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Switchable reporter enzymes based on mutually exclusive domain interactions allow antibody detection directly in solution

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Abstract

Detection of antibodies is essential for the diagnosis of many diseases including infections, allergies and autoimmune diseases. Current heterogeneous immunoassays require multiple time-consuming binding and washing steps, which limits their application in point-of-care diagnostics and high-throughput screening. Here we report switchable reporter enzymes that allow simple colorimetric detection of antibodies directly in solution. Our approach is based on the antibody-induced disruption of an intramolecular interaction between TEM1- β -lactamase and its inhibitor protein BLIP. Using the HIV1-p17 antibody as an initial target, the interaction between enzyme and inhibitor was carefully tuned to yield a reporter enzyme whose activity increased 10-fold in the presence of pM antibody concentrations. Reporter enzymes for two other antibodies (HA-tag and Dengue virus type I) were obtained by simply replacing the epitope sequences. This new sensor design represents a modular and generic approach to construct antibody reporter enzymes without the cumbersome optimization required by previous engineering strategies.

Antibody detection is essential for the diagnosis of many disease states, including infectious diseases, autoimmune diseases and allergies.¹ While a wide variety of analytical techniques have been developed for the detection of antibodies in blood, saliva and other bodily fluids, many of them come with intrinsic limitations such as the requirement for multiple time-consuming incubation steps (ELISA and other heterogeneous, sandwich-type assays), multiple reagents, and/or sophisticated equipment (e.g. surface plasmon resonance). New generic antibody detection strategies in which molecular recognition and enzyme activation are integrated within a single protein would be ideal, in particular for high-throughput screening and point-of-care applications.² From a protein engineering perspective, the key question is how antibody binding to a sensor protein can be translated into a readily detectable signal.^{3,4} The most common approach thus far has been to introduce peptide epitopes at permissive sites within reporter enzymes such as β -galactosidase,⁵ β -lactamase,⁶ and alkaline phosphatase.^{7–9} However, these hybrid enzymes are catalytically compromised and analyte binding often results in a further decrease in activity,^{6,7} which is an important drawback from an application point of view. Moreover, since their performance relies on subtle allosteric mechanisms, the development of each new sensor involves a time-consuming process of trial-and-error. Combinatorial approaches such as phage display and in vivo selection strategies have been reported in an effort to make development of these allosterically regulated reporter enzymes more efficient, but these approaches have not

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ASSOCIATED CONTENT

Supporting Information. Peptide synthesis, DNA and protein sequences, peptide binding assays, characterization of additional sensor variants, and determination of inhibition constants for BLIP mutants. This material is available free of charge via the Internet at <http://pubs.acs.org>.

solved the intrinsic problem of small changes in enzyme activity.⁶ An alternative strategy is to make use of antibody-induced oligomerization of reporter enzymes or complementation of split reporter enzymes.^{10,11} These approaches utilize the bivalent nature of antibodies to bring together two protein fragments to form an active enzyme. While more easily adaptable to different antibodies, the reconstitution of split enzyme systems typically also results in low enzymatic activities. Furthermore, these systems tend to be less robust than single protein sensors, because their performance also depends on the sensor concentration.¹¹

Here we introduce a new, highly modular sensor concept for antibody-responsive reporter enzymes that addresses many of the limitations discussed above. In our approach switchable reporter enzymes are constructed by conjugation of a full length reporter enzyme to an inhibitor domain via a long semi-flexible linker, forming a catalytically inactive enzyme-inhibitor complex in the absence of its target antibody. Binding of a single antibody to epitope sequences introduced adjacent to the enzyme and inhibitor domains separates the enzyme-inhibitor complex, resulting in an increase in enzyme activity. The feasibility of this new approach is demonstrated using TEM1 β -lactamase as a reporter enzyme, allowing detection of pM concentrations of specific antibodies using simple colorimetric or fluorescent read-outs. Moreover, the modular architecture of these reporter enzymes allows easy exchange of epitope sequences without compromising the sensors' performance.

