20DS2x and 35DS2x can be extracted from the radial distribution functions (RDFs) obtained via an inverse Fourier transformation of the scattering profiles (Figure 3.5b). These RDFs represent the probability of finding inter-atomic distances \( r \) within the protein (complex) weighted by the electron density of the corresponding atoms. The RDF of IgG1 shows a single broad maximum \( M \) at \( r = 4.5 \) nm reminiscent of the RDF profiles reported for IgG1 b12.\(^{[49]}\) This single, broad maximum at \( r \sim 5 \) nm is
Complex formation of IgG₁ with bivalent peptide-dsDNA has a significant impact on the RDF profile, shifting the maxima towards $M = 5.9$ nm and $M = 6.5$ nm for IgG₁:20DS₂x and IgG₁:35DS₂x respectively. The shape of the RDF profiles and the increase in $R_g$ and RDF peak maxima upon binding 20DS₂x and 35DS₂x confirm the structural plasticity of the IgG₁ and thus explain its ability to accommodate bivalent ligands with different DNA linker lengths.

![Figure 3.5: a) Small-angle X-ray scattering (SAXS) profiles and b) normalized radial distribution functions (RDFs) of IgG₁ (black squares) and monodisperse complexes formed between IgG₁ and the bivalent ligands IgG₁:20DS₂x (open spheres) or IgG₁:35DS₂x (open triangles). All samples were measured at a concentration of 0.8 mg mL⁻¹ in 10 mM PBS at pH 7.4.](image)

### 3.2.3 Investigation of the bivalency effect on antibody binding

Having established that the DNA-based bivalent ligands, 20DS₂x and 35DS₂x, exclusively form cyclic 1:1 complex we next investigated the effect on antibody affinity of a DNA-based bivalent ligand with respect to its monovalent counterpart. A fluorescence displacement assay was performed to assess the effectiveness of dsDNA to act as a multivalent scaffold for targeting antibodies. The affinity of the monovalent interaction was determined by direct titration of IgG₁ to 10 nM of fluorescently-labeled peptide (FPCAP) or fluorescently-labeled monovalent peptide-dsDNA (35FDS₁x). Binding of IgG₁ resulted in an increase in fluorescence anisotropy (Figure 3.6a), which could be analyzed by non-linear least-square analysis using a 2:1 binding model assuming independent binding sites resulting in dissociation constants of 29 nM and 33 nM, respectively (Appendix I). To allow a comparison of the relative affinities of the bivalent ligands (nDS₂x, with $n = 20$, 35 or 50 bp) to that of the monovalent peptide, a competition binding assay was employed (Figure 3.6b). First 30 nM of fluorescently-labeled bivalent ligand (35FDS₂x) was incubated with 62 nM IgG₁ to ensure almost complete conversion to the complexed form. Subsequently, increasing concentrations of the monovalent peptide (PCAP) or bivalent peptide-DNA conjugates (nDS₂x) were added. As expected, non-labeled 35DS₂x can effectively compete with its fluorescently labeled analog.
(35FDS2x), yielding the expected titration curve for competition between two ligands with the same affinity, i.e. 50% competition is observed at 30 nM of each ligand (Figure 3.6b). Similar titration curves were observed for bivalent peptide-DNA constructs with shorter (20DS2x) and longer (50DS2x) dsDNA linkers, indicating that the antibody can accommodate a variety of linker lengths with similar affinity (Figure 3.6c) which is in line with the observed SEC-traces. In contrast, much higher concentrations of the monovalent peptide (PCAP) are required to displace 35FDS2x from the antibody, providing clear evidence for strong multivalent binding by the bivalent nDS2x constructs (Figure 3.6b). Full equilibrium between both ligands and the antibody was reached very slowly and required overnight incubation of a separate sample for each ligand concentration. These slow exchange kinetics are not surprising, as bivalent interactions are known to exhibit slow dissociation kinetics.[51,52]

To quantify the gain in affinity between the monovalent peptide and the bivalent 35DS2x, a model was developed that describes the binding between bivalent ligands and IgG₁ in the presence of a monovalent competing ligand (Figure 3.6d and Appendix II).[53–55] The interaction between IgG₁ and 35DS2x is the product of an intermolecular and intramolecular binding step. The dissociation equilibrium constants \(K_{d\ mono}\) and \(K_{d\ inter}\) are equal to the \(K_{d}\) values determined in Figure 3.6a for monovalent binding of PCAP or 35DS1x to a single antigen binding site, (Figure 3.6a; \(K_{d\ mono} = K_{d\ inter} = 30\ nM\)). \(K_{d\ intra}\) describes the equilibrium between the ‘open’ (monovalent) and ‘cyclic’ (bivalent) 1:1 IgG₁:35DS2x complex, which is equal to \(K_{d\ inter}\) divided by the effective concentration \(C_{eff}\) (equation 3.2). \(C_{eff}\) is the effective concentration of the non-bound peptide-epitope with respect to the second antigen binding site in the open 1:1 IgG₁:35DS2x complex. The effective concentration depends on the length of the dsDNA linker and the distance between the two antigen binding sites.

\[
K_{d\ intra} = \frac{K_{d\ inter}}{C_{eff}}
\]

(3.2)

Using this model the concentration of the cyclic 1:1 complex can be described as function of \(K_{d\ inter}\), \(K_{d\ intra}\) and the total concentrations of IgG₁, 35FDS2x, and the competing ligand PCAP. Since \(K_{d\ intra}\) is the only parameter that is not known, its value could be obtained by fitting the concentration of the cyclic 1:1 complex as a function of the concentration of the competing monovalent peptide ligand, yielding \(K_{d\ intra} = 3.7\ \times\ 10^{-3}\). Based on this value, the effective concentration can be calculated to be 8 µM. The experimental binding data and the modeling support the observation by SEC of almost exclusive formation of a cyclic 1:1 IgG₁:35DS2x complex since this \(C_{eff}\) provided by the DNA linker is substantially higher than \(K_{d\ intra}\). The value of \(K_{d\ intra}\) also allows the overall affinity for the binding between IgG₁ and 35FDS2x to be calculated (equation 3.3), yielding a \(K_{d\ avidity}\) of 5.5 \(\times\ 10^{-11}\) M. Bivalent display of antibody binding peptides on a 35-bp
dsDNA linker thus resulted in a 545-fold increase in affinity for its target antibody compared to the affinity of the monovalent peptide.

\[
K_d^{\text{avidity}} = \frac{1}{4} K_d^{\text{inter}} \times 2 K_d^{\text{int-ra}}
\]

(3.3)

**Figure 3.6**: Quantification of binding of bivalent and monovalent ligands to IgG1 using fluorescence anisotropy. a) Binding of the monovalent FPCAP and 35FDS1x to IgG1 was fitted with a 2:1 binding model (dashed lines) assuming independent binding sites resulting in values of \(K_d^{\text{mono}}\) of 29 ± 3 nM and \(K_d^{\text{inter}}\) of 33 ± 4 nM (for FPCAP and 35DS1x respectively). For further analysis we assumed \(K_d^{\text{inter}} = K_d^{\text{mono}} = 30\) nM. b, c) Fluorescence displacement assay comparing the affinity of monovalent PCAP and nDS2x (with \(n = 20, 35\) or 50 bp). 30 nM of fluorescent 35DS2x (35FDS2x) was incubated with 62 nM IgG1 prior to the titration of non-fluorescent competing ligands (monovalent PCAP and nDS2x). The competitive displacement experiment between IgG1:35FDS2x and monovalent PCAP was analyzed using the model described in Appendix II using fixed values of the dissociation equilibrium constants \(K_d^{\text{inter}}\) and \(K_d^{\text{mono}} = 30\) nM. The dashed line shows the fit yielding a value of \(K_d^{\text{mono}} = 3.7 \times 10^{-3} \pm 0.6 \times 10^{-3}\). All measurements were performed in 10 mM PBS-buffer pH 7.4 at room temperature and every datapoint of the competition experiment was obtained after overnight incubation. d) Overview of equilibria formed in the titration of monovalent peptide to the complex of antibody and fluorescently labeled 35FDS2x.
3.2.4 Antibody activation by protease cleavage

Having established that bivalent peptide-dsDNA conjugates provide effective, high affinity ligands to mask the antigen binding domains of monoclonal antibodies, we next tested whether this blockage could be reversed by cleavage of the MMP2-recognition sequence that links the peptide epitope sequence to the DNA template. The MMP-2 cleavage of the PCAP-peptide at the recognition sequence (PLG|LAG) was verified by ESI-MS (Figure 3.7).

![Figure 3.7: HPLC-traces and m/z-spectra of the PCAP-peptide before (a) and after MMP-2 incubation (b). The MMP-2 incubation experiment was performed in MMP-buffer (50 mM TRIS, 200 mM NaCl, 10 mM CaCl₂, 10 µM ZnCl₂ at pH 7.4). a) The PCAP-peptide before MMP-2 incubation. Gradient RP-HPLC, 5-70% in 10 minutes. Maleimide-PLG|LAG-ELDRWEKIRLRPGG-CONH₂ ESI-MS: MW\textsubscript{calc} = 2383.7 Da; MW\textsubscript{obs} = 2384.1 Da. b) The PCAP-peptide after MMP-2 incubation. H\textsubscript{2}N-LAG-ELDRWEKIRLRPGG-CONH₂: Gradient RP-HPLC, 5-70% in 10 minutes. ESI-MS: MW\textsubscript{calc} = 1965.3 Da; MW\textsubscript{obs} = 1965.5 Da. Maleimide-PLG-COOH ESI-MS: MW\textsubscript{calc} = 436.5 Da; MW\textsubscript{obs} = 436.3 Da.]

Binding of the antibody to its native target, the HIV-1 viral coat protein p17, was assessed by immobilization of the p17 protein on a CM5 Biacore chip and monitoring association and dissociation of the antibody using Surface Plasmon Resonance (SPR). IgG\textsubscript{1} was mixed with a slight excess of 20DS2x and equilibrated for 30 minutes to allow formation of the cyclic 1:1 complex. As expected, addition of a slight excess of 20DS2x to IgG\textsubscript{1} led to an almost complete inhibition of binding to the p17 protein (Figure 3.8). Subsequent addition of MMP-2 and incubation at 37 °C resulted in a steady increase in binding activity until the original binding level of the non-blocked antibody was reached after 2 hours. The restoration of binding activity is the direct result of MMP-2 activity, as no release of inhibition was observed when the cyclic IgG\textsubscript{1}:20DS2x complex was incubated for 2 hours under similar conditions in the absence of MMP-2. This result shows that the cyclic 1:1 complex is indeed cleaved by MMP-2 resulting in the “free” IgG\textsubscript{1} that can bind to its target antigen.
3.3 Conclusions

Multivalency has long been known to be an essential property of antibody-mediated targeting and antibody-mediated aggregation and receptor clustering. Here we show that bivalent peptide ligands with dsDNA as a rigid linker can effectively bridge the relatively large distance between the two antigen binding sites within the same antibody, yielding exclusively the cyclic 1:1 antibody-ligand complex. The interaction between bivalent peptide-dsDNA conjugate and antibody is 500-fold stronger than that of the monovalent peptide, allowing effective blocking of the antigen binding sites in a non-covalent manner. In contrast to our findings, Baird and coworkers reported that bivalent DNP ligands using various lengths of dsDNA linkers did not affect its affinity to IgE significantly and resulted in a mixture of oligomeric complexes.\(^{[56,57]}\) These differences in avidity could in part be due to differences in hinge region flexibility between various antibody classes. The structural plasticity of the IgG\(_1\) class used in the present work allows a relatively wide range of dsDNA linkers to be used, but this flexibility is known to be more restricted in the IgE and IgA antibody families. Bivalent peptide-DNA conjugates may thus provide a useful system to further explore this interplay between multivalency and antibody plasticity on antibody mediated interactions.

The strong affinity enhancement observed for the bivalent peptide-dsDNA conjugates provides an efficient strategy to introduce protease sensitivity into antibody-based molecular recognition. Cleavage of the linker between peptide epitope and the DNA linker releases the bivalent interaction, resulting in complete restoration of the antibody binding activity. Because this approach takes advantage of the Y-shaped multivalent presentation of antigen binding sites common to all antibodies, our strategy is likely to be
broadly applicable. The thermodynamic analysis showed that in order for this approach to be successful, the dissociation constant for the monovalent interaction should be significantly lower than the effective concentration provided by the linker \( (C_{\text{eff}} = 8 \, \mu \text{M} \) in this case), but not so low as to also block the antigen binding site in a monovalent fashion. Finally, taking advantage of the large difference in affinity between bivalent and monovalent antibody ligands is not only an attractive approach to render antibody function sensitive to protease activity, but could also be envisioned to switch antibody activity by other input signals such as light or connect antibody activity to DNA-based circuits.

3.4 Experimental section

**Peptide synthesis.** Peptide synthesis was performed on an automated peptide synthesizer (Prelude, Protein technologies) following the standard Fmoc peptide synthesis protocol on a 100 µmol scale of NovaSyn TGR resin. The Fmoc-Lys(Mmt)-OH building block was used in order to introduce a fluorophore. Selective deprotection of the monomethoxytrityl (Mmt)-group was performed by repeatedly stirring in a mixture of trifluoroacetic acid, triisopropylsilane and dichloromethane (TFA/TIS/DCM: 2/5/93) for 5 minutes until the yellow color of the Mmt-carbocations disappeared. 5(6)-Carboxyfluorescein was coupled to the \( \varepsilon \)-amine for two times 8 hours using a 5-fold molar excess followed by a trytillation step. 3-Maleimido proprionic acid was coupled to the N-terminus of the peptide for two times 30 minutes using stoichiometric amounts of O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) and N,N-Diisopropylethylamine (DIPEA). ESI-MS spectra were recorded on an Applied Biosystems Single Quadrupole Electrospray Ionization Mass Spectrometer API-150EX in positive mode. Reversed phase HPLC was performed on a Shimadzu LC-8A HPLC system by using a VYDAC protein & peptide C18 column. Maleimide-PLGLAG-ELDRWEKIRLRPGG-CONH2 (PCAP, Protease Cleavable Antigen Peptide): Gradient RP-HPLC 20-40% in 30 min. ESI-MS: MW\text{calc}: 2383.7 Da; MW\text{obs}: 2383.7 Da. Maleimide-PLGLAG-ELDRWEKIRLRPGG-K(FAM)G-CONH2 (FPCAP, Fluorescent Protease Cleavable Antigen Peptide): Gradient RP-HPLC 20-40% in 30 min. ESI-MS: MW\text{calc}: 2927.3 Da; MW\text{obs}: 2927.3 Da.

**Synthesis and characterization of DNA-based mono- and bivalent ligands.** All non- and 5'-end thiol-functionalized oligonucleotides were designed to possess low self-complementarity and were synthesized by Eurofins MWG Operon and purified by reversed-phase HPLC (Table 3.2). The functionalized oligonucleotide sequences were synthesized with a six-carbon, thiol-modified 5'-linker. 20 µL of 100 µM thiol-functionalized oligonucleotide was mixed with 20 µL 10 M urea and 30 µL TCEP-resin and thoroughly mixed for one hour. After centrifugation, the supernatant was recovered and the resin was washed twice with 100 mM triethylammonium acetate (TEAA) at pH 7.0, followed by centrifugation in order to recover all oligonucleotide. Fifty equivalents of PCAP or FPCAP were mixed with the reduced oligonucleotide in 100 mM TEAA pH 7.0 containing 3 M urea and incubated overnight at 4 °C and subsequently analyzed with a native 15% polyacrylamide gel (Figure 3.3c). A Superdex 75 column (D = 3.2 mm, L = 300 mm), equilibrated with 100 mM TEAA at pH 7.0, was used to remove the excess peptide from the reaction mixture. Fractions containing oligonucleotide
Reversible Antibody Blocking

were collected, lyophilized and subsequently purified using an analytical GRACE RP-HPLC column (D = 2.1 mm, L = 50 mm) using a ten minute gradient of 5-70% acetonitrile in 100 mM TEAA pH 7.0 and with a flow rate of 0.2 mL min\(^{-1}\). Fractions containing oligonucleotide-peptide conjugate were lyophilized (3x) to remove excess TEAA and redissolved in MilliQ-water. After synthesis and purification typical yields of 20-40% were observed of which the lower yields corresponded to the 50 bases strand. Flow Injection Analysis (FIA) was performed using electrospray ionization mass spectrometry, Xevo G2 Q-ToF (Waters, USA). Electrospray ionization was achieved in the negative mode and by direct injection of the 10 μM solutions in 1:1 isopropanol/water + 1% triethylamine (pH 10) at room temperature at 100 μL min\(^{-1}\) and by applying -2.5 kV on the needle. The observed ions were deconvoluted using MaxEnt software. Equimolar amounts of leading and complementary strands were hybridized at room temperature for 30 minutes. The final hybridized constructs were analyzed on a native 15% polyacrylamide gel and size exclusion chromatography to check the purity of the hybridized ligands.

**Table 3.2**: Oligonucleotide (ODN) sequences used for the synthesis of mono- and bivalent ligands.

| Abbrev. | Oligonucleotide sequences (5'→3') | Bases |
|---------|----------------------------------|-------|
| ss_20   | HS-C6-GTCACGATGAACACTGTCTA      | 20    |
| ss_c20  | HS-C6-TAGACAGTTTCATCGGTGAC      | 20    |
| ss_20   | HS-C6-GTCACGATGAACACTGTCTAGACTCAGCAAGCA | 35 |
| ss_c35  | HS-C6-TGCTTGTGACTGCTAGACAGTTCATCGGTGAC | 35 |
| ss_50   | HS-C6-GTCACGATGAACACTGTCTAGACAGTTCATCGGTGAC | 50 |
| ss_c50  | HS-C6-GTCTAATCGACGCTCTGCTTAGACAGTTCATCGGTGAC | 50 |

**Fluorescence anisotropy studies.** Fluorescence anisotropy measurements were recorded on a Varian Cary Eclipse photoluminescence spectrometer using a Hellma quartz SUPRASIL cuvet. All measurements were performed at 20 °C. Samples were excited at 492 nm and monitored between 517 and 523 nm with slit widths of 10 nm. The affinity of Anti-HIV p17 (Zeptometrix, IgG1 b12 subclass) for the monovalent PCAP peptide was determined by titration of antibody to a solution of 10 nM fluorescein labeled peptide (FPCAP) in 10 mM PBS pH 7.3. Anisotropy was measured after a 20 minutes equilibration time to allow full equilibration. The fluorescence anisotropy titration data was analyzed using non-linear least square analysis using a binding model based on the assumption that the FPCAP binds to the two independent binding sites on the antibody (Appendix I).[58] The same instrumental settings were used for the competition experiments. 30 nM of fluorescent 35DS2x (35FDS2x) was incubated with 62 nM of IgG1 in 10 mM PBS pH 7.3. This concentration was chosen to ensure the optimal response upon titration of competing ligands. After addition of the non-fluorescent competing ligands, samples were incubated overnight to ensure complete equilibration of the ligands before measurement of fluorescence anisotropy. Every data point in the titration was obtained using a new sample of IgG1:35FDS2x complex. The competitive titration data were analyzed with a mathematical model (Appendix II) that includes binding of the bivalent ligand (35DS2x) to the
antibody, intramolecular cyclisation resulting in the cyclic 1:1 IgG1:35DS2x complex and competitive binding of PCAP to both binding sites of the antibody.

**Complex formation studies using size exclusion chromatography.** SEC-measurements were carried out on a Shodex KW403-4F column (D = 4.6 mm, L = 300 mm) with an isocratic solvent system of 10 mM PBS pH 7.4 and a flow rate of 0.3 mL min⁻¹. The complexes were analyzed with a UV detector and monitored at 260 and 280 nm. The complexes were formed by incubating the mono- or bivalent ligands and IgG1 (1 µM each) for 30 minutes at room temperature prior to injection on column. The concentration of the compounds was determined using the reported extinction coefficients for IgG (210,000 M⁻¹ cm⁻¹, λ = 280 nm) and dsDNA of 20, 35 and 50bp (318,878 M⁻¹ cm⁻¹, 554,956 M⁻¹ cm⁻¹, and 794,747 M⁻¹ cm⁻¹, respectively, λ = 260 nm).

**Small angle X-ray scattering experiments and data processing.** Data acquisition and data reduction. Synchrotron radiation X-ray scattering data were collected at two different beamlines. The data for the IgG1 sample were collected at the ID02 High Brilliance beamline at the European Synchrotron Radiation Facility (ESRF, Grenoble, France), operating at 12.46 keV. The data for the IgG1:20DS2x and IgG1:35DS2x samples were collected at the X12SA-cSAXS beamline at the Swiss Light Source of the Paul Scherrer Institut (PSI, Villigen, Switzerland), operating at 12.4 keV. The scattering intensity was measured as function of momentum transfer vector \( q = 4\pi\sin(\theta)/\lambda \) (where \( \lambda = 0.1 \text{ nm} \) is the radiation wavelength and \( 2\theta \) is the scattering angle) at a sample-to-detector distance of 3 m at the ID02 to cover an angular range of 0.033 < \( q < 1.69 \text{ nm}^{-1} \). At the cSAXS beamline a sample-to-detector distance of 7 m was used, covering an angular range of 0.077 < \( q < 1.68 \text{ nm}^{-1} \). Silver behenate was used for the beam center and \( q \)-scale calibrations. The IgG1 sample (0.8 mg mL⁻¹, 10 mM PBS buffer pH 7.4) was measured in a polycarbonate flow through capillary (\( d = 1.9 \text{ mm} \)) and the IgG1:20DS2x and IgG1:35DS2x samples (0.8 mg mL⁻¹, 10 mM PBS buffer pH 7.3) were measured in an Anton Paar quartz microcell capillary (\( d = 2 \text{ mm} \)) in a temperature controlled holder at \( T = 20^\circ \text{C} \). The 2D data were radially averaged and normalized to the intensity of the incident beam. After subtraction of the scattering of the buffer, the scattering curves were brought to absolute scale using the known scattering cross section of water, \( \Delta\Sigma/\Delta\Omega = 0.01632 \text{ cm}^{-1} \) for \( T = 20^\circ \text{C} \). Data collection at the ID02 beamline was performed using 10 frames of 0.3 s giving a total data collection time of 3 s per sample, and data collection at the cSAXS beamline was performed at 4 positions in the capillary with 50 cycles and 5 frames per point per cycles with an exposure time of 100 ms, giving a total data collection time of 100 seconds per sample. No radiation damage or aggregation was observed as determined by SEC before and after the SAXS measurements.

**Data processing.** A Guinier approximation (\( I(q) = I_0 \exp(-\frac{1}{2}R_g^2q^2) \)), with \( qR_g \leq 1.3 \) valid for globular proteins) was used to estimate the size (\( R_g \)) of the complex. The \( R_g \) and \( I_0 \) were determined from the slope and \( y \)-intercept of the Guinier plot \( \ln(I(q)) \) vs. \( q^2 \) using PRIMUS from the ATSAS software package. Indirect inverse Fourier transformation of the scattering data with GNOM yielded the radial distribution function (RDF), \( P(r) \), corresponding to the probable frequency of interatomic vector lengths (\( r \)) within scattering particle. The maximum linear dimension (\( D_{\text{max}} \)) was set to be \( \sim 3R_g \) and adjusted accordingly to give the best fit. The RDF was considered to be zero at \( r = 0 \text{ Å} \) and approaches zero at \( D_{\text{max}} \).
MMP2-promoted release of antibody blockage studied using SPR. Surface plasmon resonance (SPR) measurements were performed on a BiACore T100 (GE Healthcare). P17 protein (17 kDa, Fitzgerald Industries) was dissolved in 10 mM NaOAc (pH 5.0) at a concentration of 140 µg mL⁻¹. 500 RU of p17 protein was immobilized on a CM5-chip via standard EDC/NHS activation. 300 nM of IgG1 was mixed with a slight excess of 20DS2x (1:1.4) in MMP-buffer (50 mM TRIS, 200 mM NaCl, 10 mM CaCl₂, 10 µM ZnCl₂ at pH 7.4) and equilibrated at room temperature for 30 minutes. MMP-2 (Calbiochem) was added in a ratio of 1:15 (MMP2:20DS2x) and the reaction was incubated at 37 °C. Samples were taken at various time points, diluted in HBS-N buffer (10 mM HEPES and 150 mM NaCl at pH 7.4) with 1 mM ethylenediaminetetraacetic acid (EDTA) to stop enzyme activity and stored on ice until injection. All SPR experiments were performed at 25 °C using HBS-N as running buffer (10 µL min⁻¹). Samples containing 10 nM IgG1:20DS2x complexes were injected for 1500 seconds followed by a dissociation phase of 1200 seconds. Regeneration of the chip was performed by two injections of 10 mM glycine-HCl, pH 2.0 for 90 seconds. To correct for aspecific binding and buffer effects the response from a reference surface blocked with ethanolamine was subtracted.

Appendix I: Thermodynamic model to analyze the 2:1 binding of a monovalent ligand to a bivalent antibody with two independent binding sites.

Figure 3.9 shows the equilibria that need to be formulated to derive equations describing the binding of a monovalent ligand (L) to a bivalent antibody (A) with two independent but identical binding sites.

\[ A_{\text{tot}} = \text{initial concentration of bivalent antibody in mol L}^{-1} \]
\[ L_{\text{tot}}^{\text{mon}} = \text{initial concentration of monofunctional ligand in mol L}^{-1} \]
\[ K_{d}^{\text{mono}} = \text{reference dissociation constant monofunctional } A L_{\text{mon}}^{\text{mon}} \text{ in mol L}^{-1} \]
\[ L^{\text{mon}} = \text{free concentration of monovalent ligand in mol L}^{-1} \]
\[ A = \text{free concentration of bivalent antibody in mol L}^{-1} \]
\[ AL_{\text{mon}}^{\text{mon}} = \text{free concentration of antibody:ligand complex in mol L}^{-1} \]
\[ AL_{2}^{\text{mon}} = \text{free concentration of antibody:(ligand)₂ complex in mol L}^{-1} \]

Figure 3.9: Thermodynamic scheme describing the interaction between the antibody (A) and a monovalent ligand (L^{\text{mon}}).
Mass conservation equations were derived for the total concentration of antibody (\(A_{\text{tot}}\)) and monovalent ligand (\(L_{\text{tot}}^\text{mon}\)) as shown in Figure 3.9:

\[
A_{\text{tot}} = [A] + [AL_{\text{mon}}] + [AL_{2\text{mon}}]\tag{3.4}
\]

\[
L_{\text{tot}}^\text{mon} = [L] + [AL_{\text{mon}}] + 2[AL_{2\text{mon}}]\tag{3.5}
\]

The concentration of all complexes is expressed as a function of the equilibrium constant \(K_{d\text{mono}}\) and the free concentrations of antibody (\(A\)) and monovalent ligand (\(L_{\text{mon}}\)):

\[
[AL_{\text{mon}}] = \frac{2[A][L_{\text{mon}}]}{K_{d\text{mono}}}\tag{3.6}
\]

\[
[AL_{2\text{mon}}] = \frac{[A][L_{\text{mon}}]^2}{(K_{d\text{mono}})^2}\tag{3.7}
\]

The expression of all complexes (equations 3.6 and 3.7) combined with the mass conservation equations result in the following equations which were solved numerically using the Matlab function \texttt{fsolve} resulting in the concentrations of \(L_{\text{mon}}^\text{tot}\) and \(A_{\text{tot}}\) for given values of \(L_{\text{tot}}^\text{mon}\), \(A_{\text{tot}}\) and \(K_{d\text{mono}}\):

\[
A_{\text{tot}} = [A] + \frac{2[A][L_{\text{mon}}]}{K_{d\text{mono}}} + \frac{[A][L_{\text{mon}}]^2}{(K_{d\text{mono}})^2}\tag{3.8}
\]

\[
L_{\text{tot}}^\text{mon} = [L_{\text{mon}}] + \frac{2[A][L_{\text{mon}}]}{K_{d\text{mono}}} + \frac{2[A][L_{\text{mon}}]^2}{(K_{d\text{mono}})^2}\tag{3.9}
\]

Concentrations of the other complexes (\(AL_{\text{mon}}\) and \(AL_{2\text{mon}}\)) were calculated using equations 3.6 and 3.7.

