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ARTICLE

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A tailor-made, self-sufficient and recyclable monooxygenase catalyst based on coimmobilized cytochrome P450 BM3 and glucose dehydrogenase

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Abstract

Cytochrome P450 monooxygenases (P450s) promote hydroxylations in a broad variety of substrates. Their prowess in C–H bond functionalization renders P450s promising catalysts for organic synthesis. However, operating P450 reactions involve complex management of the main substrates, O_2 and nicotinamide adenine dinucleotide phosphate (NAD(P)H) reducing equivalents against an overall background of low operational stability. Whole-cell biocatalysis, although often used, offers no general solution to these problems. Herein, we present the design of a tailor-made, self-sufficient, operationally stabilized and recyclable P450 catalyst on porous solid support. Using enzymes as fusion proteins with the polycationic binding module Z_{basic2}, the P450s BM3 (from *Bacillus megaterium*) was coimmobilized with glucose dehydrogenase (type IV; from *B. megaterium*) on anionic sulfopropyl-activated carrier (ReliSorb SP). Immobilization via Z_{basic2} enabled each enzyme to be loaded in controllable amount, thus maximizing the relative portion of the rate limiting P450 BM3 (up to 19.5 $U/g_{carrier}$) in total enzyme immobilized. Using lauric acid as a representative P450 substrate that is poorly accessible to whole-cell catalysts, we demonstrate complete hydroxylation at low catalyst loading (≤0.1 mol%) and efficient electron coupling (74%), inside of the catalyst particle, to the regeneration of NADPH from glucose (27 cycles) was achieved. The immobilized P450 BM3 showed a total turnover number of ~18,000, thus allowing active catalyst to be recycled up to 20 times. This study therefore supports the idea of practical heterogeneous catalysis by P450s systems immobilized on solid support.

KEYWORDS
cytochrome P450, fatty acids, glucose dehydrogenase, heterogeneous biocatalysis, immobilization

1 | INTRODUCTION

Cytochrome P450 monooxygenases (P450s) are heme-enzymes with important applications in biocatalysis (Dong et al., 2018; Hollmann, Arends, Buehler, Schallmey, & Bühler, 2011). P450s have good reputation for promoting chemically most challenging reactions. Hydroxylation, via oxygen insertion from O_2 into a nonactivated C–H bond of the substrate, is a prominent type of P450 transformation of high synthetic interest (Hollmann et al., 2011; Urlacher & Girhard, 2012; Wei, Ang, & Zhao, 2018). P450 BM3 from *Bacillus megaterium* represents the prototype of a so-called "self-sufficient" P450. It comprises, within a single polypeptide chain, the monooxygenase and reductase domains required for the complete catalytic cycle of the enzyme. P450 BM3 hydroxylates a broad variety of substrates with

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high activity using NADPH as electron source and O_2 (air) as oxidant (Whitehouse, Bell, & Wong, 2012).

Despite their perceived potential for synthetic use, significant limitations on the applicability of P450s arise, especially at larger scale (Kaluzna et al., 2016; Lundemo & Woodley, 2015; O'Reilly, Köhler, Flitsch, & Turner, 2011). Operating P450s involves complex management of the target substrate, O_2 and nicotinamide adenine dinucleotide NADH/NADPH reducing equivalents (Lundemo & Woodley, 2015; O'Reilly et al., 2011). In addition, low operational stability of the enzyme during conversion of nonnatural substrates is a challenge. Futile reaction cycles, referred to as uncoupling, wherein NAD(P)H is oxidized and O_2 reduced but no hydroxylated product formed, can be a problem, especially when nonnatural substrates are used (Meunier, de Visser, & Shaik, 2004; O'Reilly et al., 2011; Whitehouse et al., 2012).

Whole-cell catalysis is widely used to render P450 conversions practical (Kaluzna et al., 2016). However, limitations on applicability arise from low specific activity of the catalyst and for substrates (e.g., medium and long-chain fatty acids) poorly taken up by the cells via diffusion (Schrewe, Ladkau, Bühler, & Schmid, 2013; Ströhle, Kranen, Schrader, Maas, & Holtmann, 2016). To gain versatility, isolated enzymes could be used in principle, but operational stability issues almost naturally ensue. In general, when air bubbles are entrained into the liquid for O_2 supply and a second organic phase is present from partially dissolved hydrophobic substrates, interfacial inactivation becomes a prominent factor of enzyme stability (Bommarius & Paye, 2013; Dib & Nidetzky, 2008; Kaluzna et al., 2016; Urlacher & Girhard, 2012). At high substrate concentrations, NAD(P)H must be efficiently regenerated for use in catalytic amounts (Beyer et al., 2017; Kaluzna et al., 2016; Ströhle et al., 2016). Immobilization of monooxygenases on porous carriers is promising to develop robust catalysts able to complement the scope of whole cells for process chemistry applications of enzymatic hydroxylations. Generally, enzyme immobilization enables catalyst reuse and so supports continuous process development. It will also often increase enzyme stability (Garcia-Galan, Berenguer-Murcia, Fernandez-
Lafuente, & Rodrigues, 2011; Rodrigues, Ortiz, Berenguer-Murcia, Torres, & Fernández-Lafuente, 2013). In addition, immobilization can be designed to improve coenzyme regeneration (Velasco-Lozano, Benítez-Mateos, & López-Gallego, 2017). In the case of an immobilized monooxygenase system, therefore, substrate hydroxylation would occur from O_2 and relatively inexpensive electron sources (e.g., glucose) and involve an insoluble catalyst that offers facile reuse and product recovery.