Figure 1a/b shows the schematic architecture of the antibody reporter enzymes. TEM1 β -lactamase was chosen as a reporter enzyme because it does not require oligomerization for activity and many substrates are available both for colorimetric and fluorescence detection. In our initial designs we focused on developing a sensor for the detection of the HIV1-p17 antibody. Several well-characterized linear epitope sequences are available for this antibody, which has made it a popular choice for the development of new antibody detection assays.^{12,13} The linker between the enzyme and the inhibitor modules initially consisted of two short peptide epitopes specific for the HIV1-p17-antibody (WEKIRLR, $K_d = 3.3 \mu\text{M}$; Fig. S1), which were separated by a semi-flexible linker. The linker contains three flexible blocks consisting of six GSG repeats and two α -helical blocks each consisting of six EAAAK repeats (Fig. 1a). This linker was also used in a recently developed FRET sensor protein,¹² where it was shown that introduction of the two 45 Å α -helical blocks in the flexible linker was essential for the linker to efficiently bridge the distance between the two antigen binding sites. To establish the influence of inhibitor affinity, two variants were initially constructed containing either a weak-binding RRGHYY peptide derived from phage display ($K_i \sim 140 \mu\text{M}$)¹⁴; **Abs-1**) or the natural β -lactamase inhibitor protein (BLIP), which has a K_i of 0.5 nM (**Abs-2**).¹⁵ To allow proper folding, proteins were expressed in *E. coli* BL21 (DE3) using a periplasmic leader sequence and purified using an N-terminal His-tag and a C-terminal Strep-tag. This two-step purification protocol ensures the isolation of full-length protein, without truncated versions of the sensor lacking e.g. the inhibitor domain.

Enzymatic activity assays using the colorimetric substrate nitrocefin showed that the activity of **Abs-1** was similar to that of TEM1 β -lactamase in the absence of any inhibitor (Fig. 1c). Moreover, no increase in enzymatic activity was observed upon addition of 200 nM anti-HIV1-p17. These results show that the affinity of the peptide inhibitor used in **Abs-1** is too low to result in substantial enzyme inhibition in the absence of antibody. In contrast, the enzymatic activity of **Abs-2** was strongly inhibited compared to that of TEM1 β -lactamase, but no increase in activity was observed upon addition of 200 nM of the target antibody. This result suggests that the intramolecular interaction between wild-type BLIP and β -lactamase domains is actually too strong and that antibody epitope binding strength is not sufficient to overcome this interaction.

To provide a stronger driving force for disrupting the enzyme-inhibitor interaction, Abs-3 was created in which the short WEKIRLR epitope was extended to a longer epitope with a higher affinity (ELDRWEKIRLRP; $K_d = 42$ nM; Fig. S2). The X-ray structure of the TEM1 β -lactamase-BLIP complex has been reported, and for many of the residues at the binding interface their contribution to the binding strength has been determined.^{15,16,17} To systematically attenuate the interaction between the β -lactamase and BLIP, a series of Abs-3 variants was explored with mutations either in BLIP, β -lactamase, or both. First, several single point mutations were introduced in BLIP. These mutations were previously reported to have affinities ranging from 20-150 nM, but none of them yielded antibody-responsive Abs-3 variants. A similar result was obtained for a single point mutation in β -lactamase (E104D) (Fig. S5), which in a recent study was reported to have a 3 orders of magnitude lower affinity for BLIP than wt β -lactamase ($K_i = 1500$ nM).¹⁸ However, combination of the E104D mutation in β -lactamase and single point mutations in BLIP yielded several sensor variants that showed an increase in enzymatic activity upon addition of the target antibody (Fig. 1d). The variants that displayed substantial activity in the absence of antibody typically showed only a modest increase in activity upon antibody binding. The two most promising variants, Abs-3-E104D/E31A (**Abs-3-1**) and Abs-3-E104D/F142A (**Abs-3-2**), were further characterized, as these combined a low background activity with a 5-6 fold increase in enzyme activity.

