Selective aerobic oxidation reactions via combination of photocatalytic water oxidation and enzymatic oxyfunctionalisations

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Abstract

Peroxygenases offer versatile means to address challenges in selective oxyfunctionalisation chemistry. Despite their attractiveness, the application of peroxygenases in synthetic chemistry remains challenging due to their facile inactivation by the stoichiometric oxidant (H_2O_2). To address this, we show that visible light-driven, catalytic water oxidation can be used for *in situ* generation of H_2O_2 to promote selective oxyfunctionalisation reactions catalysed by peroxygenases.

Introduction

Selective oxyfunctionalisation of carbon-hydrogen bonds still represents a dream reaction in organic synthesis.[1, 2, 3, 4, 5, 6, 7] Particularly, balancing reactivity of the oxygen-transfer reagent with selectivity is largely unsolved for (in)organic catalysts while it is an inherent feature of many oxidative enzyme such as heme-dependent monooxygenases and peroxygenases. The relevance of peroxygenases (UPO for *unspecific* peroxygenase, E.C. 1.11.2.1) for selective oxyfunctionalisation reactions in preparative organic synthesis is increasing rapidly.[8, 9, 10] Particularly the 'novel' peroxygenases from *Agrocybe aegerita* (*Aae*UPO),[11, 12, 13, 14] *Marasmius rotula* (*Mro*UPO)[15, 16] or *Coprinopsis cinerea* (*Cci*UPO)[17, 18] excel in terms of substrate scope and specific activity over the well-known chloroperoxidase from *Caldariomyces fumago* (*Cfu*UPO),[19] P450 monooxygenases and chemical counterparts. The very high turnover numbers (TONs) reported so far give reason to expect truly preparative-scale applications of these very promising biocatalysts. Additionally, crystal structures of *Aae*UPO[20] as well as directed evolution protocols[21, 22, 23] together with efficient recombinant expression systems have

been established in the past few years. Hence, current gaps in substrate scope, stability and/or selectivity will be closed.[4, 24, 25, 26, 27, 28, 29, 30, 31]

35   In contrast to P450 monooxygenases, peroxygenases do not rely on complicated and susceptible electron transport chains delivering reducing equivalents to the heme active site needed for reductive activation of molecular oxygen and therefore are not subject to the *Oxygen Dilemma*.[32] Rather, peroxygenases utilise H_2O_2 directly to regenerate the catalytically active oxyferyl heme species. At the same time, however, peroxygenases suffer (like all heme-dependent enzymes) from a pronounced instability against H_2O_2 making controlled *in situ*

40   provision with H_2O_2 inevitable. Today, the well-known glucose/glucose oxidase system to generate H_2O_2 from O_2 prevails on lab-scale; but shows little potential for larger, preparative applications due to its poor atom-efficiency.[33] More efficient electron donors such as small alcohols or electrochemical sources have recently been proposed.[34, 35, 36]

Ideally, water could serve as co-substrate and electron donor for *in situ* generation of H_2O_2. Peroxygenase

45   reactions are generally conducted in aqueous media ($c(H_2O)$ = 55 mol L^{-1}) and the sole by-product of the water oxidation reaction is molecular oxygen. A broad variety of heterogeneous water oxidation catalysts (WOCs) have been reported in recent years that could be used for the partial oxidation of water to hydrogen peroxide.[37, 38, 39, 40, 41, 42, 43, 44, 45, 46] The thermodynamic driving force for this reaction is derived from (visible) light. Mostly, this approach is evaluated with respect to catalytic water splitting into H_2 and O_2. However, under

50   aerobic conditions, electrons liberated from water can also be transferred to O_2 yielding H_2O_2. Also, incomplete oxidation of water to H_2O_2 can be conceived. This motivated us to evaluate catalytic, photochemical water oxidation to promote peroxygenase-catalysed, selective oxyfunctionalisation reactions (Scheme 1). For the proof-of-concept experiments Au-doped TiO_2 appeared promising due to its favourable redox potentials of the valence (+2.7 V) and conducting band (-0.5 V) making water oxidation ($E^{0'}$ = 2.4 V) and O_2-reduction ($E^{0'}$ = -0.16