Immobilization of P450s (including P450 BM3) was studied variously (Ducharme & Auclair, 2018). However, study focus was mostly on single enzyme immobilization without addressing coenzyme recycling (Lee et al., 2014; Tan et al., 2016; Wang, Rabe, Ahmed, & Niemeyer, 2015; Zernia et al., 2016). The immobilizate's operational performance and stability in synthetic transformations was typically not assessed. Enzyme total turnover numbers (TTNs) often were quite low (≤1,000) compared with TTNs of ~50,000 attainable with natural P450 systems (Dong et al., 2018; Ströhle et al., 2016). Only a few studies considered immobilization of P450 BM3 for development of a self-sufficient catalyst in the above-described sense, including NADPH regeneration. Sol–gel co-encapsulation of P450 BM3 and formate dehydrogenase was used. The resulting catalyst showed a TTN of 3,600 in batch conversion of 10-(4-nitrophenoxy)-decanoic acid (0.25 mM; 72%) and coenzyme could be recycled at least 14 times (Maurer, Schulze, Schmid, & Urlacher, 2003). Immobilized P450 BM3 was used in a peroxygenase reaction wherein H_2O_2 is the oxidant and NADPH therefore not needed (Weber et al., 2010). However, the peroxygenase reaction is not well developed for synthetic application with these enzymes. The commercialized Lentikat technology was applied to the immobilization of *Escherichia coli* cell extract containing P450 BM3. The specific activity of the immobilized monooxygenase was just 0.2 $U/g_{carrier}$ (Lentikats/ROBOX, 2018). The applicability of immobilized P450 BM3 systems to actual process conditions under enzyme reuse was not examined in detail. Therefore, a significant gap exists in P450 transformations of synthetic interest and well-characterized immobilized enzyme preparations suitable for their practical realization. Only to note, enzyme coimmobilization is a difficult problem and broadly applicable methods to its solution are not available. Multimeric enzymes require special attention in this context (Fernandez-Lafuente, 2009). Furthermore, it would be desirable to achieve enzyme purification and immobilization in one single step of processing.

In this study, we conceptualized a tailor-made design for P450 BM3 immobilization and applied it to the development of a self-sufficient, operationally stabilized and recyclable monooxygenase catalyst on porous solid support. The design should be adaptable readily to other self-sufficient P450 enzymes. It could thus present an immobilization platform amenable to diversification into a larger panel for off-the-shelf biocatalysts. Fusion to the binding module Z_{basic2} (small peptide of 7 kDa size; 58 amino acids; Graslund, Lundin, Uhlén, Nygren, & Hober, 2000) was used to create two chimeras of P450 BM3 and its NADPH-regenerating partner enzyme (glucose dehydrogenase IV from *B. megaterium*; *Bm*GDH), respectively. The two chimeras were conveniently coimmobilized via Z_{basic2}-mediated, noncovalent attachment to an anionic support. Due to clustered positive charges from multiple arginine residues on one of its sides, Z_{basic2} features a localized surface patch of high cation density. It is for this reason that Z_{basic2} can drive, and steer in respect to molecular orientation, the immobilization by cationic exchange with almost absolute selectivity, which omits additional purification steps before immobilization (Bolivar & Nidetzky, 2012; Wiesbauer, Bolivar, Mueller, Schiller, & Nidetzky, 2011). As we show for P450 BM3 and *Bm*GDH, this greatly benefits the coimmobilization of different enzymes directly from cell lysates through a general and simple one-step procedure. Catalyst optimization is enabled through convenient control over amount and relative proportion of the enzymes loaded on the carrier. It would be difficult to do a similar optimization in whole cells. We demonstrate application of the enzyme coimmobilizate as an off-the-shelf monooxygenase catalyst for hydroxylation of lauric acid as well as anisole.

2 | MATERIALS AND METHODS

2.1 | Materials

ReliSorb SP400/SS carrier was from Resindion (Milano, Italy). Diameter and pore size were specified by Resindion as 50–150 µm and 80–100 nm, respectively. Unless stated, chemicals were of highest purity obtainable from Sigma-Aldrich (Vienna, Austria) or Carl Roth (Karlsruhe, Germany). Bovine liver catalase (2000–5000 U/mg) was from Sigma-Aldrich (Steinheim, Germany).

2.2 | Enzymes

Gene cloning, protein expression, and purification are described in the Supporting Information Figure S1. Each enzyme was obtained in the expected subunit size, which including Z_{basic2} was 127.29 kDa for P450 BM3 and 37.67 kDa for *Bm*GDH (NCBI reference sequence: WP_013055759.1). The Z_{basic2} fusions are referred to henceforth as Z_P450 BM3 and Z_GDH.

2.3 | Enzyme immobilization

Reported procedure for immobilizing Z_{basic2} enzyme directly from cell extract was used (Bolivar & Nidetzky, 2012). Briefly, ReliSorb SP400 (100 mg) was incubated under slow stirring in loading buffer (50 mM potassium phosphate, pH 7.5, 250 mM NaCl). Cell extract containing Z_P450 BM3 (4 µM) or Z_GDH (60–90 U/ml), with total protein concentration between 18 and 24 mg/ml, was supplemented with NaCl (250 mM) and the pH was set to 7.5. The carriers (100 mg) were incubated with 1 ml cell extract in an end-over-end rotator (20 rpm) at 25°C for 1 hr. Samples from the supernatants were analyzed for active enzyme content (Z_P450 BM3; in µM) or enzyme activity (Z_GDH; in U/ml) using specific assays described later. The immobilization was repeated four times, whereby the carrier was washed in between each step with 1 ml of loading buffer. After the washing, fresh cell extract was added. For each round of immobilization, the immobilization yield (%) was calculated as $100 \times (c_0 - c)/c_0$, where c_0 is the initial enzyme activity/concentration, and c is the enzyme activity/concentration in the supernatant after the immobilization. The amount of activity immobilized was calculated from the difference (c_0–c) at each round. E_{bound} is the immobilized enzyme expressed in $U/g_{carrier}$. For Z_P450 BM3, E_{bound} is the result of the enzyme content measured and the specific activity.