To determine the affinity of each sensor for their target antibody, the rate of nitrocefin hydrolysis was measured as a function of antibody concentration using 100 pM of sensor (Fig. 2a/b). Fitting these curves assuming a 1:1 binding model yielded dissociation constants of 0.17 ± 0.03 nM for **Abs-3-1** and 0.19 ± 0.02 nM for **Abs-3-2**. The bivalent interaction between sensor and antibody thus not only provides a convenient switching mechanism, but also results in a 200-fold increase in overall affinity. To test the proposed mechanism we verified that both epitopes were required for antibody-induced activation. Two variants of **Abs-3-2** were generated in which either the epitope next to the enzyme (Abs-3-E0E2) or the epitope adjacent to the BLIP domain was deleted (Abs-3-E2E0). Indeed, both variants showed a low enzymatic activity and neither showed any increase in enzymatic activity upon addition of 100 nM of HIV1-p17 antibody (Fig. 2c). These results confirmed that the activity increase is due to bivalent binding of the antibody to the two epitope sequences. To test sensor specificity, **Abs3-2** was incubated with a random mix of IgG proteins. No significant increase in enzyme activity was observed up to the highest concentration of IgG tested (2 μ M) (Fig. 2a/b). Moreover, the presence of non-specific IgGs did not interfere with binding of the target antibody, as a similar increase in enzyme activity was observed upon addition of 10 nM anti-HIV1-p17 in the absence and presence of a large excess of IgG mix (0.1 mg/mL, i.e. 660 nM) (Fig. 2c). The latter is important because in serum specific antibodies need to be detected against a high background of non-binding antibodies.

The use of nitrocefin and other colorimetric substrates provides a straightforward means to detect sub-nM concentrations of a specific antibody directly by eye. However, assays based on light absorption require relatively high concentrations of substrate. Since substrate and BLIP compete for the same binding site on β -lactamase, using high substrate concentrations may also result in increased background activity. We therefore assessed the performance of **Abs-3-1** using the commercially available fluorescent substrate CCF2-FA, which can be used at a 50-fold lower concentration. When this FRET probe is hydrolyzed by β -lactamase, a fluorescein molecule (acceptor) is expelled from the probe which results in an increase in coumarin fluorescence (donor). Unlike nitrocefin, which is slowly hydrolyzed even in the absence of sensor (Fig. 3a), CCF2-FA was found to be completely stable providing a low background (Fig. 3b). Moreover, the enzymatic activity of the sensor protein in the absence of target antibody was found to be significantly lower using 1 μ M of CCF2-FA compared to 50 μ M nitrocefin (Fig. 3a-b, d). Therefore using CCF2-FA as a substrate resulted in an

increased dynamic range (9-fold) by suppressing the background reaction. The fluorescent substrate also proved essential to use the reporter enzyme in serum. Unlike nitrocefin, which was rapidly hydrolyzed even in the absence of any reporter enzyme, CCF2-FA was found to be completely stable in both bovine and human serum. Figure 3c shows that the dynamic range of the reporter enzyme in serum is at least as high as observed in buffer, showing a 10-fold increase in enzyme activity upon addition of 50 nM of target antibody.

To challenge the modularity of our sensor design we tested whether the original epitope sequences could be exchanged for epitope sequences targeting different antibodies. Reporter enzymes targeting an HA-tag specific antibody (**Abs-4**) or a Dengue type I specific antibody (**Abs-5**) were constructed by replacing the epitope sequences present in **Abs-3-1** by YPYDVPDYA (**Abs-4**) or EHKYSWKS (**Abs-5**)¹⁹. Fluorescence polarization titration experiments were first done to establish the affinities of both antibodies for their peptide epitopes (Fig. S3/4), yielding K_d values of ~5 nM and 70 nM, respectively. Although no sensor optimization was performed, **Abs-4** showed very similar sensor properties compared to its parent sensor **Abs-3-1**. Titration of HA antibody resulted in 7-fold increase in activity and a K_d of 0.20 ± 0.02 nM for the sensor-antibody interaction (Fig. 4a). Similarly, a 7-fold increase in enzymatic activity was observed upon titration of Dengue type I antibody to **Abs-5**, consistent with a K_d of $1.13 \text{ nM} \pm 0.46$ (Fig. 4b).