55   V) feasible at ambient conditions (i.e. neutral pH) (Scheme 1).
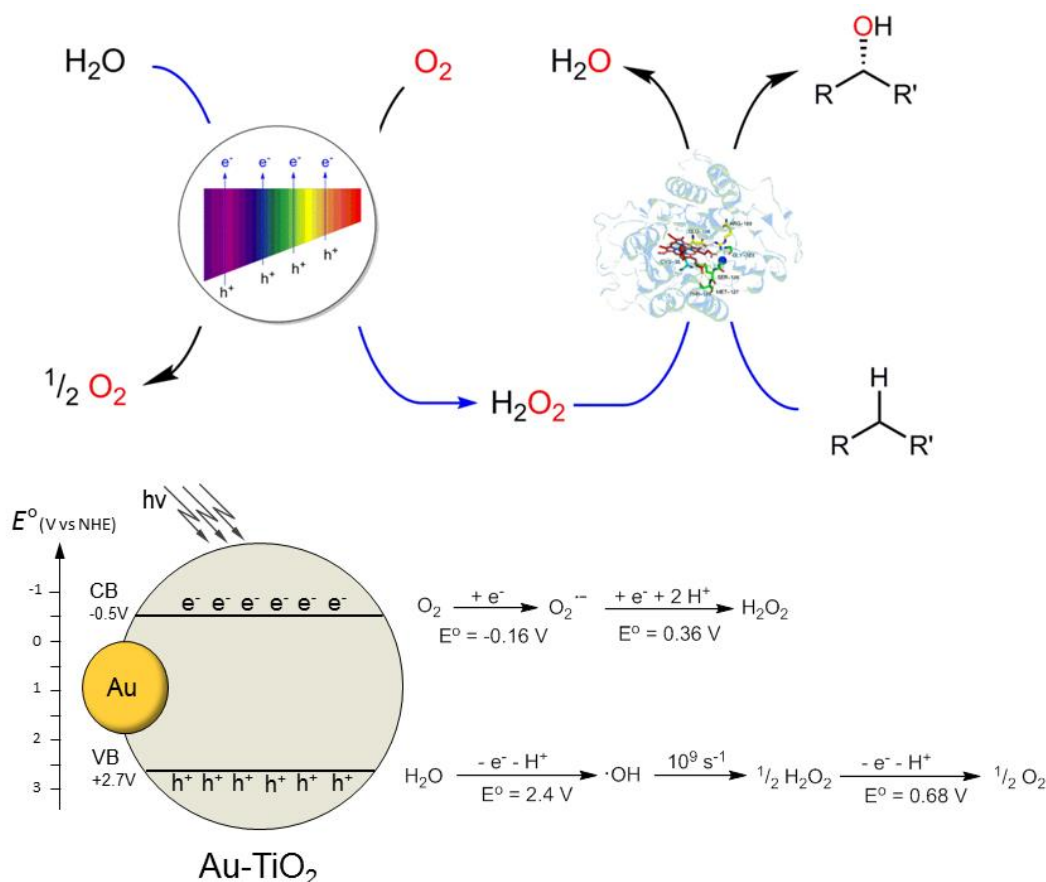
Scheme 1. Upper: Light-driven water oxidation for the *in situ* generation of H_2O_2 to promote peroxygenase-catalysed selective hydroxylation of hydrocarbons. The electron flow is shown in blue, for clarity reasons protons are omitted from the scheme, the overall reaction is pH-neutral. Lower: band energy values for TiO_2[47] and elementary redox steps occurring at the TiO_2-surface. The Au-nanoparticles modulate the absorption properties (enhancing the activity with visible light).

Results and Discussion

As model enzyme for our studies we chose an evolved expression UPO mutant from *Agrocybe aegerita*, which was recombinantly overproduced in *Pichia pastoris* (r*Aae*UPO) following a previously reported protocol.[22] The enzyme was purified to near homogeneity by a single anion exchange chromatography step (Fig S1). The enzyme preparation used herein exhibited a Reinheitszahl (Rz: A_{420}/A_{280}) value of 1.6. As model reaction we chose the stereoselective hydroxylation of ethyl benzene to (*R*)-1-phenyl ethanol as model reaction (Figure 1). Visible-light active Au-doped TiO_2 was used as photocatalysts for the proof-of-concept experiments.[48, 49]

Under arbitrarily chosen reaction conditions (Figure 1) we were pleased to observe significant accumulation of (*R*)-1-phenyl ethanol. Control reactions in the absence of the Au-TiO_2 photocatalyst or in the darkness yielded no product formation. Experiments in the absence of either the enzyme or using thermally inactivated enzyme resulted in a slow accumulation of racemic 1-phenyl ethanol (less than 0.14 mM within 24 h) and approximately the same concentrations of acetophenone. This minor background oxidation activity of the photocatalyst also explains the slightly decreased optical purity of the (*R*)-1-phenyl ethanol obtained from the

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75   photobiocatalytic oxidation reactions (90% ee) as compared to traditional reaction schemes for the provision of rAaeUPO with H_2O_2 (> 97% ee).[50]

        Particular attention was paid to the nature of the electron donor for this reaction as in principle also other reaction components may be susceptible to TiO_2 oxidation and thereby serve as sacrificial electron donor for the reduction of O_2. For this, the enzyme preparation contained phosphate only as buffer component to

80   exclude possible contributions of other sacrificial hole scavengers to H_2O_2 generation. Also experiments using immobilised enzymes were conducted to exclude rAaeUPO oxidation to promote H_2O_2 generation. To further support the assumed water oxidation-based mechanism, we performed a range of experiments using ^{18}O labelled water as reaction mixture. The occurrence of ^{18}O-labelled (R)-1-phenylethanol (Figure S16) supports the proposed mechanism. Performing this experiment in the presence of ambient air (predominantly consisting

85   of $^{16}O_2$) resulted in a minor incorporation of ^{18}O into the product, which predominantly contained ^{16}O. Using deaerated reaction mixtures (wherein only water oxidation can account for O_2) the ^{18}O-labelled product dominated. These findings strongly support the suggested TiO_2-mediated oxidation of H_2O to O_2 coupled to TiO_2-catalysed reduction of O_2 to H_2O_2 finally being used by rAaeUPO for specific incorporation into ethyl benzene. Also, a contribution of H_2O_2 originating from direct two-electron water oxidation (Scheme 1) is

90   possible.[51] In any case, the above-mentioned results make us confident that water served indeed as sole source of reducing equivalents to promote the selective rAaeUPO-catalysed oxyfunctionalisation reactions.

        Next, we advanced to characterise the reaction system in more detail, particularly investigating the effect of varying catalyst concentrations on the reaction system. It is worth mentioning here that, though the product concentrations shown in Figure 1 may appear low but significantly surpass the concentrations of H_2O_2

95   obtained from water oxidation reported so far for Au-TiO_2 and other WOCs.[38, 44, 52] We attribute this to a H_2O_2-oxidation activity of the illuminated WOCs (Fig S17) eventually leading to a low steady-state concentration of H_2O_2.[53] At first sight, this may appear as a limitation for the current system, but it also enables us to maintain low, constant in situ concentrations of H_2O_2 as required for efficient and robust peroxygenase catalysis.