Enzyme coimmobilization was performed sequentially (as described above for single enzymes), with Z_P450 BM3 immobilized first in four consecutive loading steps (each step 4 µM). The carrier with immobilized Z_P450 BM3 was washed with 1 ml loading buffer and Z_GDH (in U/ml) was immobilized subsequently in one loading step. The carrier was washed once more (1 ml loading buffer) and stored in loading buffer at 4°C until use.

2.4 | Assays for single enzymes

CO titration was used to determine the concentration of active Z_P450 BM3 (see Supporting Information Figure S2; Omura & Sato, 1964). Activity of Z_P450 BM3 was measured using anisole as substrate. Soluble enzyme (0.6 nmol) or 6 mg carrier (containing 0.6 nmol enzyme) was incubated in 1 ml potassium phosphate buffer (50 mM, pH 7.5) with 20 mM anisole for 5 min at 25°C. Anisole was dissolved in ethanol and then added to the reaction. The final ethanol concentration was 2.2% (by volume). The reaction was started with NADPH (0.2 mM) and continued under gentle mixing in an end-over-end rotator (20 rpm). Samples taken at certain times were analyzed for guaiacol using 4-aminoantipyrine (4-AAP) assay (Dennig, Lülsdorf, Liu, & Schwaneberg, 2013). The coupling efficiency (mol product/mol NADPH oxidized) was determined from the guaiacol formed after complete oxidation of 0.2 mM NADPH. The NADPH oxidation was measured spectrophotometrically. Reaction rates of soluble and immobilized Z_P450 BM3 dependent on the NADPH concentration 0.1–1.6 mM) were obtained as described above. After incubation for 5 min, the guaiacol produced was measured.

The activity and coupling efficiency of Z_P450 BM3 were also determined using lauric acid as the substrate. Except for the substrate concentration (0.4 mM lauric acid), identical reaction conditions and catalyst amount (0.6 nmol) were used as for hydroxylation of anisole. The conversion of lauric acid was started with NADPH (0.4 mM). Substrate conversion was monitored by depletion of substrate signal in gas chromatography–flame ionization detector (GC–FID) analysis. Gas chromatography–mass spectrometry (GC–MS) analysis was used to confirm hydroxy acid product formation. Further details of the GC analysis are given in the Supporting Information Figures S3–S7.

The Z_GDH activity was measured spectrophotometrically at 340 nm. Reactions were performed at 25°C in potassium phosphate buffer (50 mM, pH 7.5), containing $NADP^+$ (5 mM), and Z_GDH in appropriate amount. Glucose (200 mM) was added to start the reaction. One unit activity (U) is the enzyme amount producing 1 µmol NADPH/min. The reduction rate dependent on the concentration of $NADP^+$ (0.5–10 mM) was evaluated for soluble and immobilized preparations of Z_GDH under the conditions stated above. Reactions were performed under continuous stirring in a Thermo Scientific GENESYS 10 S UV–Vis spectrophotometer (Thermo Fisher Scientific, Vienna, Austria).

2.5 | Stability study

Using the same enzyme concentrations as above, the stability of soluble and immobilized Z_P450 BM3 was determined under resting condition in the absence of NADPH. The enzyme was incubated at 25°C under gentle mixing in a final volume of 1 ml potassium phosphate buffer (50 mM, pH 7.5). Anisole was present at 20 mM (2.2% ethanol, by volume). Samples taken certain times were

supplemented with NADPH (0.2 mM) to start the reaction. After 5 min, the released guaiacol was measured.

2.6 | Self-sufficient monooxygenase reactions

2.6.1 | Anisole hydroxylation

Reactions contained anisole (20 mM), $NADP^+$ (0.8 mM), glucose (200 mM), and enzymes in soluble or immobilized form (9 nmol Z_P450 BM3; 70 U Z_GDH; 100 mg carrier) in a final volume of 5 ml potassium phosphate buffer (50 mM; pH 7.5). Optionally, bovine liver catalase (1000 U/ml) was added in soluble form to remove H_2O_2 that could be released during reaction cycles of Z_P450 BM3. Reactions were done in 15 ml tubes under gentle mixing using an end-over-end rotator at 25°C. Guaiacol formation was analyzed with the 4-AAP assay. In addition, the O_2 concentration was measured inside the liquid phase at certain times using an optical oxygen sensor (Solvent-Resistant Oxygen Probe; PyroScience GmbH, Aachen, Germany).

2.6.2 | Lauric acid hydroxylation

Reactions contained lauric acid (2 mM), $NADP^+$ (0.1 mM), glucose (200 mM), and enzymes in soluble or immobilized form (1.8 nmol Z_P450 BM3; 14 U Z_GDH; 20 mg carrier) and soluble catalase (1000 U/ml) in a final volume of 5 ml potassium phosphate buffer (50 mM; pH 7.5). Reactions were done in 15 ml plastic tubes under gentle mixing in an end-over-end rotator at 25°C. Control reactions for GC analysis were performed with carrier not containing immobilized enzymes (100% substrate reference sample). The conversion of lauric acid into the respective hydroxy acid products was followed by GC–FID and GC–MS (see the Supporting Information).

