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Photo-controlled deactivation of immobilised lipase†

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Lipase from Candida rugosa was immobilised on a quartz surface using an azobenzene-containing, bifunctional linker, which allows deactivation of the immobilised enzyme by irradiation with visible light.

The challenge to control biological functions in a non-invasive manner, using light, has greatly stimulated the development of biocatalytic systems with intrinsic photoresponsive properties. These systems have potential applications in numerous fields, such as biosensing, diagnostics, optobioelectronics and signal amplification. Especially in applications in which the enzymatic activity has to be precisely adjusted (initiated or terminated) in time, with little perturbation to the sample, light offers unparalleled advantages as easily applied, external and orthogonal control element.

Photocontrol over biocatalytic activity has been achieved by creating a responsive environment, which can be based on a light-switchable polymer or a surfactant, or alternatively by random or site-selective incorporation of photoswitchable units within the structure of the enzyme. In general, the incorporation of photoswitches involves modification of enzymes in solution, taking advantage of the nucleophilicity of certain amino acids that can react with azobenzene or thiophenefulgide derivatives. Papain and α-chymotrypsin were modified, using this approach, with photoswitchable units bearing an activated ester moiety. Willner et al. have reported on a redox enzyme, glucose oxidase, which was randomly modified with a spiropyran derivative and subsequently immobilised on a gold electrode via a short linker, showing, in the presence of a redox mediator, a photocontrolled optoelectronic response.

The immobilisation of biocatalysts on the surface of porous materials can enhance their stability and decrease the cost involved in biotechnological processes. It is, therefore, highly desirable to take advantage of both the immobilisation of enzymes and the photoresponsive properties described above. While the random incorporation of molecular photoswitches into enzymes has proven successful in the preparation of photocontrolled biocatalysts, the photoswitches have not, to the best of our knowledge, been used as covalent linkers for the immobilisation of enzymes on solid supports.

We describe here a new approach for the immobilisation of an enzyme on a surface via a photoswitchable linker (Fig. 1). We envisioned that providing an additional attachment point for the

Fig. 1 Immobilisation of lipase on a quartz surface through a photoswitchable linker.

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photoswitches, introduced randomly onto the reactive amino acid side chains in the enzyme, would reduce the flexibility of the photoswitch and might result in highly-pronounced photoresponse. We envisioned that the photoisomerisation of the azobenzene linker might change the orientation of the active site of the enzyme with respect to the surface or, when there are multiple attachment points between the enzyme and the surface, it might result in a distortion of the active site (Fig. 1). In addition, the photoisomerisation of the azobenzene could change the polarity of modified surface, which can have a significant influence on the biocatalytic properties of the immobilised protein (Fig 1).11

Towards this end, a quartz coverslip, decorated with an azide functionality, was functionalised with an amine-bearing azobenzene 1 (Fig. 2) as the photoswitchable linker (Fig. 1). Subsequently, lipase from Candida Rugosa, was immobilised using glutaraldehyde as the connection between the amine-bearing linker and lysine-NH₂ groups on the surface of the protein.12 This modification of a widely-used amino propyltriethoxysilane (APTES)/glutaraldehyde method for the enzyme immobilization was chosen for its often-reported simplicity and efficiency.

We designed the bifunctional photoswitchable linker 1 (Fig. 2) to enable modularity in the preparation of quartz-immobilised lipase (Fig. 1). It incorporates in its structure a terminal acetylene, for the attachment to an azide-decorated quartz surface via Cu(I)-catalysed Huisgen azide-alkyne cycloaddition (CuAAC),13 and an amine group that can be attached to lysine residues in the enzyme via a glutaraldehyde spacer.14 Photoswitchable linker 1 was synthesised from 4-aminobenzoic acid in good yield (61% over 6 steps, see ESI).

Model compound 2 (Fig. 2), containing the triazole moiety, was synthesized to study the behavior of the azobenzene unit in solution. Irradiation of trans-2 at λ = 365 nm results in its photoisomerisation to the cis form, manifested by a decrease of the symmetry-allowed π-π* transition band at 352 nm and the appearance of a new absorption band at 438 nm (Fig. 3a). A high photostationary state, with ≥95% cis isomer present in the irradiated sample, was observed by 1H NMR (see ESI). Notably, the azobenzene switch does not show fatigue with irradiation over at least five switching cycles in methanol (Fig. 3b). From kinetic studies, the half-life for the cis isomer to revert to the trans isomer in methanol at RT was found to be 100 min (Fig. 3c). Azobenzene 2 fulfills all the requirements needed in our assembly, i.e. a fast switching process with high photostationary state and a relatively thermally-stable cis form.

The strategy used for the preparation of the photoswitchable immobilised enzyme is shown in Fig. 4. Quartz coverslip 3 was cleaned and activated with Piranha solution and subsequently functionalised with azido-silane 4.14 A CuAAC reaction was then used to anchor azobenzene 1 to the surface. Compound 6,16 an oligo(ethylene glycol) with an alkyn functional group, was used to obtain mixed layers.17 Compound 6 does not react with the enzyme, therefore, by using various ratios of azobenzene 1 and compound 6 (between 0.1 and 100% with respect to 1) during the reaction, it was possible to determine the dependence of the enzymatic activity on the coverage of azobenzene on the surface. Quartz coverslips 7 were then reacted with glutaraldehyde in phosphate buffer to form an imine moiety and a free aldehyde functionality (8), which can react with the free amino groups of solvent-exposed lysine residues of lipase from Candida Rugosa to form 9.

