Chapter 12

DNA-Specific Biosensors Based on Intramolecular β-Lactamase-Inhibitor Complex Formation

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Abstract

Synthetic protein switches that sequence-specifically respond to oligonucleotide-based input triggers provide valuable tools for the readout of oligonucleotide-based biomolecular systems and networks. Here, we discuss a highly modular approach to reversibly control the DNA-directed assembly and disassembly of a complex between TEM1-β-lactamase and its inhibitor protein BLIP. By conjugating each protein to a unique handle oligonucleotide, the enzyme-inhibitor pair is noncovalently assembled upon the addition of a complementary ssDNA template strand, resulting in inhibition of enzyme activity. Hybridization of an input-oligonucleotide that is complementary to a target recognition sequence in the ssDNA template strand results in the formation of a rigid dsDNA helix that mechanically disrupts the enzyme-inhibitor complex, hereby restoring enzyme activity. Following this noncovalent approach allowed straightforward tuning of the ssDNA template recognition sequence and target oligonucleotide lengths with only a single set of oligonucleotide-functionalized enzyme and inhibitor domains. Using a fluorescent substrate, as little as 10 pM target oligonucleotide resulted in a distinguishable increase in enzyme activity.

Key words β-lactamase, Reporter enzyme, DNA detection, Synthetic biology, Protein-DNA conjugation, Biosensor

1 Introduction

Synthetic protein switches are extensively used in synthetic biology, molecular imaging, and molecular diagnostics to study biological processes both *in vitro* and *in vivo* by responding to the presence of a specific input molecule. Ideally, molecular recognition (input) and signal generating functions (output) are part of separate domains in these switches, as such a modular organization allows straightforward exchange of input or output function [1]. A successful design approach that ensures efficient translation of input activation to output modulation, is to design switches that are able to adopt two conformational states; one in which two output domains can interact intramolecularly and one where their interaction is prohibited. The dynamic response of such a protein switch is

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determined by the relative affinities of the output domain interaction and the magnitude of the input-induced thermodynamic change, e.g., the strength of ligand binding [2]. Examples of output domains that have been applied for these approaches include fluorescent or luminescent proteins domains forming FRET or BRET pairs. To ensure efficient energy transfer between them in either the on or off state, fluorescent domains can be used that have an increased tendency to form intramolecular complexes, or their close proximity can be promoted by so-called helper domains [3]. Our group used these self-associating fluorescent protein domains to develop robust FRET sensor protein for a range of analytes, including metal ions, bile acids, and antibodies [4–7]. In addition, by replacing the self-associating fluorescent domains by an enzyme-inhibitor pair with similar affinity, the optical readout of the antibody FRET sensor could be replaced by a more sensitive enzymatic readout [8].

The modular organization of protein switches based on mutually exclusive domain interactions does not only allow exchange of different output functions, but is also well suited to combine with oligonucleotide-based biomolecular systems and networks. Most approaches reported thus far to control enzyme activity by DNA are based on the templated assembly of split enzyme fragments [9, 10]. Whereas split reporter enzymes have the advantage of providing a low background activity (at least when used intermolecularly), split enzymes generally suffer from poor thermodynamic stability and protein complementation is often not reversible. Therefore, our group recently introduced an alternative approach that is based on the reversible assembly of a complex between the reporter enzyme TEM1-β-lactamase and its inhibitor domain BLIP [11–13]. This design exploits the inherent difference in mechanical properties between ssDNA and dsDNA to mechanically disrupt and spatially separate the enzyme-inhibitor pair upon binding of an input oligonucleotide to the input-binding module. To facilitate straightforward exchange of input sequences, a noncovalent approach is followed by conjugating both TEM1-β-lactamase and BLIP to individual, 21 nucleotide handle oligonucleotides. By appending anti-handle sequences to each end of the synthetic ssDNA target recognition sequence, a ternary complex is formed upon mixing of the three protein switch components (Fig. 1). Formation of this ternary complex results in a large increase in effective concentration and hereby induces an intramolecular interaction between the enzyme-inhibitor pair. Hybridization of the complementary target oligonucleotide to the input module results in the formation of a rigid double-helix which mechanically disrupts the enzyme-inhibitor interaction, hereby switching on enzyme activity.

Since this system is based on two well-folded and stable protein domains, it is more robust compared to the assembly of split-protein fragments and readily reversible. Moreover, because of the