2.6.3 | Enzyme reusability study

For each reaction cycle (15 min), lauric acid (2 mM), $NADP^+$ (0.8 mM), glucose (200 mM), and catalase (1000 U/ml) were mixed in a final volume of 5 ml potassium phosphate buffer (50 mM; pH 7.5). As catalyst, 100 mg of carrier comprising 9 nmol Z_P450 BM3 and 70 U of Z_GDH were supplemented. After each reaction cycle, a sample was taken from the liquid phase, followed by extraction and derivatization of carboxylic acids for GC analysis. For the next cycle, the carrier was separated by centrifugation from the reaction mixture, washed with 4 ml loading buffer and added to a new reaction containing all compounds mentioned above.

3 | RESULTS AND DISCUSSION

3.1 | Enzyme characterization

The N-terminal Z_{basic2} fusions of P450 BM3 and *Bm*GDH were produced in *E. coli* and isolated as functional enzymes showing the expected molecular mass for the respective protein subunit including Z_{basic2} (Supporting Information Figure S1). Using anisole as a substrate, the specific activity of Z_P450 BM3 in the cell extract was 0.02 U/mg. For purified Z_P450 BM3, this was 1.7 U/mg and can be compared with the specific activity of 0.65 U/mg for the native P450 BM3 (Dennig et al., 2013). A coupling efficiency of ~50% (±10; $N = 4$) was determined for Z_P450 BM3. This was unexpected, and potentially relevant synthetically, for it exceeds by at least 4-fold the reported coupling efficiency of the native untagged enzyme (Dennig et al., 2013). Since coupling efficiency is a complex manifestation of enzyme behavior during catalysis (Meunier et al., 2004; Whitehouse et al., 2012), we content ourselves with reporting the effect for Z_P450 BM3 without making an attempt at molecular interpretation at this stage. For hydroxylation of lauric acid, a specific activity of 5.34 U/mg (11.3 s^{-1}) and a coupling efficiency of 74% (±1; $N = 3$) were determined with purified soluble Z_P450 BM3. Contrasting the results with anisole, the native enzyme showed higher specific activity (24.3 U/mg; 50 s^{-1}) and coupling efficiency (96%; Di Nardo & Gilardi, 2012; Whitehouse et al., 2012) than Z_P450 BM3 when lauric acid was used.

The specific activity of Z_GDH in cell extract was determined as 3.4 U/mg while purified soluble enzyme reached 57 U/mg. Based on the specific activities of cell extract and purified enzyme, we can calculate that Z_GDH was expressed in *E. coli* to about 6% of total protein.

3.2 | Single enzyme immobilization

ReliSorb SP400 carriers are spherical poly(methyl methacrylate) particles with sulfonate surface groups. The particle pores (80–100 nm diameter) should enable relatively unrestricted access of Z_P450 BM3 (~9 nm) and Z_GDH (7.4 nm) to the internal pore surface area. Characteristic enzyme diameters were calculated with the CalcTool protein mass calculator (CalcTool, 2018) based on amino acid sequence and known oligomeric state. The GDH is a homotetramer (Nagao et al., 1992).

Figure 1 shows results of single enzyme immobilization directly from the cell extract. In earlier work, we have shown that immobilization of Z_{basic2} proteins is highly selective under the conditions used (Bolivar & Nidetzky, 2012; Wiesbauer et al., 2011). This renders enzyme purification before the immobilization unnecessary and ensures that only the target enzyme is immobilized (Bolivar & Nidetzky, 2012; Wiesbauer et al., 2011). In the case of Z_P450 BM3 when 40 nmol of enzyme/$g_{carrier}$ was loaded in each of four rounds in total, the immobilization yield decreased from 90% in the first to 37% in the last round of immobilization (Figure 1a). About 100 nmol Z_P450 BM3/$g_{carrier}$ was bound after four rounds of immobilization. In terms of immobilized protein concentration and activity (anisole as substrate), this corresponded to 12.7 mg of protein/$g_{carrier}$ and 21.6 U/$g_{carrier}$ (E_{bound}), respectively. It is remarkable that this loading of Z_P450 BM3 was achieved directly from the bacterial cell extract. Note: the total amount of protein offered from the cell extract exceeded 700 mg/$g_{carrier}$ under the conditions used. Optimization of enzyme loading is a parameter usually not addressed in the immobilization of P450 BM3. Although we did not explore full

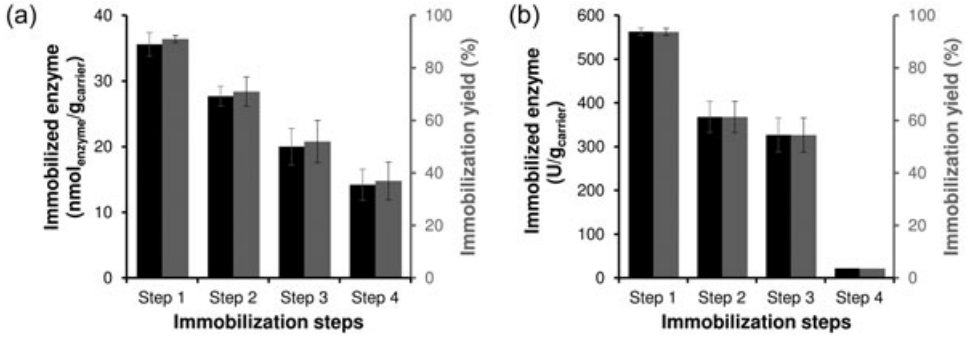

FIGURE 1 Immobilization of individual enzymes on ReliSorb SP carrier. For each step, immobilization is shown in terms of immobilized enzyme and immobilization yield (%) for Z_P450 BM3 (a) and Z_GDH (b). Error bars show standard deviations from four independent

saturation of the carrier, the P450 BM3 loading obtained here was superior (2.3-fold) to the loading for enzyme equipped with a Halotag on silica nanoparticles of ~100 nm in size (5.4 mg/$g_{carrier}$; Wang et al., 2015).