Finally, the fluorescence anisotropy titrations in which the fluorescent, monovalent peptide epitope (FPCAP) or 35DS1x (35FDS1x) was titrated to a solution of bivalent antibody were fit according to the following equation:

\[
A = A_f + (A_b - A_f) \frac{[AL_{\text{mon}}] + [2AL_{2\text{mon}}]}{L_{\text{tot}}^\text{mon}}\tag{3.10}
\]
Where $A$ is the observed anisotropy, $A_f$ is the anisotropy value for free ligand ($A_f = 0.057$) and $A_b$ the anisotropy of the solution obtained under conditions of saturating ligand ($A_b = 0.1055$). The concentrations of complexes $AL^{mon}, AL^{mon}$ and $AL^{2mon}$ dependent on $K_{d}^{mono}$ and the total concentrations ($A_{tot}, L_{tot}, I_{tot}$) and were obtained using equation 3.6 and 3.7. Non-linear least square minimization of the data using equation 3.10 was performed with the Matlab-function *lsqnonlin*. Furthermore, the total concentrations of antibody and monovalent ligand are all known as well as the value of $A_f$ and $A_b$. Therefore, the only adjustable parameter in the non-linear least square minimization of the fluorescence anisotropy titrations is the intermolecular equilibrium constant $K_{d}^{mono}$.

Appendix II: Thermodynamic model to analyze the multivalent association of a bivalent ligand to a bivalent antibody in the presence of monofunctional competing ligand.

Figure 3.10 shows the equilibria that need to be formulated to derive the equations that describe the bivalent association of the bivalent ligand ($L$) to the antibody ($A$) in presence of a monovalent competing ligand (PCAP) denoted here as a monovalent inhibitor ($I$).

$A_{tot} = \text{initial concentration of antibody in mol L}^{-1}$

$L_{tot} = \text{initial concentration of bivalent ligand in mol L}^{-1}$

$I_{tot} = \text{initial concentration of monovalent inhibitor in mol L}^{-1}$

$K_{int,er}^{d} = \text{reference dissociation equilibrium constant mono-functional } AL \text{ hetero-coupling in mol L}^{-1}$

$K_{int,ra}^{d} = \text{dimensionless equilibrium constant for cyclization of } AL \text{ to yield } AL_{cyc}$.

$K_{d}^{mono} = \text{reference dissociation equilibrium constant for mono-functional } AI \text{ hetero-coupling in mol }$.

$[L] = \text{free concentration of bivalent ligand in mol L}^{-1}$

$[A] = \text{free concentration of bivalent antibody in mol L}^{-1}$

$[I] = \text{free concentration of monofunctional inhibitor in mol L}^{-1}$

$[AL] = \text{free concentration of antibody:ligand complex in mol L}^{-1}$

$[AL_{cyc}] = \text{free concentration of cyclized antibody:ligand complex in mol L}^{-1}$

$[AL_2] = \text{free concentration of antibody:(ligand)$_2$ complex in mol L}^{-1}$

$[AI] = \text{free concentration of antibody:inhibitor complex in mol L}^{-1}$

$[AIL] = \text{free concentration of antibody:ligand:inhibitor complex in mol L}^{-1}$

$[AI_2] = \text{free concentration of antibody:(inhibitor)$_2$ complex in mol L}^{-1}$
Figure 3.10: Thermodynamic scheme describing the interaction between the antibody (A), the bivalent ligand (L) and a monovalent inhibitor (I).

Mass conservation equations were derived for the total concentration of antibody ($A_{\text{tot}}$), bivalent ligand ($L_{\text{tot}}$) and monovalent inhibitor ($I_{\text{tot}}$) as shown in Figure 3.11:

\[
A_{\text{tot}} = [A] + [AL] + [AL_{\text{cyc}}] + [AL_2] + [AI] + [AI_2] + [AIL] \tag{3.11}
\]

\[
L_{\text{tot}} = [L] + [AL] + [AL_{\text{cyc}}] + 2[AL_2] + [AIL] \tag{3.12}
\]

\[
I_{\text{tot}} = [I] + [AI] + 2[AI_2] + [AIL] \tag{3.13}
\]

The concentration of all complexes is expressed as a function of the equilibrium constant $K_{d}^{\text{mono}}$, $K_{d}^{\text{intra}}$ and $K_{d}^{\text{inter}}$ and the free concentrations of antibody (A), bivalent ligand (L) and Inhibitor (I): 

\[
[AL] = \frac{4[A][L]}{K_{d}^{\text{inter}}} \tag{3.14}
\]
The expression of all complexes (equations 3.14 to 3.19) combined with the mass conservation equations result in the following equations which were solved numerically using the Matlab function \textit{fsolve} resulting in the concentrations of $L$, $A$ and $I$ for given values of $L_{\text{tot}}$, $A_{\text{tot}}$ and $K_{d}^{\text{mono}}$:

\begin{equation}
[AL_{\text{cyc}}] = \frac{2[A][L]}{K_{d}^{\text{inter}} K_{d}^{\text{intra}}}
\end{equation}

\begin{equation}
[AL_{2}] = \frac{4[L]^{2}[A]}{(K_{d}^{\text{inter}})^{2}}
\end{equation}

\begin{equation}
[AI] = \frac{2[I][A]}{K_{d}^{\text{mono}}}
\end{equation}

\begin{equation}
[AI_{2}] = \frac{[A][I]^{2}}{(K_{d}^{\text{mono}})^{2}}
\end{equation}

\begin{equation}
[AIL] = \frac{4[A][L][I]}{K_{d}^{\text{inter}} K_{d}^{\text{mono}}}
\end{equation}

Finally, the fluorescence anisotropy competition titrations in which the fluorescent, bivalent 3FDS2x was incubated with the bivalent antibody and displaced by a titration series of monovalent peptide epitope (Inhibitor, $I$) was fitted according to the following equation:

\begin{equation}
A_{\text{tot}} = [A] + \frac{4[A][L]}{K_{d}^{\text{inter}}} + \frac{2[A][L]}{K_{d}^{\text{inter}} K_{d}^{\text{intra}}} + \frac{4[L]^{2}[A]}{(K_{d}^{\text{inter}})^{2}} + \frac{2[I][A]}{K_{d}^{\text{mono}}} + \frac{[A][I]^{2}}{(K_{d}^{\text{mono}})^{2}} + \frac{4[A][L][I]}{K_{d}^{\text{inter}} K_{d}^{\text{mono}}}
\end{equation}

\begin{equation}
L_{\text{tot}} = [L] + \frac{4[A][L]}{K_{d}^{\text{inter}}} + \frac{2[A][L]}{K_{d}^{\text{inter}} K_{d}^{\text{intra}}} + \frac{8[L]^{2}[A]}{(K_{d}^{\text{inter}})^{2}} + \frac{4[A][L][I]}{K_{d}^{\text{inter}} K_{d}^{\text{mono}}}
\end{equation}

\begin{equation}
I_{\text{tot}} = [I] + \frac{2[I][A]}{K_{d}^{\text{mono}}} + \frac{2[A][I]^{2}}{(K_{d}^{\text{mono}})^{2}} + \frac{4[A][L][I]}{K_{d}^{\text{inter}} K_{d}^{\text{mono}}}
\end{equation}
\[ A = A_{\text{min}} + (A_{\text{max}} - A_{\text{min}}) \frac{[AL] + [AL_{\text{cyt}}] + [AIL]}{L_{\text{tot}}} \]  \hspace{1cm} (3.23)

Where \( A \) is the observed anisotropy, \( A_{\text{min}} \) is the anisotropy that would be observed in the presence of saturating concentrations of monovalent inhibitor \( I \) (\( A_{\text{min}} = 0.074 \)) and \( A_{\text{max}} \) the anisotropy in the absence of any monovalent inhibitor (\( A_{\text{max}} = 0.098 \)). Non-linear least square minimization of the data using equation 3.10 was performed with the Matlab-function \textit{lsqnonlin}. The value of the reference equilibrium constant \( K_{\text{int}} \), which is equal to \( K_{\text{mono}} \) and was fixed to 30 nM as found previously by analysis of the fluorescent anisotropy data in Figure 3.6a. The only adjustable parameter in the non-linear least square minimization of the competitive displacement data is the intramolecular equilibrium constant \( K_{\text{int}}^{\text{ra}} \).

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Chapter 4

ANTIBODY ACTIVATION USING DNA-BASED LOGIC GATES

Abstract

Oligonucleotide-based molecular circuits offer the exciting possibility to introduce autonomous signal processing in biomedicine, synthetic biology and molecular diagnostics. Progress in this field is hampered by a lack of generic strategies to connect oligonucleotide circuits to protein activity, however. Here we introduce bivalent peptide-DNA conjugates as generic, non-covalent and easily applicable molecular locks that allow the control of antibody activity using toehold-mediated strand displacement reactions. Using yeast as a cellular model system, reversible control of antibody targeting is demonstrated using low nM concentrations of peptide-DNA locks and oligonucleotide displacer strands. Introduction of two different toehold strands on the peptide-DNA lock allowed signal integration of two different inputs, yielding logic OR- and AND-gates. The range of molecular inputs could be further extended to protein-based triggers by using protein-binding aptamers.

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4.1 Introduction

Their synthetic accessibility and highly predictable binding properties make oligonucleotides (ODNs) ideal building blocks to construct molecular logical circuits.\cite{1,2} The principle of toehold-mediated strand exchange\cite{3,4} has proven particularly versatile, allowing the construction of robust circuits with a broad range of functionalities, including logic gates, signal amplification, signal thresholding, feedback control, and consensus gating.\cite{5-9} Oligonucleotide-based circuits are also attractive for their potential to be integrated in biological systems, which would enable the introduction of autonomous decision making in biomedicine, synthetic biology and molecular diagnostics.\cite{10-13} Such applications are still limited by a lack of generic approaches to interface ODN circuits with protein activity, however.\cite{14,15} So far, most examples to control protein activity using ODN-based circuits are based on the streptavidin-biotin interaction\cite{16,17} or use a limited number of protein-binding aptamers.\cite{18-22}

Scheme 4.1: Blocking antibodies using bivalent peptide-dsDNA conjugates allows reversible control of antibody targeting using toehold-mediated strand displacement. Disruption of the dsDNA linker generates more weakly binding monovalent peptide ligands, which dissociate to allow target binding.

Here we introduce a generic and easily applicable approach to use the output of DNA/RNA-based logic operations to control the activity of antibodies. Antibody-based molecular recognition plays a dominant role in the life sciences ranging from applications in diagnostics and molecular imaging to antibody-based therapy and targeted drug delivery. The ability of antibody-based targeting to distinguish e.g. diseased cells from healthy cells is often far from perfect, however, resulting in toxicity in therapeutic applications and increasing the background signal in molecular imaging. To increase the specificity of antibody-based molecular recognition, new strategies are required to make antibody activity controllable by the presence of other biomarkers such as microRNAs, enzyme activity or cell surface receptors.\cite{23,24} In an impressive display of molecular engineering the group of Church used DNA-origami to construct a locked DNA barrel...
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(termed a NanoRobot) to shield specific antibodies. Binding of platelet derived growth factor (PDGF) and several receptor-based biomarkers to aptamer-based locks on the NanoRobot, resulted in the opening of the DNA box, allowing the antibodies to bind to cell surface receptors.\cite{20} In our previous work we developed a generic and non-covalent strategy to block antibody targeting by making use of bivalent peptide-DNA conjugates. These self-assembling bivalent ligands can effectively bridge the two antigen binding sites present in monoclonal antibodies, which allowed control of antibody-based targeting in a protease-reversible manner.\cite{23} We realized that the use of dsDNA as a linker in bivalent peptide ligands provides an excellent opportunity to use the output of DNA-based logic operations to control the activity of antibodies (Scheme 4.1).

4.2 Results and Discussion

4.2.1 Synthesis of peptide-ODN conjugates

To explore the design principles for DNA-based control of antibody activation, we used an IgG1-type monoclonal antibody against the hemagglutinin epitope YPYDVPDY. This peptide epitope is derived from the influenza virus and binds to an anti-HA antibody with a monovalent affinity of 5 nM.\cite{25} Peptide-ODN conjugates (POCs) were synthesized by first reacting 5'-end amine-functionalized ODN with an excess of bifunctional linker Sulfo-SMCC. Following removal of unreacted Sulfo-SMCC, the maleimide-functionalized ODN was reacted with a 10-fold excess of the thiol-containing peptide (YPYDVPDYA-GGG-C), yielding the thioether-coupled POCs (Figure 4.1a-c, Table 4.1). The single stranded POCs were subsequently purified using reversed phase HPLC and characterized by Q-ToF-MS (Figure 4.1c and d). Monovalent ligand (nDS1x, with n = 20 or 35 bp) and bivalent ligand (nSMD2x), with one of the strands containing an additional 8 base toehold sequence to facilitate strand displacement, were obtained by hybridization of complementary (peptide-functionalized) ODN strands. Analysis using native 15% polyacrylamide gel electrophoresis confirmed the formation of the expected nDS1x and nSMD2x (Figure 4.1e).
Figure 4.1: Synthesis and characterization of mono- and bivalent peptide-dsDNA conjugates. a) Synthetic strategy for conjugation of peptide to oligonucleotide (ODN). The hetero-bifunctional linker Sulfo-SMCC was reacted with a 5'-end amine functionalized ODN. The maleimide moiety reacts with the cysteine-bearing HA-epitope. b) Reversed phase HPLC-trace and m/z-spectrum of purified HA-epitope (YPYDVPDYA-GGG-C). c) Reversed phase HPLC-trace of a peptide-oligonucleotide conjugation reaction with UV-absorption at 260 nm: oligonucleotide (ODN-10, ^), peptide-oligonucleotide conjugate (POC-10, #) and HA-epitope (*). The desired POC-fraction (#) was manually collected. d) Reversed phase HPLC-trace of the collected POC-10 fractions (#) to show the purity of the collected sample. This RP-HPLC trace is illustrative for all purified POCs used in this study. The pure POC-10 fraction was analyzed using a Xevo G2 QTof mass spectrometer by direct injection to confirm successful synthesis. MaxEnt Deconvolution Software was used to perform MS-analysis. e) Native 15% polyacrylamide gel showing formation of non-, mono- and bivalent peptide-DNA ligands (35DS0x, 35DS1x and 35SMD2x) consisting of a 35 bp dsDNA and an 8 base toehold.
4.2.2 Structural characterization of antibody complexes

Size exclusion chromatography (SEC) was used to study the complex formation between the bivalent ligand and the anti-HA antibody. Prior to analysis, complexes were formed at low micromolar concentrations using equimolar amounts of DNA-based mono- or bivalent ligand (20DS1x or 20SMD2x, respectively) and anti-HA antibody (IgG1) and incubated for 30 minutes at room temperature. Figure 4.2 shows the SEC-traces for the complexes formed between the anti-HA antibody and the mono- or bivalent ligand. The SEC-traces show a lower retention time for the monovalent ligand (20DS1x) compared to the bivalent ligand (20SMD2x) in complex with the anti-HA antibody (IgG1). The lower retention time of the anti-HA antibody in complex with the monovalent ligand is attributed to the increase in hydrodynamic volume compared to the more compact structure of the bivalent ligand in complex with the antibody. The formation of a cyclic 1:1 complex is associated with strong bivalent binding and was also observed previously between DNA-based bivalent ligands and the anti-HIV antibody.[23]

Figure 4.2: SEC-traces of bivalent ligand (20SMD2x, black), anti-HA antibody (IgG1, magenta) and equimolar mixture (1 µM) of IgG1 and 20SMD2x (red) or 20DS1x (blue).

4.2.3 Oligonucleotide triggered antibody activation monitored using flow cytometry

To verify efficient blocking of antibody targeting by the bivalent peptide-DNA locks, yeast cells were used that display the HA-epitope fused to the yellow fluorescent protein Citrine (Figure 4.3a). This system allows quantitative monitoring of cell-surface antibody binding by flow-cytometric analysis of the amount of fluorescently labeled antibody binding to Citrine-positive (and thus HA-displaying) yeast cells. Different concentrations of Alexa647-labeled anti-HA antibody were incubated with yeast cells displaying the HA-epitope and Citrine at its surface. The absolute number of yeast cells was kept constant,
but the volume of the incubations was increased for lower antibody concentrations to ensure at least 10-fold excess of antibody compared to the number of HA-epitope tags. Incubation was performed at room temperature for one hour and under continuous shaking followed by a washing step to remove unbound antibody and subsequently analyzed by Fluorescence Activated Cell Sorting (FACS). Monitoring the amount of cell-bound Alexa647-labeled anti-HA antibody as a function of antibody concentration yielded a $K_d$ of 6.3 ± 0.3 nM (Figure 4.3b), which is in good agreement with titration experiments using fluorescently labeled peptide.[25]

**Figure 4.3:** a) Yeast surface display. The HA-tag and Citrine are displayed as an Aga2 fusion protein on the surface of yeast. b) Titration experiment monitoring the binding of Alexa647-labeled anti-HA antibody to yeast cells displaying the HA-epitope. The data was fitted to a one-site binding model. c) Density dot-plot of yeast cells displaying HA-tagged Citrine labelled with 10 nM Alexa647-labeled anti-HA antibody shows a correlation between Citrine fluorescence and antibody binding to the HA-tag-displaying yeast cells (10,000 cells).

Next, the concentration of anti-HA antibody was kept at 1 nM, while changing the amount of bivalent peptide-dsDNA lock. Just a slight excess of the bivalent peptide-DNA lock already completely blocked the binding of fluorescently-labeled antibody to HA-tag-displaying yeast cells, showing that the interaction between the bivalent peptide-dsDNA ligand and the antibody is very strong (Figure 4.4a and 4.4b). To test whether the bivalent interaction can be disrupted via toehold-mediated strand displacement, a fully complementary oligonucleotide strand was titrated to the blocked antibody (1 nM antibody and 1.1 nM bivalent-peptide-dsDNA) and the re-activation of antibody targeting was monitored using FACS. To ensure complete thermodynamic equilibration, the displacement reaction was incubated for 2 h prior to FACS analysis. Figures 4.4c and 4.4d show that antibody binding can be completely restored by addition of low nM concentrations of the displacer strand. The final binding level that is reached is comparable to that obtained using the same concentrations of antibody and monovalent peptide-dsDNA. Similar results were obtained for locks containing 20 bp and 35 bp dsDNA linkers, which shows that the flexibility in the hinge region allows the antibody to accommodate different length of dsDNA linker, as was also observed in our previous work using an anti-HIV1-p17 antibody.[23] This flexibility is important, because it allows more design freedom when targeting different oligonucleotide inputs or more.
complicated logic gates. In subsequent experiments we decided to continue with the 20 bp dsDNA linkers.

![Antibody activation by toehold-mediated DNA strand displacement.](image)

**Figure 4.4:** Antibody activation by toehold-mediated DNA strand displacement. a) Histogram showing Alexa647 fluorescence for yeast cells incubated with 1 nM Alexa647-labeled anti-HA antibody in the presence or absence of 1 nM 35SMD2x. b) Titration experiment showing binding of anti-HA antibody (1 nM) as a function of the concentration of the bivalent peptide-DNA ligand 35SMD2x. c, d) Titration experiments showing restoration of antibody binding as a function of displacer strand concentration. Different concentrations of displacer strands (43 or 28 nucleotides, respectively ODN-11 or -12, Table 4.2) were added to 1 nM anti-HA antibody and 1.1 nM bivalent ligand (35SMD2x (c) or 20SMD2x (d)) and incubated for 2 h prior to FACS analysis. The amount of antibody binding was determined by obtaining the mean Alexa647 fluorescence of 10,000 cells. The mean fluorescence was normalized by dividing it by the mean fluorescence observed in the presence of 1.1 nM of the corresponding monovalent ligand (35DS1x or 20DS1x).

### 4.2.4 Antibody binding does not affect kinetics of toehold-mediated strand displacement

The experiments above show that an 8 nucleotide toehold provides sufficient thermodynamic driving force to overcome the favorable bivalent interaction between the antibody and the bivalent peptide-dsDNA lock. However, the kinetics of the strand-displacement reaction might be affected by binding to the antibody. To monitor the kinetics of the strand displacement reaction, a fluorophore-quencher pair was introduced
in the bivalent-dsDNA lock (20FQ2x) to allow direct monitoring of the strand displacement by measuring the increase in fluorescence in time (Figure 4.5). The bivalent ligand 20FQ2x, containing a 15 nucleotides toehold and a fluorophore-quencher pair (Fluorescein and IowaBlack, respectively), was formed by hybridization of POC-5 and -6 (Table 4.1).

The pseudo first order rate constant of the displacement reaction was measured as a function of toehold length (using 4 nM displacer strand) and as a function of displacer strand concentration (using a fixed toehold length of 8 nt). Surprisingly, very similar reaction rates were observed in the absence and presence of antibody in both cases (Figure 4.5). When using short toehold lengths and/or low concentrations of the displacer strand, the reaction rate is determined by formation of the toehold complex, whereas the DNA-displacement reaction itself becomes rate-limiting at a toehold size of > 8 nt and/or high concentrations of the input strand.[6,26] The similar kinetics thus suggest that invasion of the displacer strand is not hampered by binding of the peptide-DNA lock to the antibody and can freely rotate around the dsDNA linker during the strand displacement reaction.

The spectrofluorimetry studies of the displacement reactions were performed in a TE/Mg\(^{2+}\) buffer (10 mM Tris-HCl, 1 mM EDTA and 12.5 mM MgCl\(_2\) pH 8.0), which are the conditions that were also used in previous studies investigating strand exchange kinetics.[6] However, the experiments involving yeast were performed in phosphate buffered saline buffer (PBS) containing 0.1% BSA, which resembles more physiological conditions. Although the displacement reaction is slower in PBS, due to the absence of high divalent cation concentrations, the reaction still saturates during the incubation time of at least two hours used in the yeast experiments.

**Figure 4.5:** Scheme illustrating the displacement reaction of 20FQ2x in absence (a) and presence of anti-HA antibody (b). 20FQ2x has a toehold of 15 nucleotides and displacement can be performed with different complementary ODN-lengths (green; Table 4.3). Kinetics of the toehold-mediated strand displacement reaction in the absence (c + e) and presence of 1 nM anti-HA antibody (d + f) as a function of toehold length (c + d) and displacement strand concentration (e + f). All displacement reactions were performed in standard TE-buffer (pH = 8.0) including 12.5 mM MgCl\(_2\) using 1 nM 20FQ2x by monitoring the increase in fluorescence in time. The effect of toehold length was studied using 4 nM of the displacer strand. The effect of displacer strand concentration was studied using a constant toehold length of 8 nucleotides (ODN-34). Pseudo first order reaction rates derived from these experiments were plotted as function of toehold length (g) or as function of displacer strand concentration (h).
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Fluorescence vs. Time for different [ODN] concentrations and Toehold (nt) lengths.

- **a)** and **b)** illustrate the logic gate activation mechanisms.

- **c)** and **d)** show the fluorescence vs. time for different Toehold (nt) lengths with and without anti-HA.

- **e)** and **f)** demonstrate the fluorescence vs. time for different [ODN] concentrations with and without anti-HA.

- **g)** and **h)** display the rate constant (kobs) vs. Toehold length and Displacer strand concentration, respectively.
4.2.5 Antibody activation by DNA-based logic gates

Having established the principle of antibody activation using a single input strand, we next rendered antibody activation conditional on the presence of two oligonucleotides, each (partially) hybridizing with one of the linker strands (Figure 4.6a). To this end we constructed DNA-based bivalent ligands containing a 10 nucleotides single strand toehold at each side of a 20 bp dsDNA-linker (20LG2x, Table 4.1). Depending on the length of the overlapping sequences, this architecture could act as either an AND- or an OR-gate. As expected, addition of one of the fully complementary 30 bp input strands was sufficient to fully restore antibody targeting, representing an OR-gate (Figure 4.6b). OR-gate behavior was also observed for 25 nucleotides input strands, but only a small amount of activation was observed using a single 20 nucleotides strand (complementary to the 10 nucleotides toehold and 10 bp in the linker) even in the presence of a 20-fold excess of the input strand (Figure 4.6b). The background of 0.2 fluorescence units that was observed even in the absence of displacer strands is a consequence of the small excess of bivalent peptide-DNA lock that was used (1.1 nM vs 1 nM antibody), which results in a small amount of non-blocked antibody (see also Figure 4.4d).