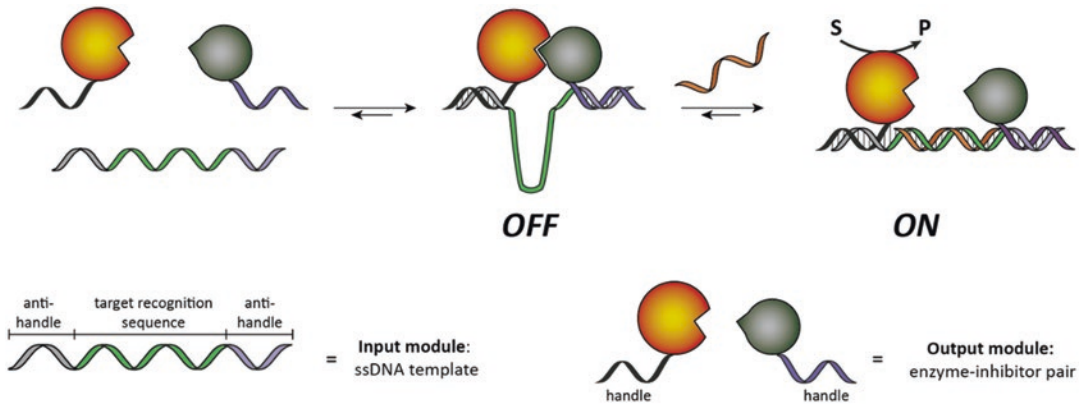

Fig. 1 Design of a modular DNA-directed biosensor based on intramolecular TEM1-β-lactamase–inhibitor complex formation. To implement full modularity, a multi-component strategy is used where the input and output modules are noncovalently assembled. Therefore, the enzyme and inhibitor proteins are conjugated to oligonucleotide handles that are complementary to anti-handle sequences appended to the target recognition sequence, allowing the assembly of the enzyme-inhibitor pair in a ternary complex ("OFF" state). Hybridization of a complementary input oligonucleotide to the target recognition sequence results in the formation of a rigid double-helix, hereby mechanically disrupting the enzyme-inhibitor pair, i.e., the enzyme is switched on ("ON" state)

catalytic conversion of substrate to product, the enzyme-based DNA detection is more sensitive than direct optical readout of conformational switching sensors such as molecular beacons [14]. Here, we provide detailed protocols for the expression, purification, and bioconjugation of the TEM1-β-lactamase- and BLIP-oligonucleotide conjugates, as well as their assembly into an easily tunable system for oligonucleotide detection in molecular diagnostics and the readout of DNA-based molecular circuits. While the DNA-directed protein switch described in this chapter is originally designed to respond to ssDNA, the range of input molecules could be extended to small molecules and proteins by intelligent redesign of the input module using conformation-switching aptamers. Since plasmids for expression of both protein components will be made accessible via AddGene, we hope that these protocols will enable the TEM1-β-lactamase/BLIP system to be widely used. In addition, both the design principles and the bioconjugation methods described here for the TEM1-β-lactamase/BLIP system should be generally applicable to other enzyme-inhibitor pairs, provided that they interact with a similar affinity.

2 Materials

2.1 Protein Expression and Purification

1. Plasmid pET29a_TEM1-β-lactamaseE104D_CtermC (encoding periplasmic leader sequence, His-tag, Thrombin cleavage site, TEM1-β-lactamase(E104D), cysteine, Strep-tag) (*see* **Notes 1** and **2**).

2. Plasmid pET29a_BLIP_NtermC (encoding periplasmic leader sequence, His-tag, Thrombin cleavage site, cysteine, GGSHG linker, β-lactamase inhibitor protein (BLIP)) (*see* **Notes 1** and **2**).
3. Competent *Escherichia coli* BL21 (DE3) cells.
4. LB medium: 10 g peptone, 5 g yeast extract and 10 g NaCl in 1 L dH_2O supplemented with 30 mg/L kanamycin.
5. 100 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) stock solution. Filtered to sterilize (0.2 μm).
6. Shaking incubator.
7. High-speed centrifuge.
8. Osmotic shock solution: 30 mM Tris–HCl, 1 mM EDTA, 20% sucrose at pH 8.0.
9. Low salt solution: 5 mM $MgSO_4$.
10. 10× Tris–HCl buffer: 200 mM Tris–HCl at pH 7.4.
11. His-tag binding resin.
12. Charge buffer: 50 mM $NiSO_4$.
13. Bind buffer: 20 mM Tris–HCl, 500 mM NaCl, 30 mM imidazole at pH 7.9.
14. Wash buffer: 20 mM Tris–HCl, 500 mM NaCl, 60 mM imidazole at pH 7.9.
15. Elution buffer: 20 mM Tris–HCl, 500 mM NaCl, 400 mM imidazole, 2 mM TCEP at pH 7.9.
16. Storage buffer: 20 mM Tris–HCl, 150 mM NaCl, 2 mM TCEP at pH 7.9.
17. Amicon centrifugal filter unit (NMWL = 10 kDa).
18. Nanodrop UV-VIS spectrophotometer.

2.2 Maleimide Functionalization of Amine-Modified Oligonucleotides

1. Oligonucleotides modified with a primary amine at their 3′- or 5′-terminus (e.g., Integrated DNA Technologies).
2. Dimethylsulfoxide (DMSO).
3. Ethanol (cooled to −30 °C).
4. Phosphate buffered saline (PBS): 100 mM sodium phosphate, 150 mM NaCl at pH 7.2.
5. 5 M NaCl.
6. Sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC, no-weigh format).

2.3 Conjugation of Maleimide-Functionalized Oligonucleotides to Proteins

1. Ligation buffer: 100 mM sodium phosphate at pH 7.0.
2. Desalting column: PD-10 (e.g., GE Healthcare, Cat No: 17-0851-01).
3. Strong anion-exchange spin column, mini (e.g., Thermo Scientific, Cat No: PI90008).

