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Surface-tethered protein switches†

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Protein switches are engineered fusion proteins with an input domain that recognizes and responds to an input signal and an output domain whose function is regulated by the state of the input domain. Here we demonstrate a fully functional surface tethered protein switch that offers a potential route to a universal biosensing platform.

Biological sensor platforms usually combine a recognition element (*e.g.* antibody, recombinant protein/peptide, or oligonucleotide/oligonucleoside) that binds a target molecule (*e.g.* protein, DNA fragment, or whole cell) with a transduction/ output scheme that provides a readable signal. A class of proteins called protein switches represent a unique platform for biosensors since they combine both input and output domains.¹

Protein switches are engineered fusion proteins with two domains: an input domain that recognizes and responds to an input signal and an output domain whose function is regulated by the state of the input domain.² This feature can be exploited to selectively switch the activity of a protein on and off through recognition and binding of the input substrate. For example, the fusion of a ligand-binding domain with an enzyme domain can result in a protein switch in which enzyme activity (the output function) is regulated by ligand concentration (the input signal) *via* ligand-induced conformational changes.^{3,4} Such modular allosteric regulation allows the possibility of developing universal biosensors in which different input domains can be coupled to the same output domain.

The response of a protein switch to an input signal is usually measured in solution, however, for sensor applications it is desirable to immobilize the recognition element. Here we demonstrate a fully functional surface tethered protein switch, the first step towards a platform for universal biosensors. For this purpose we used RG13, an engineered allosteric protein switch comprising a maltose binding protein domain (MBP) and TEM1 β -lactamase domain (BLA), a protein that hydrolyzes β -lactam antibiotics.

Scheme 1 shows a schematic illustration of the strategy for the immobilization of RG13 on the gold surface (see ESI[†] for details). To ensure proper orientation for ligand and substrate

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accessibility, RG13 was genetically engineered with a hexahistidine (His₆) tag at the C-terminus, which served as the site for attachment to the surface *via* binding to chelated Ni(II). The switch was immobilized on a gold surface using a nitrilotriacetic acid (NTA)-terminated thiol chelated with nickel(II) (see Scheme 1). First, a mixed monolayer of OH-terminated thiol (SH(CH₃)₁₁OH) and NH₂-terminated thiol (SH(CH₃)₁₁NH₂) was formed on ultra-smooth template stripped gold.⁵ A terminal aldehyde group was then formed on the NH₂-terminated thiols (switch attachment sites) by reaction with glutaraldehyde. Subsequent reaction with *N*-(5-amino-1-carboxypentyl) iminodiacetic acid (AB-NTA) couples the NTA group to the thiol through formation of a schiff base with the aldehyde group. After immersing in NiSO₄ solution, the functionalized surface was incubated with



Scheme 1 Schematic illustration of the fabrication of a surface tethered protein switch. (a) Incubation of gold surface with OH-terminated thiol and NH₂-terminated thiol, (b) reaction with glutaraldehyde and AB-NTA, (c) incubation with Ni(II), (d) incubation with the protein switch that has been genetically engineered with a single hexahistidine (His₆) tag at the C-terminus. The tethered switch can be regenerated by incubation in EDTA followed by steps (c) and (d).

 His_{6} -tagged RG13. The surface coverage of the NH₂-terminated thiol was 10%, corresponding to a complete monolayer of the switch. The OH-terminated thiol prevents non-specific adsorption on the exposed surface.

The activity of RG13 on the surface was measured through reaction with the colorimetric substrate nitrocefin (NCF), which undergoes a color change from yellow ($\lambda_{max} = 390$ nm, pH 7.4) to red ($\lambda_{max} = 486$ nm, pH 7.4) upon hydrolysis of the amide bond in the β -lactam ring by the β -lactamase.⁶ The selective binding of maltose to RG13 amplifies the enzymatic activity of BLA in solution by a factor of about 25 (a combination of a 3-fold increase in k_{cat} and 8-fold decrease in K_m) via maltose induced conformational changes.^{4,7}

Fig. 1 shows the evolution of the absorbance spectra upon nitrocefin hydrolysis by the tethered RG13 protein switches. The absorbance decrease at 390 nm corresponds to substrate consumption, whereas the absorbance increase at 500 nm is due to the formation of the hydrolyzed product. The absorbance for 50 μ m NCF without maltose after 20 min incubation with the tethered protein switch shows a small increase in absorbance at 500 nm CF (Fig. 1a). This small increase is due to the low enzymatic activity of the switch in the absence of maltose. Importantly, the presence of 10 mM maltose in the solution resulted in a significant increase in the rate of absorbance change at 500 nm.



Fig. 1 (a) Absorbance spectra for: 50 μ M NCF, the tethered RG13 switch with 50 μ M NCF (no maltose) after 20 min incubation, and the tethered RG13 switch with 50 μ M NCF and 10 mM maltose after 2, 20, 30, and 60 min incubation. (b) Absorbance change at 500 nm (absorbance of the hydrolyzed NCF referenced to the absorbance of 50 μ M NCF) *versus* time for the tethered RG13 switch in 50 μ M nitrocefin with and without 10 mM maltose. *Inset*: Nitrocefin hydrolysis by the tethered RG13 switch in the presence of 10 mM maltose plotted as M/C_0 versus $t^{1/2}$. *M* is the amount of nitrocefin hydrolyzed by the switch (molecules cm⁻²) and C_0 is the initial concentration of the nitrocefin (molecules cm⁻³).