Biomolecular logic gates do not provide a true/false output but a continuous output. Therefore we adopted the approach described by Nikitin and coworkers. The threshold between 0 and 1 was defined as the square root of the product of the maximum and minimum responses (equation 4.1).[27]

\[
\text{Output}_{\text{Threshold}} = \sqrt{\text{Output}_{\text{min}} \times \text{Output}_{\text{max}}} \quad (4.1)
\]

To identify the regime in which the system shows AND-gate behavior, we systematically varied the lengths of the two input strands between 15 and 20 nucleotides in steps of 1 nt. Figure 4.6c shows that addition of two 15 nucleotide strands is not sufficient to disrupt antibody-blockage. In this case strand invasion probably occurs, but the 10 bp that remain between the two linker strands are sufficient to preserve the bivalent ligand. However, stepwise addition of more nucleotides rapidly increases the amount of antibody activation, showing partial antibody activation by two 16 nucleotide strands and nearly complete activation using two 17 nucleotide strands. The amount of antibody activation is highly tunable and dependent on the number of remaining base pair interactions in the original linker strands, since a combination of a 16 nucleotides strand with an 18 nucleotides strand gives a comparable level of activation as two 17 nucleotide strands. The sharp transition between inhibition and activation is attractive because it allows the design of AND-gates with minimal leakage. It also provides maximal flexibility with respect to the length and sequence of the two input strands, allowing the integration of the outputs from two different DNA-logic circuits or two different miRNA sequences as triggers in AND- or OR-gate designs.
Figure 4.6: a) Control of antibody activity using 2 inputs yielding logic OR- or AND-gates. 1 nM anti-HA antibody was blocked by addition of 1.1 nM of a bivalent ligand consisting of a 20 bp linker (20LG2x), flanked by two 10 nt single strand overhangs. Following incubation with 24 nM input strand(s) for 2 h (Table 4.2), yeast cells were added and antibody binding was monitored by measuring the mean cellular Alexa647 fluorescence. b) OR-gate behaviour is observed for input strands with more than 20 nucleotides. c) AND-gate behaviour for combinations of two input strands depends on the length of the strands. All experiments were performed in duplo. Strand A length of 0 nt means that no displacer strand was added. The amount of antibody binding was determined by obtaining the mean Alexa647 fluorescence of 10,000 cells. The mean fluorescence was normalized by dividing it by the mean fluorescence observed in the presence of 30 nt strand A and B (30/30) and experiments were performed in duplo.
4.2.6 Controlling antibody activity using a protein binding aptamer

To further expand the type of molecular triggers, we also explored the possibility to control antibody activity using protein-binding aptamers.[28–30] A previously reported high-affinity thrombin aptamer was extended at its 5’-end with 6 nucleotides (A) such that it could partially hybridize with the displacer strand (D) forming an aptamer lock (A:D). The aptamer lock prevents D from binding to the toehold in the bivalent peptide-DNA lock (20Apt2x, Table 4.1). Binding of thrombin to the aptamer should result in release of the displacer strand to bind to the toehold on the peptide-DNA lock, resulting in strand displacement and antibody activation (Figure 4.7a and 4.7b).

Figure 4.7: Aptamer-mediated control of antibody-reactivation. a) Thrombin binding to A:D releases D resulting in the release of the toehold complement initiating the toehold-mediated strand displacement with the 8 nucleotide toehold present at the bivalent ligand (20Apt2x). b) A:D complex including the thrombin binding aptamer sequence GGTTGGTTGGTG (A, orange) the blocked toehold complement (purple, D) flanked by the remaining complement (green) of the bivalent ligand. c) A*:D complex is the full aptamer lock allowing the binding of thrombin to the aptamer sequence (orange, A*), but is not able to release D due to the stable duplex flanking the aptamer site. A*:D was used as a negative control. d) Control experiments to test the specificity of thrombin-mediated antibody activity. 1 nM of Alexa647-labeled anti-HA antibody and 1.1 nM of 20Apt2x were incubated prior to the addition of lock and / or thrombin. The amount of antibody binding was determined by obtaining the mean Alexa647 fluorescence of 10,000 cells.
Initially, the specificity of the system was tested by performing control reactions with thrombin that lacked the aptamer-displacer complex or when the aptamer-displacer was made so stable that thrombin binding could not release the displacer strand (A*:D, Figure 4.7c). As expected, the blocked Alexa647-labeled anti-HA antibody cannot be activated by thrombin in the absence of A:D or in presence of 100 nM full aptamer lock A*:D (Figure 4.7d). Addition of 100 nM displacer strand D alone resulted in a strong increase in antibody binding. This level of fluorescence represents the maximum level of antibody binding that can be expected following thrombin-induced aptamer switching. Mixing 100 nM A:D with an excess of thrombin does not result in the maximum level of fluorescence as was observed for the displacer strand D only control. Furthermore, addition of the aptamer-displacer complex (A:D) in the absence of thrombin also resulted in a small increase in antibody activation, which could be partially repressed by using a 3-fold excess of aptamer over displacer strand (300 nM A and 100 nM D). In an effort to further reduce background displacement we tried to elongate either A or D at the 5'-end by one base. Although the background displacement could be reduced, the binding of thrombin did not result in the release of strand D. Thrombin binding to this, one additional basepaired, lock does not provide enough driving force to release strand D and therefore favours the double helix and locked state.

The amount of antibody activation as a function of thrombin concentration was tested using the lock consisting of a 3-fold excess of aptamer over displacer strand (A:D). The titration of thrombin to the antibody which was pre-incubated with the bivalent peptide-DNA lock and aptamer lock showed a 2-fold increase in antibody targeting between 300 nM and 1 µM (Figure 4.8). The modularity of the approach used here should allow the same locked antibody to be activated by different aptamers. For future applications the aptamer could also be directly included in the dsDNA linker, although this may require extensive reengineering of the peptide-DNA lock for each new aptamer.

Figure 4.8: Titration experiment showing re-activation of antibody binding as a function of thrombin concentration. 1 nM of Alexa647-labeled anti-HA antibody and 1.1 nM 20Apt2x were incubated prior to the addition of the A:D complex (300 nM A and 100 nM D) and different concentrations of thrombin. The titration experiment was performed in duplo.
Chapter 4

4.3 Conclusions

Bivalent peptide-dsDNA conjugates provide effective non-covalent molecular locks that allow control of antibody activity using toehold-mediated strand displacement reactions. This new approach takes advantage of the large difference in affinity between a bivalent peptide-DNA ligand and the monovalent peptide-DNA ligand that forms as a result of a strand displacement reaction. The most important design constraint is that the monovalent affinity of the antibody-epitope interaction should be significantly lower than the effective concentration provided by the dsDNA linker, which has been estimated to be \( \sim 8 \, \mu\text{M} \),\(^{[23]} \) but not so low as to block antibody binding to a cell-surface receptor as a monovalent ligand. Our design compares favorably with strategies based on 3D DNA origami\(^{[20,31]} \) in its ease of construction and the possibility to use hydrolytically stable ODN analogs such as phosphorothioate oligonucleotides\(^{[32]} \) or peptide nucleic acids.\(^{[33]} \) Our approach uses the characteristic Y-shaped molecular architecture shared by all antibodies and provides a generic strategy to introduce autonomous signal processing in antibody-based targeting and exploit their molecular recognition properties in the field of DNA-based computing.

4.4 Experimental section

Peptide synthesis. The HA-epitope was synthesized manually following a standard Fmoc peptide synthesis protocol on a 100 µmol scale of Rink Amide MBHA resin. Peptide was cleaved from the resin by using a mixture of TFA / TIS / H₂O / EDT (92.5: 2.5: 2.5: 2.5% v/v) and continuous stirring for 3 hours followed by precipitation in ice cold ether. ESI-MS spectra were recorded on an Applied Biosystems Single Quadrupole Electrospray Ionization Mass Spectrometer API-150EX in positive mode. Reversed phase HPLC was performed on a Shimadzu LC-8A HPLC system by using a VYDAC protein & peptide C18 column. YPYDVPDYA-GGG-C (HA-epitope): Gradient RP-HPLC 20-40% in 20 min. ESI-MS: \( \text{MW}_{\text{calc}} = 1417.50 \text{Da} \); \( \text{MW}_{\text{obs}} = 1417.75 \text{Da} \).

Synthesis and characterization of DNA-based mono- and bivalent ligands. Oligonucleotides functionalized at their 5'-end with a primary amine via a C6-linker were purchased from Eurofins MWG operon or Eurogentec (Table 4.1). The oligonucleotides were dissolved in PBS (100 mM NaPi, 150 mM NaCl, pH 7.2) to a final concentration of 1 mM and 20 equivalents of Sulfo-SMCC (Thermo Scientific, solid) were added. Following incubation for 2 hours at room temperature with continuous shaking (850 rpm), excess Sulfo-SMCC was removed by (repeated) ethanol precipitation of the conjugate. The precipitated SMCC-oligonucleotides were pelleted by centrifugation (15,000 g for 15 minutes) and dried to air overnight at room temperature. Ten equivalents of HA-epitope peptide (1 mg mL⁻¹) were mixed with the SMCC-oligonucleotide in 100 mM triethyl ammonium acetate (TEAA) pH 7.0 and incubated for 2 hours at room temperature. RP-HPLC was performed using a GRACE Alpha-C18 (\( D = 4.6 \text{ mm}, L = 250 \text{ mm} \)), equilibrated with 100 mM TEAA at pH 7.0 to obtain pure POCs. A 5-50% acetonitrile gradient in 20 minutes was used at a flow rate of 1.0 mL min⁻¹ and monitored at 260 nm. POC-fractions were lyophilized (3x) to remove excess TEAA and redissolved in MilliQ-water. Mass spectrometry was performed as reported...
previously.\cite{23} In short, flow injection analysis (FIA) and electrospray ionization mass spectrometry was performed using a Xevo G2 Q-ToF mass spectrometer (Waters, USA). Electrospray ionization was achieved in the negative mode and by direct injection of the 10-50 µM pure POC-solutions in 1:1 isopropanol/water + 1% triethylamine (pH 10) at room temperature at 100 µL min\(^{-1}\) and by applying -2.5 kV on the needle. MaxEnt software was used to deconvolute the m/z-spectra. POC-1 (MW\(_{\text{calc}} = 7948.2\) Da; MW\(_{\text{obs}} = 7948.6\) Da), POC-2 (MW\(_{\text{calc}} = 10,357.0\) Da; MW\(_{\text{obs}} = 10,357.5\) Da), POC-3 (MW\(_{\text{calc}} = 12,600.2\) Da; MW\(_{\text{obs}} = 12,600.2\) Da), POC-4 (MW\(_{\text{calc}} = 15,046.0\) Da; MW\(_{\text{obs}} = 15,045.6\) Da), POC-5 (MW\(_{\text{calc}} = 13,006.7\) Da; MW\(_{\text{obs}} = 13,006.6\) Da), POC-6 (MW\(_{\text{calc}} = 8430.6\) Da; MW\(_{\text{obs}} = 8431.0\) Da), POC-7 (MW\(_{\text{calc}} = 11,006.2\) Da; MW\(_{\text{obs}} = 11,006.4\) Da), POC-8 (MW\(_{\text{calc}} = 10,920.1\) Da; MW\(_{\text{obs}} = 10,919.6\) Da), POC-9 (MW\(_{\text{calc}} = 10,478.8\) Da; MW\(_{\text{obs}} = 10,479.3\) Da) and POC-10 (MW\(_{\text{calc}} = 7860.0\) Da; MW\(_{\text{obs}} = 7859.8\) Da). Monovalent and bivalent peptide-dsDNA ligands were obtained by hybridizing equimolar amounts of leading and complementary strands at room temperature for 30 minutes. The purity of the final hybridized constructs was analyzed on a native 15% polyacrylamide gel.

Table 4.1: Oligonucleotides used for synthesis of peptide-oligonucleotide conjugates (POC). Toehold sequences are shown in bold.

| Abbrev. | Oligonucleotide sequences (5' \(\rightarrow\) 3') | nt | POC | Hybrid |
|---------|-----------------------------------------------|----|-----|--------|
| ODN-1   | H2N-C6-TAGACAGTTTCATCGGTGAC                    | 20 | 1   | 20SMD 2x |
| ODN-2   | H2N-C6-GTACCCGATGAAACTGTCTACAGACTCA            | 28 | 2   | 35SMD 2x |
| ODN-3   | H2N-C6-TGCTTGTGAGTCTGTAGACAGTTTCATCGGTGAC      | 35 | 3   | 20FQ2x |
| ODN-4   | H2N-C6-GTACCCGATGAAACTGTCTACAGACTCA            | 43 | 4   | 20LG2x |
| ODN-5   | H2N-C6-dT(FAM)CCACCGATGAAACTGTCTACAGACTCAGCAAGCA | 35 | 5   | 20Apt2 x |
| ODN-6   | H2N-C6-TAGACAGTTTCATCGGTGGA-IowaBlackFQ        | 20 | 6   |        |
| ODN-7   | H2N-C6-TAGACAGTTTCATCGGTGAGCTCAATCA            | 30 | 7   |        |
| ODN-8   | H2N-C6-GTACCCGATGAAACTGTCTACAAACCTCA           | 30 | 8   |        |
| ODN-9   | H2N-C6-CTAGAAGGACGTTGCAAGGCGACACTGT            | 28 | 9   |        |
| ODN-10  | H2N-C6-GCCTTGCAACGTCCTCTTAG                    | 20 | 10  |        |

Complex formation studies using size exclusion chromatography. SEC-measurements were carried out on a Shodex KW403-4F column (D = 4.6 mm, L = 300 mm) with an isocratic solvent system of 10 mM PBS pH 7.4 and a flow rate of 0.3 mL min\(^{-1}\). The complexes were analyzed with a UV detector and monitored at 260 and 280 nm. The complexes were formed by incubating the mono- or bivalent ligands and IgG\(_1\) (1 µM each) for 30 minutes at room temperature prior to injection on column. The concentration of the compounds was determined using the reported extinction coefficient for IgG (210,000 M\(^{-1}\) cm\(^{-1}\), \(\lambda = 280\) nm) and the pre-hybridized stocks of 10 µM for the dsDNA based mono- and bivalent ligands (20DS1x and 20SMD2x, respectively).
**Alexa Fluor 647 labeling of anti-HA antibody.** 150 µL of anti-HA antibody (1 mg mL⁻¹, IgG₁, clone: 2-2.2.14, Thermo Scientific #26183) was incubated with a 50-fold molar excess of NHS-Alexa647 (Life Technologies) and reacted for 45 minutes at room temperature by continuous shaking at 900 rpm, followed by immediate removal of the excess dye using MWCO-filters of 50 kDa (Millipore) and repeated washing steps resulting in an average labeling of 2.5 Alexa647-dyes per HA-antibody.

**FACS-settings.** All FACS measurements were done using a FACS Aria III equipped with a 70 µm nozzle. Citrine was excited by a 488 nm laser and detected through a 530/30 bandpass filter with a PMT voltage of 445 mV. Alexa647 was excited by a 633 nm laser and detected through a 660/30 bandpass filter with a PMT voltage of 670 mV. For all analyses, doublet cells were excluded by standard doublet discrimination with forward- and side scatter height versus width plots. Alexa 647 intensities were recorded for cells that were positive for Citrine. No spectral interference was observed between Citrine and Alexa 647. Therefore, no compensation was necessary. All samples were recorded with a flow rate of 1.0 mL min⁻¹.

**Cloning of HA-tag fused to Citrine and transformation of yeast strain.** The pCT-CON2 plasmid and the EBY100 yeast strain were a kind gift from Prof. dr. K. Dane Wittrup (MIT). The pCT-CON2 plasmid was digested with NheI and BamHI (New England Biolabs). Citrine was PCR-amplified from a previously described plasmid[34] using Phusion Polymerase (NEB) and 200 nM of forward (TAGTACGCTAGCATGGTGAGCAAGGGCGAG) and reverse primer (TACACGGATCCCTTGTACAGCTCGTCCATGCC). Thermo cycling was done using an initial 30 s denaturation step at 98 °C followed by 35 cycles of 10 s denaturation at 98 °C, 30 s annealing at 67 °C and 30 s extension at 72 °C and a final extension of 10 min. at 72 °C. The PCR product was purified using a commercial PCR purification kit (Qiagen) and digested with BamHI and NheI (NEB) according to the manufacturer’s instructions. Finally the Citrine-insert was ligated C-terminal to the HA-epitope into the linearized pCT-CON2 vector with T4 DNA ligase (NEB). The correct sequence of the resulting pCT-Citrine plasmid was verified by Sanger sequencing (BaseClear, Leiden, The Netherlands). pCT-Citrine was transformed into the EBY100 yeast strain with the LiAc/PEG/ssDNA method[35] and plated on SD-CAA Agar (15 g L⁻¹ Bacto agar (BD Biosciences); 6.7 g L⁻¹ Difco yeast nitrogen base without amino acids (BD Biosciences); 5.0 g L⁻¹ Bacto Casamino Acids (BD Biosciences); 20 g L⁻¹ D-(+)-glucose (Sigma Aldrich). A single colony was amplified in liquid medium, aliquotted and frozen in 5% glycerol/10% DMSO and used for all subsequent yeast display experiments.

**Yeast surface display.** Initial yeast surface display was performed following the protocol of Wittrup and coworkers.[36] Typically, yeast was grown overnight in 5 mL SDCAA medium (20 g dextrose, 6.7 g Difco yeast nitrogen base, 5 g Bacto casamino acids, 5.4 g Na₂HPO₄ in deionized H₂O to a volume of 1 liter and sterilized by filtration) at 30 °C, 220 rpm and passed (the next morning) 1 : 20 in fresh SDCAA-medium and was allowed to grow for another 3 hours at 30 °C to ensure that the yeast cells were in exponential growth when the induction was performed (OD₆₀₀nm = 1, absorbance of 1 equals 1 x 10⁷ cells per mL).
Yeast grown in SDCAA-media was pelleted by centrifugation (5 minutes at 2,500 g), SDCAA-medium was removed and the yeast cells were resuspended in SGCAA-medium (same composition as SDCAA, but 20 g galactose instead of dextrose) in order to induce the desired protein display at the yeast surface. Induction was performed at 20 °C, 220 rpm and for at least 20 hours. Prior to antibody titration or activation experiments, yeast was pelleted by centrifugation (5 minutes at 2,500 g) and the SGCAA-medium was removed. 1 mL of Phosphate Buffered Saline solution (1 L of PBS+: 8 g NaCl, 0.2 g, 1.44 g Na2HPO4 and 0.24 g KH2PO4 in ultrapure water and sterilized by filtration, pH 7.4) containing 0.1 % of BSA (Bovine Serum Albumin, Sigma Aldrich) was added to the pelleted yeast cells and they were thoroughly re-suspended. Cells were pelleted again by centrifugation for 1 minute at 12,000 g. The PBS+ was removed and the yeast cells were re-suspended in a fresh solution of PBS+. The OD of a 10-fold dilution in water was measured at 600 nm and the yeast cells were then diluted to an OD = 1 (1 x 10^7 cells per mL) in a typical volume of 1 mL PBS+.

**General incubation and washing procedures involving yeast cells.** All experiments involving yeast cells were performed in PBS+. Typical incubation times were one hour, unless stated otherwise, at room temperature and under continuous agitation. Washing of the yeast cells was performed by pelleting the yeast cells (1 minute, 12,000 g) and subsequently resuspending the cells in PBS+ (cold, 4 ºC) followed by an additional centrifugation step. Finally, the PBS was removed and the pelleted yeast cells were put on ice (also in between washing steps). Individual samples were resuspended in cold PBS+ prior to FACS-analysis.

**Bivalent ligand titration experiment.** 1 nM of Alexa647-labeled anti-HA antibody (200 µL) was incubated with different concentrations of bivalent ligand (35SMD2x) for one hour at room temperature. Subsequently, 10 µL of yeast cells (1 x 10^7 cells mL^-1) were added followed by an additional incubation step of one hour at room temperature under continuous shaking. A washing step was performed, as described above, prior to the FACS-analysis.

**Toehold-mediated strand displacement titration.** 1 nM of Alexa647-labeled anti-HA antibody (200 µL) was incubated with 1.1 nM of bivalent ligand (20SMD2x or 35SMD2x) for 30 minutes. The toehold strand displacement reaction was initiated by the addition of fully complementary ODN-strands ODN-11 (20SMD2x) or ODN-12 (35SMD2x) in a concentration range of 300 pM to 300 nM and incubated for one hour at room temperature. Subsequently, 10 µL of yeast cells (1 x 10^7 cells mL^-1) was added and the entire mixture was incubated for another hour at room temperature under continuous agitation to allow binding of the reactivated anti-HA antibody to the yeast cells. Washing steps were performed, as described above, prior to the FACS-analysis.

**Logic gate based toehold-mediated strand displacement (AND/OR gate).** The bivalent ligand used for this experiment consisted of POC-7 and POC-8 (20LG2x) creating a 10 nucleotides single strand toeholds at each side of the 20 bp dsDNA-linker. 1 nM of the Alexa647-labeled anti-HA antibody (200 µL) was incubated with 1.1 nM of bivalent ligand (20LG2x) for 30 minutes. 24 nM of one or two displacer ODN strands (ODN-13 to ODN-28) was added to the anti-HA antibody-20LG2x complex and incubated for one hour at room temperature. Subsequently, 10 µL of yeast cells (1 x 10^7 cells mL^-1) was added and the entire mixture was incubated for another hour at room temperature.
temperature under continuous agitation to allow binding of the reactivated anti-HA antibody to the yeast cells. Washing steps were performed, as described previously, prior to the FACS-analysis.

Table 4.2: Oligonucleotides used for toehold-mediated strand displacement experiments (Figure 4.4 and 4.6).

| Abbrev. | Oligonucleotide sequences (5’→3’) | nt | Experiment |
|---------|----------------------------------|----|------------|
| ODN-11  | TGAGTCTGTAGACGATTTCACTCGGTGAC    | 28 | Displacement of 20SMD2x |
| ODN-12  | CGACGCTCTGCTTGTAGCTGTAGACAGTTTCACTCGGTGAC | 43 | Displacement of 35SMD2x |
| ODN-13  | TGATTGAGCTGTACCGGATGAAACTGTCTA  | 30 |
| ODN-14  | GTTGAAGTTGTAGAGACAGTTTCACTCGGTGAC | 30 |
| ODN-15  | TGATTGAGCTGTACCGGATGAAACT      | 25 |
| ODN-16  | TGATTGAGCTGTACCGGATG            | 20 |
| ODN-17  | GTTGAAGTTGTAGACAGTTTCACTCGGTGAC | 20 |
| ODN-18  | TGATTGAGCTGTACCGGAT             | 19 |
| ODN-19  | GTTGAAGTTGTAGACAGTTCACTCGGTGAC | 19 |
| ODN-20  | TGATTGAGCTGTACCGGAT             | 18 |
| ODN-21  | GTTGAAGTTGTAGACAGTTCACTCGGTGAC | 18 |
| ODN-22  | TGATTGAGCTGTACCGGAT             | 17 |
| ODN-23  | GTTGAAGTTGTAGACAGTTCACTCGGTGAC | 17 |
| ODN-24  | TGATTGAGCTGTACCGGAT             | 16 |
| ODN-25  | GTTGAAGTTGTAGACAGTTCACTCGGTGAC | 16 |
| ODN-26  | TGATTGAGCTGTACCGGAT             | 15 |
| ODN-27  | GTTGAAGTTGTAGACAGTTCACTCGGTGAC | 15 |
| ODN-28  | GTTGAAGTTGTAGAC                 | 10 |

Thrombin-mediated antibody activation. Hybridization of ODN-29: CCAACCACAGTGGCGCTTTGCAACGCTCTTTCTAG and ODN-30: CACTGTGGTTGGTGTGGTTGG or ODN-31: CTAGAAGGACGTTGCAACGCGACACGTTGTTGGTGTGGTTGG yielded the locks A:D and A*:D, respectively. Annealing was performed in standard TE-buffer (10 mM Tris-HCl, 1 mM EDTA including 12.5 mM MgCl₂) by an initial heating step of 2 minutes at 95 °C and subsequently a ramp cool to 15 °C over a time course of 45 minutes using a standard thermal cycler. The Alexa647-labeled anti-HA antibody concentration was kept constant at 1 nM and incubated with a slight excess of bivalent ligand (20Apt2x) at 1.1 nM for 30 minutes. Subsequently, 100 nM displacer strand D, the A:D complex or the A*:D complex was added, either in the absence or presence of thrombin (30 nM to 6 µM) and incubated for 90 minutes at room temperature followed by the addition of the yeast cells. The entire mixture was incubated for another hour at room temperature under continuous
agitation to allow binding of the reactivated anti-HA antibody to the yeast cells. Washing steps were performed, as described above, prior to the FACS-analysis.

**Fluorescence measurements of displacement kinetics.** Fluorescence measurements were recorded on a Varian Cary Eclipse fluorescence spectrometer using a Hellma quartz Suprasil cuvette. All measurements were performed at 20 °C. Samples were excited at 495 nm (slit width of 5 nm) and monitored at 520 nm (slit width of 10 nm). 2 mL of 1 nM of 20FQ2x was incubated for 10 minutes in the presence or absence of 1 nM anti-HA antibody in TE-buffer (10 mM Tris-HCl, 1 mM EDTA and 12.5 mM MgCl2 pH 8.0) and the displacement reaction was monitored in time.

A fixed concentration of 4 nM of the displacer strand (ODN-32 to ODN-38, Table 4.3) was added to both samples (20FQ2x with or without anti-HA antibody), mixed and the fluorescence was immediately monitored. In a second set of experiments the effect of displacer strand concentration on the kinetics of the displacement reaction was studied in absence or presence of 1 nM anti-HA antibody, using a displacer strand with an 8 nucleotide toehold (ODN-34). After addition and brief mixing the fluorescence was monitored and plotted as a function of time. After subtracting the initial fluorescence, equation 4.2 was used to fit the displacement reactions, where $a$ is the maximum fluorescence obtained over the time course of the experiment and $k_{\text{obs}}$ represents the pseudo first order rate constant describing the displacement reaction.

$$v = a(1 - e^{-k_{\text{obs}}t})$$

(4.2)

**Table 4.3:** Oligonucleotides used in kinetics experiments in order to displace 20FQ2x (Figure 4.5).

| Abbrev. | Oligonucleotide sequences (5' → 3') | nt |
|--------|-----------------------------------|----|
| ODN-32 | TCTGTAGACAGTTTCATCGGTGGA          | 4  |
| ODN-33 | AGTCTGAGACAGTTTCATCGGTGGA         | 6  |
| ODN-34 | TGAGTCTGAGACAGTTTCATCGGTGGA       | 8  |
| ODN-35 | GCTGAGTCTGAGACAGTTTCATCGGTGGA     | 10 |
| ODN-36 | TTTCTGAGTCTGAGACAGTTTCATCGGTGGA   | 12 |
| ODN-37 | GCTTGTAGTCTGAGACAGTTTCATCGGTGGA   | 14 |
| ODN-38 | TGCTTGAGTCTGAGACAGTTTCATCGGTGGA   | 15 |

**4.5 Acknowledgements**

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Chapter 5

REVERSIBLE CONTROL OF CETUXIMAB BINDING USING (HETERO)BIVALENT PEPTIDE-DNA LOCKS

Abstract

Monoclonal antibodies have become important therapeutics for the treatment of a range of human diseases. However, their inability to distinguish antigens displayed on diseased and healthy cells can result in severe side-effects. Controlling the binding mode of therapeutic antibodies by a dual-specific targeting strategy increases the tissue specificity and can lower off-target side-effects. Here we report two different approaches to control the activity of the therapeutic antibody cetuximab using non-covalent peptide-dsDNA locks. The first approach employs a recently reported cyclic peptide mimotope to construct a homobivalent peptide-dsDNA ligand that effectively bridges the two antigen binding sites of cetuximab. In the second approach a different cyclic peptide was used that was recently shown to bind in a unique pocket at the interface of the variable and constant domains of the light and heavy chains. By linking this framework-binding cyclic peptide with the cyclic peptide mimotope, a novel hetero-bivalent peptide-dsDNA lock was constructed that controlled the activity of cetuximab at the level of the Fab-fragment. Both strategies showed effective blocking of the antigen-binding sites in a non-covalent manner. Cetuximab blocking could be reversed by matrix metalloprotease 2 induced cleavage or by toehold-mediated DNA strand displacement.
Chapter 5

5.1 Introduction

Over the past decade, monoclonal antibodies became an important class of targeted therapeutics for the treatment of a range of human diseases. More than 30 monoclonal antibodies have been FDA-approved for the treatment of different diseases, in particular oncologic and inflammatory diseases. Despite the high specificity and affinity of antibodies, their ability to distinguish diseased cells from healthy cells is sometimes limited due to the presence of the target receptor on both cells. For example, a common target such as the EGFR (epidermal growth factor receptor) shows up-regulation in various tumor types and can be targeted by cetuximab (IMC-225, Erbitux). However, the presence of high levels of EGFR in healthy skin leads to a severe acne-type rash when treated with cetuximab which limits the therapeutic window for treatment of these patients. A strategy to further increase the potency of EGFR-targeting therapeutic antibodies was described by Roovers and coworkers. A biparatopic anti-EGFR antibody that target distinct binding sites on EGFR, by combining fragments of cetuximab and matuzumab, showed improved potency. However, this increased potency does not address the off-target effects.

