Co-immobilization of P450 BM3 and glucose dehydrogenase on different supports for application as a self-sufficient oxidative biocatalyst

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Abstract

BACKGROUND: The oxy-functionalization of non-activated carbon bonds by the bacterial cytochrome P450 BM3 from *Bacillus megaterium*, presents a promising field in biosynthesis and it has gained much interest in recent decades. Nevertheless, the need for the expensive cofactor NADPH, together with low operational stability of the enzyme have made the implementation of this biocatalyst unfeasible in most cases for industry.

RESULTS: P450 BM3 and glucose dehydrogenase (GDH), as a cofactor regeneration enzyme, were successfully co-immobilized obtaining a bi-functional self-sufficient oxidative biocatalyst. First, a broad screening of 13 different supports was carried out. Five selected agaroses with three different functionalities (epoxy, amine and aldehyde) were studied and their immobilization processes optimized. Finally, P450 BM3 and GDH were co-immobilized on those supports showing the best performance for P450 BM3 immobilization: epoxy-agarose (epoxy-agarose-UAB) presenting 83% and 20% retained activities respectively; AMINO-agarose presenting 28% and 25%, and Lentikats[®] with which both enzymes retained 100% of the initial activity. Furthermore, the re-utilization of the self-sufficient immobilized derivatives was tested in five repeated cycles.

CONCLUSIONS: P450 BM3 and GDH have been successfully immobilized on three supports and their re-usability has been tested in a model reaction. It represents a step forward for future P450 BM3 industrial implementations.

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Keywords: immobilization; biocatalysis; enzymes; oxidation

INTRODUCTION

Cytochromes P450 (CYPs) are versatile monooxygenases able to hydroxylate non-activated carbon bonds with the only requirement being molecular oxygen and an electron donor. CYPs are found as: (i) double domain proteins, presenting a heme-containing oxidative part and a FAD-/FMN-containing reductase; as (ii) triple domain enzymes with a third Ferredoxin subunit; and (iii) as fusion proteins with just one enzymatic unit.¹ These enzymes have been the focus of research in recent decades due to interest in their application as catalysts for the efficient production of fine chemicals, polymers, active pharmaceutical ingredients or nutritional supplements. They display a broad substrate range as well as the capability to catalyse a variety of oxidations including epoxidations, hydroxylation of aromatics, N-oxidation, deamination, dehalogenation and others.²

P450 BM3 (EC 1.14.14.1) from *Bacillus megaterium* was discovered in 1986 and excels among CYPs because it presents the highest turnover numbers (17 000 min⁻¹ for arachidonic acid).^{3,4} In contrast with eukaryotic CYPs, P450 BM3 is a self-sufficient soluble protein that contains both oxidative and reductase domains in the same polypeptide chain. Its natural substrates are medium to long chain fatty acids, however, as with other CYPs, P450 BM3 is a

promiscuous enzyme. It accepts fatty amides and alcohols, hydroxylated fatty acids and ω -oxo fatty acids. It also displays, not only hydroxylating activity, but olefin epoxidation, ring expansion, heteroatom oxidation and dealkylation, and dehydrogenation across C—O, C—N and C—C bonds, as well as carbon–carbon bond formation and cleavage.⁵

There are some limitations to the large-scale application of P450 BM3, such as low operational stability, a dependence on the expensive NADPH electron donor and low retained activity in organic media.⁶ Thus, there have been few cases where it has been attempted to use it industrially.^{7,8} In order to tackle these

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bottlenecks, protein engineering, cofactor recycling systems, surface modifications, reaction media engineering or immobilization are the main strategies usually followed in biocatalytic processes.

Regarding the low operational stability, immobilization has proven to be an efficient methodology for a broad type of oxidoreductases, not only for improving their stability but also for enhancing process metrics by biocatalyst recycling.^{9,10} In addition, immobilization can confer operational advantages such as the possibility to operate in continuous mode, reduction of foam formation, increased stability in organic solvents or simplified product purification.^{11,12} Immobilization of P450 BM3 has been pursued by other authors. For example, Maurer *et al.* tried to immobilize it on a variety of commercially available supports.¹³ They succeeded using the positively charged resins DEAE and SuperQ; and a Sol-Gel, but only the last one was found suitable for bioconversions. Axarli *et al.* immobilized a triple mutant onto epoxy-sepharose, achieving retained activities of 81% and improved stabilities at 37°C.¹⁴ Weber *et al.* entrapped the heme-domain of the P450 BM3 into mesoporous molecular sieves (MCM-41 and SBA-15) and found a correlation between the activity observed from the derivative and pore diameter.¹⁵ Furthermore, Zhao *et al.* immobilized a mutant on DEAE-650S, further entrapped into k-carrageenan together with catalase and zinc dust (Zn/Co(III)sep) which served as electron source. They could operate the reactor over 10 batch cycles with continued conversions above 80%.¹⁶ Other successful and more recent examples involved the fusion of the enzyme to linkers that enabled immobilization on solid surfaces.^{17–19} From all the work present in the literature, there are few cases where P450 BM3 has been successfully immobilized covalently for bioconversion purposes. Many of the above works focused on bio-sensing, in which low amounts of enzyme are required, or on the electrochemical study of the enzyme.²⁰ For large-scale applications, the attachment of large quantities of the enzyme to solid matrices by covalent bonds is a robust method that often confers novel properties to the enzyme and it does not present leaching.