The immobilization of Z_GDH was similar in overall trend to the immobilization of Z_P450 BM3, in that >90% of the offered enzyme was immobilized in the first round, whereas the immobilization yield decreased substantially in later rounds to <10% (Figure 1b). Contrary to Z_P450 BM3 immobilization, the immobilization of Z_GDH appeared to have reached saturation of the carrier after the third round. In total 1280 U/$g_{carrier}$ or 22.5 mg Z_GDH protein/$g_{carrier}$ were bound after the three rounds of immobilization. In summary, these results give good indication to practical upper limits of individual immobilization Z_P450 BM3 and Z_GDH on ReliSorb SP400. However, the apparent carrier binding capacities for the two Z_{basic2} enzymes seem adequate for the envisioned application in biocatalytic substrate hydroxylation.

3.3 | Immobilized enzyme characterization

The effectiveness factor η describes the ratio between the actual activity of the immobilizate (E_{obs}) and the activity effectively bound (E_{bound}). An ideally active immobilized enzyme would yield η of unity, which is typically not reached ($\eta < 1$). To determine η for immobilized Z_P450 BM3, we measured the time course of guaiacol production from anisole (Figure 2a) and calculated E_{obs} from the initial reaction phase. A η value of 0.48 (±0.03; $N = 3$) was calculated for Z_P450 BM3, which falls into a range of η values (≥0.3–0.9) for a series of other Z_{basic2} enzymes immobilized on anionic supports (Bolivar & Nidetzky, 2012; Valikhani, Bolivar, Pfeiffer, & Nidetzky, 2017). Considering the large dimeric structure and functional complexity of native P450 BM3, the obtained value of η is remarkable. Good retention of specific activity in the immobilized enzyme is ascribable to oriented immobilization via the Z_{basic2} module. The coupling efficiency of the Z_P450 BM3 immobilizate was 50% (±5; $N = 3$), thus unchanged compared with the soluble enzyme. This additionally supports the notion of useful conservation of enzyme properties in immobilized Z_P450 BM3. Figure 2b shows the activity of immobilized Z_P450 BM3 dependent on the NADPH concentration and reveals maximum activity at 0.4 mM. Activity declined at higher NADPH concentrations. The activity dependence on the NADPH concentration was similar for soluble Z_P450 BM3. Taking 75% of the maximum activity as practical threshold a broad range of NADPH concentrations between 0.3 and 1.0 mM appeared to be usable for synthesis with immobilized Z_P450 BM3.

When comparing the activities of soluble and immobilized Z_GDH based on measurements with the standard assay, η was

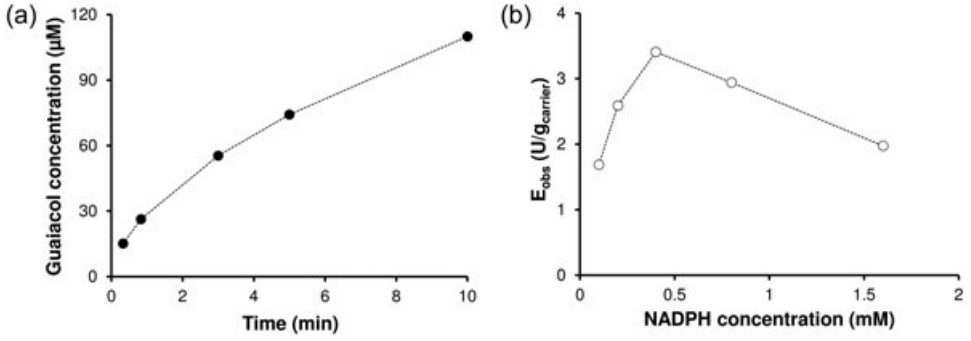

FIGURE 2 Characterization of immobilized Z_P450 BM3. (a) Shows the time course of anisole hydroxylation from the initial reaction phase using 0.2 mM NADPH. (b) Shows the dependency of immobilized Z_P450 BM3 activity on the NADPH concentration. The reaction mixture contained 20 mM anisole and 6 mg carrier (containing 0.6 nmol enzyme) in 1 ml of 50 mM potassium phosphate; 25°C, pH 7.5.
NADPH: nicotinamide adenine dinucleotide phosphate reduced form