A promising approach to suppress off-target effects is to control the binding mode of a monoclonal antibody such that it is inactive in healthy tissue and only becomes active at the site of the disease by the action of proteases. Several groups have recently reported site-specific targeting by antibodies controlled by disease-associated proteases. Protease activity is upregulated in diverse pathologies including oncological and cardiovascular diseases. Williams and coworkers developed the first antibody prodrug by introducing a protease cleavable linker between a fusion of a single chain fragment (scFv) of cetuximab and its cognate protein, domain III of EGFR. Proteolytic activation resulted in an 8-fold increase in binding to the EGFR. Daugherty and coworkers introduced a protease-cleavable linker in between a fusion of a peptide epitope and anti-VCAM antibody and thereby targeting atherosclerotic plaques more specifically. In a similar approach, Desnoyers and coworkers developed an EGFR probody by fusing a 21-mer peptide mimotope, which masks the antigen binding site, and a cleavable substrate linker directly to the N-terminus of the full length cetuximab antibody. The masked cetuximab showed a 400-fold reduced binding to EGFR-expressing cells and reduced the toxicity of cetuximab to healthy tissue. Proteolytic activation of the masked cetuximab by proteases specific for the tumor tissue showed suppression of tumor growth in mice which was as effective as cetuximab itself. However, these strategies require substantial antibody engineering for every new target and are not easily applied to current therapeutic strategies that still mostly depend on full length monoclonal antibodies. Church and coworkers introduced a different strategy to control antibody binding by encapsulating the antibody (in this case human leukocyte antigen (HLA) specific scFv) in a DNA-origami based barrel. Instead of protease activation, aptamer-based locks were employed such that the barrel would only open in the presence of several receptor-based biomarkers, thereby allowing the scFv to bind to their cellular target.
previous work we developed a non-covalent strategy to block antibody targeting by making use of bivalent peptide-dsDNA locks.\textsuperscript{[16,17]} This approach takes advantage of the bivalent Y-shaped molecular architecture shared by all full length antibodies and does not require extensive protein or complex 3D DNA-engineering. Bivalent peptide-dsDNA locks of 20 or 35 bp were shown to effectively bridge the distance between the antigen binding sites of two different IgGs, which prevented binding to their target receptors. The blockage could be reversed by either protease cleavage\textsuperscript{[16]} or toehold-mediated DNA strand displacement.\textsuperscript{[17]} Although these proof-of-concept studies suggested that the approach is generic, its application for a clinically relevant therapeutic antibody remained to be demonstrated.

\textbf{Scheme 5.1:} Blocking by bivalent peptide-dsDNA locks prevents cetuximab binding to the epidermal growth factor receptor (EGFR). The blockage is reversible and cetuximab can be activated via protease cleavage. The transition from a tight binding and stable bivalent ligand to a weaker monovalent ligand allows cetuximab to bind to the EGFR.

Here we report the successful application of bivalent peptide-dsDNA locks to control the activity of the clinically relevant antibody cetuximab (C225). Cetuximab binds to the extracellular domain III of the epidermal growth factor receptor (EGFR) and prevents the binding of the natural ligands EGF and TGF\textsubscript{α}.\textsuperscript{[18]} Cetuximab is FDA-approved (in combination with chemo- or radiation-therapy) for the treatment of late stage colon cancer and metastatic head and neck cancer.\textsuperscript{[8,19,20]} Binding of cetuximab prevents growth-factor induced dimerization and autophosphorylation of EGFR, which results in cell growth inhibition or even shrinkage of the tumor (Scheme 5.1).\textsuperscript{[18,21]} Two cyclic peptides were used in this study to create homo- and heterobivalent peptide-dsDNA locks. One of these peptides acts as a mimotope and blocks the antigen binding site, whereas the other was recently found to bind at a cetuximab-specific binding site in the constant part of the Fab-framework. The use of these disulfide-containing peptides required the development of a different synthetic strategy for the synthesis of peptide-oligonucleotide conjugates (POCs) based on copper(I)-catalyzed alkyne-azide
cycloaddition (CuAAC). The homo- and hetero-bivalent peptide-dsDNA locks assembled from the POCs were shown to allow reversible control over cetuximab activity towards the EGFR-overexpressing tumor cell line A431.

5.2 Results

5.2.1 Characterization of cetuximab mimotopes

Using peptide phage display Riemer and coworkers identified several cyclic peptide sequences that showed specific binding to cetuximab.[22] The phage clone of the most prominent sequence, C-QFDLSTRRLK-C, was shown to compete for binding with cetuximab when mixed with cell extract of the EGFR-overexpressing cell line A431 suggesting binding to the antigen binding site of cetuximab. Since the affinity of these putative mimotope peptides was not reported, we started by synthesizing the three reported mimotope peptides by Fmoc-mediated solid phase peptide synthesis (SPPS). The peptides were elongated at the N-terminus with the MMP-2 cleavable linker, PLG|LAG, a flexible linker (GGS- or pentanoic acid) and fluorescein (Table 5.1). Following purification using reversed phase HPLC, correct formation of the disulfide-linked cyclic peptides was confirmed using ESI-MS (cQFD, cQYN and cVWQ).

The affinity of the cyclic mimotopes was determined by monitoring the increase in fluorescence polarization upon titration of cetuximab to 10 nM fluorescently labeled mimotope (Figure 5.1). The highest affinity ($K_d$ of $264 \pm 10.6$ nM, per antigen binding site) was observed for C-QFDLSTRRLK-C (cQFD), while C-QYNLSSRALK-C (cQYN) and C-VWQRWQKSYV-C (cVWQ) showed 3- and 4-fold weaker binding with a $K_d$ of $1.09 \pm 0.10 \mu M$ and $812 \pm 131$ nM, respectively. The observation that cQFD is the cetuximab mimotope with the highest affinity is in accordance with the findings of Riemer and coworkers who observed that this sequence was most prominent after multiple rounds of selection using phage display. Although this affinity is 9-fold weaker than the peptide used in our initial proof-of-concept study,[16] based on the effective concentration provided by the DNA-linker ($C_{eff} = 8 \mu M$) this affinity would be expected to still result in a ~60-fold increase in affinity for the bivalent ligand. cQFD was therefore initially selected as the mimotope of choice for the construction of bivalent ligands.
Figure 5.1: Determination of cetuximab affinities for three different peptide mimitopes using fluorescence polarization measurements. Titrations were performed in duplo using 10 nM of fluorescent peptide in a phosphate buffer at pH 7.0 (50 mM NaPi, 100 mM NaCl and 1 mg mL⁻¹ BSA). The individual binding curves were fitted to a one-site binding model.

5.2.2 Synthetic strategies for the conjugation of disulfide-containing cyclic peptides to oligonucleotides

In our previous work we used thiol-maleimide chemistry to conjugate a cysteine-containing linear peptide to a maleimide-functionalized oligonucleotide (ODN).[17] Since the presence of additional cysteines for cyclization prevents direct application of this approach here, several alternative strategies were explored (Scheme 5.2). The first strategy that was explored still relies on thiol-maleimide conjugation, but uses an N-terminal thiazolidine as a protected form of an N-terminal cysteine (Thz_cQFD, Table 5.1). The use of thiazolidine allows the formation of a disulfide bridge by the cysteines flanking the mimitope sequence prior to the selective deprotection of thiazolidine resulting in an N-terminal cysteine that can react with the maleimide functionalized ODN (Scheme 5.2a).
Scheme 5.2: Synthetic strategies for the conjugation of the cyclic mimitope (cQFD) to an oligonucleotide (ODN). The peptide substrate for MMP-2 (PLGLAG) is introduced between the N-terminal functional group and cQFD. 

a) Thiol-maleimide approach I: a protected cysteine, thiazolidine, is the functional group at the N-terminus of the peptide. The cysteines flanking the mimitope sequence are able to form a cyclic peptide by oxidation, followed by the conversion of a thiazolidine to a cysteine by methoxyamine. The maleimide-functionalized ODN enables the reaction with the N-terminal cysteine of the cyclic peptide.

b) Thiol-maleimide approach II: the cysteines flanking the mimitope sequence are able to form a cyclic peptide upon the addition of dibromomethyl-benzene (DBMB) followed by the conversion of a thiazolidine to a cysteine by methoxyamine. The maleimide-functionalized ODN enables the reaction with the N-terminal cysteine of the cyclic peptide.

c) Copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) approach: an azidophenyl-moiety (AzPhe) is the functional group at the N-terminus of the peptide. The cysteines flanking the mimitope sequence are able to form a cyclic peptide. The CuAAC-reaction between the azide-functionalized peptide and the commercially available alkyne-functionalized ODN can be performed.

Synthesis of the peptide and formation of the disulphide bond was successful as judged by ESI-MS. However, upon treatment with methoxyamine (MeONH₂) at pH 4.0 to convert the N-terminal thiazolidine to cysteine, we observed the formation of two isomeric products (Figure 5.2b). The isomerization was attributed to the disulfide shuffling of the three cysteines, despite the formation of the disulfide bridge prior to the deprotection of the thiol at acidic pH. Efforts were made to prevent the rapid disulfide shuffling by optimizing the reaction conditions (time, pH and temperature) or
by performing the cysteine-deprotection and the thiol-maleimide reactions simultaneously.\textsuperscript{[23]} Unfortunately, the disulfide exchange reaction was found to be faster than the reaction with maleimide at the acidic pH required for the reaction of MeONH\textsubscript{2} with thiazolidine.

\[ \text{AA}_{10} = Q-F-D-L-S-T-R-R-L-K \]

**Figure 5.2:** a) Schematic view of disulfide bridged mimotope flanked by MMP-2 substrate sequence (Thz_cQFD, Table 5.1) showing the conversion of a N-terminal thiazolidine to a cysteine upon treatment with methoxyamine. b) RP-HPLC trace of the Thz_cQFD before (dashed line) and after 2 hours (solid line) of the reaction in 250 mM MeONH\textsubscript{2}, 50 mM NaOAc and 2 M Gu\textsubscript{•}HCl at pH 4.0 and 37 °C. The reaction yielded two major products (# and *) with an identical mass of 2193.1 Da corresponding to the loss of carbon.

Therefore an alternative cyclization strategy that makes use of dibromomethylbenzene (DBMB) was explored. Previous studies used this strategy to cyclize peptides derived from phage display libraries to find tight binders to protein targets.\textsuperscript{[24,25]} Five equivalents of DBMB were mixed with the reduced peptide and incubated for one hour. ESI-MS confirmed the successful formation of a DBMB-bridged cQFD mimotope. The conversion of the thiazolidine to cysteine was performed as described previously and the crude reaction showed a major product corresponding to the DBMB-bridged QFD mimotope and the deprotected N-terminal cysteine (Figure 5.3a). To check whether the presence of the DBMB-bridge affects binding to cetuximab, the N-terminal cysteine of the DBMB-bridged mimotope was conjugated with maleimide-functionalized fluorescein followed by RP-HPLC purification and characterization (cQFD_DBMB(FAM), Figure 5.3b and Table 5.1). Titration of cetuximab using 10 nM fluorescent DBMB-bridged QFD mimotope revealed a 20-fold attenuation of binding affinity to cetuximab ($K_d$ of 5.4 ± 2.5 µM) compared to the disulfide bridged mimotope (Figure 5.3c). Since this $K_d$ is similar to the effective concentration provided by the DNA linker ($C_{\text{eff}} = 8$ µM), this affinity will not be sufficient to yield a bivalent ligand that effectively blocks cetuximab.
Figure 5.3: a) Crude RP-HPLC trace and m/z-spectrum of the DBMB-cyclized peptide after converting the N-terminal thiazolidine into a cysteine using 250 mM MeONH₂, 50 mM NaOAc and 2 M Gu-HCl at pH 4.0 and incubated for 2 hours at 37 °C. The reaction yielded one major product corresponding to the thiol-deprotected DBMB-bridged peptide (MW\text{calc} = 2296.7 Da, MW\text{obs} = 2296.4 Da). b) RP-HPLC trace and m/z-spectrum of the purified fluorescein (FAM) functionalized DBMB-bridged mimicope, cQFD\textsubscript{(FAM)}DBMB (MW\text{calc} = 2724.1 Da, MW\text{obs} = 2723.8 Da and Table 5.1). c) Fluorescence polarization measurements of cetuximab binding to DBMB-bridged cQFD. Titrations were performed in duplo using 10 nM of fluorescent peptide in a phosphate buffer at pH 7.0 (50 mM NaPi, 100 mM NaCl and 1 mg mL\textsuperscript{-1} BSA). The binding curve was fitted to a one-site binding model resulting in a \( K_d \) of 5.4 ± 2.5 µM.

As the disulfide-bridge was found to be important for binding, we decided to change our conjugation method and replaced the thiazolidine at the N-terminus of the cQFD mimicope by an azidophenyl-moiety to allow copper(I)-catalyzed [3+2] cycloaddition with an alkyne-functionalized ODN (Scheme 5.2c and Table 5.1). Optimal conjugation was observed when performing the reaction in a 1:1 mixture of H\textsubscript{2}O and dimethylsulfoxide (DMSO) for 150 minutes at 37 °C with 10-fold excess of peptide (AzPhe\textsubscript{cQFD}(FAM), Figure 5.4a), 0.1 mM coppersulfate, 5 mM sodium ascorbate and 0.5 mM THPTA-ligand to protect the peptide and ODN from copper-byproducts and radicals and/or peroxides.\[^{26}\] Figure 5.4b shows a characteristic reversed phase HPLC trace of the crude reaction mixture. Reaction yields were 50 - 90 % depending on the length of the ODN. The peptide-ODN conjugates (POC-1 to 6, Table 5.2) were purified using RP-HPLC and the peak corresponding to the POC was characterized by Q-ToF-MS. Figure 5.4c and d shows the characteristic analysis by mass spectrometry for the POC resulting from a reaction of a 35 nt ODN and the fluorescein- and azide-functionalized cQFD mimicope.
5.2.3 Characterization of DNA-based mono- and bivalent ligands binding to cetuximab

Fluorescently-labeled monovalent (35FDS1x) and bivalent dsDNA conjugates (35FDS2x) were obtained by hybridization of complementary peptide-oligonucleotides (fluorescent cQFD-functionalized, POC-4 and POC-6 in Table 5.2). Analysis using native 15% polyacrylamide gel electrophoresis confirmed the formation of 35FDS1x and 35FDS2x (Figure 5.5a). The affinities of the mono- and bivalent ligands were determined...
by direct titration of cetuximab to 20 nM fluorescently labeled cQFD mimitope, 35FDS1x or 35FDS2x. Surprisingly, both the mono- and bivalent dsDNA-based ligands bind weaker to cetuximab (\(K_d\) of 4.51 ± 1.59 µM and 901 ± 355 nM, respectively) than the monovalent cQFD mimitope (\(K_d\) of 278 ± 42 nM). This ~10-fold attenuation could be due to electrostatic repulsion between cetuximab and the negatively charged dsDNA-based ligands. This hypothesis was confirmed, since cetuximab binding to 35FDS1x was found to be 9-fold stronger in the presence of 1.5 M NaCl (\(K_d\) of 544 ± 46 nM), whereas the affinity for cQFD was not affected. As expected, under these conditions of high ionic strength the affinity for the bivalent ligand 35FDS2x was even further increased, yielding a \(K_d\) of 70 ± 14 nM (Figure 5.6).

\[\text{Figure 5.5: a) Native 15\% polyacrylamide gel showing the hybridization of (fluorescent peptide functionalized) oligonucleotides resulting in non-, mono- and bivalent ligands of 35 bp (35DS0x, 35FDS1x and 35FDS2x, respectively). b) Fluorescence polarization assay monitoring cetuximab binding to monovalent cQFD, mono- and bivalent dsDNA-based ligands, 35FDS1x and 35FDS2x. Titrations were performed in duplo using 20 nM of fluorescent ligand in a phosphate buffer at pH 7.0 (50 mM NaPi, 100 mM NaCl and 1 mg mL\(^{-1}\) BSA). The individual binding curves were fitted to a one-site binding model corrected for the number of antigen binding sites resulting in a \(K_d\) of 278 ± 42 nM for the monovalent peptide AzPhe_cQFD(FAM) and a \(K_d\) of 4.51 ± 1.59 µM and 901 ± 355 nM and for the mono- and bivalent ligand, respectively.}\]
Control Cetuximab Activity

Figure 5.6: a) Fluorescence polarization assay monitoring cetuximab binding to monovalent cyclic peptide, mono- and bivalent dsDNA-based ligands, 35FDS1x and 35FDS2x in presence of 1.5 M NaCl. Titrations were performed in duplo using 20 nM of fluorescent ligand in a phosphate buffer at pH 7.0 (50 mM NaPi, 1.5 M NaCl and 1 mg mL⁻¹ BSA). The individual binding curves were fitted to a one-site binding model corrected for receptor depletion resulting in a $K_d$ of 284 ± 28 nM for the monovalent Az_cQFD(FAM) and a $K_d$ of 544 ± 46 nM and 70 ± 14 nM for the mono- and bivalent ligand, respectively.

5.2.4 Alternative strategies to control the binding mode of cetuximab

In an effort to increase the affinity of the original cQFD peptide, yeast display was used to screen a library of single-point mutation variants, yielding three mutations that individually provided only a small increase in affinity. Fluorescence polarization titrations using a triple mutant of the cQFD peptide (combining all three mutations, Table 5.1) showed that the mutations were additive, providing an increase in cetuximab affinity to a $K_d$ of 62 ± 4 nM (Figure 5.7a). This 4-fold increase in monovalent affinity would translate in a substantial increase in bivalent affinity. However during the course of this work Williams and coworkers reported that the cQFD peptide does not bind to the antigen binding site of cetuximab. In contrast to previous assumptions, the crystal structure of a cQFD-Fab fragment complex revealed that the cyclic peptide targets a unique pocket formed by the interface between the 2 variable domains and the (first) constant domains of the heavy and light chains, which was confirmed by the fact that binding of cQFD did not interfere with the binding of cetuximab to EGFR. [27] Fortunately, at about the same time Desnoyers and coworkers reported a different peptide sequence, QGQSGQCSPRGCPDGYVMY, that apparently did bind to the antigen binding site of cetuximab. [14] While the two cysteines in the sequence suggested the presence of a disulfide-bridge also in this peptide, the affinity of this peptide (QGQ) to cetuximab was not reported. To determine the affinity of the QGQ-peptide, a fluorescence polarization
assay was used to monitor the binding of cetuximab to cQGQ peptide functionalized with an N-terminal fluorescein, yielding a $K_d$ of 293 ± 89 nM (Figure 5.7b).

**Figure 5.7:** a) Fluorescence polarization assay to determine the difference in affinity between the wild-type cQFD and the triple mutant cQFD(3m) (Table 5.1) to the unique pocket within the Fab-framework of cetuximab (C225) resulting in a $K_d$ of 264 ± 9.2 nM and 61.6 ± 3.9 nM, respectively. b) Fluorescence polarization assay to determine the affinity of cQGQ-mimotope to the antigen binding site of cetuximab (C225) resulting in a $K_d$ of 293 ± 89 nM. Titrations were performed in duplo using 10 nM of fluorescent peptide in a phosphate buffer at pH 7.0 (50 mM NaPi, 100 mM NaCl and 1 mg mL$^{-1}$ BSA). The individual binding curves were fitted to a one-site binding model.

Having confirmed that the cQGQ peptide binds to the antigen binding site of cetuximab with sufficient affinity, two different types of peptide-dsDNA molecular locks were designed (Scheme 5.3a). In the first approach the cQGQ was used to construct a homo bivalent peptide-dsDNA ligand following the design reported previously. Two different constructs were designed in which the cQGQ peptide was connected by a 20 bp or 35 bp dsDNA-linker. To allow reactivation of cetuximab via protease activity, an MMP-2 cleavage-site (PLG|LAG) was introduced between the cQGQ-peptide and the dsDNA-linker. In addition, 10 nucleotide single-strand toeholds were introduced at each side of the dsDNA linker to allow control of cetuximab activity via toehold-mediated strand displacement.

The availability of a peptide that targets the pocket in the Fab-framework (cQFD and triple mutant), provides an opportunity to explore a second strategy to reversibly block the antigen binding site of cetuximab using hetero bivalent dsDNA-based ligands (Scheme 5.3b). In this new approach, a hetero bivalent peptide-dsDNA ligand was used in which cQGQ- and cQFD(mutant)-peptides were coupled to a 10 nucleotides single strand at each side of a 20 bp dsDNA-linker. The 20 bp dsDNA-linker was expected to be sufficient for bridging the distance between the antigen binding site and the unique pocket within the Fab-framework of cetuximab. The 10 nt single strands create flexibility to allow the simultaneous binding of cQGQ- and cQFD-peptide at the two separate positions and should enable cetuximab activation via toehold-mediated DNA strand displacement or protease activity (Scheme 5.3b).[17]
Scheme 5.3: Blocking of cetuximab using homo-bivalent (a) or hetero-bivalent peptide-dsDNA locks (b). Cetuximab can be reactivated using MMP-induced cleavage of the linker or toehold-mediated strand displacement. Cetuximab activation by protease is possible due to the presence of the PLG|LAG sequence between the mimotope and DNA linker. Addition of a full complementary ODN-strand initiates toehold-mediated DNA strand displacement and the stable bivalent ligand undergoes a transition to the weaker monovalent ligand and thereby activating cetuximab. a) The homo-bivalent lock contains solely the cetuximab masking peptide: QGQSGQCISPRGCPDYVMY (orange triangle) flanked by the MMP-substrate peptide sequence PLG|LAG. b) The hetero-bivalent ligand contains the cetuximab masking peptide cQGQ as well as the Fab-framework binding peptide cQFD(wt) or cQFD(3m) (green triangle).

The conjugation of the azide-functionalized cQFD(wt)-, cQFD(3m)- and cQGQ-peptides (Table 5.1) to alkyne-functionalized ODN was achieved using the same CuAAC-reaction conditions as described above. POC-7 to -12 were purified using RP-HPLC and monovalent ligand and bivalent ligands were obtained by hybridization of complementary (peptide-functionalized) ODN strands. Analysis using native 15% polyacrylamide gel electrophoresis confirmed successful formation of cQGQ-functionalized homobivalent DNA-ligands (Figure 5.8a) and cQGQ- and cQFD(3m)-based heterobivalent DNA-ligands (Figure 5.8b) as well as the monovalent ligands.
Figure 5.8: Native 15% polyacrylamide gels showing the hybridization of mono- and bivalent ligands. a) Mono- and homobivalent ligands (nDS1x or nDS2x) based on the cetuximab’s antigen binding site binding peptide cQGQ and different dsDNA-linkers (n = 20 or 35 bp). b) Mono- and heterobivalent ligands (DS1x or DS2x) based on the wild-type (cQFD) and triple mutant (cQFD(3m)) combined with the antigen binding site binding peptide cQGQ (20bp DNA-linker).

To verify efficient blocking of cetuximab by the homo- and heterobivalent peptide-dsDNA locks, the EGFR-overexpressing A431 cell line (human epidermoid carcinoma) was used. Binding of Alexa647-labeled cetuximab (C225) to A431-cells was quantified by flow cytometric analysis. Different concentrations of Alexa647-labeled cetuximab were incubated with A431-cells for 30 minutes at room temperature, to minimize cetuximab-EGFR internalization by the A431-cells, and subsequently analyzed by Fluorescence Activated Cell Sorting (FACS). Monitoring the amount of A431-cell bound Alexa647-labeled cetuximab as a function of cetuximab concentration yielded an apparent $K_d$ of 407 ± 76 pM (Figure 5.9a), which is in good agreement with previous reported affinities ($K_d$ of 390 pM) of cetuximab towards EGFR present on A431-cells.[28]

Figure 5.9: a) Titration experiment monitoring the binding of Alexa647-labeled cetuximab (C225) to A431-cells with EGFR expressed at the surface. The binding curve was fitted according to a one-site binding model resulting in a $K_d$ of 407 ± 76 pM. b) Binding of 1 nM Alexa647-labeled Cetuximab to A431-cells when pre-incubated with 100 nM monovalent ligand (20DS1x_cQGQ) or 50 nM bivalent peptide-dsDNA lock (homo or hetero). The triple mutant, cQFD(3m), was used in combination with the cQGQ peptide for the heterobivalent peptide-dsDNA lock. The amount of cetuximab binding was determined by obtaining the mean Alexa647-fluorescence of 10,000 cells and the experiment was performed in duplo. The mean fluorescence was normalized by dividing it by the mean fluorescence observed in the presence of 100 nM of the corresponding monovalent ligand (20DS1x_cQGQ).
Next, the inhibition of cetuximab by the various ligands was assessed by incubating 1 nM cetuximab with 100 nM of the monovalent or 50 nM of the homo- and heterobivalent peptide-dsDNA locks, followed by addition of a constant amount of A431-cells and FACS-analysis. Figure 5.9b shows that both bivalent peptide-DNA locks block cetuximab binding at these concentrations, whereas much less efficient inhibition is observed for the monovalent ligand. The most efficient blocking is observed for the homobivalent ligand under these conditions, resulting in 80% reduction in cetuximab binding. Blockage by the heterobivalent peptide-DNA lock appeared to be slightly less efficient (70%), which may be due to the fact that binding of two peptide-DNA locks is required to block antibody binding.