        Interestingly, the concentration of the WOC had only a minor influence on the initial rate of the

100   reaction (Figure 1 A). We attribute this to WOC-concentration-independent in situ H_2O_2 concentrations, most probably due to the simultaneous water- and H_2O_2-oxidation activity of the WOCs mentioned above. The WOC concentration, however, had a very significant influence on the robustness of the overall reaction. In general, no more product accumulation was observable after approx. 6 h. Varying the Au content (0.6-1.8 wt%) and particle size (2.8-7.9 nm) on the TiO_2 surface hardly influenced the time course of the photobiocatalytic

105   hydroxylation reaction with the exception of plain TiO_2. Here, the overall rate was approximately half of the rates obtained with Au-TiO_2 (Figure S9).
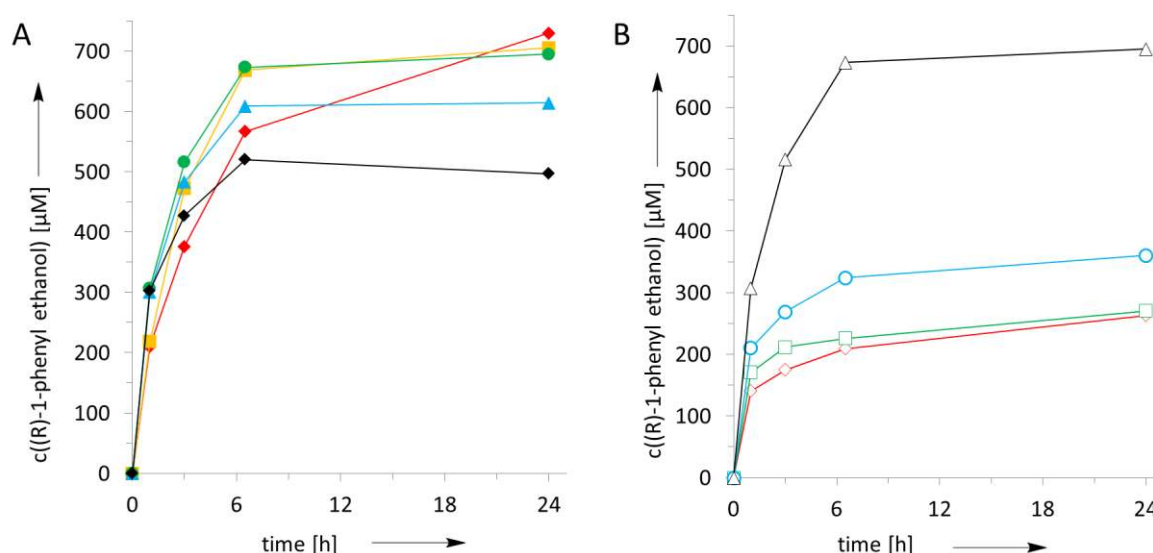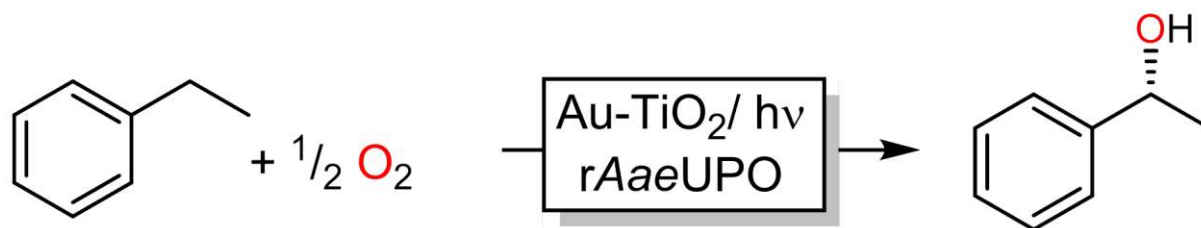
Figure 1. Time courses of the photochemoenzymatic hydroxylation of ethyl benzene with varying photocatalyst (A) and enzyme (B) concentrations. General conditions: reactions were performed in 60 mM phosphate buffer (pH 7.0) under visible light illumination ($\lambda > 400$ nm), T= 30 °C, c(ethyl benzene) = 15 mM. A: c(rAaeUPO) = 350 nM, c(Au-TiO$_2$) = 1 gL^{-1} (◆), 2.5 gL^{-1} (■), 5 gL^{-1} (●), 10 gL^{-1} (▲), 15 gL^{-1} (◆); B: c(Au-TiO$_2$) = 5 gL^{-1}, c(rAaeUPO) = 10 nM (◇), 50 nM (□), 150 nM (○), 300 nM (△).

In contrast, the enzyme concentration directly influenced the overall reaction rate (Figure 1 B) and a linear dependency of the initial (R)-1-phenyl ethanol accumulation on the concentration of rAaeUPO applied was observed. However, again, the reactions ceased after 6-7 h.

Apparently, the robustness of the overall reaction (as judged from the accumulation of (R)-1-phenyl ethanol) correlated with the ratio of photo- and bio-catalyst. We hypothesised that rAaeUPO may be inactivated by the Au-TiO$_2$-WOC. It should be mentioned here, that in the experiments reported so far, only TiO$_2$ mostly composed of anatase (91.1%) had been used as WOC. Given the rather hydrophilic surface of anatase TiO$_2$, adsorption of the glycoprotein rAaeUPO (with a glycosylation degree of 30 %)[22] appears likely. Therefore, we performed a range of control experiments to shed light on the inactivation of the biocatalyst: incubation of the enzyme with the photocatalyst in the darkness resulted in a minor reduction of its catalytic activity as compared to the same experiment in the presence of light (Figure 2). Therefore, we conclude that adsorption of rAaeUPO to the photocatalyst *per se* does not lead to inactivation of the enzyme.
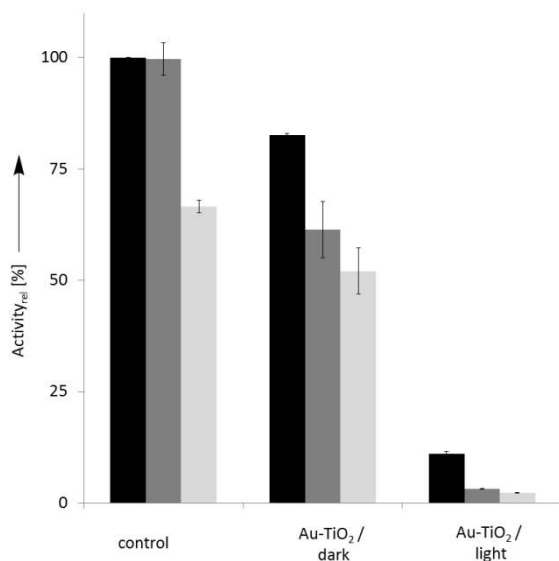
Figure 2. Residual r*Aae*UPO activity after incubation in the presence of the photocatalyst for 1 (black), 5 (dark grey) and 24 h (light grey). General conditions: phosphate buffer (60 mM, pH 7.0), T= 30 $^{\circ}$C, c(anatase Au-TiO$_2$) = 0 (control, under illumination) or 10 gL^{-1}, c(r*Aae*UPO) = 150 nM. The samples were either kept in the darkness or illuminated under visible light illumination (λ > 400 nm). At intervals, samples were withdrawn from the incubation mixtures and analysed for peroxygenase activity.