As can be seen from Fig. 1a, a measurable absorbance change was detected after 2 min incubation with the tethered switch. The removal of the solution from the modified surface halted the absorbance change in solution, indicating that the measured enzyme activity was that of the tethered switch and not that of any desorbed switches.

Fig. 1b shows the time dependence of switch activity with and without maltose. The inset in Fig. 1b shows the integrated amount of nitrocefin hydrolyzed by the tethered switch plotted *versus* $t^{1/2}$. The linear behavior shows that the rate of nitrocefin hydrolysis is controlled by diffusion to the surface. Assuming a constant concentration of nitrocefin in bulk solution C_0 , the total amount of nitrocefin hydrolyzed by the switch M(molecules cm⁻²) is given by:⁸

$$\frac{M}{C_0} = 2\left(\frac{Dt}{\pi}\right)^{1/2}$$

where *D* is the diffusion coefficient. From a least squares fit to the data, we obtain a diffusion coefficient of 1.3×10^{-6} cm² s⁻¹, in good agreement with the value of about 5×10^{-6} cm² s⁻¹ reported for β -lactam antibiotics of similar size in aqueous solution.⁹ Experiments, where RG13-modified gold surfaces were incubated with maltose solution for 30 min prior to adding nitrocefin (data not shown), confirmed that nitrocefin hydrolysis does not depend on maltose diffusion to the maltose binding protein domain.

The effect of maltose on the initial reaction rate of the tethered switch was estimated by comparing the absorbance change after 2 min incubation in nitrocefin solution with and without maltose (Fig. 1b). The binding of maltose to the MBP domain of the tethered switch triggered BLA domain enzymatic activity resulting in a 9-fold increase in activity (see ESI† for details). This is very close to the 15-fold increase in activity measured in bulk solution in 50 μ M NCF.⁴ The small difference in activity may be due to differences in the switching mechanism, limited substrate diffusion (nitrocefin), or restricted accessibility of the active sites of both domains.

Next, we checked if the RG13-modified surface could be used as a platform for detection of maltose. Fig. 2 shows the change in absorbance upon adding different concentrations of maltose in presence of 50 μ M nitrocefin. The absorbance change increases with maltose concentration, from the lowest concentration measured (50 μ M), reaching a maximum at a concentration of about 20 mM.



Fig. 2 Absorbance change at 500 nm for 50 μ M nitrocefin and different concentrations of maltose incubated with surface tethered RG13 switch for 20 min.



Fig. 3 Absorbance change at 500 nm (after 20 min) for (a) 50 μ M NCF, 10 mM maltose, and RG13 switch incubated with an NTAmodified gold surface (no Ni(II)), (b) 50 μ M NCF and 10 mM maltose incubated with an NTA/Ni(II)-modified gold surface (no RG13 switch), (c) 50 μ M NCF and 10 mM sucrose incubated with tethered RG13 switch, (d) 50 μ M NCF incubated with tethered RG13 switch, (e) 50 μ M NCF and 10 mM maltose incubated with tethered RG13 switch and, (f) 50 μ M NCF and 10 mM maltose incubated with a regenerated surface-tethered RG13 switch after removing the original Ni(II)-switch complex by incubation with EDTA.

A series of control experiments was performed to confirm that our system was functioning as expected (see ESI† for details). (1) When the NTA-functionalized surface was not treated with Ni(II) prior to incubation with RG13, no absorbance change was detected (Fig. 3). This result confirms that NCF hydrolysis is due to specific immobilization of the His-tagged protein switch on the surface, and not due to nonspecific adsorption. (2) No changes in NCF absorbance were observed after exposure to a functionalized surface without RG13, demonstrating that NCF hydrolysis is due to the enzymatic activity of the tethered RG13 switch (Fig. 3). (3) Ligand-activation of RG13 activity was observed only in the presence of sugars that are known to bind to MBP; the activity in the presence of sucrose—a disaccharide that neither binds nor activates RG13 in solution—(Fig. 3) was equivalent to the activity in the absence of maltose (Fig. 3). (4) The sensor surface could be regenerated through the use of EDTA (Fig. 3), indicating that this platform can be reused with different His-tagged switches.

We have demonstrated that the protein switch RG13 can be tethered to a surface while retaining the ability to bind maltose to the MBP domain and to elicit the associated conformational changes that switches the activity of the TEM1 β -lactamase domain. The response of the tethered RG13 platform to maltose is controlled by diffusion of nitrocefin to the surface. The tethered switch platform responds to maltose concentrations over a wide range, up to 20 mM. Our platform offers oriented tethering of protein switches in a reusable format. Combined with the potential modularity of switches created by domain fusion, our system offers a potential route to a universal biosensing platform in which different input domains can be coupled to the same output domain.

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