We next tested whether the inhibition of cetuximab binding to EGFR by the dsDNA-based peptide locks is reversible by means of protease cleavage[16] and/or toehold-mediated DNA strand displacement.[17] To test whether the antibody-inhibitor complexes can be unlocked by toehold-mediated strand displacement, 500 nM of a full complementary oligonucleotide strand was added to 1 nM cetuximab that had been pre-incubated with 50 nM of a bivalent peptide-dsDNA lock. Following incubation for 90 minutes, a constant amount of A431-cells was added and cetuximab binding was quantified using FACS after an additional 30 minutes incubation at room temperature. Figure 5.10a shows that homobivalent peptide-DNA ligands with a 20 bp linker more effectively block cetuximab binding than that with a 35 bp dsDNA linker, but in both cases toehold-mediated strand displacement can be used to trigger reactivation of cetuximab binding to the same level. We also tested whether the blockage by homo-bivalent dsDNA-peptide lock could be reversed by cleavage of the MMP-2 recognition sequence that links the cQGQ-peptide to the ODN. MMP-2 was added to the pre-incubated homo-bivalent peptide-dsDNA lock (20 bp) with cetuximab and incubated at 37 °C for 90 minutes followed by the addition of A431-cells. After a 30 minute incubation step at room temperature FACS-analysis was performed showing the partial restoration of cetuximab activity. The level of cetuximab binding after MMP-2 treatment was comparable to that observed upon toehold-mediated strand displacement, suggesting essentially complete disruption of the bivalent ligand in both cases.

For the hetero-bivalent peptide-dsDNA locks we observed more effective blocking of cetuximab for the hetero-bivalent lock containing the triple mutant cQFD(3m) compared to the wild-type cQFD(wt) (Figure 5.10b). For both ligands, efficient restoration of cetuximab binding was observed following incubation with a 10-fold excess of a displacer oligonucleotide. The final binding levels that are observed are similar for the two heterobivalent ligands and even slightly higher than that of the monovalent control, which may be explained by the 2-fold higher concentration of monovalent cQGQ-dsDNA ligand in the latter case (100 nM vs 50 nM). It is therefore somewhat surprising that the final cetuximab binding levels obtained after strand displacement or MMP2 cleavage for the homobivalent peptide-dsDNA ligand are slightly lower than that of the monovalent peptide-dsDNA control. This could be explained if the cQGQ peptide binds
less tightly to cetuximab when conjugated to dsDNA (as in the monovalent control) compared to the cQGQ peptide alone or the cQGQ peptide conjugated to single-strand DNA. More detailed titration and kinetics experiments will need to be done to test this hypothesis and provide a complete thermodynamic description of the interaction of these bivalent peptide-dsDNA locks. Nonetheless, these preliminary results convincingly show the feasibility of using homobivalent and heterobivalent peptide-dsDNA locks to reversibly controlling cetuximab binding to the EGFR receptor on A431-cells.

5.3 Discussion and Conclusions

This work represents the first successful example of controlling the activity of a therapeutic antibody, cetuximab, in a non-covalent manner and avoids the extensive protein engineering to the antibody as shown previously by Desnoyers and coworkers.\[14\] The use of homo- and hetero-bivalent dsDNA-ligands resulted in effective inhibition of cetuximab binding to the EGFR expressed at the surface of A431-cells. The blocking of cetuximab by the peptide-dsDNA locks could be reversed by protease cleavage as well as toehold-mediated DNA strand displacement reactions. To obtain peptide-oligonucleotide conjugates with a disulfide-bridged peptide a new conjugation strategy based on copper(I)-catalyzed azide-alkyne [3+2] cycloaddition reaction was developed.

\[\text{Figure 5.10: Control of cetuximab activation using different peptide-dsDNA locks.} \]
a) 1 nM Alexa647-labeled cetuximab was blocked by the addition of 50 nM homo-bivalent lock consisting of a 20 or 35 bp linker, flanked by a 10 nt single strand overhang. Following incubation with 500 nM of a full complementary ODN-displacer strand for 90 minutes at room temperature, A431-cells were added and cetuximab binding was monitored by measuring the mean cellular Alexa647-fluorescence. In addition, cetuximab activation was tested by incubating the 20 bp homo-bivalent lock with 5 nM MMP-2 for 90 minutes at 37 °C followed by the addition of A431-cells. b) Similar experiment as shown in (a) but blockage of cetuximab was tested with the hetero-bivalent peptide-dsDNA locks containing either cQFD(wt) or cQFD(3m). All experiments were performed in duplo and the amount of cetuximab binding was determined by obtaining the mean Alexa647 fluorescence of 10,000 cells.
The subsequent hybridization of complementary conjugates resulted in the formation of homo- and hetero-bivalent dsDNA-ligands in a simple and straightforward fashion.

The cQFD peptide was initially tested and was expected to bind to the antigen binding site of cetuximab. The cyclic nature of the peptide was important and two cyclization strategies were evaluated for cetuximab binding and showed a 20-fold attenuated binding for the benzene-bridged cQFD when compared to the disulfide-bridged cQFD. Unexpected at that time was also the 10-fold attenuation of the disulfide-bridged cQFD when conjugated to dsDNA. During the course of this work Williams and coworkers reported that the cQFD peptide binds to a unique pocket in the Fab-fragment and does not bind to the antigen binding site of cetuximab.[27] This remarkable finding explains the decreased binding for the benzene-bridged cQFD which was attributed to the steric hindrance in the unique Fab-fragment binding pocket caused by the benzene-moiety. In addition, the disulfide-bridged cQFD conjugated to dsDNA may experience steric as well as electrostatic repulsion with the Fab-fragment of cetuximab causing a decrease in binding.

Fortunately a different peptide was reported that did bind to the antigen binding site of cetuximab, cQGQ.[14] The binding of cQFD and cQGQ at distinct parts of cetuximab enabled the design of two different peptide-dsDNA locks, homo-bivalent and hetero-bivalent locks. This is the first example of a non-covalent blocking strategy that only uses the Fab-fragment. Controlling the binding mode of Fab-fragments using the hetero-bivalent approach could be beneficial for targeting purposes when better tissue penetration is required, which has been shown to be limited when using the classical large and Y-shaped antibodies.[29] Furthermore, Williams and coworkers showed that it is possible to graft the unique Fab-framework of cetuximab onto a different monoclonal antibody (Trastuzumab)[27], which would enable this hetero-bivalent approach also to be applied to other therapeutic antibodies.

This and previous work showed that the affinities between the dsDNA-based mono- and bivalent ligand, antibody and target protein must be carefully tuned to obtain well-defined ON / OFF states of antibody activity. The difference between monovalent and bivalent affinity must be sufficiently large such that the antibody remains effectively blocked in presence of the bivalent ligand but favours the target protein when the ligand becomes monovalent. In this study, the homo- and hetero-bivalent dsDNA ligands showed effective inhibition of cetuximab binding to EGFR. However, the monovalent affinity of the cQGQ binding to the antigen binding site of cetuximab is still rather weak ($K_d$ of 293 nM) compared to mimitope/epitope-antibody interactions used in previous studies ($K_d$ of 30 and 5 nM, respectively).[16,17] Therefore, blocking of cetuximab required the addition of 50 equivalents of bivalent ligand, whereas in our previous work a slight excess of bivalent ligand was sufficient to completely block antibody binding. Effective inhibition of cetuximab without the need of a large excess of bivalent ligand would require improving the monovalent affinity of cQGQ. We showed that the affinity of a peptide to cetuximab (Fab-fragment binder cQFD), which was derived from phage
display screening, could be systematically increased by 4-fold. We propose that a similar strategy could aid in increasing the affinity of the antigen binding site peptide binder cQGQ. Alternative approaches would be the construction of dsDNA-based bivalent ligands containing (attenuated) cognate protein domains to control antibody activity or connecting two heterobivalent ligands used in this study as such that they can bind simultaneously to both Fab-fragments of one cetuximab antibody.

Our results showed the reversible blocking of cetuximab upon addition of MMP-2 or complementary DNA-strands restoring the binding of cetuximab towards the EGFR on the surface of the A431-cells. In this study we focused on the binding levels of blocked and activated cetuximab to A431-cells, however, the binding of cetuximab to EGFR has downstream effects on cell proliferation and differentiation arrest or even the induction of apoptosis. The next step is to characterize the blocking and activation of cetuximab, using cell viability assays. To test the reversible blocking of cetuximab by peptide-dsDNA based locks in situ, the stability of our peptide-dsDNA locks needs to be considered further. The use of hydrolytically stable DNA analogs such as phosphorothioate oligonucleotides or peptide nucleic acids would prolong the half-life of the peptide-DNA locks in vivo and ensure the inactive state of cetuximab upon arrival at the diseased site. Several endogenous triggers could be considered to induce activation of cetuximab near the tumor site. The current MMP-2 cleavage site could be substituted by the LSGRSDNH protease cleavable linker, which proved to be a good substrate for proteases that are specifically up-regulated at the tumor site. Altered expression levels of miRNAs have been linked to human cancers. Endogenous and extracellular miRNAs could be used to initiate toehold-mediated strand displacement reactions to selectively activate cetuximab. Finally, several studies showed the autonomous targeting of diseased cells by using aptamer-based DNA-structures that release their payloads due to specific aptamer-protein interactions at the cell membrane. The incorporation of aptamer sequences in our bivalent dsDNA-locks may trigger the selective binding of cetuximab to tumor cells by cell surface receptors other than the EGFR.

5.4 Experimental section

General. Unless stated otherwise, all reagents and chemicals were obtained from commercial sources and used without further purification.

Peptide synthesis. Peptide synthesis was performed on an automated peptide synthesizer (Intavis, Miltipep RSi) following the standard Fmoc peptide synthesis protocol on a 100 µmol scale of NovaSyn TGR resin (Novabiochem). All amino acids were coupled for two times 30 minutes each. Different on-resin fluorescein-labeling strategies of peptides used for polarization assays were performed throughout the course of this project. A 12-fold molar excess of fluorescein isothiocyanate (Sigma-Aldrich) was reacted overnight to the N-terminus of the cQYN- and cVWQ-mimotope as well as the wild-type and triple mutant of the cQFD-mimotope. The introduction of a pentanoic acid linker (O1PEN, Iris Biotech GmbH) allowed the efficient labeling of the fluorophore.
The strategy to use the Fmoc-Lys(Mmt)-OH building block to introduce a 5,6-Carboxyfluorescein (on resin) at the C-terminus of the cQFD mimotope was reported previously.\[16\] Fmoc-thiazolidine-carboxylic acid and p-azidobenzoic acid were coupled to the N-terminus of (C-terminal fluorescently labeled) peptides for two times one hour using 8-fold molar excess. All peptides were cleaved from the resin by using a mixture of TFA / TIS / H2O / EDT (92.5: 2.5: 2.5: 2.5% v/v) and continuous stirring for 3 hours followed by precipitation in ice cold ether. Cyclic peptides were prepared by dissolving the crude peptides in 1-3 M Gu•HCl containing 100 mM Tris pH 8.0 at a concentration of 1 mg mL\(^{-1}\). Oxidation of the disulphide bond by air was accomplished by stirring at 4 ºC in an open tube and was tracked by ESI-MS. The excessive salts were removed by making use of a solid phase extraction column (Strata-XL, Phenomenex) and the peptides were eluted using a 1:1 mixture of acetonitrile in H2O (both containing 0.1% TFA) prior to preparative RP-HPLC purification (Table 5.1).

**Table 5.1:** Peptides synthesized for fluorescence polarization assays and conjugation purposes. cQFD\* was synthesized prior to the start of this project by E. Lempens and B. Janssen, whereas cQGQ\* was commercially obtained (CASLO ApS).

| Peptide abbrev.     | Peptide sequences (N’→C’)                  | MW\(_{\text{calc}}\) (Da) | MW\(_{\text{obs}}\) (Da) |
|---------------------|--------------------------------------------|-----------------------------|----------------------------|
| cQFD\*             | FAM-GGSGGS-PLGLAG-GGSGGS-C-QFDLSTRRLK-C   | 3138.0                      | 3138.9                     |
| cQYN               | FITC-O1PEN-G-PLGLAG-C-QYNLSRLAK-C-G       | 2495.8                      | 2495.9                     |
| cVWQ               | FITC-O1PEN-G-PLGLAG-C-VWQRWQKSYV-C-G      | 2696.1                      | 2696.1                     |
| cQFD_DBMB(FAM)      | FAM-C-G-PLGLAG-C-QFDLSTRRLK-C-G           | 2724.1                      | 2723.8                     |
| AzPhe_cQFD(FAM)    | AzPhe-G-PLGLAG-C-QFDLSTRRLK-C-G-GGGK(FAM)G | 2892.2                      | 2892.0                     |
| cQFD(wt)           | FITC-O1PEN-G-PLGLAG-C-QFDLSTRRLK-C-G     | 2580.5                      | 2580.4                     |
| cQFD(3m)           | FITC-O1PEN-G-PLGLAG-C-VFDLGTRRLR-C-G     | 2549.5                      | 2549.7                     |
| cQGQ\*             | FITC-Ahx-QGQSGQCISPRGCPDGYVMY-GSSGGS      | 3178.5                      | 3179.1                     |
| Thz_cQFD           | Thz-G-PLGLAG-C-QFDLSTRRLK-C-G            | 2204.6                      | 2205.4                     |
| AzPhe_cQFD         | AzPhe-G-PLGLAG-C-QFDLSTRRLK-C-G          | 2234.6                      | 2235.0                     |
| AzPhe_cQFD(3m)     | AzPhe-G-PLGLAG-C-VFDLGTRRLR-C-G          | 2203.6                      | 2204.0                     |
| AzPhe_cQGQ         | AzPhe-G-PLGLAG-QGQSGQCISPRGCPDGYVMYG     | 3008.4                      | 3008.8                     |

**Synthetic approaches and characterization of DNA-based mono- and bivalent ligands.**

**Approach I, Thiazolidine and disulfide-bridged peptide:** The disulfide-bridged Thz_cQFD peptide was selectively converted from a thiazolidine to N-terminal cysteine by dissolving the protected peptide (1 mg mL\(^{-1}\)) in 250 mM MeONH\(_2\), 50 mM sodium acetate and 2 M Gu•HCl at pH 4.2 and incubated for 2 hours at 37 ºC.\[23,36\] ESI-MS showed the conversion into a N-terminal cysteine since the loss of 12 Da was observed which corresponds to a carbon-atom. Treatment of the protected Thz_cQFD (MW\(_{\text{calc}}\) = 2204.6 Da; MW\(_{\text{obs}}\) = 2205.4 Da) with methoxyamine resulted in two isomeric products (MW\(_{\text{calc}}\) = 2192.6 Da; MW\(_{\text{obs}}\) = 2193.1 Da).
**Approach II, Thiazolidine and DBMB-bridged peptide:** The disulphide-bridged Thz_cQFD (1 mM) was reduced by incubation with 4 equivalents of TCEP (2 mM) in 20 mM NH₄HCO₃ containing 5% v/v DMSO at pH 8.0 for 1 hour at room temperature. Subsequently 5 equivalents of dibromo-p-xylene (DBMB), dissolved in acetonitrile, were added to the reduced peptide to cyclize the cQFD mimotope via the DBMB-moiety.[25] The final composition of the reaction mixture was 5% v/v DMSO, 65% v/v 20 mM NH₄HCO₃ and 30% v/v acetonitrile. The reaction mixture was incubated for one hour at room temperature under continuous agitation (700 rpm) and analyzed using ESI-MS. Reduced Thz_cQFD (MW<sub>calc</sub> = 2206.6 Da; MW<sub>obs</sub> = 2206.1 Da) and thiazolidine protected cQFD_DBMB (MW<sub>calc</sub> = 2308.7 Da; MW<sub>obs</sub> = 2308.1 Da).

Excess TCEP and DBMB were removed by making use of a solid phase extraction column (Strata-XL, Phenomenex) and the thiazolidine protected cQFD_DBMB was eluted using a gradient of acetonitrile in H₂O (both containing 0.1% TFA) and subsequently lyophilized. The selective conversion of the N-terminal thiazolidine to cysteine was performed as described above followed by an additional solid phase extraction column and lyophilization step to remove excessive methoxyamine and sodium acetate. The major product corresponded to the DBMB-bridged cQFD with a N-terminal free cysteine (MW<sub>calc</sub> = 2296.7 Da; MW<sub>obs</sub> = 2296.4 Da). Four equivalents of fluorescein-5-maleimide were pre-dissolved in DMF and subsequently added to the DBMB-bridged cQFD bearing the free cysteine (5 mg) in 100 mM HEPES-buffer at pH 7.0. The mixture was incubated for 2 hours at room temperature and under continuous agitation. RP-HPLC was performed to obtain the pure cQFD_DBMB(FAM) peptide (MW<sub>calc</sub> = 2724.1 Da; MW<sub>obs</sub> = 2723.8 Da), gradient: 25 to 40% in 20 minutes.

**Approach III, Azidophenyl and disulfide-bridged peptide:** The alkyne-functionalized ODN (commercially obtained from Baseclick or Integrated DNA technologies, Table 5.2), were dissolved together with 10-fold molar excess of azide-functionalized peptide in argon bubbled 1:1 mixture of H₂O and DMSO and subsequently added to a premixed solution of CuSO₄, THPTA and sodium ascorbate.[26,37] The final concentrations of all components were as follows: 20 µM ODN, 200 µM peptide, 0.1 mM CuSO₄, 0.5 mM THPTA (tris(3-hydroxypropyltriazolylmethyl)amine) and 5 mM sodium ascorbate.[26] The reaction mixture (500 µL) was incubated for 2.5 hours at 37 ºC in a 500 µl eppendorf tube fixed to a rotating wheel to allow mixing and to prevent excessive formation of oxygen (when shaking) which may hamper the reaction (reactive oxygen species). In addition, the amount of dead air in the tube was minimized for the same reason.[26] The reaction was quenched by addition of excess EDTA (10 equivalents) and stored on ice prior to RP-HPLC purification.

RP-HPLC was performed using a GRACE Alpha-C18 (D = 4.6 mm, L = 250 mm), equilibrated with 100 mM TEAA at pH 7.0 to obtain pure POCs. A 5-50% acetonitrile gradient in 20 minutes was used at a flow rate of 1.0 mL min⁻¹ and monitored at 260 nm. POC-fractions were lyophilized (3x) to remove excess TEAA and redissolved in MilliQ-water. Pure POC-fractions were analyzed using Q-ToF-MS in negative mode and direct injection of pure POC-solutions in 1:1 isopropanol/water + 1% triethylamine (pH 10). MaxEnt software was used to deconvolute the m/z-spectra. POC-1 (MW<sub>calc</sub> = 9,728.3 Da; MW<sub>obs</sub> = 9,729.2 Da), POC-2 (MW<sub>calc</sub> = 9,759.8 Da; MW<sub>obs</sub> = 9,760.9 Da), POC-3 (MW<sub>calc</sub> = 13,346.1 Da; MW<sub>obs</sub> = 13,345.9 Da), POC-4 (MW<sub>calc</sub> = 13,411.2 Da; MW<sub>obs</sub> = 13,412.0 Da), POC-5 (MW<sub>calc</sub> = 14,003.5 Da; MW<sub>obs</sub> = 14,003.8 Da), POC-6 (MW<sub>calc</sub> = 14,068.6 Da; MW<sub>obs</sub> = 14,068.8 Da).
Control Cetuximab Activity

14,069.4 Da), POC-7 (MW<sub>calc</sub> = 12,358.9 Da; MW<sub>obs</sub> = 12356.9 Da), POC-8 (MW<sub>calc</sub> = 12,272.9 Da; MW<sub>obs</sub> = 12,271.9 Da), POC-9 (MW<sub>calc</sub> = 13,952.9 Da; MW<sub>obs</sub> = 13,952.1 Da), POC-10 (MW<sub>calc</sub> = 16,904.9 Da; MW<sub>obs</sub> = 16,903.6 Da), POC-11 (MW<sub>calc</sub> = 11,561.1 Da; MW<sub>obs</sub> = 11,559.7 Da) and POC-12 (MW<sub>calc</sub> = 11,530.1 Da; MW<sub>obs</sub> = 11,529.7 Da), Monovalent and bivalent peptide-dsDNA ligands were obtained by hybridizing equimolar amounts of leading and complementary strands at room temperature for 30 minutes. The purity of the final hybridized constructs was analyzed on a native 15% polyacrylamide gel.

**Table 5.2:** Oligonucleotides used for synthesis of peptide-oligonucleotide conjugates (POC) via the CuAAC-reaction. ODN-1 to -4 contained an Alkyne-C8-dC at the 5'-end whereas ODN-5 to -9 contained a hexynyl-linker at the 5'-end.

| ODN abbrev. | Peptide sequences (N' → C') | nt  | Poc | AzPhe | Hybrid       |
|-------------|-----------------------------|-----|-----|-------|-------------|
| ODN-1       | GTCACCGATGAAACTGTCTA        | 21  | 1   | cQFD  | 20DS2x      |
| ODN-2       | TAGACAGTTTCATCGGTGAC        | 21  | 2   | cQFD  |             |
| ODN-3       | GTCACCGATGAAACTGTCTACAGACTCAGCAAGCA | 36  | 3, 5| cQFD (FAM) | 35(F)DS 2x |
| ODN-4       | TGCTTGCTGAGTCTGAGACAGTTCGTTGAC | 36  | 4, 6| cQFD (FAM) |             |
| ODN-5       | TAGACAGTTTACTGCAGACGCTCAATCA | 30  | 7   | cQGQ  | 20SMD2 x_homo |
| ODN-6       | GTCACCGATGAAACTGTCTACCACTCAAC | 30  | 8   | cQGQ  |             |
| ODN-7       | TGCTTGCTGAGTCTGAGACAGTTCGTTGAC | 35  | 9   | cQGQ  | 35SMD2 x_homo |
| ODN-8       | GTCACCGATGAAACTGTCTACAGACTCAGCAAGCACCACAAATACAT | 45  | 10  | cQGQ  |             |
| ODN-9       | CCACACATGATTGGACTGTCACCGATG | 30  | 11, 12| cQFD (3m) | 20DS2x _hetero |

**Fluorescence polarization.** Fluorescence polarization measurements were recorded on a Tecan Safire monochromator microplate reader or the Tecan Infinite F 500 microplate reader (Figure 5.7b). Samples were excited at 470 nm and monitored between 514 and 524 nm. Unless stated otherwise, fluorescent peptides or conjugates were diluted from a concentrated stock to a concentration of 10 nM in 50 mM NaPi, 100 mM NaCl at pH 7.0 containing 1 mg mL<sup>-1</sup> BSA. Different concentrations of cetuximab were added to the sample and allowed to equilibrate for one hour at 28 °C prior to measurement. Fluorescence polarization data of the cetuximab titration experiments were fitted according to a one-site binding model and corrected for the number of antigen binding sites of cetuximab (Equation 5.1).

\[
P = \frac{P_{\text{max}} \times 2 \times \text{[Cetuximab]}}{K_d + 2 \times \text{[Cetuximab]}}
\]  

(Equation 5.1)

Equation 5.2 was used to fit the polarization data of the DNA-based mono- and bivalent ligands in presence of 1.5 M NaCl (Figure 5.6) to correct for receptor (antibody) depletion.
\[
P = P_f + (P_b - P_f) \times \frac{(L_f + K_d + R_f) - \sqrt{(-L_f - K_d - R_f)^2 - 4L_f R_f}}{2L_f}
\] (5.2)

**P** is the measured polarization, **P**_\(f\) is the polarization value of the free ligand (peptide), **P**_\(b\) is the polarization value of the bound ligand, **L**_\(f\) is the total concentration of ligand (20 nM) and **R**_\(f\) is the concentration of antigen binding sites of cetuximab.

**Culturing of A431-cells.** Human A431 carcinoma cells were cultured according to previous protocols described by Sonntag.[36] In brief, A431-cells were cultured in RPMI-1640 media supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin / streptomycin at 37 °C, 5% CO₂. Experiments were done with cells that were passaged at least 7 times and at most 20 times. For FACS experiments, a 175 cm² flask was cultured to 80% of confluency A431-cells, trypsinized and centrifuged for 5 minutes at 1250 g and the supernatant was discarded. Cells were then washed with PBS containing 0.1% BSA (PBS+), centrifuged again and resuspended in 1 mL fresh PBS (A431-stock) with BSA and stored at room temperature before addition to Alexa647-labeled cetuximab. The absolute number of A431-cells was kept constant throughout all samples on one day, but the volume of the incubations was increased for lower cetuximab concentrations to ensure excess of cetuximab compared to the EGFR present at the A431-cells.

**Alexa Fluor 647 labeling of cetuximab.** NHS-Alexa647 (Life Technologies) was added in a 20-fold molar excess to cetuximab (C225, Erbitux) and reacted for 1 hour at room temperature followed by immediate removal of the excess dye using MWCO-filters of 10 kDa (Millipore) and repeated washing steps resulting in an average labeling of 5 Alexa547-dyes per cetuximab. Flow cytometry was performed on a FACS Aria III with a 70 µm nozzle. Alexa-Fluor 647 was excited with a 633 nm laser and detected with a 660/20 bandpass filter. FACS data were analyzed with FACS Diva 8.0 and all samples were recorded with a flow rate of 3.0 mL min⁻¹.

**Homo- and heterobivalent experiment.** Homo- and heterobivalent ligands were assembled prior to the experiment. POC-7 and -8 were used to construct the homo-bivalent ligand with a 20 bp-linker (20SMD2x_homo) whereas POC-9 and -10 were used to construct the homo-bivalent ligand with a 35 bp linker (35SMD2x_homo). POC-7 and -11 were used to construct the hetero-bivalent ligand with a 20 bp-linker and cQFD(wt) mimotope (20SMD2x_hetero(wt)) whereas POC-7 and -12 were used to construct the hetero-bivalent ligand with a 20 bp linker and cQFD(3m) mimotope (20SMD2x_hetero(3m)). 1 nM of Alexa647-labeled cetuximab (200 µL) was incubated with 50 nM of bivalent ligand for one hour at room temperature. Subsequently, 50 µL of A431 cells (A431-stock) were added followed by an additional incubation step of 30 minutes at room temperature under continuous shaking. Finally, the A431 cells were pelleted (5 minutes, 1250 g at room temperature) and the PBS was removed. Individual samples were resuspended in PBS+ prior to FACS-analysis.
Toehold-mediated strand displacement reaction. The toehold strand displacement reaction was initiated by the addition of 500 nM fully complementary ODN-strands GTTGAGGTTGAGACAGTTTCATCGGTGAC (20SMD2x_homo), ATGTATGTGGCTTGC-TGAGTCTGTAGACAGTTTCATCGGTGAC (35SMD2x_homo) or CATCGGTGACAGCTCAAT-CAATGTATGTGG (20SMD2x_hetero(wt/3m)) and incubated for one hour at room temperature in PBS+. Subsequently, 50 µL of A431 cells (A431-stock) were added and the entire mixture was incubated for another 30 minutes at room temperature under continuous agitation to allow binding of the reactivated cetuximab antibody to the A431 cells. Finally, the A431 cells were pelleted (5 minutes, 1250 g at room temperature) and the PBS was removed. Individual samples were resuspended in PBS+ prior to FACS-analysis.