We hypothesised that reactive oxygen species (ROS) generated at the surface of the water oxidation catalyst[54] may account for this observation via oxidative inactivation of the enzyme. In fact, using the spin trap technique in electron paramagnetic resonance spectroscopy, significant amounts of mainly hydroxyl (HO•) radicals (a_H = 1.495 mT; g = 2.0050) could be detected in illuminated anatase Au-TiO$_2$ samples (Figure 3 A).[54] These hydroxyl radicals may originate from water oxidation (Scheme 1), from the reaction of superoxide (•O$_2^-$, from O$_2$ reduction) or other steps in the complex redox chemistry of ROS.[55, 56] Though more detailed mechanistic studies will be necessary to fully understand this inactivation mechanism, we hypothesise a major role of the hydroxyl radical over the superoxide radical. First, addition of superoxide dismutase did not improve the robustness of the overall reaction. Second, •O$_2^-$ should react with native peroxygenase leading to the formation of the so-called Compound III of the catalytic cycle, for which we have not found any spectroscopic evidence (no characteristic absorption peak at 625 nm, Figure S10).[57]
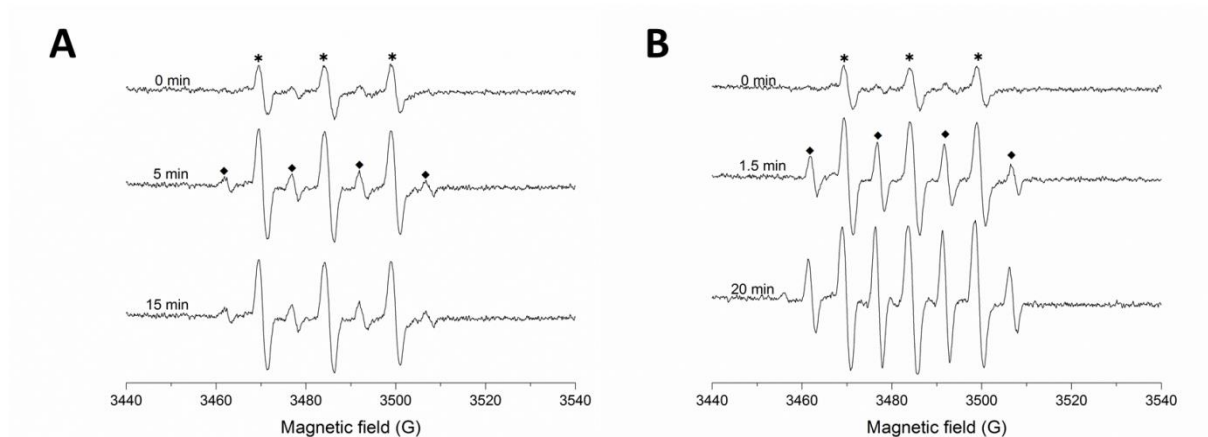
Figure 3. Electron paramagnetic resonance (EPR) spectra recorded during the illumination of anatase (A) and rutile (B) Au-TiO$_2$ in water. Signals marked with asterisk belong to the existing oxidation product of DMPO, 5,5-dimethyl-2-oxopyrroline-1-oxyl (DMPOX);[58] signals marked with solid diamonds belong to the spin-adduct •DMPO-OH, which is not overlapping the signals of DMPOX and therefore provides sufficient quality for analysis. Reaction condition: Au-TiO$_2$ = 5.0 mg mL^{-1}, c(DMPO) = 30 mM, RT, hν > 400 nm. DMPO = 5,5-Dimethyl-1-pyrroline *N*-oxide.

Given the rather short half-life time of hydroxyl radicals (approx. 10^{-9} s in aqueous media) we envisioned that simple spatial separation of the WOC (at which's surface the HO• radicals are being formed) and the biocatalyst may circumvent this limitation. Therefore, we evaluated (1) spatial separation of anatase Au-TiO$_2$ from r*Aae*UPO using immobilised enzymes and (2) avoiding r*Aae*UPO adsorption to the WOC surface by using hydrophobic surfaces.