4. Buffer A: 20 mM Tris–HCl, 50 mM NaCl at pH 8.0.
5. Buffer B: 20 mM Tris–HCl, 1 M NaCl at pH 8.0.

2.4 Semi-Native SDS-PAGE of Reconstituted DNA-Directed Enzyme-Inhibitor Complex

1. Complexation buffer: 50 mM sodium phosphate, 100 mM NaCl at pH 7.0.
2. 2× sample buffer: 125 mM Tris–HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 0.01% bromophenol blue.
3. 30% (w/v) acrylamide/bisacrylamide solution (29:1, w/w).
4. *N,N,N′,N′*-tetramethylethylenediamine (TEMED).
5. 10% (w/v) ammonium persulfate solution in water.
6. Isopropanol.
7. Running buffer: 187 mM glycine, 19 mM Tris–HCl, 3.5 mM SDS.
8. Protein molecular weight marker, prestained.
9. Coomassie Brilliant Blue.

2.5 Enzyme Activity Assay of Reconstituted DNA-Directed Enzyme-Inhibitor Complex

1. PBS^+: 50 mM sodium phosphate, 100 mM NaCl, 1 mg/mL bovine serum albumin (BSA) at pH 7.0.
2. 50 μM ssDNA template stock solution dissolved in H_2O (Table 1).
3. 50 μM ssDNA target stock solution dissolved in H_2O (Table 1).
4. 500 μM nitrocefin, chromogenic β-lactamase substrate (e.g., EMD Millipore, VWR, Cat No: 80,017–707) dissolved in PBS.
5. Multiwell plate reader (with absorbance mode).
6. Transparent 96-well plates.

2.6 Enzyme Activity Assay at Different Input Oligonucleotide Concentrations

1. PBS^+: 50 mM sodium phosphate, 100 mM NaCl, 1 mg/mL BSA at pH 7.0.
2. 50 μM ssDNA template stock solution dissolved in H_2O (Table 1).
3. 50 μM ssDNA target stock solution dissolved in H_2O (Table 1).
4. 20 μM CCF2-FA, fluorescent β-lactamase substrate (e.g., Invitrogen, Cat No: K1034) dissolved in PBS.
5. Multiwell plate reader (with fluorescence mode).
6. Black 96-well plate.

Table 1
Sequences of handle oligonucleotides, ssDNA templates, and ssDNA targets

Name	Sequence
ODN1	5′-TGTCACCGATGAAACTGTCTA-NH_2–3′
ODN2	5′-H_2N-GTGATGTAGGTGGTAGAGGAA-3′
Template 0	5′-TTCCTCTACCACCTACATCACTAGACAGTTTCATCGGTGACA-3′
Template 10	5′-TTCCTCTACCACCTACATCACCACCACCGCATAGACAGTTTCATCGGTGACA-3′
Template 20	5′-TTCCTCTACCACCTACATCACCACACCACCACCGCAACCACTAGACAGTTTCATCGGTGACA-3′
Template 30	5′-TTCCTCTACCACCTACATCACCACAACACACCACCACCGCAACCACCCACCTAGACAGTTTCATCGGTGACA-3′
Template 40	5′-TTCCTCTACCACCTACATCACCAACCCACAACACACCACCACCGCAACCACCCACCACCAATAGACAGTTTCATCGG TGACA-3′
Template 50	5′-TTCCTCTACCACCTACATCACCAACACAACCCACAACACACCACCACCGCAACCACCCACCACCAACACCATAGACAGT TTCATCGGTGACA-3′
Target 10	5′-TGCGGTGGTG-3′
Target 20	5′-GTGGTTGCGGTGGTGGTGTG-3′
Target 30	5′-GGTGGGTGGTTGCGGTGGTGGTGTGTTGTG-3′
Target 40	5′-TTGGTGGTGGGTGGTTGCGGTGGTGGTGTGTTGTGGGTTG-3′
Target 50	5′-TGGTGTTGGTGGTGGGTGGTTGCGGTGGTGGTGTGTTGTGGGTTGTGTTG-3′

3 Methods

To facilitate hybridization-induced assembly of the enzyme-inhibitor pair the oligonucleotide handles have to be conjugated to the individual protein domains. In particular, cysteine is an attractive target for protein conjugation due to its low prevalence in natural proteins and ease of insertion using site-directed mutagenesis. In contrast to non-natural amino acids, which allow fully orthogonal protein conjugation chemistries, the incorporation of a nonnative cysteine typically does not result in a significant decrease in expression yield (~0.3 mg/L culture for both TEM1-β-lactamase and BLIP). To allow straightforward protein expression while ensuring site-specific conjugation of the oligonucleotide handles to the proteins (yielding homogeneous ODN-protein conjugates), thiol-maleimide coupling was used targeting a genetically inserted cysteine on the proteins. To install the maleimide moiety on the oligonucleotide handles, the heterobifunctional crosslinker Sulfo-SMCC, consisting of an NHS-ester, cyclohexyl spacer, and maleimide, was coupled to a 5′- or 3′-amine-modified oligonucleotide for BLIP and TEM1-β-lactamase, respectively. Subsequent ethanol precipitation of the maleimide-functionalized oligonucleotides allowed the removal of non-reacted Sulfo-SMCC. Finally, the oligonucleotides were conjugated to the cysteine inserted in the proteins (Fig. 2).