MMP2-promoted release of cetuximab blockage. The MMP2-promoted release of cetuximab blockage (20SMD2x_homo, 50 fmol) was initiated by the addition of 5 fmol MMP-2 and incubated for 90 minutes at 37 °C in 100 µL MMP-buffer (50 mM TRIS, 200 mM NaCl, 10 mM CaCl2, 10 µM ZnCl2 at pH 7.4) under continuous agitation. The mixture was 10 times diluted in PBS+ resulting in final concentrations of 1 nM cetuximab, 50 nM 20SMD2x_homo and 5 nM MMP-2 followed by the addition of 50 µL of A431 cells (A431-stock) and subsequently incubated for 30 minutes at room temperature under continuous shaking followed by an centrifugation step to pellet the A431-cells (5 minutes, 1250 g at room temperature). Individual samples were resuspended in PBS+ prior to FACS-analysis.

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Chapter 6

EFFICIENT SYNTHESIS OF PEPTIDE AND PROTEIN FUNCTIONALIZED PYRROLE-IMIDAZOLE POLYAMIDES USING NATIVE CHEMICAL LIGATION

Abstract

The advancement of DNA-based bionanotechnology critically depends on the availability of efficient strategies to functionalize DNA nanostructures in a specific manner with other biomolecules, most importantly peptides and proteins. Common DNA-functionalization methods rely on laborious and covalent conjugation between DNA and proteins or peptides. Pyrrole-Imidazole polyamides, based on natural minor groove DNA-binding small molecules, can bind to DNA in a non-covalent but sequence specific fashion. In this study we explore the use of Pyrrole–Imidazole (PI) polyamides for addressing proteins and peptides to DNA in a sequence specific and non-covalent manner. Native chemical ligation was used as a generic approach to couple peptide epitopes and proteins to the PI-polyamide resulting in a set of different PI-polyamide conjugates. The effect of PI-polyamide conjugation on DNA binding was investigated by Surface Plasmon Resonance (SPR). Although the synthesis of different protein-PI-polyamide conjugates was successful, attenuation of DNA affinity was observed, in particular for the protein-PI-polyamide conjugates. The practical use of protein-PI-polyamide conjugates for addressing DNA structures in an orthogonal but non-covalent manner therefore remains to be established.

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6.1 Introduction

In addition to its natural role in the storage of genetic information, DNA has become a versatile building block for a wide range of applications in bionanotechnology. The synthetic accessibility and highly predictable binding properties of DNA in combination with its structural properties enable the bottom up construction of complex 3-dimensional DNA nanostructures with well-defined geometries, topologies and mechanical properties. The development of efficient strategies to address DNA nanostructures with functional proteins and peptides increases their application potential in biomedical research. The most common strategy to spatially address DNA-nanoarchitectures with proteins is via covalent conjugation of the protein with ssDNA, which subsequently hybridizes to complementary handles present on the DNA nanostructure. A variety of chemoselective conjugation methods have been used for this purpose, including thiol-maleimide chemistry, azide-alkyne Huisgen cycloaddition (‘click chemistry’) and several enzyme-mediated conjugation reactions. These approaches require the introduction of single-strand DNA handles during the design of the DNA architectures and inevitably introduce a rigid dsDNA linker of > 5 nm between the protein and the DNA-architectures. Another popular strategy is to use biotinylated-oligonucleotides and the very strong streptavidin-biotin interaction to immobilize biotinylated proteins or peptides. However, in addition to the introduction of the relatively large streptavidin linker, this strategy often yields heterogeneous complexes due to the tetravalent nature of streptavidin and the essentially irreversible nature of the interaction. Self-assembly of protein-DNA assemblies has also been achieved by introduction of DNA aptamer sequences, but this strategy is restricted to the limited number of proteins for which high affinity aptamers have been developed such as thrombin.

Instead of the introduction of specific protein binding oligonucleotide tags, protein functionalization of DNA-based nanostructures can also be achieved using sequence-specific dsDNA binders such as zinc finger domains or pyrrole-imidazole polyamides. Zinc finger domains are natural occurring DNA binding domains that bind in the major groove of DNA and specifically target 3 consecutive bases. Recent examples of zinc fingers include the functionalization of DNA-origami structures with fluorescent proteins and the re-assembly of a split-luciferase for DNA-detection and logic-gated protein function. Whereas zinc finger domains can be connected to the protein of interest by genetic fusion, functional expression of the cysteine-rich zinc binding proteins can be challenging. Pyrrole-Imidazole (PI) hairpin polyamides were developed by Dervan and coworkers as a low molecular weight alternative to DNA binding proteins. Inspired by the natural products netropsin and distamycin, these polyamide oligomers of pyrrole, imidazole and hydroxypyrrole can be designed to bind in a sequence specific manner in the minor groove of dsDNA with nanomolar affinity. Paired imidazole (Im) and pyrrole (Py) recognize G-C basepairs whereas paired Py/Im recognize C-G. Py/Py recognizes both A-T and T-A basepairs. Research on PI-polyamides initially focused on the
control of gene transcription by targeting transcription factor recognition sites.\textsuperscript{[18–20]} Functionalizing the PI-polyamide with a chlorambucil-moiety enabled site-specific DNA alkylation resulting in a covalent bond between the PI-polyamide and the DNA sequence of interest, which inhibited the binding of transcription factors and prevented gene transcription.\textsuperscript{[21]} Gene transcription could also be activated by synthesizing peptide-PI-polyamide conjugates of which the peptide was able to recruit transcription factors and thereby initiate gene transcription at the site of interest.\textsuperscript{[22–25]} More recently PI-polyamides have been used as specific dsDNA binders in other applications, including DNA-detection based on the aggregation of gold nanoparticles\textsuperscript{[26]} or microcontact printing,\textsuperscript{[27]} as a construction element for DNA architectures\textsuperscript{[28,29]} or controlled energy transfer along a DNA wire.\textsuperscript{[30]} The use of PI-polyamides for the sequence specific patterning of proteins on a DNA-scaffold has been achieved by attaching a biotin group via a PEG\textsubscript{3} linker to PI-polyamides. The complexes formed with streptavidin were assessed by atomic force microscopy and showed alignment of the complexes in a regular spacing.\textsuperscript{[31–33]}

**Scheme 6.1:** Synthesis of PI-polyamide conjugates via native chemical ligation. Schematic representation of the PI-polyamide sequence used in this study (ImImPyPy-γ-PyPyPyPy-Py-β-Dp) to target the DNA sequence 5’-WWGGWWW-3’ (W represents either A or T). The introduction of a cysteine at the N-terminus of the PI-polyamide allows native chemical ligation of peptides and proteins bearing a C-terminal thioester.
PI-polyamide conjugates are typically obtained by using classic amine coupling reactions, yielding PI-polyamide conjugates containing (protected) peptides\textsuperscript{[22,25]} or small molecules like fluorophores,\textsuperscript{[34,35]} alkylating anti-cancer agents (chlorambucil)\textsuperscript{[33,36,37]} and chromatin modifying histone deacetylase inhibitor (SAHA).\textsuperscript{[38,39]} Native chemical ligation has also been explored as a more chemoselective reaction to couple non-protected peptides to PI-polyamides. In that work the C-terminus of the PI-polyamide was treated with benzyl bromide to produce a thioester and subsequent ligation to a N-terminal cysteine present on a glutamate-rich peptide resulted in peptide-PI-polyamide conjugates that were able to recruit transcription factors to specific DNA sites.\textsuperscript{[22,40]}

The aim of this study was to develop a generic synthetic strategy for the conjugation of PI-polyamides to peptides and recombinant proteins. Unlike the previous reported strategy of introducing a thioester on the PI-polyamide, our strategy was to functionalize PI-polyamides with an N-terminal cysteine. Introduction of the more stable cysteine on the PI-polyamide is advantageous because synthesis of the PI-polyamide is the most labor intensive step (Scheme 6.1). These cysteine-functionalized PI-polyamides were shown to readily react with peptides and proteins containing a C-terminal thioester. Characterization of the DNA binding properties of these PI-polyamide conjugates using Surface Plasmon Resonance revealed a significant attenuation of DNA affinity, in particular for the protein-PI-polyamide conjugates.

6.2 Results

6.2.1 Synthesis of cysteine-functionalized PI-polyamide

In this work we used ImImPyPy-γ-PyPyPyPy-Dp as a model PI-polyamide. This sequence was chosen because it avoids the use of the hydrolytically sensitive hydroxyprrole,\textsuperscript{[41]} but is still reasonably specific because of the use of two consecutive imidazoles. Furthermore, this PI-polyamide was reported to bind dsDNA 5’-WWGGWWW-3’ (W represents either A or T) with low nM affinity.\textsuperscript{[42]} Native chemical ligation of peptides and proteins with a C-terminal thioester requires the introduction of a cysteine with a free N-terminal amine in the PI-polyamide. This cysteine could be introduced at either terminus of the PI-polyamide (Scheme 6.2). Introduction at the C-terminal part of the PI-polyamide requires cleavage from the resin by N,N-bis(aminopropyl)methylamine (BDp), followed by a coupling step in solution to the pre-synthesized PEGylated and tert-Butylthiol protected cysteine. Although the final product was obtained in pure form, synthesis of the C-terminal cysteine-modified PI-polyamide was rather cumbersome, resulting in low overall yield of 3%. A synthetically more straightforward approach is to introduce the cysteine at the N-terminus of the PI-polyamide, since in this case all coupling steps can be done on the resin. First we synthesized the polyamide ImImPyPy-γ-PyPyPyPy manually using Fmoc-β-alanine-Wang resin.\textsuperscript{[41]} It appeared to be critical, prior to the addition to the resin, to pre-incubate the building block with activator followed by the addition of base (DIPEA). Since the amine in
imidazole is a relatively poor nucleophile, the coupling steps to imidazole were performed for 15 hours, whereas reaction times of 1.5 hours were used for coupling to pyrrole. To prevent steric hindrance between the protein and the DNA, we chose to first introduce a short PEGₙ-linker at the N-terminus \((n = 3, \text{PEG}_3)\). Following deprotection of the Fmoc-group, the amine of the PEG₃-linker was coupled to Fmoc- and t-BuSH-protected cysteine on solid phase, followed by Fmoc-deprotection and cleavage from the solid-phase.

**Scheme 6.2:** Synthetic scheme for the preparation of PI-polyamide bearing a PEGylated and tert-Butylthiol protected cysteine for native chemical ligation purposes. The tert-Butylthiol protected cysteine can be introduced at the C-terminus (A) or N-terminus (B) of the PI-polyamide. Introduction at the C-terminus requires cleavage from the resin using N,N-bis(aminopropyl)methylamine (BDp) followed by conjugation of the pre-synthesized PEG₃-Cys(StBu) to the free amine of BDp. Introduction of PEGylated and tert-Butylthiol protected cystein at the N-terminus can be performed on resin followed by cleavage from the resin using 3-(dimethylamino)-propylamine (Dp).

Figure 6.1 shows the chemical structure and ESI-MS characterization of the final product, Cys-PI-polyamide, as well as the PI-polyamide reference compound with an N-terminal acetyl group. Both compounds were cleaved via aminolysis of the ester on the resin using 3-(dimethylamino)-propylamine (Dp). The positively charged tertiary amine that is formed at the C-termini of the PI-polyamides provides an additional electrostatic interaction between the PI-polyamide and DNA. Typical overall yields after RP-HPLC were 20% for PI-polyamide and 14% for Cys-PI-polyamide, which is comparable to yields reported previously for PI-polyamides of similar sequences.\[^{41}\]
Chapter 6

6.2.2 Native chemical ligation of thioester peptides and proteins to Cys-PI-polyamides

To test the efficiency of the native chemical ligation (NCL) to Cys-PI-polyamide, a C-terminal thioester functionalized peptide epitope (ELDRWEKIRLRP) was synthesized that binds to the anti-HIV p17 antibody. The C-terminal thioester peptide was synthesized according to the method of Dawson and coworkers and involved Fmoc-solid phase synthesis using a Rink Amide AM resin containing a diaminobenzyl linker.\(^\text{[44]}\) The resin-bound N-acyl-benzimidazolinone (Nbz) peptide was deprotected and cleaved from the resin using trifluoroacetic acid. The desired peptide AELDRWEKIRLRPA-Nbz was obtained in high purity and a final yield of 15% after RP-HPLC purification (Figure 6.2a). Since the Nbz-moiety is a poor leaving group, the NCL-reaction with Cys-PI-polyamide was performed using 4-mercaptophenylacetic acid (MPAA) as a catalyst.\(^\text{[46]}\) Cys-PI-polyamide was mixed with a slight excess of peptide-Nbz with final concentrations of 2 and 2.2 mM, respectively. Analysis by ESI-MS showed full conversion of the Cys-PI-polyamide to the epitope-PI-polyamide conjugate after overnight (15 hours) incubation at room temperature (Figure 6.2b). The HPLC-trace actually showed two peaks with the expected mass for the epitope-PI-polyamide product, which we attribute to the formation of a minor amount of a diastereomer.
NCL-reactions with full-sized proteins tend to be less efficient compared to those involving peptide thioesters, if only because of the lower concentrations that can be used. Following successful NCL with a peptide thioester, we therefore next tested the efficiency of the reaction between Cys-PI-polyamide and three different recombinant proteins with a C-terminal MESNA-thioester. Enhanced yellow fluorescent protein (EYFP) and cyan fluorescent protein (ECFP) were chosen as they form a useful Förster Resonance Energy Transfer (FRET) pair to allow assessment of protein-protein distances on DNA structures.[45–47] The collagen binding protein CNA35 has been successfully applied as a recognition domain for collagen imaging and was successfully used to construct CNA35-micelles,[48] liposomes,[49] and dendrimers.[50] C-terminal fusion proteins with intein and chitin binding domains were expressed in E. coli and purified using the Intein Mediated Purification Affinity Chitin Tag (IMPACT) method.[49] ESI-MS analysis showed the successful formation of 4-mercaptophenylacetic acid (MESNA) functionalized proteins (Figure 6.3). The NCL-reaction was performed by mixing a 10-fold excess of a Cys-PI-polyamide (3.0 mM) to protein-MESNA (0.3 mM). ESI-MS analysis showed full conversion to the ligation product after 36 hours for all three proteins. Excess of Cys-PI-polyamide, TCEP and MPAA were extensively removed by exchanging buffer using centrifugal filters.
Figure 6.3: Native chemical ligation of proteins with a C-terminal MESNA-thioester and Cys-PI-polyamide. a, e) RP-HPLC and m/z-spectra of the purified EYFP-MESNA (MW$_{\text{calc}}$ = 27,845 Da, MW$_{\text{obs}}$ = 27,852 Da) (a), ECFP-MESNA (MW$_{\text{calc}}$ = 27,763 Da, MW$_{\text{obs}}$ = 27,765 Da) (c) and CNA35-MESNA (MW$_{\text{calc}}$ = 36,028 Da, MW$_{\text{obs}}$ = 36,025 Da) (e). b, d, f) RP-HPLC and m/z spectra of protein-PI-polyamide conjugates of EYFP (MW$_{\text{calc}}$ = 29,254 Da, MW$_{\text{obs}}$ = 29,252 Da) (b), ECFP (MW$_{\text{calc}}$ = 29,167 Da, MW$_{\text{obs}}$ = 29,165 Da) (d) and CNA35 (MW$_{\text{calc}}$ = 37,427 Da, MW$_{\text{obs}}$ = 37,425 Da) (f). The additional peak at 27,637 Da corresponds to ECFP (d) in which the MESNA-thioester was hydrolysed to a carboxylic acid. Reactions were performed at room temperature in 200 mM Na$_2$HPO$_4$, 100 mM MPAA, 20 mM TCEP•HCl at pH 6.8.

6.2.3 Effect of conjugation on DNA binding properties

Previous work showed that the peptide conjugation can affect both the affinity and specificity of PI-polyamides to DNA.[22–24,51] To study the influence of peptide and protein conjugation, the DNA binding properties of the PI-polyamide conjugates were investigated using Surface Plasmon Resonance (SPR). SPR provides not only information about the thermodynamics, but also about the kinetics of the interaction. Commercially
available streptavidin-functionalized SPR chips were immobilized with biotin-functionalized oligonucleotides (biotin-ODN) that form a hairpin loop resulting in a piece of double stranded DNA containing the cognate sequence for the PI-polyamide of interest. \cite{52} Besides the cognate DNA-sequence, we chose to immobilize a scrambled DNA sequence and a sequence containing 3 mismatches (see experimental section). Increasing concentrations of the reference PI-polyamide were injected and flown over the different channels containing one of the three DNA-sequences. The sensorgram of the matching DNA-sequence showed a low dissociation rate indicating tight binding of the non-functionalized PI-polyamide to the DNA. The maximum response level of approximately 40 RUs indicates that the PI-polyamide molecule binds to the DNA in a 1:1 stoichiometry (Figure 6.4a and b). Fitting the steady state response levels with a 1:1 binding Hill equation (n=1, no cooperativity) yielded a $K_d$ of $1.6 \pm 0.1$ nM for the cognate DNA-sequence, which is comparable to the $K_d$ of 2.0 nM reported previously for this PI-polyamide sequence using quantitative footprint titrations. \cite{42} In accordance with previous work, an ~100-fold decreased affinity was observed for a sequence that contained 3 mismatches compared to the cognate DNA sequence (Figure 6.4b). \cite{53}

**Figure 6.4:** Binding of PI-polyamide (a) and Cys-PI-polyamide (c) to the cognate DNA sequence during 12 minute injections (25 µL min$^{-1}$) over a concentration range of 24 pM to 200 nM in HBS-EP + 0.1% DMSO, pH 7.4. b) Steady-state binding levels of non-functionalized PI-polyamide to a surface modified with approximately 450 RUs of cognate, 3 bp mismatch or scrambled DNA sequence were fitted individually to a one-site binding model yielding an overall $K_d$ of $1.6 \pm 0.1$ nM for the non-functionalized PI-polyamide binding to the cognate DNA sequence and a $K_d$ of $314 \pm 160$ nM for the 3 bp mismatch sequence. d) The steady-state binding levels of Cys-PI-polyamide to the cognate DNA sequence were fitted according to a one-site binding model yielding an overall $K_d$ of $9.3 \pm 1.6$ nM.
Interestingly, introduction of the cysteine-functionalized PEG₃-linker at the N-terminus of the PI-polyamide resulted in a 6-fold decrease in affinity towards the cognate DNA sequence (Figure 6.4c and d), yielding an apparent $K_d$ of 9.3 ± 1.6 nM. The decreased affinity observed for Cys-PI-polyamide was at least partially due to an enhanced dissociation rate (Figure 6.4c).

**Figure 6.5:** Binding of the epitope-PI-polyamide conjugate only (a + b) or in combination with anti-HIV (c + d) to a surface modified with the cognate DNA sequence. a) Response of the epitope-PI-polyamide conjugate binding to the cognate DNA sequence during 8 minute injections (25 µL min⁻¹) over a concentration range of 6 nM to 400 nM. b) The steady-state values were fit to a one-site binding model yielding an overall $K_d$ of 17.4 ± 0.5 nM for the epitope-PI-polyamide conjugate binding to the cognate DNA sequence. c) Response of pre-incubated mixtures containing 100 nM epitope-PI-polyamide conjugate (black line) and anti-HIV antibody binding in a concentration range of 0 to 200 nM (grey lines) to the cognate DNA sequence during 10 minute injections (25 µL min⁻¹). d) Binding level as a function of antibody concentration for the cognate and scrambled DNA sequences. Experiments were performed in HBS-EP + 0.1% DMSO, pH 7.4.

A further 2-fold decrease in DNA affinity to a $K_d$ of 17.4 ± 0.5 nM was observed for the epitope-PI-polyamide conjugate (Figure 6.5a and b). This relatively small additional effect of peptide conjugation suggests that most of the attenuation in DNA affinity is due to local steric and/or repulsive effects also present in Cys-PI-polyamide. To test whether the peptide epitope is still available for antibody binding when the epitope-PI polyamide is bound in the DNA minor groove, a fixed concentration of 100 nM epitope-PI-polyamide was pre-incubated with different anti-HIV antibody concentrations ranging from 0 to 200 nM. Figure 6.5c shows that there is an antibody concentration dependent increase in
response. Although complex formation is not complete under these conditions, this experiment shows that the epitope-PI-polyamide conjugate enables the functionalization of DNA with, in this case, antibodies in a non-covalent and a sequence specific manner.

Finally, the SPR binding assay was used to assess the DNA-binding properties of the protein-PI-polyamide conjugates (Figure 6.6). Although DNA binding was clearly attenuated, complex association kinetics were observed for the ECFP-PI-polyamide as well as the CNA35-PI-polyamide conjugate. At relatively high concentrations the initial rapid increase in response is followed by an unexpected drop in response levels until a steady-state level is reached. A possible explanation for this complex binding behavior could be competitive binding by a compound with a higher affinity and lower molecular weight. Care was taken to remove the excess of non-conjugated Cys-PI-polyamide following NCL, either by repeated concentration/dilution cycles using centrifugal filters (for ECFP and EYFP) or using Ni-affinity chromatography (for CNA35). Nonetheless, we cannot exclude the possibility that some of the Cys-PI-polyamide sticks to the protein due to its strongly hydrophobic nature. Although the steady-state levels are higher for the protein-PI-polyamide conjugates compared to the smaller Cys-PI-polyamide or epitope-PI-polyamide conjugate, it is also clear that the binding is not saturated even at the highest concentration of protein-PI-polyamide tested (400 nM), which indicates that the DNA affinity is further attenuated for the protein-PI-polyamide conjugates compared to the Cys-PI and epitope-PI-polyamide conjugates.

![Figure 6.6: Binding of protein-PI-polyamide conjugate to a surface modified with the cognate DNA sequence.](image)
a) Response of ECFP-PI-polyamide conjugate binding in a concentration range of 1.56 nM to 400 nM to the cognate DNA sequence.
b) Response of CNA35-PI-polyamide conjugate binding in a concentration range of 1.56 nM to 400 nM binding to the cognate DNA sequence. Experiments were conducted in HBS-EP + 0.1% DMSO, pH 7.4 and using 10 minute injections (25 µL min⁻¹).

### 6.3 Discussion and Conclusions

A novel synthetic strategy was developed that allows native chemical ligation of both peptides and proteins to PI-polyamides. The introduction of an N-terminal and thiol-protected cysteine on the PI-polyamide combined with established strategies to
synthesize thioester-peptides and recombinantly express thioester proteins avoids the cumbersome introduction of a C-terminal thioester on the PI-polyamide upon treatment with benzyl bromide.[22,40] Our synthetic approach also compares favourably with previous work that used standard peptide coupling conditions to conjugate short side-chain protected peptide fragments (5-mers) to a free amine on the PI-polyamide. Although the conjugation was successful, this strategy showed lower conversion (maximum of 60%) and required the use of excess peptide and an additional cleavage step after conjugation to obtain unprotected peptide-PI-polyamide.[23,25]

Our finding that the DNA binding of the epitope-PI-polyamide was 10-fold weaker compared to the reference PI-polyamide is consistent with previous work on PI-polymamide conjugation.[22] Both the chemical nature and the length of the linker used for the peptide-PI-polyamide conjugates are known to be important for maintaining the DNA-binding properties of the PI-polyamide. A 100-fold affinity loss was observed upon conjugation of a gene-expression promoting peptide sequence to the N-methyl position of imidazole of a PI-polyamide via a linker consisting of a single glycine.[21,22] In a follow up study the same peptide was connected at the C-terminus of the PI-polyamide via a PEG3-linker or equivalent alkane linker containing a tertiary amine. The peptide-PI-polyamide containing the tertiary amine was suggested to bind stronger to the DNA, since the recruitment of the transcription factor protein was more efficient compared to the conjugate containing the PEG-linker. Despite the indirect nature of the assay, Stafford and coworkers attributed this effect to the presence of a positively charged ammonium ion in the linker possibly interacting with the negatively charged DNA backbone, whereas PEG would be unfavourable due to electrostatic repulsion with the DNA backbone.[24] The introduction of a PEG4-linker between a small molecule (camptothecin) and a PI-polyamide sequence resulted in a 10-fold loss in affinity compared to the non-functionalized, whereas an equivalent alkane-linker resulted in 25-fold loss in affinity.[51] The further decrease in DNA affinity observed upon conjugation of full sized proteins to Cys-PI-polyamide may be partially due to the ineffective removal of excess and hydrophobic Cys-PI-polyamide. In addition, the current PEG2-linker might be insufficient to separate the protein from the PI-polyamide, thereby hampering the binding of the latter to the DNA. To increase the spatial separation between the protein and the PI-polyamide one could introduce longer PEG-linkers or linkers with an increased persistence length. Another strategy would be to connect the protein to a tandem-PI-polyamide. The affinity of these tandem-PI-polyamides has been reported to be in the picomolar-range, but their synthesis is more laborious.[54] Finally, the Cys-PEG PI-polyamide could be functionalized with a chlorambucil-moiety.[33] Incubation of this PI-polyamide would result in a covalent bond with the DNA sequence of interest. In a subsequent step the N-terminal cysteine on the PI-polyamide-DNA construct could react with thioester proteins (or peptides) to obtain protein-functionalized DNA structures. This approach would circumvent the attenuated affinity and dissociation of the non-covalent protein-PI-polyamide conjugate from the DNA.
6.4 Experimental section

**Fmoc-mediated solid phase synthesis of the PI-polyamide main structure.** The synthesis of the PI-polyamide was performed manually on Fmoc-β-alanine-Wang resin (200 µmol scale, Novabiochem) following previously reported procedures. The deprotection of the Fmoc-group was performed using 20% piperidine in N-methyl-2-pyrrolidone (NMP) for two times 10 minutes. Four equivalents of Fmoc-N-methylpyrrole carboxylic acid (Fmoc-Py, Wako Chemicals GmbH), Fmoc-N-methylimidazole carboxylic acid (Fmoc-Im, Wako Chemicals GmbH) or Fmoc-γ-aminobutyric acid (Fmoc-GABA, Novabiochem) were preactivated with 3.9 equivalents of O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) for 5 minutes followed by the addition of 8 equivalents of N,N-diisopropylethylamine (DIPEA) before addition to the resin. The final concentration of the building block was 0.15 mM. In general, the coupling reactions were performed at room temperature and reacted for 90 minutes except for the coupling to the amine of an imidazole, in that case the reaction was performed for 15 hours. After each coupling step, an 8 mL solution of acetic anhydride/pyridine/NMP (3:5:2, respectively) was added to the resin in order to cap the remaining unreacted amines. Typical cleavage of the synthesized PI-polyamide was achieved by the addition of 500 µl of either N,N-bis(aminopropyl)methyamine (BDp, TCI Europe) or 3-(dimethylamino)-propylamine (Dp, Sigma Aldrich) to the dried resin followed by 15 hours of incubation at 37 °C under continuous agitation. The resin was removed by filtration through a glass filter and washed with 1.5 mL dichloromethane (DCM, two times) and 1.5 mL methanol. The collected organic layers were reduced by evaporation on a rotary evaporator. 2 mL of DCM was added and the solution was precipitated via drop wise addition (0.030 mL s⁻¹) to ice cold diethyl ether using an automated syringe pump (Harvard apparatus). The precipitate was centrifuged at 3700 rpm for 10 minutes. The pellet was resuspended in 2 mL DCM and 0.5 mL methanol and the precipitation step was repeated to obtain a white precipitate. The precipitate was dried with argon flow and dissolved in 20% acetonitrile in water mixture. Purification was done by preparative RP-HPLC using a gradient of acetonitrile in H₂O (both containing 0.1% TFA). Gradient RP-HPLC: 20-40% in 10 minutes.