To achieve physical separation of the WOC and r*Aae*UPO, we covalently immobilised the latter to a poly(methyl methacrylate) resin activated by glutardialdehyde. Covalent linkage to the spacer unit occurred through imine formation with surface-exposed lysine residues (see SI for more detailed information). To test the second option, i.e. avoidance of enzyme adsorption by less hydrophilic WOC surfaces, rutile Au-TiO$_2$ was evaluated. Rutile exhibits a far more hydrophobic surface as compared to the previously used anatase catalyst. This is corroborated also by the lack of the characteristic IR-absorptions of surface-bound H$_2$O and Ti-OH at 3422 and 1632 cm^{-1}, respectively (Figure S19). This leads to the assumption that the heavily glycosylated r*Aae*UPO may be less prone to adsorption to rutile than to anatase surfaces. Hence, while the photoelectrochemical properties (i.e. the redox potential and energy levels of conducting- and valence band)[59] of both crystal phases are comparable, rutile should be preferable due to its expected lower adsorption tendency for proteins. Indeed, r*Aae*UPO adsorbed approximately 10 times less to rutile as compared to anatase catalyst (Figures S11 & S 12). Furthermore, this effect does not appear to be limited to glycoproteins such as r*Aae*UPO as also a bacterial enzyme (the old yellow enzyme homologue from *Bacillus subtilis*, YqjM)[60] showed a similar adsorption behaviour as r*Aae*UPO (Figure S13). Overall, both strategies appeared to be suitable to minimise oxidative inactivation of r*Aae*UPO at the photocatalyst surface and therefore should lead to more

robust photobiocatalytic hydroxylation reactions. Figure 4 compares the time courses of these catalytic systems.

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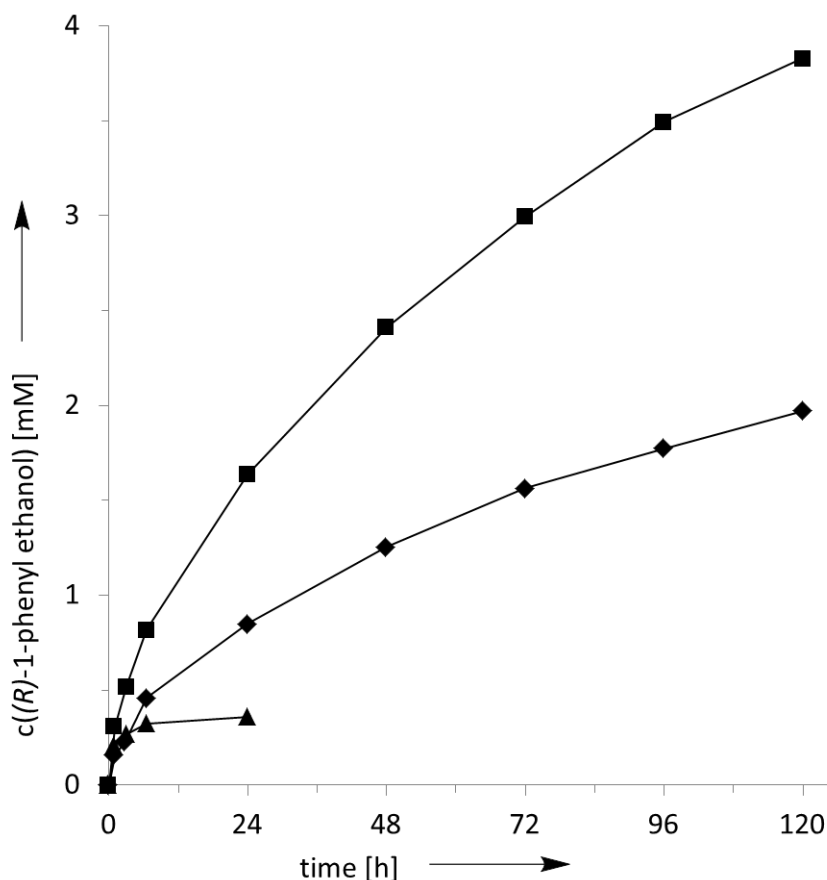
Figure 4. Time courses of the photobiocatalytic hydroxylations of ethyl benzene using the original reaction setup with dissolved r*Aae*UPO and anatase-Au-TiO$_2$ (▲) and systems with reduced interaction between r*Aae*UPO and the Au-TiO$_2$ by using immobilised enzyme (◆) or by using hydrophobic rutile-Au-TiO$_2$ (■). General conditions: c(r*Aae*UPO) = 150 nM (dissolved) 120 nM (immobilised), c(Au-TiO$_2$) =5 g L^{-1}, c(ethyl benzene)$_0$ = 15 mM ethyl benzene in 60 mM phosphate buffer
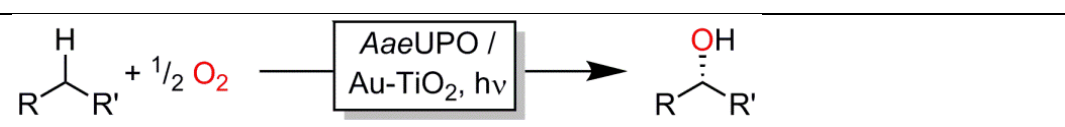180   (pH 7.0) under visible light illumination (λ > 400 nm).

In both cases steady product accumulation was observed for at least 120 h thereby representing a more than 20-fold increase of the robustness as compared to the starting conditions (Figure 4 ▲). Consequently, also the turnover number of the enzyme increased f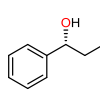rom approx. 2000 using dissolved enzyme and anatase-Au-TiO$_2$ to more than 16000 or 21000 using immobilised r*Aae*UPO (Figure 4 ◆) or rutile-Au-TiO$_2$
185   (Figure 4 ■), respectively. The latter system also provided *(R)*-1-phenyl ethanol in much higher optical purity (>98% ee) as compared to the starting conditions. The reaction using immobilised r*Aae*UPO was considerably slower than the reaction using free r*Aae*UPO and rutile-Au-TiO$_2$. This may, at least to some extent, be attributed to diffusion limitations originating from the double heterogeneous character of the catalysts. Also, partial loss of enzyme activity as a consequence of the immobilisation may contribute to this.[61, 62] Systematic
190   immobilisation studies with r*Aae*UPO are currently ongoing to clarify this.
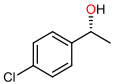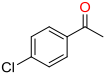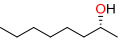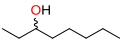
The average turnover frequency of r*Aae*UPO of 2.9 min^{-1} (average over 4 days) indicates that there is room for improving the efficiency of this reaction system. Indeed, increasing the rutile-Au-TiO$_2$ concentration linearly increased the initial rate of the overall reaction (Figure S20). Surprisingly, an EPR investigation of the rutile-Au-TiO$_2$ catalysed water oxidation (Figure 3 B) revealed that this catalyst generates significantly higher amounts of •HO radicals than anatase-Au-TiO$_2$. In fact, as already stated, a higher amount of superoxide may be formed by rutile Au-TiO$_2$. At first sight this is in contrast to the higher compatibility of rutile-Au-TiO$_2$ with the enzymes investigated. It may, however, be rationalised by the poor adsorption tendency of proteins to the rutile-TiO$_2$ surface and the very short half life time of the hydroxyl radical resulting in very short diffusion distances.[63, 64]