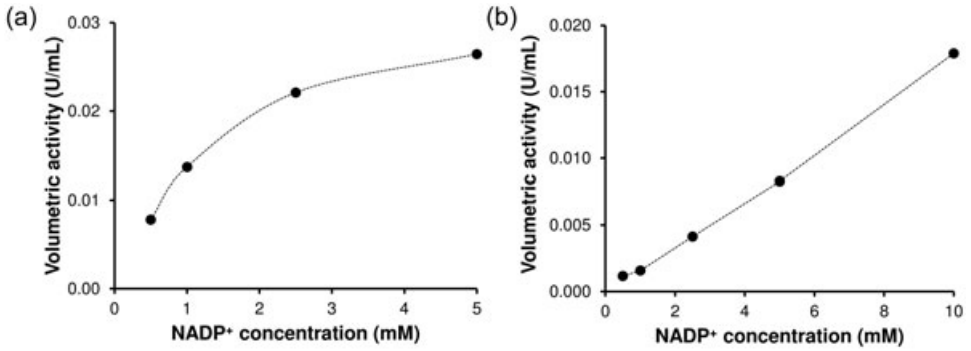

FIGURE 3 Characterization of immobilized Z_GDH. The dependency of Z_GDH activity on the $NADP^+$ concentration is shown in (a) for soluble Z_GDH and in (b) for immobilized Z_GDH. The reaction mixture contained 200 mM glucose and 0.03 U (0.014 nmol) of soluble or immobilized enzyme (0.02 mg carrier) in 1 ml of 50 mM potassium phosphate; 25°C, pH 7.5. $NADP^+$: nicotinamide adenine dinucleotide phosphate

found to be 0.31 (±0.06; $N = 3$). To examine possible origins of this relatively low η value, we measured Z_GDH activity dependent on the $NADP^+$ concentration. Results in Figure 3b show a linear dependence for the activity of immobilized Z_GDH, clearly different from the saturation characteristics found for the soluble enzyme (Figure 3a). For soluble Z_GDH, an apparent K_m of 1.6 mM for $NADP^+$ was calculated that is in agreement with literature for native *Bm*GDH (Nagao et al., 1992). In light of prior evidence indicating largely unperturbed behavior of various Z_{basic2} enzymes immobilized on anionic supports (Bolivar & Nidetzky, 2012), dramatic change of the intrinsic properties of Z_GDH in consequence of the immobilization would be unexpected, although it cannot, of course, be ruled out. At the high value of E_{obs} measured, diffusional limitations for the $NADP^+$ might become a relevant factor. Moreover, the anionic nature of carrier surface and its effect on the availability of $NADP^+$ to the immobilized enzyme have to be considered. However, further inquiry for clarification was beyond the scope of the current study.

Figure 3 shows that the soluble Z_GDH was nine-fold more efficient than the immobilized enzyme. This was also reflected by the enzyme's low η in apparent catalytic efficiency. These characteristics of immobilized Z_GDH thus indicate room for further optimization of the enzyme preparation. Nonetheless, the activity of immobilized Z_GDH was 33-fold higher than Z_P450 BM3 and therefore suitable for the envisioned application as coimmobilizate.

3.4 | Enzyme coimmobilization

Considering the specific activity of purified Z_P450 BM3 with anisole, 33-fold lower than that of Z_GDH, enzyme coimmobilization was performed with the goal of maximizing the amount of Z_P450 BM3 in total enzyme immobilized. Relying on the Z_{basic2} module as common mediator for the immobilization, exactly the same immobilization conditions could be applied to each enzyme, rendering difficult case-specific optimizations unnecessary. In addition, issues of compatibility of immobilization method used for one enzyme with activity and stability of the respective other enzyme are avoided. Therefore, Z_P450 BM3 was immobilized in four consecutive steps to a final loading of 100 $nmol/g_{carrier}$. This was followed by a single-step of Z_GDH immobilization, offering 900 $U/g_{carrier}$ (~42 nmol). Figure 4 shows Z_GDH immobilization to about 700 $U/g_{carrier}$, implying an immobilization yield of 78%. This result indicates that, despite loading the carrier with high amount of Z_P450 BM3 in single enzyme immobilization (Figure 1a), a sufficient surface area remains on ReliSorb SP400 for efficient immobilization of Z_GDH. Note that: only 10% (10 nmol) leakage of Z_P450 BM3 (determined by CO-titration) occurred during immobilization of Z_GDH. It would be an interesting future study to assess the relative distribution of the two enzymes within ReliSorb SP400 particles and determine its effect on the catalyst performance.

In summary, facile and practical coimmobilization of Z_P450 BM3 and Z_GDH was demonstrated. An off-the-shelf insoluble catalyst was provided that comprised (per gram carrier) 90 nmol Z_P450 BM3 (19.5 U; anisole) and 700 U Z_GDH. The approach could be expanded to create a versatile platform of immobilized P450s or multienzymatic systems in general (Schrittwieser, Velikogne, Hall, & Kroutil, 2018).

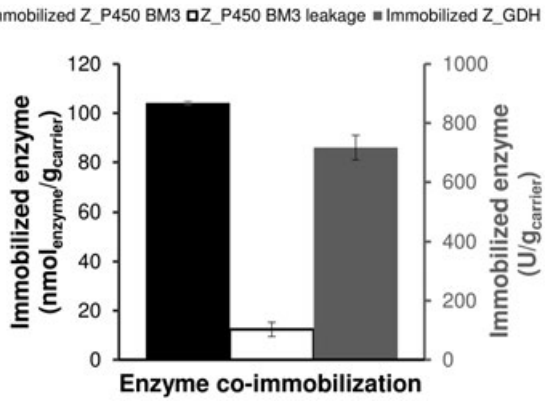

FIGURE 4 Coimmobilization of Z_P450 BM3 and Z_GDH. The immobilization of the individual enzymes is shown

3.5 | Coimmobilized Z_P450 BM3 and Z_GDH as a self-sufficient monooxygenase system