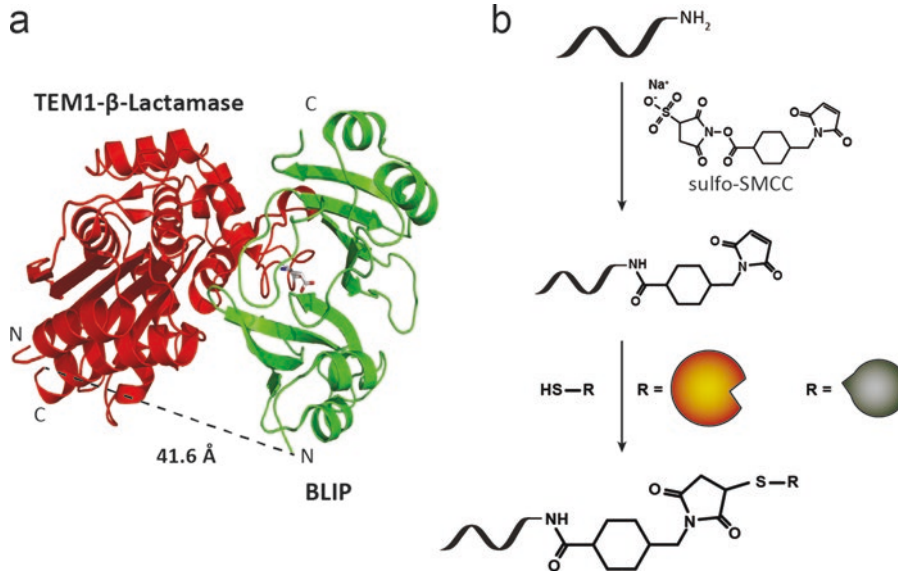

Fig. 2 (**a**) Crystal structure of a complex between TEM1-β-lactamase and BLIP (PDB: 3C7V). (**b**) ODN-protein conjugation strategy. Amine-modified oligonucleotides are reacted with the NHS-ester of the heterobifunctional crosslinker Sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carbocylate (Sulfo-SMCC) installing a thiol-reactive maleimide moiety on the oligonucleotides. After the removal of the excess crosslinker by ethanol precipitation, the maleimide-activated oligonucleotides are reacted with a cysteine in the proteins. Adapted with permission from ref. 13. Copyright 2015 American Chemical Society

Conversion yields (typically 50–90%) can be estimated by SDS-PAGE analysis. Subsequent Ni^{2+}-affinity chromatography and anion-exchange chromatography to remove excess oligonucleotides and unconjugated proteins, respectively, resulted in pure ODN-protein conjugates.

Formation of the intermolecular ternary complex is performed by mixing ODN1-TEM1-β-lactamase, ssDNA template, and ODN2-BLIP in a 1:1.2:2 molar ratio. Using a slight excess of ssDNA template and ODN2-BLIP ensures that no free ODN1-TEM1-β-lactamase or ODN1-TEM1-β-lactamase-template remains, both of which would contribute to background activity. In order to confirm the formation of homogeneous ternary complexes upon hybridization of the individual components, the mixtures were analyzed by semi-native PAGE. Quantitative conversion to the ternary complex was observed upon the addition of the ssDNA template to a mixture of the ODN-enzyme and ODN-inhibitor conjugates. A gel-shift to higher molecular weight after the addition of a complementary input oligonucleotide indicates the formation of the desired quaternary complex. Whereas semi-native PAGE suggests successful formation of the ternary DNA-directed enzyme-inhibitor complex, enzyme-activity assays were performed to test whether the enzyme-inhibitor interaction is indeed induced upon hybridization of both ODN-protein conjugates to the ssDNA template strand. To this end, the complexes (containing templates with target recognition sequences of 0 to 50 nucleotides) were diluted to a final concentration of 1 nM. After the addition of the colorimetric substrate nitrocefin, substrate conversion was monitored by measuring the optical absorbance of the formed product as a function of time. Whereas a strong inhibition of enzyme activity was observed with all target recognition sequence lengths, inhibition efficiencies increased as the target recognition sequence length increases. This observation suggests that a certain degree of flexibility between the protein partners is preferable to allow more efficient intramolecular complex formation. In contrast, full recovery of enzyme activity was only achieved upon the addition of input sequences of >40 nucleotides, suggesting that a double-helix of at least 40 nucleotides is required to overcome flexibility in the protein termini and SMCC-linker and spatially separate the interacting protein partners.

Making use of an enzyme as output domain provides the advantage over conventional fluorescent domains that substrate molecules are catalytically converted, hence the output signal accumulates. This allows the use of much lower concentrations of the sensor complex, hereby increasing the sensitivity of the input oligonucleotide detection. When using 100 pM of the DNA-specific biosensor together with the more sensitive fluorescent substrate CCF2-FA, as little as 10 pM of input oligonucleotide results in a distinguishable increase in enzyme activity.