Encouraged by these promising results, we further explored the product scope of the photochemoenzymatic hydroxylation reaction using dissolved r*Aae*UPO and rutile-Au-TiO$_2$. As shown in Table 1, a broad range of aliphatic and aromatic compounds was converted into the corresponding alcohols. The enantioselectivities and relative activities corresponded to the values reported previously indicating that the natural reactivity and selectivity of the enzyme were not impaired.[65, 66] Similar results were also observed in the system utilising anatase-Au-TiO$_2$ and immobilised enzyme (Table S2). It is worth mentioning here that the current reactor setup chosen for these experiments suffers from severe issues in mass balance of the reagents due to their volatility. Hence, many examples from Table 1, though exhibiting seemingly low yields actually represent full conversion of all starting material. Reaction setups avoiding direct contact of the reaction mixtures with ambient atmosphere will minimise this loss of reagents due to evaporation. Also semipreparative-scale reactions proofed to be feasible with this setup (Figures S39-S41). Hence, approx. 110 mg of highly enantioenriched (ee = 97.4%, 31% isolated yield) (*R*)-1-phenyl ethanol was produced.

Table 1. Substrate scope of the photobiocatalytic hydroxylation reaction.[a]

Entry [a]	product	mM[b]	ee [%][b]	*Other products*	mM[b]	Yield, %[b]	TON, 10^{3}[b]
1		4.1	/		0.5	45.2	30.1
2		4.2	/		0.1	43.1	28.7
3		2.6	/		0.1	26.7	17.8
4		2.3	>99		0.5	28.2	18.8

5		3.6	95.2		1.0	45.8	30.5
6		5.0	75.0		0.8	58.2	38.8
7		0.3	78.5		0.2	4.8	3.2

[a] Conditions: $c(\text{substrate})_0$ = 10.0 mM, c(rutile Au-TiO$_2$) = 10 g L^{-1}, c(r*Aae*UPO) = 150 nM (dissolved), in phosphate buffer (pH 7.0, 60 mM), T = 30 °C, 70 h, visible light illumination (λ > 400 nm). n.d. = not determined. [b] based on the concentration of both products.

The regioselectivity of all reactions was very high except for entry 7 where ω-2 and ω-3 hydroxylation products were observed. This observation is in line with previous reports on *Aae*UPO-selectivity towards linear alkanes.[67]

Generally, the only by-product observed was the 'overoxidation' product, i.e. the corresponding ketone. We suspected WOC-catalysed further oxidation of the primary r*Aae*UPO-product (*(R)*-1-phenyl ethanol) to account for this. Indeed, the concentration of acetophenone linearly increased with increasing concentrations of Au-TiO$_2$ (Table S3). Hence, the overoxidation degree can be controlled by adjusting the concentration of the photocatalyst.

The dual activity of the photocatalyst (water- and alcohol oxidation) motivated us to evaluate more elaborate photochemoenzymatic cascades to extend the product scope beyond (chiral) alcohols. In particular, we coupled the photochemoenzymatic oxidation of toluene to benzaldehyde to an enzymatic benzoin condensation using the benzaldehyde lyase from *Pseudomonas fluorescens* (*Pf*BAL) (Scheme 2 A).[68, 69] Also, acetophenone, formed by the photoenzymatic oxyfunctionalisation of ethyl benzene was submitted to a reductive amination using the ω-transaminases from *Aspergillus terreus* (*R*-selective, *At*-ω-TA) and *Bacillus megaterium* (*S*-selective, *Bm*-ω-TA) (Scheme 2 B). [70, 71] Both cascades were performed in a one-pot-two-step fashion, i.e. the photochemoenzymatic oxidation to the corresponding aldehyde or ketone was performed first, followed by addition of the biocatalysts needed for the second transformation (details can be found in SI). Recently, Flitsch and coworkers reported a similar transformation (ethyl benzene to enantiomerially pure (*R*)- or (*S*)-1-phenyl ethyl amine) attaining very similar product titers.[72] It is worth mentioning that also a one-pot-one-step procedure was possible in case of cascade B, albeit at somewhat lower product yields (0.7 mM, 37 % ee and 0.5 mM, 99 % ee for (*R*)- and (*S*)-1-phenyl ethyl amine, respectively).