3.5.1 | Anisole hydroxylation

The production of guaiacol from anisole by the coimmobilizate was studied. Applying 0.23 mg/ml (0.36 nmol) of immobilized Z_P450 BM3 on carrier gave volumetric activities of 0.39 U/ml for Z_P450 BM3 and 14 U/ml for Z_GDH. Air was entrained from the liquid surface and facilitated by stirring. Measurement of the dissolved O_2 at different times confirmed that the overall conversion was not limited by O_2 availability (≥25% air saturation) in the liquid phase. The pH was also monitored, but no significant change was observed. In Figure 5 we compare reaction time courses recorded in the absence and presence of 1000 U/ml catalase. In both experiments, guaiacol production was fast in the beginning (≤2 hr), but leveled off rapidly afterwards. When no catalase was present, the reaction stopped after 4 hr yielding 2 mM guaiacol (10% conversion). The addition of catalase seemed to attenuate the slowdown of the reaction. It was effective probably because of the degradation of the peroxide formed in the reaction (Hernandez, Berenguer-Murcia, Rodrigues, & Fernandez-Lafuente, 2012). It thus benefited the maximum concentration of guaiacol produced which was increased about two-fold to 4 mM (±0.26; $N = 3$). Although Z_P450 BM3 showed higher specific activity and coupling efficiency for anisole hydroxylation compared to earlier work (Dennig et al., 2013), the conversion was not improved. To examine limitations on immobilizate activity/stability, we eluted Z_P450 BM3 and Z_GDH from carrier beads recovered after the conversion. Controls showed that the elution conditions used did not inactivate either enzyme. The specific activities of both enzymes as recovered from the carrier were decreased substantially, interestingly that of Z_GDH more (≥85% loss) than that of Z_P450 BM3 (63% loss). Enzyme inactivation during conversion clearly is a critical factor of catalyst performance. Nonetheless, a (TTN; $mol_{product}$ per $mole_{enzyme}$) of 2,220 was calculated for immobilized Z_P450 BM3 from these experiments.

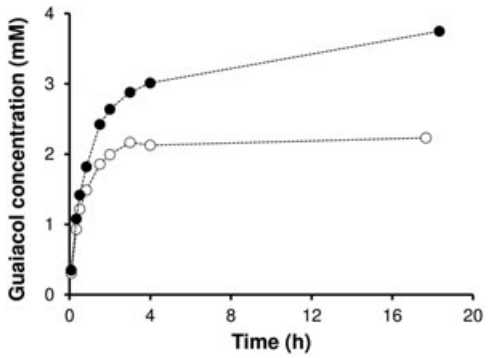

FIGURE 5 Time courses of anisole hydroxylation by the enzyme coimmobilizate. The reaction was performed in the presence (•) and in the absence (○) of catalase. The reaction mixture contained 0.39 U/ml Z_P450 BM3 (1.8 μM), 14 U/ml Z_GDH, 1000 U/ml catalase, 20 mM anisole, 0.8 mM $NADP^+$, and 200 mM glucose in 50 mM potassium phosphate, 25°C, pH 7.5

This is a useful value for conversion of a nonnatural substrate (Dennig et al., 2013, Dennig, Busto, Kroutil, & Faber, 2015, Dennig et al., 2017; Whitehouse et al., 2012), in particular considering that no further optimization of catalyst or reaction engineering was done. Assuming a coupling efficiency of 50%, we calculate that $NADP^+$ (0.8 mM) was regenerated at least 10 times in the reaction. Using soluble Z_P450 BM3 and Z_GDH in amounts corresponding exactly to the enzyme loading from the solid immobilizate, we studied anisole hydroxylation under conditions of catalase added (Supporting Information Figure S8). A maximum guaiacol concentration of 2.3 mM (±0.4; $N = 2$) was obtained and the reaction effectively stopped already after 1 hr. Comparing this result to anisole hydroxylation by the enzyme immobilizate, we find that the operational enzyme stability during conversion of anisole was enhanced about two-fold in consequence of the immobilization. A generally stabilizing effect of immobilization was confirmed for Z_P450 BM3 incubated under resting conditions in the presence of anisole (Supporting Information Figure S9). A P450 BM3 fusion with the poly(3-hydroxybutyrate) binding protein phasin also showed improved stability upon immobilization on poly(3-hydroxybutyrate) granules (Lee et al., 2014). Uncoupling, and the formation of reactive oxygen species associated with it, has generally been considered a main factor of low operational stability of P450 BM3 in reactions with nonnatural substrates. In the reaction with anisole using the native enzyme, uncoupling is a high as 90% (Dennig et al., 2013; Whitehouse et al., 2012). Interestingly, therefore, despite the decrease in uncoupling in Z_P450 BM3 as compared with the native enzyme, and the improved stability of the coimmobilizate, the TTN of immobilized Z_P450 BM3 remained largely unaffected. Of note, therefore, the ReliSorb SP400 carriers are conveniently regenerated for reuse through a simple washing off of the immobilized Z_{basic2} enzymes (Bolivar & Nidetzky, 2012; Wiesbauer et al., 2011).