With regard to electron donors, there have been attempts to substitute the expensive NADPH co-substrate for less costly products, e.g. NADH or H₂O₂ by protein engineering.^{21–23} In a different way, electrochemical-driven catalysis has also proved a successful method to bypass the use of NADPH. In various works, the immobilization of P450s on electrode surfaces that served as electron sources led to enzymatically catalysed product formation.^{24–27} Strategies for regenerating the expensive NADPH have also been studied both in enzymatic and non-enzymatic manners.^{28–30} The use of whole cell biocatalyst expressing P450 has been considered as a suitable and much cheaper strategy by taking advantage of the cellular metabolism for NADPH regeneration.^{31,32} However, the use of whole cells entails several drawbacks such as secondary undesired reactions or low reaction rates due to substrate or product mass transfer limitation. Among the different strategies for cofactor regeneration, the utilization of secondary enzymes that make use of sacrificial substrates is one of the most attractive systems. The use of enzymatic NADPH regeneration systems in P450-catalysed reactions has been reported by other authors. Different enzymes such as glucose dehydrogenase, formate dehydrogenase or phosphite dehydrogenase proved to be efficient and suitable biocatalysts for this purpose.^{13,33–35}

In this work, a bi-functional self-sufficient oxidative biocatalyst is obtained by the successful co-immobilization of P450 BM3 and a GDH from *Thermoplasma acidophilum* as cofactor-regenerating enzyme (Fig. 1). First, P450 immobilization was analysed prior to

Figure 1. Scheme of the bi-functional self-sufficient oxidative biocatalyst obtained by the co-immobilization of P450 BM3 from *Bacillus megaterium* and a GDH from *Thermoplasma acidophilum* as cofactor-regenerating enzyme.

selection of the best co-immobilization strategy since P450 is a less robust enzyme compared with GDH. Thus, deeper understanding of P450 BM3 immobilization by two different enzyme-support interactions (adsorption or covalent attachment) is presented. In this sense, different supports presenting a broad range of features were tested to gain insight to the optimal immobilization procedure that should be followed. Finally, co-immobilization of P450 BM3 and GDH was performed by applying the best methodologies resulting for the P450 BM3. The encapsulation in polyvinyl alcohol particles (Lentikats®) was also studied for the confinement of both P450 BM3 and GDH as an alternative to adsorption and covalent attachment strategies.

The resulting self-sufficient biocatalysts were tested in terms of operational stability in the hydroxylation of sodium laureate as a model reaction for proof-of-concept.

EXPERIMENTAL

Materials and supports

Nicotinamide adenine dinucleotide phosphate sodium salts in its oxidized and reduced form (NADP⁺ disodium salt and NADPH tetrasodium salt) was purchased from BONTAC Bioengineering (Shenzhen, Guangdong, China). All the other reagents were purchased from Sigma Aldrich® (St. Louis, MO, USA) and were of analytical grade if not stated otherwise.

The supports used in the screening section were donated by Purolite® Life Sciences (Bala Cynwyd, PA, USA) and their features are summarized in Table 2.

The epoxy-agarose-SIGMA was purchased from Sigma-Aldrich®. According to the supplier's specifications it presented spherical shape, it had an activation degree of $\geq 20 \mu\text{mol mL}^{-1}$ and 12 atoms spacer.

Non-functionalized agarose (spherical beads $\varnothing 50 - 150 \mu\text{m}$) purchased from Agarose Bead Technologies® (ABT®, Madrid, Spain) was used to obtain epoxy-agarose-UAB by support functionalization carried out as described by Axarli *et al.*¹⁴ The quantification of the epoxy groups present on the supports was done following the method described by Gupta.³⁶

Amino functionalized agarose (AMINO-agarose) presenting amino ethyl groups and aldehyde functionalized agarose (GLYOXYL-agarose) with an extent of labelling of $40 - 60 \mu\text{mol mL}^{-1}$ both were also purchased from ABT®. All this information was always according to the supplier's specifications.