3.1 Expression and Purification of Recombinant Proteins

1. Transform competent *E. coli* BL21 (DE3) cells separately with the pET29a_TEM1-β-lactamaseE104D and pET29a_BLIP plasmids (*see* **Note 3**).
2. For each construct, inoculate 20 mL of LB medium supplemented with 30 mg/L kanamycin with a single colony of transformed bacteria and incubate overnight at 37 °C in a shaking incubator at 250 rpm.
3. Transfer the seed culture to 2 L of LB medium supplemented with 30 mg/L kanamycin.
4. Grow the bacteria in a shaking incubator at 200 rpm at 37 °C. At an optical density at λ = 600 nm of 0.6, add 2 mL of IPTG stock yielding a final concentration of 100 μM IPTG and incubate at 16 °C for 20 h.
5. Harvest the cells by centrifugation at 10,000 × *g* for 10 min at 4 °C.
6. Resuspend the pelleted cells in 300 mL osmotic shock solution and incubate for 10 min at room temperature.
7. Pellet the cells by centrifugation at 12,000 × *g* for 20 min.
8. To extract the periplasmic fraction, resuspend the pelleted cells in 300 mL ice-cold low salt solution and incubate for 10 min on ice prior to centrifugation at 40,000 × *g* for 40 min at 4 °C.
9. Add 10× Tris–HCl buffer to the supernatant (i.e., the periplasmic fraction) to yield a final concentration of 20 mM Tris–HCl.
10. Charge a 2 mL His-bind column (gravity flow) with Ni^{2+} ions by washing the column with 10 column volumes of charge buffer and subsequently equilibrate with 10 column volumes bind buffer.
11. Load the periplasmic fraction on the His-bind column and allow it to enter the column bed completely. Wash the column with 10 column volumes of wash buffer and subsequently elute the protein of interest with 10 column volumes of elution buffer.
12. Concentrate the eluted proteins to a final volume of ~2.5 mL using the centrifugal filter unit.
13. Exchange the buffer to storage buffer by gel filtration using a PD-10 desalting column and determine the concentration by measuring the optical absorption at λ = 280 nm with the Nanodrop UV-VIS spectrophotometer and calculated extinction coefficients (*see* **Note 4**).
14. Flash-freeze the purified proteins in liquid nitrogen and store at −80 °C.

3.2 Maleimide Functionalization of Amine-Modified Oligonucleotides

1. Dissolve the amine-modified oligonucleotides in PBS to a concentration of ~1 mM.
2. Dissolve 2 mg of Sulfo-SMCC (1 vial) in 300 μL DMSO to yield a stock concentration of ~20 mM.
3. Mix an equal volume of Sulfo-SMCC with the amine-modified oligonucleotides yielding a 20-fold molar excess of Sulfo-SMCC in 1:1 PBS:DMSO.
4. Incubate the mixture at room temperature under continuous shaking at 850 rpm for 2 h.
5. To precipitate the oligonucleotides, add 10% (v/v) 5 M NaCl and 300% (v/v) ethanol precooled to −30 °C, and store the mixture at −30 °C for at least 1 h.
6. Pellet the precipitated oligonucleotides by centrifugation at 19,000 × *g* rpm for 15 min at 4 °C.
7. Dissolve the oligonucleotides in PBS to a concentration of ~1 mM and repeat **steps 5** and **6** of the ethanol precipitation procedure twice (*see* **Note 5**).
8. Finally, dry the pelleted oligonucleotides to air overnight at room temperature.

3.3 Conjugation of Maleimide-Functionalized Oligonucleotides to Proteins

1. Immediately prior to oligonucleotide conjugation, desalt the proteins to ligation buffer to remove TCEP by gel filtration using a PD-10 desalting column and dilute to ~50 μM (*see* **Note 6**).
2. Add the appropriate volume of protein solution to the dried oligonucleotide to yield a threefold molar excess of maleimide-functionalized oligonucleotide.
3. Incubate the mixture at room temperature under continuous shaking at 850 rpm for 2 h.
4. To remove excess oligonucleotides load the reaction mixture on a 1 mL His-bind column charged with Ni^{2+} ions and equilibrated with bind buffer. Wash with 10 column volumes of bind buffer.
5. Elute the oligonucleotide-protein (ODN-protein) conjugates in 500 μL fractions using 10 column volumes of elution buffer.
6. Analyze the reaction efficiency and purity of the elution fractions by 12% SDS-PAGE.
7. Combine all elution fractions that contain ODN-protein conjugates.
8. Exchange the buffer to buffer A by gel filtration using a PD-10 desalting column.

9. Prewash a strong anion-exchange spin column by applying 400 μL buffer A and spinning at 2000 × *g* for 5 min and discarding the flow-through.
10. Repeatedly add 400 μL of desalted ODN-protein conjugates and centrifuge for 5 min at 2000 × *g* until the entire sample is loaded on the anion-exchange spin column. Discard the flow-through.
11. Remove non-conjugated protein by adding 400 μL buffer A and centrifuging for 5 min at 2000 × *g*. Discard the flow-through. Repeat this step ten times.
12. Elute the purified ODN-protein conjugate by adding 200 μL buffer B and centrifuging for 5 min at 2000 × *g*. Repeat this step ten times.
13. Analyze the elution fractions by 12% SDS-PAGE and combine all fractions containing the purified ODN-protein conjugates (Fig. 3).
14. Quantify the concentrations of the ODN-protein conjugates by measuring the absorbance at λ = 260 nm and using extinction coefficients of the oligonucleotides.
15. Aliquot the purified ODN-protein conjugates in 20 μL fractions and flash-freeze in liquid nitrogen. Store the ODN-protein conjugates at −80 °C.