3.5.2 | Lauric acid hydroxylation in batch and with enzyme reuse

Conversion of lauric acid was initially studied using similar conditions as for anisole hydroxylation. Compared with anisole, lauric acid displays significantly higher coupling efficiency (74%) in the reaction with immobilized Z_P450 BM3. For that reason, and considering a 8.2-fold higher specific activity of soluble Z_P450 BM3 for lauric acid, we decreased the enzyme loading in the carrier (20 mg) to 0.24 U/ml Z_P450 BM3 (1.8 nmol) and 2.8 U/ml Z_GDH. The $NADP^+$ concentration was also decreased to 0.1 mM. In Figure 6 we compare reaction time courses for the soluble enzymes and the coimmobilizate. The soluble enzymes were about 2.5-fold more active than the coimmobilizate, which is in good agreement with the η value of 0.48 for immobilized Z_P450 BM3. The reaction gave a useful turnover number of 5,550 for Z_P450 BM3. It involved at least 27 cycles (=2 mM substrate/0.1 mM $NADP^+$/0.74 coupling efficiency) of NADPH regeneration, which compares favorably to similar reactions (Beyer et al., 2017; Maurer et al., 2003).

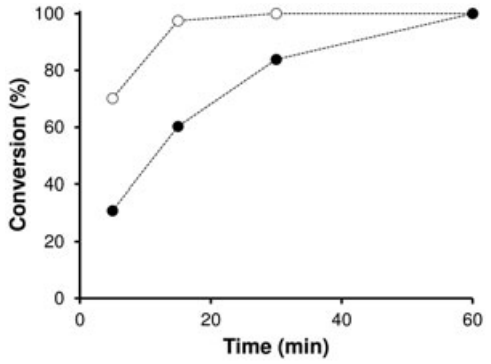

FIGURE 6 Time courses of lauric acid hydroxylation for the soluble enzymes and the enzyme coimmobilizate. The symbols show soluble enzymes (○) and the coimmobilizate (•). The reaction mixture contained 0.24 U/ml Z_P450 BM3 (0.36 μM), 2.8 U/ml Z_GDH, 1000 U/ml catalase, 2 mM lauric acid, 0.1 mM $NADP^+$, and 200 mM glucose in 50 mM potassium phosphate, 25°C, pH 7.5

Figure 7 shows the recycling of the solid catalyst for multiple rounds of reaction. Lauric acid was converted for 15 min in 20 repeated batches. Lauric acid (2 mM; 40 mg) was used at the upper limit of its solubility in water. Substrate conversion ran to completion for nine consecutive batches and decreased gradually afterwards. A space-time yield of 8 mM/hr was attained in the first 2 hr of reaction. An operational half-life for the heterogeneous catalyst after 18 repeated batches was determined from these experiments, an outstanding value employing an immobilized P450 (Ströhle et al., 2016; Tan et al., 2016). In each round, NADPH was regenerated at least 3.5 times. The total recycling number for coenzyme was 70. Taking into account only the first 10 rounds of reaction in which substrate conversion was 95% or higher, a turnover number of 10,000 was calculated for Z_P450 BM3. This falls into the TTN range

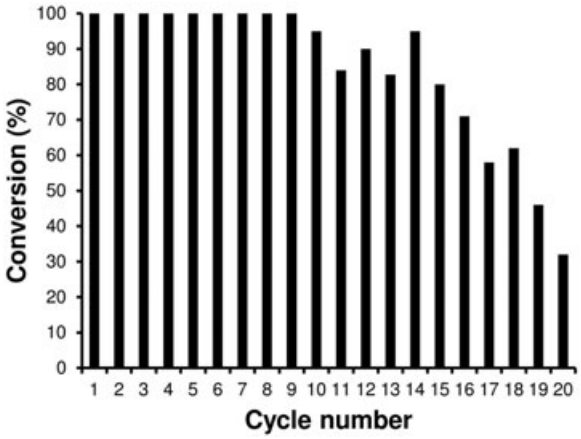

FIGURE 7 Reuse of the enzyme coimmobilizate. The reaction mixture contained 1.2 U/ml Z_P450 BM3 (1.8 μM), 14 U/ml Z_GDH, 1000 U/ml catalase, 2 mM lauric acid, 0.8 mM $NADP^+$, and 200 mM glucose in 50 mM potassium phosphate, 25°C, pH 7.5. For each cycle, the carrier was separated by centrifugation from the reaction mixture, washed with 4 ml loading buffer and added to a new reaction mixture

of P450 systems, immobilized enzymes included, showing the highest operational performance (Dong et al., 2018; Ströhle et al., 2016; Whitehouse et al., 2012). After 20 rounds of reaction, the TTN of the immobilized Z_P450 BM3 was ~18,000. A TTN as high as this is considered useful for synthesis of pharmaceuticals or fine chemicals (Dong et al., 2018). It is worth mentioning that reasonable amounts of solid catalyst (<0.1% loading) and substrate (0.4 g/L lauric acid) were used to highlight the synthetic potential of the immobilized P450.

4 | CONCLUSIONS

Design of a self-sufficient, recyclable monooxygenase catalysts based on P450 BM3 immobilized on solid support is presented. Fusion with the binding module Z_{basic2} enabled convenient coimmobilization of P450 BM3 with its GDH partner enzyme for robust and efficient NADH/NADPH regeneration. The immobilization was characterized by retention of native-like functional characteristics in P450 BM3 and provided enhanced robustness under operational conditions. The coimmobilization strategy conceptualized here for P450 BM3 should be applicable broadly to the development of other immobilized monooxygenase systems or NAD(P)H dependent enzymes. For biocatalytic applications, thus immobilized P450 BM3 constitutes an interesting alternative and complementary option to whole cell approaches. The immobilized catalyst may be useful to carry out synthetically relevant hydroxylations at larger scale. Its high TTN in lauric acid conversion, plus the fact that the enzyme carrier is conveniently regenerated for multiple rounds of fresh coimmobilization, are prerequisites of a technically robust and economically viable process. Demonstration of the applicability of P450s reactions for full-scale biocatalytic production remains an important task.

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