Lentikats® polymer was purchased from GeniaLab® (Braunschweig, Germany) with composition consisting of a mixture of polyvinyl alcohol (10%), polyethylene glycol (6%) and demineralized water (84%).

Recombinant expression of P450 BM3 and glucose dehydrogenase in *E. coli*

P450 BM3 (CYP102A1 from *Bacillus megaterium* BM3) was recombinantly produced in *Escherichia coli* in a 20 L scale batch fermentation employing an *E. coli* K12 derivative and a pBAD/myc-HisC based expression vector. Glucose dehydrogenase (GDH) from *Thermoplasma acidophilum* (GDH-Tac) was co-expressed from the same vector in a poly-cistronic arrangement of the GDH gene downstream of the P450 BM3 gene. A 500 mL pre-culture were used to inoculate 20 kg main culture medium with 100 µg mL⁻¹ neomycin. The pre-culture was prepared in standard Luria-Bertani (LB) medium supplemented with 100 µg mL⁻¹ neomycin. The fermentation was performed using terrific broth (TB) medium with glycerol. 2.5 h after inoculation of the fermenter as inducer, pre-sterilized L-arabinose was added to the fermenter to final concentration 0.02% (w/v). Twenty-four hours after inoculation of the fermenter, the cell material was harvested by centrifugation. Cell-free extract was prepared by adding 2 weight equivalents of 100 mmol L⁻¹ potassium phosphate (KP_i) buffer (pH 7.0) to 1 weight equivalent of *E. coli* wet cells and sonication of this cell suspension with an ultrasound probe for 20 min with cooling on ice and centrifugation to remove the cell-debris.

Total protein and enzyme content

The cell lysate was pre-clarified by centrifugation (3220 g for 15 min.) and analysed accounting for total protein content by means of Bradford Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA) using bovine serum albumin as standard.³⁷

Enzyme content was assessed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (NuPage 12%, Invitrogen, USA) run in a Mini-PROTEAN II apparatus (BioRad, USA) following the protocol of Laemmli et al.³⁸ Low range protein markers were used for molecular weight determination. Gels were stained using Coomassie G250 colloidal stain solution [34% (v/v) ethanol, 2% (v/v) H₃PO₄, 17% (w/v) (NH₄)₂SO₄ and 0.066% Coomassie G250] and the Image LAB™ software (BioRad, USA) was used for image processing.

Active P450 BM3 content determination

P450 BM3 active form concentration was determined using the CO-differential spectra analysis described by Omura and Sato, using $\epsilon = 91 \text{ mM}^{-1} \text{ cm}^{-1}$.^{39,40}

P450 BM3 activity measurement

The activity of the P450 BM3 was measured spectrophotometrically ($\lambda = 340 \text{ nm}$) following the consumption of NADPH ($\epsilon = 6.22 \text{ mmol L}^{-1} \text{ cm}^{-1}$, 200 µmol L⁻¹), using sodium dodecanoate (sodium laurate) as substrate (1.3 mmol L⁻¹ dissolved in sodium phosphate buffer 50 mmol L⁻¹ pH = 7.5). 25 µL of enzyme sample were added to the total volume of the assay (500 µL). The basal consumption of NADPH by unspecific enzymes present in the lysate or/and by P450 uncoupling in absence of substrate was determined by this same test but avoiding the addition of the substrate and adding buffer instead. This consumption rate, that represents about 2–3%, is subtracted from the measurement

with sodium dodecanoate. One unit of activity (U) is defined as the enzyme required to convert 1 µmol of NADPH per minute at those given conditions (30°C and pH = 7.5). The absorbance was recorded using a spectrophotometer Cary 50 Bio UV–visible (Palo Alto, USA). The equipment allows temperature control and stirring. Plastic micro-cuvettes BRAND® UV (Sigma Aldrich®) were used for soluble enzyme characterization and 3.5 mL quartz cuvettes HELLMA® 100-QS (Hellma Analytics, Müllheim, Germany) with magnetic stirring, were used for the immobilized derivatives (suspension). In the first case a total volume of 500 µL was reached while in the second it was 2 mL.