Surface modification was monitored by contact angle measurements and UV-vis absorption spectroscopy. The contact angle for the clean quartz surface was 61° and, upon functionalisation with 4, the contact angle increased to 62°, which is associated with an increase in hydrophobicity. When the enzyme is anchored, as in 9, the contact angle decreased slightly to 56°, although it shows a strong dependence on the mol% of 6 used in the CuAAC reaction: the higher the content of 6 on the surface, the lower the contact angle, as expected for a mixed layer with an increased proportion of hydrophilic oligo(ethylene glycol) units.

UV-vis absorption spectroscopy provides additional evidence of surface functionalisation. The characteristic azobenzene absorption at 348 nm was observed for the quartz surface 7a functionalised with the switch (Fig. 5a). By applying sequential irradiation with λ = 365 nm and visible light (>400 nm), the trans-cis isomerisation of the azobenzene 1 anchored to the surface was achieved (Fig. 5b). The process was reversible over at least 2 cycles with little fatigue observed. Importantly, these changes were also observed when the enzyme was anchored via the azobenzene linker to the surface i.e. 9a (Fig. 5c,d), which confirms that the reversible isomerisation of the photoswitch is retained in the presence of the enzyme. The reversible photoswitching behaviour has been shown for the coverslip 9a, fully modified with 1: this proves that even an overcrowded environment makes the photoswitching possible and we assumed that, in the systems in which compound 1 is diluted on the surface with 6 (9b-d), the photosomeration will also occur.

The standard enzyme activity assay used in this study is based on the lipase-catalysed hydrolysis of p-nitrophenyl butyrate to p-
nitrophenol, which can be quantified by following the increase in absorbance at 442 nm.\(^5\)

Lipase-modified coverslip 9a does not show a significant difference in enzymatic activity after irradiation with \(\lambda = 365\) nm and after irradiation with white light (Fig. 6a). With a mixed surface, comprising the oligo(ethylene glycol) and the enzyme bound via an azobenzene linker, it was possible to decrease the enzyme loading on the surface and a significant difference in enzymatic activity between the surfaces irradiated with \(\lambda = 365\) nm and with white light was observed (Fig. 6a). This phenomenon is probably associated with the less dense packing of the enzyme on the surface, which increases its conformational flexibility and enables more pronounced changes upon irradiation. For lipase-modified coverslip 9d the enzymatic activity is comparable to that of the non-catalysed hydrolysis of \(p\)-nitrophenyl butyrate in buffer. The largest change in enzymatic activity was obtained for lipase-modified coverslip 9c, comprising a mixed layer system with 1% of compound 1.

These conditions for immobilization were used for further investigation. The enzymatic activity was measured directly for at least three different lipase-modified coverslips (9e) and the average of these measurements are the enzymatic activity values reported in Fig. 6b. The activity was measured after immobilisation, after irradiation with visible light and after irradiation at \(\lambda = 365\) nm. The average enzymatic activity of modified lipase-modified coverslips is \(0.28 \pm 0.14 \mu M \text{cm}^2 \text{min}^{-1}\). After irradiation at \(\lambda = 365\) nm, the enzymatic activity for lipase-modified coverslip is \(0.23 \pm 0.08 \mu M \text{cm}^2 \text{min}^{-1}\), showing no significant change. On the contrary, the enzymatic activity drops to \(0.04 \pm 0.01 \mu M \text{cm}^2 \text{min}^{-1}\) after subsequent white light irradiation (Fig. 6b), what constitutes an approximately an 8-fold change (Fig. 6b). Although the photosisomerisation of azobenzene occurs for at least 2 cycles (Fig. 5d), a reversible change in enzyme activity was not observed (ESI).

Given that the azobenzene is initially anchored on the surface mainly in its \(trans\)-form, we expected that irradiation at \(365\) nm, which induces the \(trans-cis\) isomerisation, should effect the orientation of the enzyme with respect to the surface and therefore the activity. Instead, deactivation of the enzyme on the surface was observed after irradiation with white light, i.e. upon \(cis-trans\) isomerisation. Irradiation selectively at \(\lambda = 400\) nm, instead of white light, resulted in \(~50\%\) isomerisation to \(trans\) isomer, and proportional decrease in enzymatic activity was observed (see Supporting Information). Neither the use of white light, nor the irradiation at \(\lambda = 365\) nm influences the activity of the unmodified enzyme in solution and irradiation does not result in a desorption of the enzyme from the surface (for control experiments, see ESI). Therefore, the irreversible change in activity of lipase on the quartz surfaces after irradiation with white light can be attributed only to the isomerisation of the photoswitchable linker or to the changes that this induces in the surface-bound enzyme (Figure 1).

There are many factors that can affect the activity of a biocatalyst upon immobilisation, including hydrophobic/hydrophilic interactions, hydrogen bonding and salt bridges, and the effect of limited diffusion.\(^12\) In the presented system, in view of the reports on the influence of the surface polarity on the activity of immobilised enzymes,\(^12\) we attribute the irreversible change in activity to the change in polarity of the surface upon the \(cis-trans\) isomerisation of the linker, rather than to a change in orientation of the enzyme with respect to the surface.

To our knowledge, this system constitutes the first example of covalent immobilisation of an enzyme with a photoswitchable linker. The design of the linker with two groups that can react in an orthogonal fashion enables a modular approach to be taken to immobilise enzymes on surfaces. The photoswitch formed in the CuAAC reaction has a high photostationary state (95% \(cis\)) and high thermal stability of the \(cis\) form. Upon attachment of the enzyme, a stable immobilised is formed, and, although not yet in a reversible manner, its activity can be changed by white light irradiation to a significant extent. The present results open up new approaches for biosensor construction, especially in analytical applications\(^2\) where the duration of enzymatic reaction is crucial and it has to be terminated in a manner that is mild and orthogonal to other elements of the system.

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