**Fmoc-mediated solid phase synthesis of the tert-Butylthiol protected Cys-PI-polyamide.** The PI-polyamide main structure was synthesized as previously described. Four equivalents of Fmoc-12-amino-4,7,10-trioxadodecanoicacid (PEG3, PolyPeptide Group) was preactivated with 3.9 equivalents of HCTU followed by the addition of DIPEA prior to the addition to the resin and allowed to react for 90 minutes. After a capping and deprotection step, four equivalents of Fmoc-S-tert-butylthio-L-cysteine (t-BuSH) (Novabiochem) were activated and added to the resin as discussed previously and reacted for two times 30 minutes. No capping step was performed after deprotection of the Fmoc-Cys(t-BuSH) coupled to the PEG3-PI-polyamide. The resin was dried under vacuum cleaved with Dp as described previously. Gradient RP-HPLC: 20-40% in 10 minutes.

**Fmoc-mediated solid phase peptide synthesis of Nbz-peptide.** The synthesis of the thioester peptide was performed manually on a Dawson Dbz AM resin (Novabiochem) on a 100 µmol scale. Deprotection of the Fmoc-group attached to the resin was performed two times for 20 minutes using 20% piperidine in NMP followed by three consecutive NMP washing steps. The first
amino acid (6 equivalents), in this case alanine, was pre-activated with 5.9 equivalents of 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) followed by the addition of 12 equivalents of DIPEA prior to the addition to the resin. The coupling of Fmoc-protected alanine was performed twice and reacted for 90 minutes at room temperature. The subsequent amino acids were coupled using HCTU instead of HATU using a single coupling step of one hour and 6 equivalents per amino acid. The final amino acid was introduced as a Boc-protected amino acid. After completion of the synthesis, the peptide was washed with dichloromethane (DCM) and dried under vacuum. The resin was swollen in DCM prior to the cleavage. 100 mg of p-nitrophenylchloroformate (Sigma-Aldrich) was dissolved in 6 ml DCM was added to the resin and agitated gently for one hour. The peptide was washed three times with DCM and twice with NMP followed by the addition of 0.5 M DIPEA and agitated for 30 minutes. After activation the peptide was washed with NMP and DCM. A mixture of TFA/triisopropylsilane/water (95: 2.5: 2.5% v/v) was added to the resin and agitated for 3 hours followed by precipitation of the Nbz-peptide in ice cold diethyl ether. After a centrifugation step the pellet was dissolved in a mixture of acetonitrile and water frozen, and lyophilized. Purification was done by preparative RP-HPLC using a gradient of acetonitrile in H2O (both containing 0.1% TFA). Gradient RP-HPLC: 18-28% in 10 minutes.

Native chemical ligation of Cys-Pl-polyamide to the thioester Nbz-peptide. Cys-Pl-polyamide was mixed in a slight excess (1.1 to 1 ratio) with the thioester peptide at a final concentration of 2.0 mM in a ligation buffer containing 200 mM sodium phosphate, 6 M Guanidine·HCl, 20 mM TCEP, 200 mM MPAA at pH 6.8 and reacted for 15 hours. Excess MPAA and TCEP were removed using a solid phase extraction column (Strata-XL, Phenomenex) and the conjugate was eluted using a gradient of acetonitrile in H2O (both containing 0.1% TFA) and subsequently lyophilized.

Protein-expression and purification. The expression plasmids pTXB1-EYFP, pTXB1-ECFP and pTXB1-CNA35 were transformed in E. coli BL21 (DE3) cells. Similar expression conditions were used for both proteins and were reported previously. In short, bacteria from the glycerol stock were added to a 5 mL LB medium containing 0.1 mg mL\(^{-1}\) ampicillin and were grown overnight at 37 °C and 225 rpm. The culture was transferred to a 2 L LB medium containing 0.1 mg mL\(^{-1}\) ampicillin and incubated at 37 °C and 225 rpm until an optical density was reached of 0.6 (OD\(_{600}\) nm). Protein expression was induced by addition of 0.25 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the cultures were grown overnight at 225 rpm at 15 °C. The cells were harvested by centrifugation at 8,000 rpm at 4 °C for 15 minutes. The cytoplasmic fraction was obtained by chemical lysis of the cell pellet by addition of 5 mL Bugbuster (Novagen) and 5 µl benzonase (Novagen) per gram cell pellet and agitated for 40 minutes at room temperature. The lysate was centrifuged at 16,000 g for 20 minutes at 4 °C. The supernatant containing ECFP, EYFP or CNA35 was directly applied to a column containing chitin beads (New England Biolabs), equilibrated with 10 column volumes of wash buffer (25 mM sodium phosphate, 0.5 mM EDTA, 0.5 M NaCl, pH 8.0). After loading of the supernatant, the column was washed with 10 column volumes of wash buffer. The column was quickly flushed with 3 column volumes of cleavage buffer (20 mM Sodium Phosphate, 0.12 mM EDTA and 0.5 M NaCl and 400 mM sodium 2-mercaptopethanesulfonate, MESNA, at pH 6) and the flow was stopped and incubated overnight at room temperature to allow intein cleavage and thioester
PI-Polyamide peptide and protein conjugates

formation. The protein thioester was collected by washing the column with 3 column volumes of cleavage buffer. The pooled protein fractions were buffer-exchanged into wash buffer (at pH 7.0) and concentrated using Amicon ultra centrifuge tubes (MWCO of 10 kDa). The purity of the MESNA-thioester proteins was analyzed by SDS-PAGE and ESI-MS analysis. The concentrations of EYFP, ECFP and CNA35 containing a C-terminal MESNA thioester (EYFP-MESNA, ECFP-MESNA and CNA35-MESNA) were determined by UV-vis using \( \varepsilon_{514\text{nm}} = 83,400 \text{ M}^{-1} \text{ cm}^{-1} \), \( \varepsilon_{433\text{nm}} = 32,500 \text{ M}^{-1} \text{ cm}^{-1} \) and \( \varepsilon_{280\text{nm}} = 33,167 \text{ M}^{-1} \text{ cm}^{-1} \). 1 L of culture resulted in yields of 10 mg for EYFP, 9 mg for ECFP and 20 mg for CNA35 The protein was frozen with liquid nitrogen and stored in the freezer until the native chemical ligation was performed.

Native chemical ligation of Cys-PI-polyamide to ECFP-, EYFP- or CNA35-MESNA. Typically, high protein concentrations are desirable in order to achieve near complete reaction yields. Prior to ligation, the tert-butylthiol protected Cys-PI-polyamide was dissolved at mM concentrations and incubated for one hour in ligation buffer containing 200 mM sodium phosphate, 180 mM tris(2-carboxyethyl)phosphine (TCEP), 100 mM MPAA and 2.5% DMSO at pH 6.8 to deprotect the cysteine followed by the addition of MESNA-protein resulting a 10-fold excess of Cys-PI-polyamide over the MESNA-protein (3.0 : 0.3 mM, respectively). The reaction was performed at room temperature for 36 hours. Excess Cys-PI-polyamide, MPAA and TCEP were removed extensively by repeated exchanging buffer (200 mM sodium phosphate, 0.5 M NaCl at pH 6.8) using centrifugal filters (MWCO of 10 kDa).

Surface Plasmon Resonance. Sensor grams were obtained on a Biacore T100 (GE Healthcare) using streptavidin-coated sensor chips (SA-chip, GE Healthcare). Immobilization of biotin-functionalized DNA sequences (MWG Eurofins) was achieved by starting a sensorgram for the flow channel (FC) of interest with HBS-EP + 0.1% DMSO as running buffer (GE healthcare) at a flow rate of 20 µL min\(^{-1}\) at 25 °C. The carboxymethylated dextran chip with immobilized streptavidin was initially washed with a mixture of 1 M NaCl and 50 mM NaOH and injected seven times for one minute at 20 µL min\(^{-1}\). The running buffer was flown over the chip for 5 minutes, followed by the injection of 25 nM biotin-functionalized DNA at a flow rate of 2 µL min\(^{-1}\) until the desired immobilization level of approximately 450 RUs (1 RU = 1 pg mm\(^{-2}\)) was reached. Finally the flow rate was changed back to 20 µL min\(^{-1}\) and the activation buffer was injected again two times for one minute and the final immobilization level was determined. These steps were repeated for different DNA sequences in different flow channels. Flow channel 1 was typically chosen as the reference channel and was left unmodified. Cognate DNA sequence: CGCATATGGTATGTGCCGCGAA-AAACCCGCGCACATACCATATGC; 3 bp mismatch: CAGTCATGAGCATGGATGCGGA-AAACCCGCGCATCCATTGCTCATGACTG; scrambled: CGCATCTACGACGTGCGCGCAAAA-CGCACGACCTTAGATCGG.

The maximum response of the PI-polyamide (conjugate) binding to the immobilized DNA can be calculated according to equation 6.1 assuming a stoichiometric ratio of 1. All SPR-experiments shown in this chapter were performed with DNA immobilization levels of approximately 400 to 450 RU\(\text{s}\) unless stated otherwise.
All experiments were performed at 25 °C. PI-polyamide (conjugate) was dissolved in HBS-EP + 0.1% DMSO running buffer and serial dilutions were made. The PI-polyamide conjugates were injected from low to high concentration. Before every sample injection, a 5 minute flow (25 uL min⁻¹) was performed with running buffer to obtain a stable baseline. Every sample was injected for 8 or 10 minutes and followed by a dissociation phase of 10 minutes. Regeneration of the chip was performed using 10 mM glycine pH 2.5 for 30 seconds. Before the next injection, a stable baseline was obtained by washing for 5 minutes with running buffer. The collected data was analysed using the BIAcore T100 Evaluation Software and plotted in Origin using a 1:1 binding Hill equation \((n=1)\) to obtain \(K_d\)-values (equation 6.2). Aspecific binding and buffer effects were taken into account by subtracting the response from a reference channel not containing DNA.

\[
RU = \frac{[PI - polyamide]}{[PI - polyamide] + K_d}
\] (6.2)

It appeared that the regeneration condition used after every binding step was not optimal in terms of not complete removal of the analyte after binding high concentrations of PI-polyamide conjugate. Different regeneration conditions (injection times, more acidic, ionic (NaCl) or even basic conditions) did not improve the regeneration of the chip. Therefore the initial regeneration solution containing 10 mM Glycine and pH 2.5 remained the regeneration solution of choice, but a new SA-chip was functionalized with DNA for each new titration experiment.

6.5 Acknowledgements

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Chapter 7

EPILOGUE
Chapter 7

7.1 Introduction

The work described in this thesis showed that DNA provides an attractive scaffold to reversibly control the activity of antibody binding as well as enzyme activity. Both strategies make use of the predictable nature of Watson-Crick base pairing and the tuneable structural properties of DNA to create highly modular systems. Bivalent epitopes connected by a double stranded DNA-linker were shown to effectively span the relatively large distance (~ 12 nm) between the antigen binding sites in antibodies, yielding predominantly 1:1 closed complexes. Our proof-of-principle-study using the anti-HIV antibody revealed a 500-fold increase in binding to the antibody when using the bivalent peptide-dsDNA ligand compared to the monovalent ligand. This large difference in affinity is the key principle that allows the reversible blocking of antibodies. Since the Y-shaped bivalent presentation of two antigen binding sites is shared by all antibodies, the non-covalent blocking of the antigen binding sites by bivalent peptide-dsDNA locks should be generic applicable, as was subsequently shown for two different antibodies of the IgG1 subclass; anti-HA and cetuximab.

Several other approaches have recently been reported to control the activity of a monoclonal antibody such that it is inactive in healthy tissue and only becomes active at the site of the disease in the presence of specific biomarkers, e.g. proteases or miRNA sequences. However, these approaches all require extensive antibody or DNA-based engineering,[1–4] whereas the non-covalent self-assembly strategy explored in this thesis can be applied directly to the native monoclonal antibody. The potential of antibody activation by specific biomarkers was initially investigated by the introduction of protease sensitivity in the DNA-linker. The presence of the MMP2-protease reversed the blocking of the anti-HIV antibody allowing the antibody to bind to its target antigen. Furthermore, the use of specific oligonucleotides as triggers for antibody activation was investigated. The introduction of a toehold sequence in the bivalent peptide-dsDNA lock enabled toehold-mediated DNA strand displacement reactions to activate antibodies. This was shown for the anti-HA antibody as well as for the clinically approved therapeutic antibody cetuximab. Besides using the DNA-based locks to control antibody targeting, these DNA-locks also provide an excellent opportunity to harness the great molecular recognition properties of antibodies for DNA-based biomolecular computing, providing a generic molecular hub to combine the programmability of DNA with protein activity as in- or output. Current DNA-based computing systems or circuits are predominantly based on DNA-exchange reactions or the use of specific DNA sequences (aptamers) that recognize a limited number of proteins and not so much in controlling protein function.

This chapter elaborates on the key design principles and the future directions of using bivalent peptide-dsDNA locks for antibody targeting purposes. In addition the societal impact and possible risks associated with this novel technology will be assessed as part of the Risk Analysis and Technology Assessment (RATA) program within the NanoNextNL consortium.
7.2 Design principles for bivalent peptide-DNA locks

Antibodies typically show higher affinities towards native proteins or protein domains in comparison to peptide epitopes or mimotopes. To obtain effective reversible blocking of an antibody towards its native target, three important thermodynamic prerequisites need to be considered: (1) the bivalent binding of the peptide-DNA lock to the antibody must be significantly stronger than the binding between the antibody and the native protein. (2) The transition from a stable bivalent to the weaker monovalent ligand must result in a large attenuation of antibody affinity and (3) the monovalent affinity of the peptide to the antibody should be weaker compared to that of the native target protein, thus allowing the antibody to bind to the native protein upon ligand monomerization.

The results described in Chapter 3 to 5 together provide a comprehensive insight in these design requirements. The binding of an antibody to a bivalent peptide-dsDNA ligand is described in Scheme 7.1. The initial step is the intermolecular interaction between the ligand and the antibody ($\frac{1}{4} K_d^{\text{inter}}$) followed by an intramolecular binding step ($2 K_d^{\text{intra}}$). The overall affinity, or avidity, of the bivalent ligand depends on the monovalent interaction, but also on the nature and the length of the linker which together determine the effective concentration ($C_{\text{eff}}$). The effective concentration is the concentration of the unbound ligand near the unbound antigen binding site, when the other ligand and antigen binding site are bound.\[5\]

\[ K_d^{\text{avidity}} = \frac{1}{2} K_d^{\text{inter}} K_d^{\text{intra}} \]
\[ C_{\text{eff}} = K_d^{\text{inter}} / K_d^{\text{intra}} \]
\[ K_d^{\text{avidity}} = \frac{1}{2} \left( K_d^{\text{inter}} \right)^2 / C_{\text{eff}} \]

**Scheme 7.1:** Thermodynamic model describing the binding of the dsDNA-based bivalent ligand from the bivalent antibody.

In our model study using the anti-HIV antibody we observed that a 35 bp DNA-linker provided an effective concentration of ~ 8 µM, resulting in a $K_d^{\text{avidity}}$ of 55 pM. Since the monovalent affinity was 30 nM ($K_d^{\text{inter}}$), the use of the dsDNA linker resulted in a 500-fold increase in overall affinity towards the antibody. In our study that used the anti-HA antibody (Chapter 4), the avidity of the bivalent peptide-dsDNA locks was not directly determined. Tight binding was observed for both the 20 bp and 35 bp linker lengths, however, since a slight excess of bivalent lock (1.1 nM vs 1 nM) was sufficient to completely block the anti-HA antibody. In this case assuming an effective concentration of 8 µM and a monovalent affinity of the HA-epitope ($K_d^{\text{inter}}$) of 5 nM would yield an
estimated bivalent affinity ($K_d^{\text{avidity}}$) of 1 pM, corresponding to a 5000-fold theoretical gain in affinity. The importance of the monovalent affinity of the peptide for the avidity of the bivalent ligand became also clear in our work on cetuximab (Chapter 5). The initial peptide mimitope obtained from literature\cite{6} showed a rather weak affinity ($K_d^{\text{inter}} = 264$ nM) towards cetuximab and was even more weakened when conjugated to an oligonucleotide due to electrostatic repulsion ($K_d^{\text{inter}} = 4.5$ µM). As expected in this case only a minor gain (5-fold) in bivalent affinity was observed ($K_d^{\text{avidity}}$ of 900 nM), since the monovalent affinity was similar to the expected effective concentration provided by the dsDNA-linker. The bivalent peptide-dsDNA-lock based on the peptide mimitope that did bind to the antigen binding site of cetuximab showed an increase in bivalency effect with respect to cetuximab blocking compared to the monovalent ligand. The affinity enhancement concerning the DNA-based bivalent lock was not yet experimentally determined. Conditional on an effective concentration of \(\sim 8\) µM provided by the DNA-linker and a monovalent affinity of 290 nM corresponds to a theoretical $K_d^{\text{avidity}}$ of 4 nM and an affinity enhancement of approximately 60-fold. The blocking of cetuximab was less efficient and required a fifty-fold excess of bivalent locks which was in contrast to the slight excess of bivalent locks that were needed to effectively block the anti-HIV and anti-HA antibodies.

A recent study of Bjorkman and coworkers using dsDNA linkers to construct bivalent Fab ligands showed similar affinity enhancements as observed for the bivalent peptide-dsDNA locks for the anti-HIV and anti-HA antibodies. In their work, homo and heterobivalent ligands were constructed by connecting Fab fragments with different length of dsDNA that could span distances of 10 nm up to 30 nm.\cite{7} A strong linker length dependency was observed resulting in a more than 100-fold gain in affinity for the bivalent ligand when the dsDNA-linker exactly matched the distance between the binding sites on the protein (14 nm). This strong dependency on linker length was attributed to the well-defined target protein that possessed limited flexibility. In our work, the linker length dependency is less pronounced, as linker lengths of 20 bp and 35 bp (7 nm and 12 nm) are both accommodated. This difference can be explained by the structural plasticity of antibodies in the hinge region, as also was observed in the SAXS-measurements described in Chapter 3. The flexibility observed in the hinge region of the IgG1 subclass suggests that the effective concentration of 8 µM may not be enhanced much further by optimizing the dsDNA-linker length connecting the peptide ligands. However, other antibody classes (IgA and IgE) are known to have a more constrained hinge region and are therefore interesting targets to investigate the dependence of dsDNA-linker length on the effective concentration and bivalent affinity to antibodies.\cite{8}

The strong bivalent binding of the peptide-dsDNA lock was important to effectively block antibody binding to the native protein. The blockage of the antibody to the native protein can be completely reversed when the transition of the stable bivalent ligand to the monovalent ligand results in a large attenuation in antibody affinity and when the affinity of the monovalent ligand ($K_d^{\text{inter}}$) is sufficiently weaker than the native protein. In
Chapter 3 we observed that binding of the anti-HIV antibody to its native protein (p17) was completely restored after proteolytic cleavage of the bivalent peptide-dsDNA lock. This indicated that indeed the affinity of the monovalent ligand is sufficiently weaker than the native p17 protein. This was not the case in our study using the anti-HA antibody, since the HA-epitope at the surface of yeast that was used as the target receptor was the same as the HA-epitope used in the bivalent ligand. Toehold-mediated strand displacement reversed the blocking of the anti-HA antibody and the resulting monovalent ligand competed with the HA-epitope at the surface of yeast for binding to the anti-HA antibody. Cetuximab binding was evaluated using the A431 cell line that displays the epidermal growth factor receptor (EGFR) at the surface. The affinity of cetuximab to the EGFR was approximately 0.5 nM. The monovalent affinity of the peptide \(K_d^{\text{inter}} = 290\) nM to cetuximab is more than 700-fold weaker than the affinity of cetuximab to EGFR, allowing complete restoration of cetuximab binding to EGFR. However, in this case the affinity of the bivalent peptide-dsDNA lock was probably weaker than that of native EGFR target, requiring the use of excess bivalent ligand to effectively block cetuximab binding to EGFR. If the effective concentration provided by the DNA-linker cannot be increased much further due to the flexibility of the IgG1-class antibodies, the only way to increase the blocking efficiency is to develop a higher affinity peptide binder. For cetuximab, a peptide with a monovalent affinity of 5 to 30 nM would ideally result in a theoretical overall affinity of the bivalent peptide-dsDNA lock of 1.5 to 56 pM. Such affinities for the monovalent peptide would be at least 10-fold weaker compared to cetuximab binding to the EGFR, whereas the bivalent ligand would be at least 10-fold stronger providing sufficient blocking of the cetuximab in absence of any trigger.

7.3 Future directions

7.3.1 Increasing the stability of peptide-DNA locks using chemically modified DNA

DNA-nanostructures provide attractive opportunities for more controlled and precise therapeutic strategies such as antibody-mediated targeting and antisense therapies. However, for \textit{in situ} or \textit{in vivo} applications the hydrolytic stability of nucleic acids remains a challenge. Recently, Perrault and coworkers addressed the stability of different DNA-based origami structures (curved octahedron, tube and rod) in tissue culture with respect to nuclease degradation. The curved octahedron was fully degraded after 24 hour incubation in serum at 37 °C, but degradation could be partially prevented by adding actin as a nuclease inhibitor. Furthermore, the relatively low concentration of magnesium in tissue culture media (0.4 mM MgCl\(_2\)) was also observed to result in instable structures.[9] Since high magnesium concentrations (> 10 mM) are necessary in the folding process of DNA-origami based structures, the use of actin and the presence of 6 mM MgCl\(_2\) under physiologic conditions were proposed to maintain the integrity of the DNA nanostructure in the biological environment.
An alternative and more robust approach to increase the stability of DNA-structures in vivo is the use of chemically modified oligonucleotides. The use of hydrolytically stable oligonucleotide analogues increases the stability towards nucleases, but can also alter the thermodynamics of hybridization. Natural nucleotides are composed of three chemical moieties: an aromatic nucleobase, a sugar and the anionic phosphodiester linkage between the monomers. Resistance towards nuclease degradation can be obtained by replacing the phosphodiester linkage by a phosphorothiate linkage, which replaces a phosphate oxygen by a sulphur atom (Figure 7.1a). The phosphorothioate modification preserves a negative charge on the DNA backbone, which retains the aqueous solubility of the oligonucleotide. However, it has been observed that phosphorothioate modification results in a decrease in hybridization stability. In comparison with a 24-mer DNA-DNA duplex ($T_m = 67.5 \, ^\circ C$), a duplex containing 24 phosphorothioate-modifications resulted in a $T_m$ decrease of 7.8 $^\circ C$, whereas a duplex containing only phosphorothioate modifications (48 nt) resulted in a decrease in $T_m$ of 15.7 $^\circ C$. The phosphorothioate linkages may be introduced throughout the oligonucleotide, but the most crucial positions to inhibit exonuclease degradation are the last 3 to 5 nucleotides at the 5'- or 3'- end.

The phosphodiester bond can also be replaced by a completely different backbone. A widely used alternative is the grafting of nucleobases on a polypeptide backbone, yielding so called peptide nucleic acids (PNA). Nielsen and coworkers replaced the deoxyribose phosphate backbone by a pseudopeptide N-(2-aminoethyl)glycine) with the nucleobases attached through a methylene carbonyl linker (aegPNA). This architecture improved the biological stability, while preserving the ability to form a stable double helix based on Watson-Crick basepairing (Figure 7.1b). The absence of a negatively charged phosphate group avoids the electrostatic repulsion between complementary strand resulting in a more stable duplex formation (e.g. 10 nt duplex PNA-PNA $T_m = 67 \, ^\circ C$; PNA-DNA $T_m = 51 \, ^\circ C$; DNA-DNA $T_m = 33.5 \, ^\circ C$). A major advantage of PNA is that it can be synthesized using standard solid phase peptide synthesis, allowing direct synthesis of PNA-peptide conjugates. However, PNA has a relative poor water solubility compared to DNA.

Instead of altering the linker connecting the deoxyribose-moieties the ribose itself may also be modified to increase the resistance towards nucleases. These locked nucleic acids (LNAs) consist of modified RNA nucleotides that contain a methylene bridge that connects the 2'-O with the 4'-C of the furanose ring. The more restricted conformational freedom of the sugar results in an improved stability towards nucleases. The aqueous solubility of LNA is comparable to that of natural DNA or RNA. More interestingly is the increase in thermodynamic stability upon complementation of natural nucleic acids (DNA and in particular RNA) with LNA due to the entropic favourable duplex formation. The $T_m$ of a LNA complex with DNA/RNA is increased by 3 to 9 $^\circ C$ per LNA monomer. The LNA:LNA duplex is more stable than the LNA:DNA or LNA:RNA duplex.
7.3.2 Alternative triggers for antibody activation

Current antibody-based therapies often display off-target effects, which limits their therapeutic window. This thesis introduced the use of bivalent peptide-dsDNA locks as a non-covalent approach to block antibodies, which could subsequently be activated at the diseased or tumor site. Several tumor-specific molecular triggers can be envisioned to allow tumor-specific antibody activation. An interesting trigger that has already been exploited to locally activate drugs and antibodies is proteolytic activity.\(^2,3\) Matrix metalloproteinases (MMPs) are the most prominent proteinases associated with oncogenesis and are released in the extracellular space around the tumor. The major MMPs that have been associated with multiple human cancers are MMP-2, -9 and -14.\(^{16}\)

Another potentially interesting trigger is miRNA, since altered expression profiles of certain miRNAs have been linked to human cancers.\(^{17}\) The majority of miRNAs are found intracellular, however significant amounts of miRNA related to tumors and cardiovascular diseases have been observed outside the cell, providing potential biomarkers for diagnostic or selective targeting purposes.\(^{18}\) Therefore, endogenous miRNAs could be used to initiate DNA strand displacement reactions to selectively activate therapeutic antibodies.