Glucose dehydrogenase activity measurement

GDH activity is measured spectrophotometrically ($\lambda = 340 \text{ nm}$) following the formation of NADPH. A specified amount of sample (1/20 of the V_T) was added to a solution containing D-glucose (200 mmol L⁻¹ dissolved in sodium phosphate buffer 100 mmol L⁻¹ pH = 8) and NADP⁺ (400 µmol L⁻¹). The same procedure explained above was applied for measuring the non-GDH formation of NADPH and the same consideration is taken to obtain the U_s. Moreover, it was checked that if NADPH is used instead of NADP⁺, no consumption of cofactor by P450 is detected under the test conditions. The absorbance was also recorded using the aforementioned equipment and conditions.

Immobilization metrics

In every immobilization, a characterization was pursued in order to obtain the retained activity (Eqn (1)) and immobilization yield (Eqn (2)). Supernatant and suspension activities were analysed over time until a steady state was reached and in all cases the activity of a blank (no support) was also monitored to ensure that the enzyme activity was not affected by protocol conditions. A 10% w/v relation between the support and the total volume was always established and small amounts of enzyme were loaded (4–5 U of P450 BM3/g of support and 5–6 U of GDH/g of support) so that diffusional limitations were minimized.

Retained activity (%) =

$$\frac{\text{Suspension activity} \left(\frac{\text{U}}{\text{g}} \right) - \text{Supernatant activity} \left(\frac{\text{U}}{\text{g}} \right)}{\text{Initial offered activity} \left(\frac{\text{U}}{\text{g}} \right)} \times 100 \quad (1)$$

Immobilization yield (%) =

$$\left(1 - \frac{\text{Supernatant activity} \left(\frac{\text{U}}{\text{g}} \right)}{\text{Initial offered activity} \left(\frac{\text{U}}{\text{g}} \right)} \right) \times 100 \quad (2)$$

For assessment of the loading capacity the supports were saturated with enzyme and the activity was calculated taking into account that diffusional limitations do not allow the actual visualization of the activity in the support (Eqn (3)).

$$\text{Loading capacity} \left(\frac{\text{U}}{\text{g}} \right) = \left(\text{Offered initial activity} \left(\frac{\text{U}}{\text{g}} \right) - \text{Supernatant activity} \left(\frac{\text{U}}{\text{g}} \right) \right) \times \frac{\text{Retained activity} (\%)}{\text{Immobilization yield} (\%)} \quad (3)$$

Moreover, the amount of protein per gram of support was also calculated, analysing the initial protein content and subtracting from it the concentration still remaining in the supernatant at the end of the process. In all cases protein concentration was determined by means of Bradford Protein Assay Kit, as already described.

Immobilization of P450 BM3 onto Purolite® resins

From Purolite®, a set of commercially available resins presenting a variety of matrices, pore sizes, linker lengths, and functional groups (Table 2) were tested for the immobilization of P450 BM3. The conditions in which immobilizations were carried out in each case were those specified by the supplier's protocols. In all cases, the relation of 10% w/v between support and total volume was maintained. The immobilizations were done at 25°C and mild agitation. The incubation times ranged from 0.5 to 5 h depending on the speed at which the enzymes were attached. The epoxy containing methacrylates (ECR8204F, ECR8215F and ECR8285) were used in 50 mmol L⁻¹ sodium phosphate buffer pH 8 containing 0.5 mol L⁻¹ NaCl. The amino functionalized resins (ECR8309F, ECR8315F, ECR8409F and ECR8415F) were mixed with the enzyme in 50 mmol L⁻¹ sodium phosphate buffer pH 6. The supports made of a mixture of styrene and methacrylate (ECR1061M and ECR1030M) were utilized in 50 mmol L⁻¹ sodium phosphate buffer pH 7.5; finally, immobilizations using the epoxy agaroses (Praesto 45, 65 and 90) were performed in 1 mol L⁻¹ potassium phosphate buffer pH 8.

Immobilization of P450 BM3 onto epoxy-agarose-SIGMA and epoxy-agarose-UAB supports

For the other epoxy-agaroses (epoxy-agarose-SIGMA and epoxy-agarose-UAB), the enzyme was dissolved in 1 mol L⁻¹ (pH 8) potassium phosphate buffer, mixed with the resin (10% w/v) and left at 25°C with mild agitation. The epoxy-agarose-UAB was incubated for 4 h and epoxy-agarose-SIGMA was incubated for 0.5 h. At the end of the process, in order to eliminate the unreacted epoxy groups, the samples were incubated with 0.2 M β -mercaptoethanol for at least 2 h at 25°C.