Although the antibody affinity is slightly attenuated in **Abs-5**, this K_d still reflects a 60-fold increase in affinity compared to the monovalent interaction between antibody and peptide epitope. These results show that the framework developed for the HIV1-p17 antibody allows the antibody specificity to be changed simply by replacing the epitope sequences without the subsequent sensor optimization required by other by protein engineering strategies. To provide a better insight into the factors that determine the binding properties of these protein switches, a thermodynamic model has been derived that describes the bivalent binding of the antibody and the sensor in 3 steps (Supplementary Fig. S8). This analysis shows that the overall affinity of the reporter enzyme depends on the square of the antibody-epitope interaction ($K_{d, AP}$), the enzyme-inhibitor interaction ($K_{d, EI}$) and two effective concentrations terms ($C_{eff, EI}$ and $C_{eff, AP}$) according to equation 1.

$$K_{d, overall} = 0.5 * \left(\left(K_{d, AP} \right)^2 / K_{d, EI} \right) * \left(C_{eff, EI} / C_{eff, AP} \right) \quad (1)$$

In conclusion, a new design concept was introduced that allows the construction of switchable antibody reporter enzymes to detect antibodies directly in solution. The modular architecture of these reporter enzymes allows their target specificity to be changed by simple exchange of epitope sequences, without the cumbersome optimization/screening procedures required by strategies based on subtle allosteric modulation of enzyme activity. In contrast to biosensors based on split reporter enzymes that depend on proximity-induced reconstitution of enzyme activity, the current design is based on the antibody-induced disruption of the intramolecular interaction between a reporter enzyme and its inhibitor protein. Because it uses fully active and stable protein domains in a single protein format, this approach is inherently more robust, easily tunable and independent of sensor concentration. This new sensor principle also takes advantage of a unique structural property that is shared by all antibody classes, the presence of two identical antigen binding sites separated by a distance of approximately 100 Å. The approach should therefore be applicable to in principle any antibody, provided a linear epitope of sufficient affinity is available. Because of the bivalent interaction between reporter enzyme and target antibody, the overall affinity of the reporter enzyme depends very strongly on the monovalent antibody-epitope interaction. Equation (1) predicts that a 10-fold difference between the

monovalent affinities for specific and non-specific binding should translate into a 100-fold difference in the overall affinity of the reporter enzyme. These reporter enzymes are therefore expected to show less cross-reactivity than systems based on monovalent peptide-antibody interactions. More general, these results demonstrate that designing protein switches based on mutually exclusive interactions between input and output domains provides an attractive modular approach for engineering biosensors. As such, the β -lactamase-BLIP system developed here could guide the construction of similar output functions using other enzyme-inhibitor pairs.