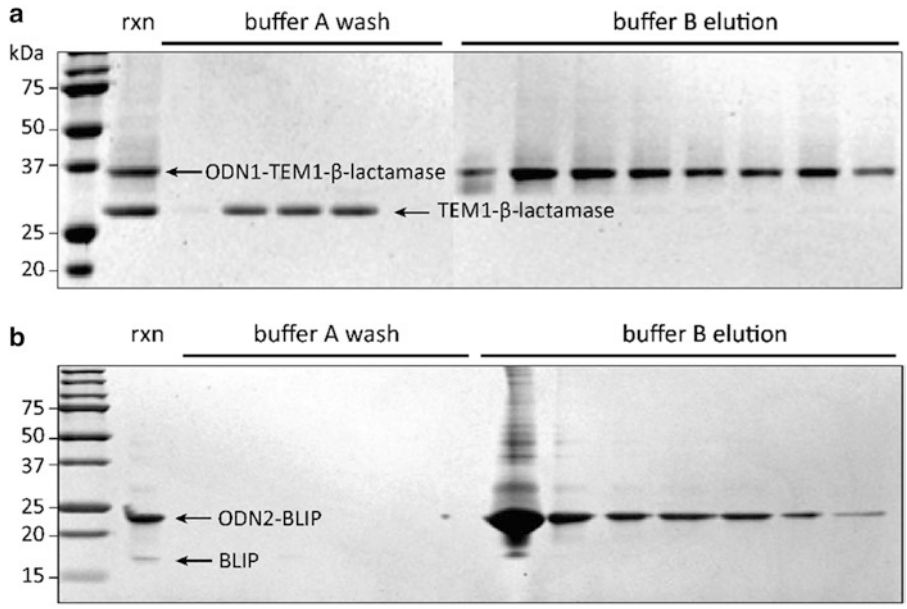

Fig. 3 SDS-PAGE analysis of the conjugation and anion exchange purification of a maleimide-functionalized oligonucleotide to a cysteine in (**a**) TEM1-β-lactamase and (**b**) BLIP on a 12% SDS-PAGE gel stained with Coomassie blue. (rxn: reaction mixture, buffer A wash: first five wash fractions with buffer A (low ionic strength) from the anion-exchange spin column, buffer B elution: first eight elution fractions with buffer B (high ionic strength) from the anion-exchange spin column

3.4 Semi-native SDS-PAGE of Reconstituted DNA-Directed Enzyme-Inhibitor Complex

1. Prepare a 0.75-mm-thick 12% resolving gel by mixing 4 mL 30% (w/v) acrylamide, 2.5 mL 1.5 M Tris–HCl pH 8.9, 3.3 mL dH_2O, 100 μL 10% (w/v) SDS, 100 μL 10% (w/v) ammonium persulfate and 6 μL TEMED. Cast the gel up to 2 cm below the top and cover with a layer of isopropanol. Allow the gel to polymerize for 30 min.
2. Prepare a stacking gel by mixing 830 μL 30% (w/v) acrylamide, 630 μL 1 M Tris–HCl pH 6.8, 3.4 mL dH_2O, 50 μL 10% (w/v) SDS, 50 μL 10% (w/v) ammonium persulfate, and 3 μL TEMED. Pour the mixture on the top of the resolving gel and insert the comb. Allow the gel to polymerize for 30 min.
3. To ensure efficient complex formation and inhibition of the enzyme in the assembled complex, sequentially mix ODN1-TEM1-β-lactamase, ssDNA template (with a target recognition sequence length of 0, 10, 20, 30, 40, or 50 nucleotides) and ODN2-BLIP in a 1:1.2:2 ratio at low micromolar concentrations in complexation buffer and incubate for 30 minutes at room temperature.
4. Mix the hybridized samples prepared in **step 2** with an equal volume of 2× sample buffer and load it on a semi-native 12% SDS-PAGE gel prepared in **step 1**. Run the gel for 15 min at 90 V followed by 1 h at 125 V.
5. Remove the gel from the glass plates and wash with dH_2O for 5 min. Subsequently stain the gel with Coomassie blue for 30 min. Then destain the gel with dH_2O until clear bands appear and image the gel (Fig. 4).