A

B
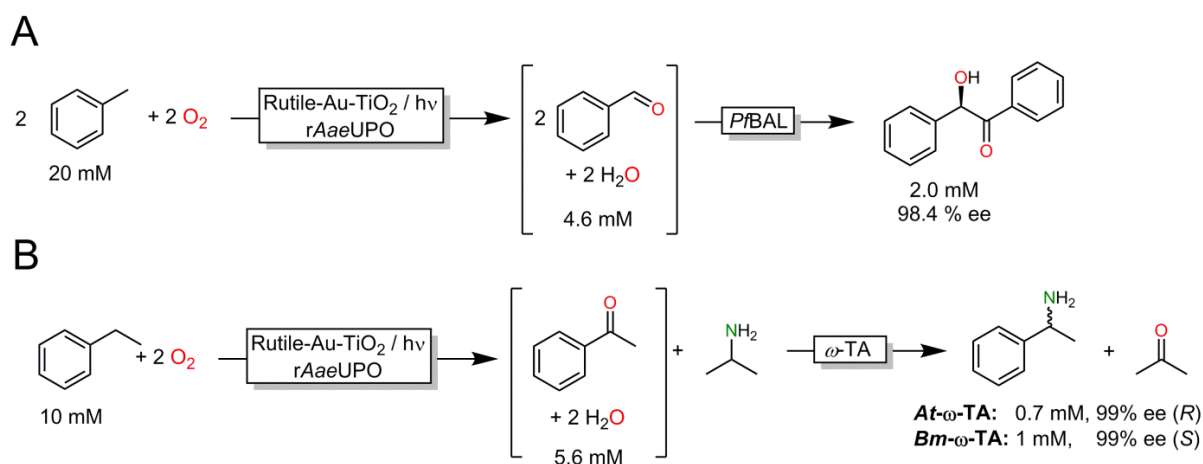
Scheme 2. Photochemoenzymatic cascade reaction for the transformation of toluene to (*R*)-benzoin (**A**) and ethyl benzene to (*R*)- or (*S*)-1-phenyl ethyl amine (**B**). Conditions: cascade A: c(toluene) = 20.0 mM, c(rutile Au-TiO$_2$) = 30 g L^{-1}, c(r*Aae*UPO) = 150 nM in phosphate buffer (pH 7.0, 60 mM), T = 30 °C, 96 h, visible light illumination (λ > 400 nm). In the second step, 100 μL of mixture in phosphate buffer (500 mM, pH 8.5) containing 5 mM of thiaminpyrophosphate (TPP), 25 mM of MgCl$_2$ and 10 mg of crude cell extract containing *Pf*BAL were added. Cascade B: c(ethyl benzene) = 10.0 mM, c(rutile Au-TiO$_2$) = 30 g L^{-1}, c(r*Aae*UPO) = 150 nM in phosphate buffer (pH 7.0, 60 mM), T = 30 °C, 96 h. In the second step, 105 μL of isopropylamine, 130 μL of phosphoric acid (5 M), 100 μL of pyridoxal phosphate (PLP, 10 mM) and 10 mg of crude cell extract containing ω-transaminase were added. The pH of above mixture was adjusted to approx. 9.0. The dilution factor of the reaction system was 1.0/1.335 = 0.75. After the first steps under illumination and initiation of the second steps the resulting reaction mixture of both cascades was shaken at 30 °C for 40 h in the darkness

These results demonstrate that the proposed photochemoenzymatic cascades enable synthesis of a broader range of value-added products (chiral alcohols, amines and acyloins) from simple starting materials. While these reactions undoubtedly still need further improvement to reach preparative feasibility, they nevertheless demonstrate the principal feasibility and broad product scope of the envisioned photochemoenzymatic cascade reactions.

The proposed *in situ* H$_2$O$_2$ generation system can also be applied to other peroxidases such as the V-dependent haloperoxidase from *Curvularia inaequalis* (*Ci*VCPO).[73, 74, 75, 76] Gratifyingly, the *Ci*VCPO-catalysed halogenation of thymol proceeded smoothly yielding 2- and 4-bromothymol with more than 70 % conversion (Scheme 3). The product distribution was comparable to previous haloperoxidase-catalysed halogenation reactions.[77, 78, 79, 80, 81] In the absence of either *Ci*VCPO, rutile-Au-TiO$_2$ or light, no conversion of thymol was observed. It is also worth mentioning that again rutile-Au-TiO$_2$ gave better results than anatse-Au-TiO$_2$ with this enzyme under otherwise identical conditions.
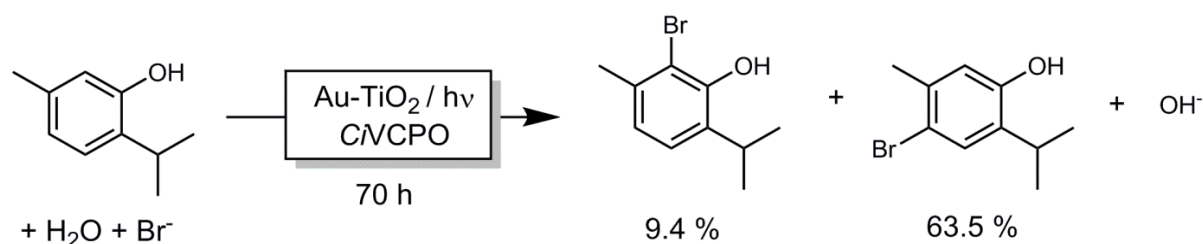
Scheme 3. Photochemoenzymatic halogenation of thymol. Conditions: c(rutile Au-TiO$_2$) = 5 gL^{-1}, c(*Ci*VCPO) = 150 nM, c(thymol) = 3 mM, c(KBr) = 6 mM, c(Na$_3$VO$_4$) = 50 μM in 1.0 mL citrate buffer (50 mM, pH 5.0) T = 30 °C, t = 70 h. The reaction mixture was irradiated by visible light (λ > 400 nm).

So far, we have focussed on TiO_2-based photocatalysts. Photocatalysis, however, is an extremely dynamic area of research and novel, potentially useful, WOCs are reported on an almost weekly basis. Therefore, we finally evaluated the scope of different WOCs for the in situ generation of H_2O_2 to promote peroxygenase-catalysed hydroxylation reactions. Amongst them visible light-active Au-BiVO$_4$[38] and g-C$_3$N$_4$[82] showed some promising characteristics (Figure S43). The product formation with Au-BiVO$_4$ as photocatalyst was rather modest. g-C$_3$N$_4$ exhibited a higher product formation rate together with a pronounced 'overoxidation activity' (approx. 10 times higher than Au-TiO$_2$ under comparable conditions).[83] Therefore, the latter catalyst may be particularly suitable for further photobiocatalytic cascades.