METHODS

Cloning and mutagenesis

Synthetic DNA sequences encoding Abs-1, BLIP and linker 2 (linker with longer epitope sequence, E2) were ordered from Genscript. The DNA sequence encoding for the inhibitor peptide in Abs-1 was replaced with the BLIP sequence by cloning with *NcoI* and *EcoRI* restriction enzymes to generate Abs-2. Linker 1 (linker with short epitope sequence, E1) in the Abs-2 construct was replaced with the linker 2 sequence by cloning with *SpeI* and *NcoI* restriction enzymes to generate Abs-3 sensors. Abs-4 and Abs-5 were constructed from Abs-3 using a strategy described previously.²⁰ Briefly, the Abs-3 vector was opened with PCR using primers HA-open-FW and HA-open-RW for Abs-4 and Deng1-open-FW and Deng1-open-RW for Abs-5 (see table S2 for list of primers used). The linker part of Abs-3 without HIV1 epitopes was PCR-amplified with the HA-linker-FW and HA-linker-RW primers for Abs-4 and Deng1-linker-FW and Deng1-linker-RW for Abs-5. This PCR-generated linker contains sequences encoding for either the HA-epitope or Dengue-1 epitopes and sequences that overlap with the opened vector. After agarose gel purification, both opened vector and linker were mixed and PCR was performed. The PCR mixture was treated with *DpnI* to remove any remaining parental DNA. Transformation and then sequencing of colonies showed successful exchange of the epitope sequences. All constructs were cloned into pET29a vectors. A plasmid encoding β -lactamase was obtained from Abs-1 by introducing a stop codon after the β -lactamase sequence and a plasmid for BLIP was obtained from Abs-2 by deleting the β -lactamase and linker parts. The QuikChange site-directed mutagenesis kit (Stratagene) was used in accordance with the manufacturer's instructions to introduce the mutations of interest by using the primers listed in Table S1. All cloning and mutagenesis results (see supplementary Fig. S9-S14) were confirmed by DNA sequencing (BaseClear).

Protein expression and purification

All proteins were expressed and purified using standard protocols. Briefly, *E. coli* BL21(DE3) cells were transformed with the appropriate pET29a vector. The bacteria containing plasmid DNA were grown in LB (2 L) media at 37 °C and induced at OD₆₀₀ ~0.6 with isopropyl- β -D-thiogalactoside (IPTG; 0.3 mM). Induced cells were grown overnight at 15 °C, and harvested for 10 min at 8,000g. The protein which is located in the periplasm was extracted by osmotic shock. The bacterial pellet was resuspended in 100 mL 30 mM Tris/HCl (pH 8.0), 20% (w/v) sucrose, and 1 mM EDTA, and incubated at room temperature for 10 min under continuous shaking. After centrifugation for 10 min at 8,000g, the pellet was resuspended in 100 mL of ice-cold 5 mM MgSO₄. After incubation for 10 min at 4 °C with continuous shaking, the suspension was centrifuged for 20 min at 12,000g. The supernatant (which contains the periplasmic protein fraction) was adjusted to pH 7.4 by adding 2 mL of 1 M Tris/HCl (pH 7.4). The supernatant was first loaded onto an immobilized metal-affinity column packed with His-bind resin in accordance with the manufacturer's instructions (Novagen). The eluted fractions were further purified on a Strep-Tactin superflow column (IBA) according to the instructions of the supplier. The

purified proteins were dialyzed against 50 mM Tris/HCl (pH 7.1) containing 150 mM NaCl using 3.5 kD MWCO membranes (SpectraPor 3). The protein concentration was determined by measuring the absorbance at 280 nm using extinction coefficients calculated from the protein sequence (<http://web.expasy.org/protparam/>). Protein aliquots were stored at -80°C .

Activity assays

Antibodies were purchased from commercial sources, anti-HIV-1-p17 (clone 32/1.24.89) from Zeptometrix, HA monoclonal antibody (clone 2-2.2.14) from Thermo Scientific and anti-Dengue virus type I antibody (clone 15F3-1) from Merck Millipore. Nonspecific IgG mix isolated from human serum was purchased from Sigma–Aldrich. Fetal bovine serum (FBS) was purchased from BioChrom AG. For activity assays, sensor proteins were pre-incubated with antibody for 15 min at room temperature, and then 50 μM of nitrocefin was added. For responsive sensors, 100 pM of sensor was treated up to 100 nM of antibody. For non-responsive sensors 300 pM of sensor was treated with up to 200 nM antibody and incubated for 1 h. The assays were performed in 50 mM phosphate buffer (pH 7.0, containing 100 mM NaCl and 1 mg mL^{-1} BSA). Measurements were performed in 96-well plates. Both absorbance (486 nm) with nitrocefin substrate, and fluorescence with CCF2-FA (Ex. 409 nm; Em. 447 nm) were recorded on a Safire2 spectrofluorimeter (Tecan). The data were plotted and analyzed using Graphad Prism5 software. Assays using β -lactamase-E104D showed that the enzyme is substantially less active in serum compared to PBS, suggesting the presence of inhibitory compounds in serum. To compensate for this decreased activity, assays in serum were done using 5 nM of reporter enzyme.