3.5 Enzyme Activity Assay of Reconstituted DNA-Directed Enzyme-Inhibitor Complex

1. Mix ODN1-TEM1-β-lactamase, ODN2-BLIP, and ssDNA template (with target recognition sequences of 0, 10, 20, 30, 40, and 50 nucleotides) in PBS^+ at low micromolar concentrations in a ratio of 1:2:1.2 and incubate for 30 min at room temperature.
2. Dilute the mixtures in PBS^+ to a final concentration of 50 nM ODN1-TEM1-β-lactamase.
3. In a 96-well plate, mix 4 μL of the diluted complexes with 152 μL PBS^+ and 24 μL target oligonucleotides (from 100 nM stock solution in PBS^+) and incubate for 1 h at room temperature.
4. Add 20 μL nitrocefin (from 500 μM stock solution in PBS) yielding a final concentration of 1 nM DNA-directed enzyme-inhibitor complex, 12 nM target oligonucleotide, and 50 μM nitrocefin.
5. Directly place the plate in the platereader and proceed with measuring the optical absorbance in time of the formed product at 486 nm.

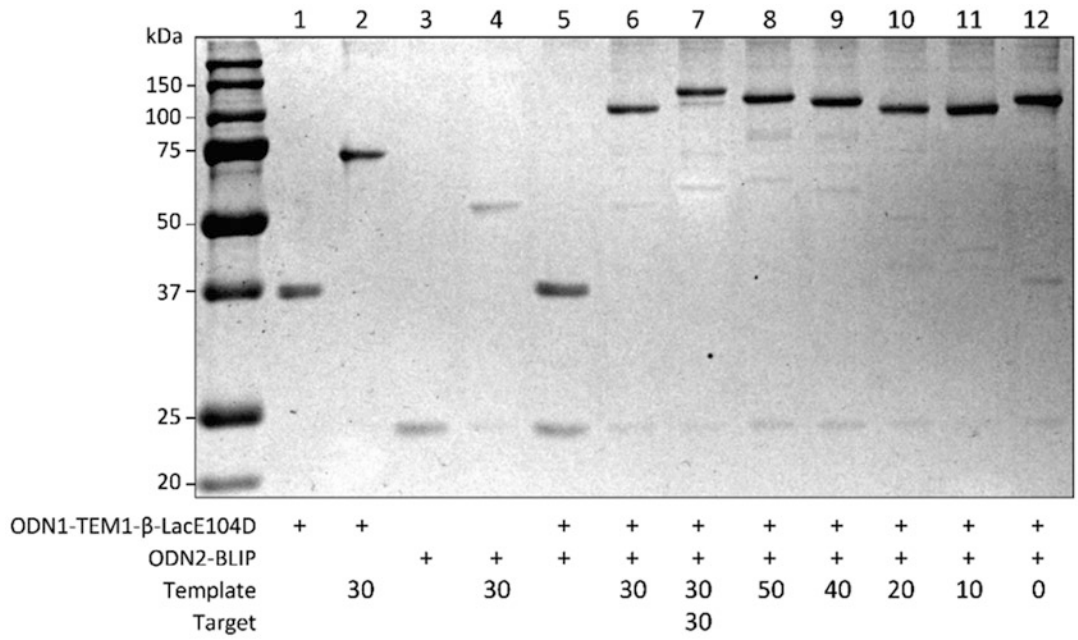

Fig. 4 Semi-native 12% SDS-PAGE analysis of the reconstitution of the DNA-directed enzyme-inhibitor pair. Low micromolar concentrations of the synthetic protein switch components were sequentially mixed at a ratio of 1:1.2:2 of ODN1-TEM1-β-lactamaseE104D, ssDNA template, and ODN2-BLIP. *Lanes 6, 8–12* show efficient formation of the ternary complex containing different lengths of target recognition sequence. A gel-shift to higher molecular weight is observed upon the addition of 10 equivalents of target oligonucleotide (30 nucleotides), indicating efficient binding of the input oligonucleotide to the synthetic protein switch. Adapted with permission from ref. 13. Copyright 2015 American Chemical Society

6. Derive the hydrolysis rate by calculating the slope of the absorbance in time of the initial, linear regime. Correct for background hydrolysis of nitrocefin in the absence of enzyme (Fig. 5).

3.6 Enzyme Activity Assay at Sub-nanomolar Input Oligonucleotide Concentrations

1. Mix ODN1-TEM1-β-lactamase, ODN2-BLIP, and ssDNA template (with a target recognition sequence length of 40 nucleotides) in PBS^+ at low micromolar concentrations in a ratio of 1:2:1.2 and incubate for 30 min at room temperature.
2. Dilute the mixture in PBS^+ to a final concentration of 5 nM ODN1-TEM1-β-lactamase.
3. In a 96-well plate, mix 4 μL of the diluted complex with 152 μL PBS^+ and 24 μL target oligonucleotide (concentrations ranging from 100 pM to 20 nM) and incubate for 1 h at room temperature.
4. Add 20 μL CCF2-FA (20 μM in PBS), yielding a final concentration of 100 pM DNA-directed enzyme-inhibitor complex, 10 pM to 2 nM target oligonucleotide, and 2 μM CCF2-FA. Incubate for 45 min at room temperature.