Finally, recently described carbon nano dot (CND) photocatalysts caught our attention as easy-to-prepare and biocompatible photocatalysts.[84, 85, 86] As CND-mediated reduction of molecular oxygen to H_2O_2 is impaired[84] we used riboflavin monophosphate (flavin mononucleotide, FMN) as co-catalyst for the generation of H_2O_2 (Figure 5). Visible light illumination of a mixture of CND and FMN in deaerated phosphate buffer resulted in fast and complete reduction of FMN as judged by the decrease of the characteristic absorption band of FMN^{Ox} at 450 nm (Figure 5 A). Exposure to ambient atmosphere resulted in complete restoration of this absorbance indicating aerobic reoxidation of FMN^{Red} yielding H_2O_2.
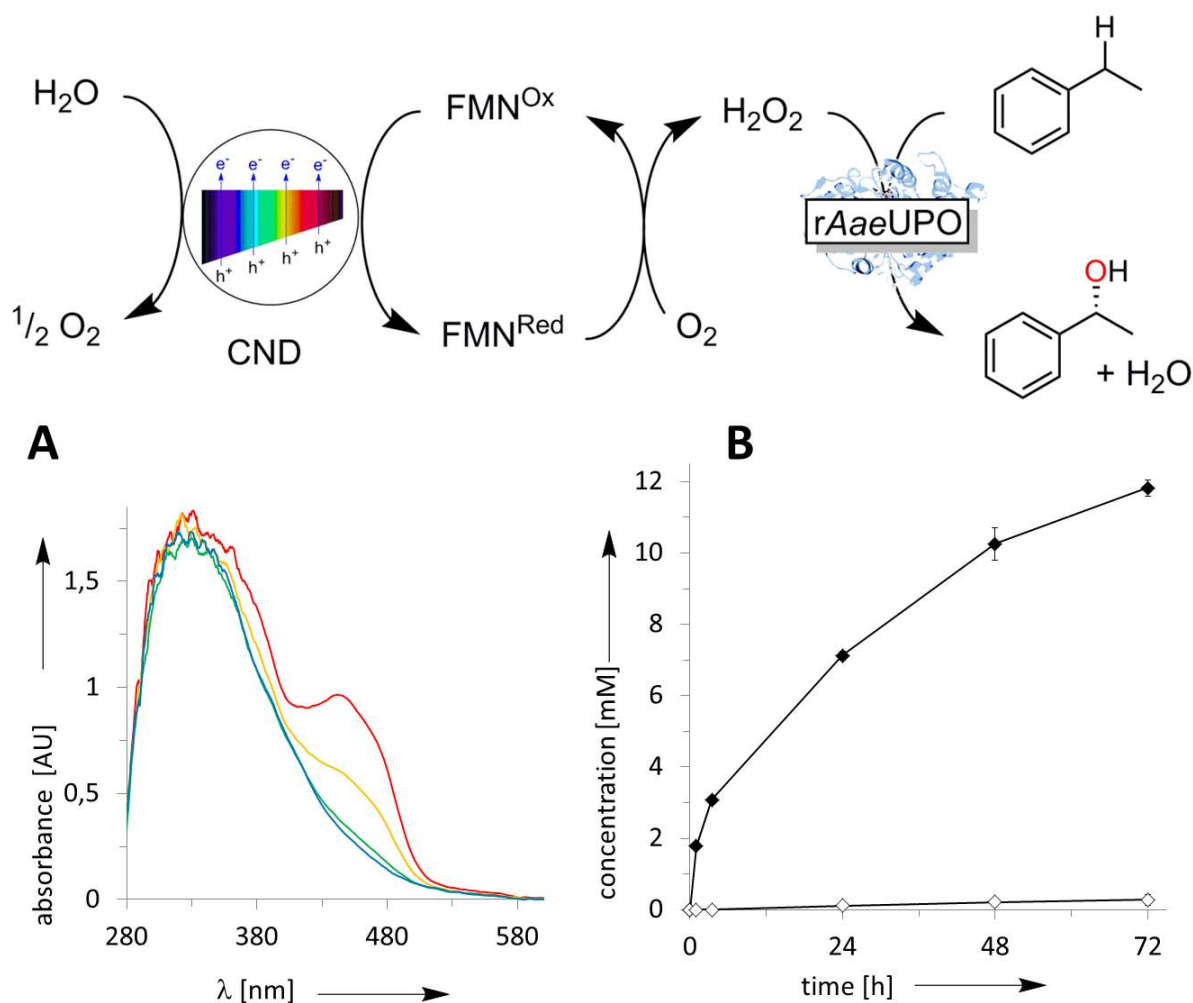
Next, we tested the photocatalytic reduction of FMN and its aerobic, H_2O_2-forming reoxidation to promote r*Aae*UPO-catalysed hydroxylation. Experiments in the absence of either CND or FMN gave no significant product formation whereas the whole system produced enantiomerically pure *(R)*-1-phenyl ethanol (98 % ee) (Figure 5 B). Compared to the previously used Au-TiO_2 the overall reaction rates were significantly higher as compared by the initial rates of 0.16 mM h^{-1} and 0.81 mM h^{-1} for Au-TiO_2 and CND, respectively. Hence, already under non-optimised conditions, almost 100000 turnovers for r*Aae*UPO and more than 100 for FMN were estimated. Similar results were achieved under the same conditions for the hydroxylation of cyclohexane (Figure S44). It is also worth noting that the overoxidation rate was reduced significantly.

Overall, we have combined photochemical water oxidation-catalysis with peroxygenase-catalysis to achieve visible light-driven, aerobic oxidation of hydrocarbons. Combined with further (enzymatic) reaction

steps this method gives access to a broad range of functionalised building blocks starting from simple alkanes. Admittedly, the system reported here falls short in terms of space-time-yields to be economical or environmentally benign.[87, 88] But the catalytic turnover achieved for the biocatalyst compares well with the
305 state-of-the-art in peroxygenase reactions and surpasses the performance of the established P450 monooxygenases and chemical catalysts (Table S6). Further improvements may be expected in the near future from optimised reaction schemes, particularly from more active WOCs.

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