Antibody titrations experiments

Antibody titrations were performed as mentioned above, the change in absorbance during the first 10 min was used to calculate the hydrolysis rate. Dissociation constants were obtained by using equation 2 to fit the hydrolysis rate as a function of antibody concentration. In this equation, A and B are constants, and [sensor] and [Ab] are the total sensor and antibody concentrations, respectively.

$$\text{hydrolysis rate} = A + B * \frac{([\text{sensor}] + K_d + [\text{Ab}]) - \sqrt{([\text{sensor}] + K_d + [\text{Ab}])^2 - 4 [\text{sensor}] [\text{Ab}]}}{2 [\text{sensor}]} \quad (2)$$

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Construction and characterization of TEM1 β -lactamase-inhibitor fusion proteins. A/B) Schematic structure of the antibody reporter enzymes described in this work. **Abs-1/2/3** target an HIV1-p17 antibody, **Abs-4** binds an HA-tag specific antibody, and **Abs-5** targets a Dengue type I specific antibody. C) Enzymatic activity of 0.3 nM of **Abs-1**, **Abs-2** or β -lactamase alone, in the absence and presence of 200 nM HIV1-p17 antibody. D) Enzymatic activities of **Abs-3** variants (0.1 nM) containing β -lactamase-E104D and various mutations in BLIP in the absence (white bars) and presence (grey bars) of 100 nM HIV1-p17 antibody. All assays were done using 50 μ M nitrocefin in 50 mM phosphate, 100 mM NaCl and 1 mg/mL BSA, pH 7.0

Figure 2.

Enzymatic activities of **Abs3-1** (a) and **Abs3-2** (b) as a function of antibody concentration. Solid lines represent best fits to equation 2, yielding K_d values of 0.17 nM (A) and 0.19 nM (B). C) Influence of the addition of IgG mix (0.1 mg/mL; 660 nM) on the performance of **Abs3-2** and control experiments showing the lack of antibody response for **Abs3-2** variants containing a single copy of the epitope. All assays were done using 0.1 nM sensor, 50 μM nitrocefin in 50 mM phosphate, 100 mM NaCl and 1 mg/mL BSA, pH 7.0.

Figure 3.

Comparison of the **Abs-3-1** performance using either a colorimetric substrate (a; 50 μ M nitrocefin) or a fluorescent substrate (b; 1 μ M CCF2-FA). Enzymatic activities were determined for substrate alone, and for **Abs-3-1** (0.1 nM) in the absence and presence of saturating amount of anti-HIV1-p17. (C) Activity of **Abs-3-1** (5 nM) in 10% Fetal Bovine Serum using 10 μ M CCF2-FA in the absence and presence of antibody. D) Comparison of the dynamic range of **Abs-3-1** under different conditions. For each condition the activity in the presence of antibody was taken as 100%.

Figure 4.

Enzymatic activities of **Abs-4** (a) and **Abs-5** (b) as a function of antibody concentration. Solid lines represent best fits to equation 2, yielding K_d values of 0.20 nM (a) and 1.13 nM $\pm$ 0.46 (b). C) Photograph taken after the activity measurements in (a) showing that antibody concentrations can be easily distinguished by eye. All assays were done using 0.1 nM sensor, 50 μ M nitrocefin in 50 mM phosphate, 100 mM NaCl and 1 mg/mL BSA, pH 7.0.