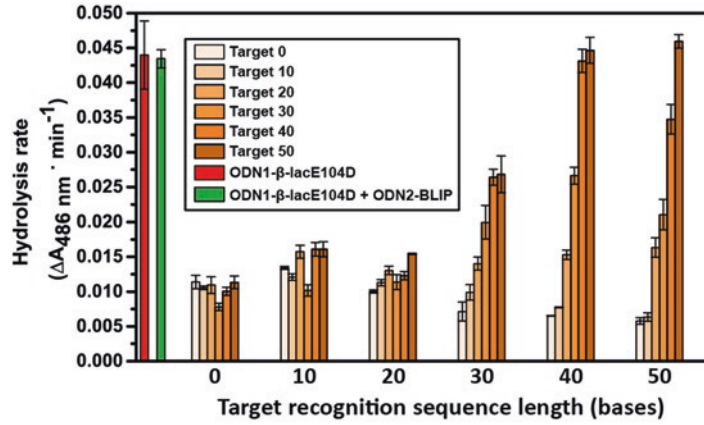

Fig. 5 Influence of target recognition sequence length and target oligonucleotide lengths on enzyme inhibition and enzyme activation, respectively. ODN1-TEM1-β-lactamaseE104D, template oligonucleotide (with various target recognition sequence lengths), and ODN2-BLIP were incubated in a 1:1.2:2 ratio at low micromolar concentrations. After subsequently diluting the mixtures to 1 nM concentration of ODN1-TEM1-β-lactamaseE104D in the presence of 12 nM target oligonucleotides of various lengths, the colorimetric substrate Nitrocefin was added and substrate conversion was measured. Adapted with permission from ref. 13. Copyright 2015 American Chemical Society

5. Place the plate in the platereader and monitor the formation of the fluorescent product at 409 nm excitation and 447 nm emission.
6. Derive the hydrolysis rate by calculating the slope of the fluorescence in time within the linear regime (Fig. 6).

4 Notes

1. Previous work has shown that conjugation efficiencies greatly depend on the steric availability of the inserted cysteine. In case of low conversion efficiencies insert a flexible GGSGG linker between the protein and cysteine [10].
2. Plasmids encoding for TEM1-β-lactamase and BLIP will be made available via AddGene.
3. Based on previous work using the intramolecular complex formation between TEM1-β-lactamase and BLIP as output module for genetically encoded protein switches, we here use the E104D mutant of TEM1-β-lactamase (K_i = 1500 nM) as the wild-type proteins bind too strongly (K_i = 0.5 nM), which might result in intermolecular complex formation and oligomerization of the DNA-directed protein switch.
4. TCEP is added to the storage buffer to reduce intermolecular disulfide bonds that render the genetically inserted cysteine

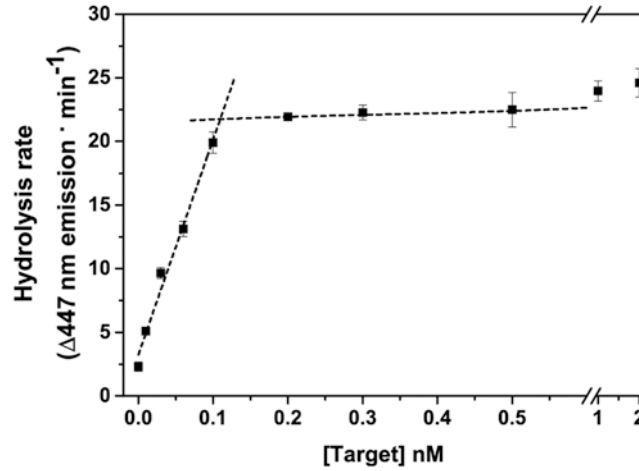

Fig. 6 Enzyme activity as a function of target oligonucleotide concentration. ODN1-TEM1-β-lactamaseE104D, template oligonucleotide (with a target recognition sequence of 40 nucleotides), and ODN2-BLIP were incubated at low micromolar concentrations to form the inhibited ternary complex. Subsequently, the complex was diluted to 100 pM of ODN1-TEM1-β-lactamaseE104D in the presence of various concentrations of target oligonucleotide. After the addition of the fluorescent substrate CCF2-FA product formation was measured by measuring the increase in fluorescence in time (λ_{ex} = 409 nm, λ_{em} = 447 nm). A linear increase in enzyme activity as a function of target concentration is observed up to 1 equivalent of the template oligonucleotide, suggesting tight binding between the ternary complex and target oligonucleotide. Adapted with permission from ref. 13. Copyright 2015 American Chemical Society

nonreactive toward maleimides. TCEP can also reduce native, intramolecular disulfide-bonds. However, the BLIP and TEM1-β-lactamase proteins did not show any (irreversible) reduction of intramolecular disulfides.

5. To ensure efficient conjugation of maleimide-functionalized oligonucleotides to the proteins of interest, it is crucial to perform at least three rounds of ethanol precipitation after reacting the amine-modified oligonucleotides with Sulfo-SMCC to ensure that all excess Sulfo-SMCC is removed since this will compete for conjugation to the protein. A single round of ethanol precipitation has shown to significantly reduce final conjugation efficiencies.
6. To avoid the formation of nonreactive disulfide-linked protein dimers directly proceed with mixing the desalted proteins (i.e., after removing TCEP) with the maleimide-functionalized oligonucleotides. Various reports have published mixed results with TCEP interfering with maleimide conjugation. In our experience, TCEP significantly lowers conversion yields and is therefore removed prior to the reaction with the maleimide-functionalized oligonucleotide.

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