The structure-switching properties of DNA aptamers have been used to create autonomous diagnostic as well as targeting devices.\(^4,19\) Human acute lymphoblastic leukemia cells (CEM) overexpress specific cell surface markers, e.g. protein tyrosine kinase 7 (PTK7). Tan and coworkers developed a DNA device bearing three different aptamer sequences including the PTK7-specific aptamer sequence (Sgc8c) to selectively release a fluorophore from a quencher.\(^{19}\) The release of the fluorophore was used to distinguish CEM cells from different cell types such as HeLa or Ramos cells based on cell surface protein expression. Church and coworkers used a combination of aptamer sequences as locks to selectively reveal the molecular payload that was shielded in a

**Figure 7.1:** Chemically modified DNA analogues to increase the stability towards nucleases.

![Chemically modified DNA analogues](image-url)
DNA-origami based barrel only in the presence of cell surface proteins characteristic for specific cell types ranging from T-cell leukemia to neuroblastoma cells. In their work, the PTK7-specific aptamer sequence (Sgc8c) was used as well as an aptamer sequence against the platelet-derived growth factor (PDGF). These aptamer sequences (30 to 40 nt) may be used to release a specific DNA strand that can initiate a toehold-mediated DNA strand displacement reaction as was shown in Chapter 4 for thrombin-induced activation of the anti-HA antibody. An alternative would be the direct incorporation of these aptamers in the bivalent peptide-dsDNA lock.

Figure 7.2: Potential triggers for activation of bivalent peptide-dsDNA locks.

Hydrogen peroxide has also been suggested as a tumor-specific biomarker. Chang and coworkers were able to chemoselectively monitor H$_2$O$_2$ fluxes in testosterone-stimulated prostate tumor xenografts in mice using boronic acid caged luciferin. The H$_2$O$_2$-mediated oxidation of aryl boronates to phenols has been used as a bioorthogonal approach to image the presence of reactive oxygen species and to activate prodrugs. Van Duijnhoven and coworkers proposed a mechanism of H$_2$O$_2$ mediated self-immolation of an aryl boronic pinacol ester-alanine wedge for radiolabeled activatable cell penetrating peptides. Based on their design for H$_2$O$_2$-triggered cell penetrating peptides one could envision the incorporation of an aryl boronic pinacol ester in between the peptide and the oligonucleotide to facilitate H$_2$O$_2$-triggered antibody activation.

Besides reactive oxygen species to trigger antibody activation, one may also consider using pH as a trigger for antibody activation. The extracellular pH in the vicinity of tumor tissues is mildly acidic compared to healthy tissues and in the range of 6.5 – 6.8. The lower pH is caused by the enhanced production of lactic acid by glycolysis and has been used as a stimulus for pH-responsive delivery of drugs and therapeutic agents. pH-dependent formation of DNA secondary structures could be exploited to switch between locked and open state of the antibody bivalent ligand interaction. For example, Krishnan and coworkers functionalized nicked dsDNA and used cytosine-rich overhangs that undergo a conformational change at low pH. The protonation of the cytosine residues results in a four-stranded cytosine-quadruplex known as an I-motif of which the stability can be tuned as a function of pH (Figure 7.3a). I-switches have
recently been tested in living cells to image pH differences by FRET.\cite{27} Whether I-motif formation at lower pH is sufficiently strong to compete with bivalent binding of the peptide-dsDNA lock to the antibody remains to be determined (Figure 7.3b).

**Figure 7.3:** pH-dependent formation of I-motif structures. **a)** Cytosine-rich strands can form stable four-stranded DNA secondary structures in acidic conditions. Two DNA duplexes are held together by cytosine-cytosine+ base pairs. **b)** Proposed scheme of antibody activation in an acidic environment based on cytosine-quadruplex formation of the DNA-bivalent linker containing a nicked site.

The previous discussed triggers are endogenous and located near the diseased site. However, light can also be used to turn on specific (biological) processes at a controlled location and at a specific time. This is often achieved by making use of light-removable protecting groups.\cite{28} Deiters and coworkers showed the incorporation of photolabile caging groups in DNA-strands to allow spatial and temporal control of displacement reactions in cells using UV-irradiation as a trigger.\cite{29} A light-removable protecting group may also be incorporated in the bivalent peptide-dsDNA lock to render antibody activity conditional on light irradiation. For instance, a nitrobenzyl-containing linker can be introduced between the peptide and the dsDNA-linker. This cleavable linker is commercially available and can be introduced at the 5’-end of the oligonucleotide and subsequently coupled to the peptide (Figure 7.4a). The cleavage occurs by irradiation with near UV-light (300-350 nm) and thereby disrupts the bivalent peptide-dsDNA lock resulting in an active antibody. DNA duplex formation can also be controlled by using azobenzene-modified oligonucleotides (Figure 7.4b and c). When irradiated at 300-380 nm, azobenzene undergoes a reversible isomerisation from trans to cis and from cis to trans at wavelengths > 400 nm.\cite{30} The presence of even a single azobenzene-moiety can lower the $T_m$ of a duplex by 15 °C when going from a trans to cis isomer.\cite{31} The position and the number of azobenzene moieties can be varied and this strategy has been used to control hybridization states of oligonucleotides for the controlled release of drugs.\cite{32} The incorporation of one or multiple azobenzene moieties could drastically influence the DNA duplex of the peptide-dsDNA lock and thereby switch between a monovalent or bivalent ligand and thus controlling antibody activity.
Chapter 7

Figure 7.4: Chemical strategies to install photochemical control of DNA hybridization. a) Peptide coupled to an oligonucleotide via a nitrobenzyl-linker allows photo cleavage by UV-light. b) Reversible isomerization by azobenzene modified oligonucleotide upon light excitation. c) The cis-conformation of the azobenzene-modified oligonucleotide hampers the hybridization to a complementary strand. Adapted with permission from[31].

7.4 Risk Analysis and Technology Assessment

The focus of the research described in this thesis was to develop novel approaches for improving antibody-based therapeutics. This research was part of the BioNano-program within the NanoNextNL-consortium. The Bio-Nano program aims to develop novel technologies that can control the interactions between biomolecules at the nanoscale and study them in the biological environment. NanoNextNL encourages academia and industrial research to collaborate and develop nanotechnology-based innovative solutions that are beneficial to society. Since society relies on the knowledge and judgement of scientists to evaluate the consequences of current research developments, researchers within the NanoNext program have the responsibility to identify and address potential risks which may arise from their research involving nanotechnology. But what is nanotechnology?

Nanotechnology is a word to grasp all the research areas involving engineering or technology at the nanometer scale (10^{-9} m). In a lecture that inspired many researchers, Richard Feynman initiated the field of nanotechnology in 1959 by creating awareness that there is plenty of room at the bottom.[33] The control and manipulation at the atomic and molecular level was expected to result in numerous advances in various research areas including chemistry and biology. DNA-based nanotechnology is an excellent example as Watson-Crick base pairing allows precise control of molecular structures and processes at the sub-nanometer scale.

The public perception of nanotechnology is subject to concern. This fear partially originates from recent examples of nanotechnology that appeared hazardous to society, e.g. carcinogenic carbon nanotubes,[34] but may also be due to the unawareness of the general public of what nanotechnology comprises. In addition, DNA-based research can have a negative connotation because it is associated with genetically modified organisms and biosafety issues. However, we do not expect major societal resistance for the use of DNA-based locks to improve antibody-based therapeutics since it does not involve modified organisms and the potential application explicitly lies in the medical field. The
use of the DNA-based locks combined with therapeutic antibodies may decrease the side-effects and thereby become beneficial for patients who undergo these treatments. Therapeutics in general may show severe side-effects or even toxicity, but are considered beneficial for the patient when the actual hazard is contained or suppressed.

Our approach to control antibody activity consists of two main components: the antibody and a DNA-based lock. The self-assembly of the DNA-based lock can be considered as a major advantage since it allows the use of FDA-approved antibodies. The majority of antibodies currently used in the clinic have a narrow therapeutic window due to off-target effects or so called side-effects like acne-form rash. The strategy described in this thesis may limit the side-effects resulting in an increase of the therapeutic window, yielding more effective antibody-based therapeutics and thereby be of societal benefit. In fact, controlling antibody activity using DNA-based locks could result in the re-evaluation of antibodies that have been discarded in the past, despite their effectiveness, due to severe side-effects.

The other components of our targeting concept are the bivalent peptide-DNA locks, which at present are constructed using natural nucleic acids. However, these are known to be susceptible towards nuclease degradation in blood serum and may result in an adverse immune response. To the best of our knowledge, only unmethylated DNA-sequences that contain cytosine followed by guanine and linked via a phosphodiester (CpG motif) are known to initiate an immune response. In our design for the lock we must specifically circumvent the unmethylated CpG motif (5'-GTCGTT-3') to prevent adverse immune responses. Furthermore, the stability of the DNA-based lock towards nucleases can be increased by using nucleic acid analogs. For example, locked nucleic acids (LNA) show stability towards nucleases and also increased thermodynamic stability when hybridized to a complementary LNA-strand. LNAs are currently considered as 3rd generation oligonucleotide analogs for antisense therapies and have recently been evaluated in Phase I clinical trials in patients with advanced and metastatic cancer. Although still in an early stage, the first trials reported good tolerability and minimal hepatic toxicity (liver) of LNA-based antisense therapies. A 2nd generation DNA analog containing the 2-O-methoxyethylribose modification (MOE) has already entered late stage clinical trials for the treatment of prostate cancer showing overall survival benefits.

The research described in this thesis started with the fundamental question whether double stranded DNA would be an effective linker to bridge the large distance between the antigen binding sites of an antibody to control antibody activity. The research trajectory has developed from a conceptual study towards the exciting possibility of using DNA-based locks to control antibody activity in novel therapeutic approaches. Although we are still at an early stage of development, reflecting at the current status of our research may help to anticipate societal embedding and address potential risks. In this way we hope to at least partially circumvent the Collingridge dilemma, which states...
that impacts cannot easily be predicted until the technology is widely used, but cannot easily be changed once the technology is embedded in society.\[39\]

7.5 References

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Summary

DNA-based control of protein activity

Deoxyribose nucleic acids or DNA stores the blueprint for life, but the highly predictable Watson-Crick base pairing also makes DNA an attractive material for bottom-up nanotechnology. In the past decade, nucleic acids were proven to be promising building blocks to develop molecular nanostructures and dynamic nanodevices that may find applications in biomedicine and molecular diagnostics. An important challenge in this field is to develop generic approaches that interface DNA-based logics with protein activity. This dissertation describes novel biomolecular approaches to use DNA to control protein-based interactions, in particular to reversible control the activity of enzymes and antibodies.

Chapter 1 describes recent advances in DNA-based nanotechnology including the use of DNA as a construction material, DNA-based computing, possible biomedical applications and the use of DNA as a scaffold for multivalent ligands. The long persistence length and the possibility to easily tune the distance by the number of base pairs makes DNA an attractive scaffold for multivalent ligands. Commonly used conjugation strategies are discussed that have been used to decorate DNA-structures with peptides and proteins. Finally, an overview of antibody-based therapeutics is given.

Chapter 2 describes a generic approach to control enzyme activity in a reversible manner by DNA-templated assembly of an enzyme and an inhibitor protein using semi-synthetic DNA-protein hybrids. TEM1-β-lactamase and its inhibitor protein BLIP were conjugated to different oligonucleotides resulting in an inactive enzyme upon the presence of a DNA template strand. The enzyme-inhibitor complex could be disrupted upon hybridization of a target strand to the template strand, resulting in a rigid dsDNA linker and the restoration of enzyme activity. The non-covalent and modular assembly of the enzyme-inhibitor complex allowed the screening of different lengths and sequences of both template and target strands. The system was able to detect as little as 2 fmol of target DNA and the modularity of the system allowed the rapid screening for different viral DNA sequences, illustrating its potential use for molecular diagnostic applications. DNA displacement reactions were used to reversibly switch between active and inactive states illustrating the possibilities of this system to interface DNA-based circuits with protein activity.

Monoclonal antibodies have become an important class of targeted therapeutics for the treatment of a range of human diseases. While the intrinsic affinity and specificity of antibodies for their target can be very high, the ability to distinguish diseased cells from healthy cells is often limited by the presence of a substantial background of target receptors in healthy tissues. The core of the work described in this thesis involves the development of a generic targeting concept to reversibly block the antigen binding sites of antibodies and thereby increase their specificity towards targets at the site of interest. We developed a non-covalent approach involving bivalent peptide-DNA ligands to block the antigen binding sites of an antibody. Chapter 3 shows that double stranded DNA provides an attractive rigid linker to effectively span the large distance between antigen binding sites. In this proof of concept study we used anti-HIV-1 as a model antibody and investigated the influence of different dsDNA-linker lengths with respect to antibody complex formation. Bivalent peptide-DNA ligands with 20 and 35 bp linkers were able to form 1:1 closed complexes with the anti-HIV-1 antibody, showing the structural plasticity of the antibody. Binding studies were performed to
investigate the difference in affinity between monovalent peptide and bivalent peptide-dsDNA ligand. Using a thermodynamic model, the interaction between bivalent peptide-dsDNA ligand and antibody was found to be 500-fold stronger than that of the monovalent peptide, allowing effective blocking of the antigen binding sites in a non-covalent manner. Introduction of a protease cleavage site allowed the antibody blocking to be reversed by treatment with matrix metalloproteinase 2.

The use of DNA as a linker also provided an excellent opportunity to control antibody activity by DNA-based logic operations. In Chapter 4 the introduction of a toehold DNA sequence on the bivalent peptide-dsDNA lock is described to enable antibody activation by toehold-mediated DNA strand displacement reactions. The anti-HA antibody that was used in this study confirmed the generic applicability of peptide-DNA locks to control antibody activity. Anti-HA antibody activation could be quantitatively monitored by flow cytometric analysis of yeast displaying the HA-epitope at its surface. The introduction of two different toehold sequences on the peptide-dsDNA lock rendered antibody activation conditional on the presence of two oligonucleotides yielding OR- and AND-gates. The kinetics of the toehold-mediated strand displacement reaction was not affected when the peptide-dsDNA lock was bound to the antibody. The range of molecular inputs could be further extended to protein-based triggers by using protein-binding aptamers.

In Chapter 5, the use of bivalent peptide-dsDNA locks was applied to control the activity of the therapeutic antibody cetuximab. Cetuximab targets the epidermal growth factor receptor (EGFR) which is upregulated in various tumor types. However, the presence of high levels of EGFR in healthy skin leads to a severe acne-type rash when treated with cetuximab, which limits the therapeutic window for treatment of these patients. Two different approaches were explored based on non-covalent peptide-dsDNA locks to control the binding of cetuximab. A homobivalent peptide-dsDNA lock was constructed that blocked cetuximab by binding to the antigen binding sites, whereas a heterobivalent peptide-dsDNA lock was developed that controlled the activity of cetuximab at the level of the Fab-fragment. The heterobivalent peptide-dsDNA lock contained two different cyclic peptides, one binding to the antigen binding site of cetuximab and the other targeting a unique pocket within the Fab-framework of cetuximab. Flow cytometric analysis revealed efficient blocking of cetuximab binding to EGFR-overexpressing cells for both types of ligands. Blockage of cetuximab could be reversed by both protease cleavage and toehold-mediated strand displacement reactions.

In the last part of this thesis (chapter 6), the use of Pyrrole-Imidazole (PI) polyamides was explored as an alternative non-covalent approach to functionalize DNA structures with peptides and proteins. These PI-polyamides are attractive small molecule DNA binders that bind to the minor groove in a sequence-specific manner with nanomolar affinity. Native chemical ligation was introduced as a generic approach to conjugate PI-polyamides to peptide and proteins. However, the peptide-PI-polyamide conjugates showed a 20-fold attenuation in DNA binding strength compared to non-functionalized PI-polyamide, whereas a further attenuation in binding affinity was observed for protein-PI-polyamide. The practicality of using PI-polyamides to address proteins on DNA in a non-covalent manner therefore remains to be established.

Chapter 7 contains a general discussion of the obtained results and elaborates on possible applications and suggestions for further research.
De activiteit van een eiwit reguleren met behulp van DNA

Desoxyribonucleïnezuur, beter bekend als DNA, bevat de blauwdruk van het leven. De structurele eigenschappen van DNA, waarbij een dubbele helix ontstaat door vorming van waterstofbruggen tussen comple mentaire basen, maakt DNA ook een aantrekkelijke moleculaire bouwsteen voor de constructie van goed gedefinieerde objecten op de nanoschaal. Dit gebied van DNA nanotechnologie heeft zich de afgelopen 10 jaar snel ontwikkeld wat heeft geresulteerd in de constructie van complexe 3D nanostructuren met gecontroleerde dynamische eigenschappen en mogelijke toepassingen in de moleculaire diagnostiek en de gecontroleerde afgifte van medicijnen. Een belangrijke uitdaging in dit veld is het ontwikkelen van algemene concepten om DNA nanotechnologie te koppelen aan eiwit functionaliteit. Dit proefschrift beschrijft nieuwe biomoleculaire methoden, gebaseerd op synthetische DNA-constructen, die de activiteit van eiwitten kunnen controleren. Hierbij hebben we ons gericht op de controle van antilichamen en enzymen.

Hoofdstuk 1 geeft een overzicht van de recente ontwikkelingen op het gebied van DNA nanotechnologie, waarbij extra nadruk is gelegd op het gebruik van DNA als constructie materiaal voor bijvoorbeeld multivalente liganden, de toepassing van DNA netwerken voor het uitvoeren van moleculaire berekeningen en logische operaties en mogelijke biomedische toepassingen. Bij deze toepassingen wordt gebruik gemaakt van het feit dat de DNA dubbele helix een rigide structuur vormt waarvan de lengte nauwkeurig kan worden bepaald door het variëren van het aantal basenparen. Daarnaast worden enkele veelgebruikte conjugatie strategieën besproken voor het decoreren van DNA-structuren met peptiden en eiwitten. Tot slot worden de laatste ontwikkelingen op het gebied van therapeutische antilichamen besproken.

Hoofdstuk 2 beschrijft een generiek systeem om de activiteit van een enzym te reguleren door enzym en remmer DNA-conjugaten te plaatsen op een complementaire DNA-keten (template). Het enzym (TEMI-β-Lactamase) en zijn remmer eiwit (BLIP) werden gekoppeld aan verschillende oligonucleotiden die in aanwezigheid van een DNA-template een complex kunnen vormen, resulterend in een inactief enzym. Het enzym-remmer complex kon worden verbroken door de hybridisatie van een DNA target streng met de template streng. De vorming van deze rigide DNA-linker verbreekt de interactie tussen enzym en remmer, wat resulteert in het herstel van enzym activiteit. Door de niet-covalente en modulaire assemblage van het enzym-remmer complex konden zowel de lengte als de sequentie van template en target strand gemakkelijk worden gevarieerd. Hierbij bleek het mogelijk om een zeer kleine hoeveelheid, 2 femto-mol, van het target DNA te detecteren. Ook bleek dit modulaire systeem uitstekend geschikt te zijn voor het snel screven van verschillende virale DNA sequenties, wat mogelijkheden biedt voor moleculair diagnostische toepassingen. Door handig gebruik te maken van competentere DNA hybridisatietoepassingen kon het enzym herhaaldelijk worden aan en uitgeschakeld, wat het tevens geschikt maakt om de activiteit van DNA netwerken te koppelen aan enzym activiteit.

Monoklonale antilichamen worden steeds vaker gebruikt voor de behandeling van ziektes zoals kanker en chronische ontstekingsreacties. Dergelijke antilichamen binden met een hoge affiniteit aan een specifiek (receptor) eiwit. Een nadeel is dat deze therapieën vaak gepaard gaan met bijwerkingen, omdat het betreffende ‘target’ eiwit ook bij gezonde cellen aanwezig is. In dit proefschrift wordt een
nieuw en generiek concept geïntroduceerd waarbij bivalente peptide-DNA constructen worden
gebruikt om de antigeen bindende domeinen van een antilichaam te blokkeren en uitsluitend te
activeren in de nabijheid van ziekte cellen. In hoofdstuk 3 laten we zien dat dubbel streng DNA
uitarm onderzocht. Bivalente peptide-DNA liganden met 20 en 35 basenpaar linker bleken
beiden een stabiel 1:1 complex te vormen met het anti-HIV-I antilichaam. Dit toont aan dat het
antilichaam flexibel genoeg is om liganden met een verschillende linkerlengte te binden.
Thermodynamische analyse van binding studies, met behulp van fluorescentie anisotropie, lieten zien
dat de interactie van het bivalent peptide-DNA ligand 500 keer zo sterk was als die van de
monovalente variant. De sterke bivalente interactie maakte het mogelijk om de antigeen bindende
domeinen van het antilichaam effectief te blokkeren. Deze blokkering kon worden opgeheven door
het inbouwen van een aminozuursequentie die herkend wordt door het enzym matrix
metalloproteinase 2. Toevoeging van dit enzym resulteerde in verbreking van de verbinding tussen
het peptide epitoop en de DNA linker, waarna het anti-HIV-I antilichaam weer kon binden aan het
p17 eiwit van het HIV-I virus.

Het gebruik van DNA als linker in een bivalente ligand biedt ook een uitstekende mogelijkheid om
de activiteit van een antilichaam te controleren door toevoeging van oligonucleotiden die de
hybridisatie van de twee DNA ketens in de linker kunnen verbreken (hoofdstuk 4). Om deze
oligonucleotide trigger de kans te geven één van de twee strands in de DNA linker te verdringen,
werd een extra stuk enkelstrengs DNA (toehold) toegevoegd aan het bivalente peptide-dsDNA slot.
De deblokkering van het anti-HA antilichaam in aanwezigheid van een volledig complementaire
oligonucleotide werd gevolgd door gebruik te maken van flowcytometrie en gistcellen die het HA-
epitoop op hun celoppervlak presenteren. Door aan weerszijden van het bivalente peptide-DNA slot
een toehold sequentie te introduceren werd de activatie van het antilichaam afhankelijk van de
aanwezigheid van twee oligonucleotide. Dit systeem kan worden gebruikt voor het uitvoeren van
simpele logische OR en AND operaties. De kinetiek waarmee de verdringingsreactie plaats vindt
bleek niet te worden beïnvloed door binding van het bivalente peptide-DNA aan het antilichaam.
Tenslotte hebben we laten zien dat de activatie van het antilichaam ook kon worden gecontroleerd
door gebruik te maken van een eiwit-bindend aptameer.

In hoofdstuk 5 wordt de ontwikkeling van bivalente peptide-DNA liganden voor het therapeutisch
antilichaam cetuximab beschreven. Cetuximab bindt specifiek aan de epidermale groeifactor receptor
(EGFR) die in verhoogde mate aanwezig is op het celoppervlak van verschillende typen tumoren. De
verhoogde aanwezigheid van EGFR in bijvoorbeeld huidcellen kan leiden tot bijwerkingen zoals
ernstige huiduitslag, wat de maximale dosis van cetuximab voor de behandeling van deze patiënten
bepaalt. Dit hoofdstuk beschrijft twee verschillende benaderingen om de activiteit van cetuximab te
kunnen controleren. De eerste benadering is analoog aan de strategie die in hoofdstukken 3 en 4
werd gevolgd en maakt gebruik van twee antigeen domein bindende, cyclische peptiden gekoppeld
aan een DNA linker van 20 basenparen. De tweede strategie bestaat uit een heterobivalente peptide-
DNA construct dat twee verschillende cyclische peptiden via een DNA linker verbindt, de één bindt
aan het antigen bindende domein en de ander bindt in een specifieke pocket in het constante
gedeelte van het Fab-fragment. De binding van cetuximab aan EGFR-presenteerende kankercellen
werd geanalyseerd met behulp van flowcytometrie en toonde aan dat beide bivalente peptide-DNA
constructen cetuximab effectief blokkeerden. Deze blokkade kon vervolgens weer worden
opgeheven door toevoeging van het enzym MMP-2 of door toevoeging van complementaire
oligonucleotiden.

Het laatste experimentele hoofdstuk van dit proefschrift (hoofdstuk 6) beschrijft het gebruik van
zogenaamde Pyrrole-Imidazole (PI) polyamide als een alternatieve en niet-covalente manier om
DNA structuren te functionaliseren met peptiden en eiwitten. Deze PI-polyamide zijn relatief kleine
moleculen die met hoge affiniteit en sequentie-specifiek in de ‘minor groove’ van de DNA dubbel
helix binden. Natieve chemische ligatie wordt geïntroduceerd als een efficiënte en algemeen
toepasbare methode om PI-polyamide te koppelen aan peptiden en eiwitten. Koppeling van een
peptide aan het PI-polyamide bleek te resulteren in een 20-keer zwakkere DNA binding. De binding
aan DNA werd nog verder verzwakt na koppeling van een groot eiwit. Hoewel natieve chemische
ligatie dus een effectieve methode is voor de conjugatie van peptiden en eiwitten aan PI-polyamides
vereist het ontrafelen van de verstoorde DNA binding en het voorkomen ervan nog meer
onderzoek.

Hoofdstuk 7 bevat een verdere discussie van de in dit proefschrift beschreven resultaten en
beschrijft mogelijke toepassingen en suggesties voor verder onderzoek.
Brian Janssen was born on August 23rd, 1987 in Heerlen, the Netherlands. After finishing his secondary education in 2005 at the Sintermeerten College in Heerlen, he studied Biomedical Engineering at the Eindhoven University of Technology. During his studies, he performed an internship of three months in the lab of dr. W. Shih at Harvard University and the Wyss Institute (United States), where he studied the self-assembly of 3D DNA nanostructures and novel site-specific DNA conjugation methods. During his final research project he worked in the group of prof. dr. E. W. Meijer and dr. M. Merkx on protein bioconjugation strategies to attach synthetic groups. After receiving his master’s degree in 2011, he started as a PhD student under supervision of prof. dr. ir. L. Brunsveld en dr. M. Merkx. The main goal of his project was to develop generic approaches to allow DNA-based control of protein activity, in particular the activity of enzymes and antibodies. The most important results of his PhD research are described in this thesis.
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Dankwoord

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Dankwoord

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-Brian