Immobilization of P450 BM3 onto GLYOXYL-agarose support

For the immobilization using GLYOXYL-agarose different pHs were tested (6.5–8.5) using 50 mmol L⁻¹ sodium phosphate buffers. The immobilizations lasted 6 h and were done at 25°C and mild agitation. For the reduction of the Schiff base, two different reducing agents were studied: sodium borohydride (1 mg mL⁻¹) added at the end of the immobilization and sodium cyanoborohydride (0.05 mg mL⁻¹ for characterization and 0.4 mg mL⁻¹ for high loads of enzyme) added at the beginning of the immobilization.

Immobilization of P450 BM3 onto AMINO-agarose support

For AMINO-agarose, 50 mmol L⁻¹ sodium phosphate buffer (pH 6) was used to dissolve the enzyme and mix it with the support. The enzyme was left to ionically adsorb onto the support for 0.5–1 h. After that, a 200 mmol L⁻¹ stock solution of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) was prepared dissolving it in 50 mmol L⁻¹ phosphate buffer and adjusting to pH 6 with HCl. It was then added at different concentrations and incubated for 30 min. to activate the carboxylic groups and promote the covalent binding. The concentration of EDC was optimized in the characterization phase (1, 2, 3, 4, 5, 10, 15,

20 mmol L⁻¹) and also when using high loads of enzyme (3, 6, 12, 30, 60 mmol L⁻¹). Finally, 0.5 mol L⁻¹ NaCl was introduced to desorb all the protein attached non-covalently. The immobilization was carried out at 25°C with mild agitation.

Co-immobilization of P450 BM3 and GDH in AMINO-agarose and epoxy-agarose-UAB

The conditions in which immobilizations were carried out were exactly the same as previously described for P450 BM3 for AMINO-agarose and epoxy-agarose-UAB. The activity of the GDH present in the lysate was also monitored in this stage together with the P450 BM3. Retained activities and immobilization yields were obtained for GDH and P450 BM3.

Co-immobilization of P450 and GDH into polyvinyl alcohol lenses (Lentikats®)

Co-immobilization of GDH and P450 BM3 by entrapment was carried out using an organic polymer commercialized as Lentikats®. The solid mixture was heated to 95°C until it turned into a transparent liquid. Then it was cooled down to 40°C and 8 mL of the polymer were mixed with 2 mL of a suspension containing the desired amount of biocatalyst. Afterwards the mixture was poured on a petri dish and the lenses were printed using the LentiPrinter®. The resulting plates were left to dry at 25°C for 1 h until they lost 80% of their weight approximately. Once printed, the support adopted a lentil-like shape of 1–2 mm diameter and 200–300 μ m wide where the proteins were entrapped inside.

Re-usability of the biocatalyst for the hydroxylation of sodium laurate

The utilization of the immobilized enzymes in repeated reaction cycles was studied following the consumption of sodium laurate. The reactor ($V_T = 5$ mL) contained 1.53 mmol L⁻¹ sodium laurate, 0.2 mmol L⁻¹ NADP⁺ and 12.5 mmol L⁻¹ D-glucose. All dissolved in 50 mmol L⁻¹ sodium phosphate buffer pH 7.5. The enzyme added was the minimum necessary to convert at least 90% of the initial substrate in 1 h. The reactors used were purchased from Multi-SynTech (Witten, Germany) and consisted of 10 mL plastic vessels with a porous plate at the bottom. It allowed the extraction of the whole reactor liquid content by filtering with this same porous plate without losing any fraction of the immobilized derivative. The reaction took place at 25°C and 1000 rpm using a Multi-Therm H5000-HC-E thermo-shaker (Benchmark Scientific Inc., Sayreville, NJ, USA). After each cycle, the immobilized enzymes were washed twice with sodium phosphate buffer 50 mmol L⁻¹ pH 7.5 then new reaction medium was added and substrate conversion was quantified. Experiments were carried out in duplicate.

GC-FID analysis of sodium laurate

Samples containing sodium laurate and its products were analysed using a 7890A gas chromatograph (Agilent Technologies, USA) equipped with a HP-INNOWAX 19095 N-123 column (30 m, 0.53 mm, 1 μ m, Agilent Technologies). Reaction samples were filtered ($\varnothing$ 0.22 μ m) and 50 μ L of hexanoic acid (2.8 mg mL⁻¹), that served as internal standard, were added to 300 μ L of sample. The column temperature started at 150°C, increased to 240°C at 24°C min⁻¹, and was held at final temperature for 11 min. The injector temperature was kept at 300°C; for the flame ionization detector, the temperature was 320°C. Helium was used as carrier gas at a flow rate of 8 mL min⁻¹. All analyses were carried out in duplicate.

Figure 2. SDS-PAGE gel containing a lysate co-expressing P450 BM3 and GDH (left lane) and a molecular weight standard Bio-Rad Precision Plus (right lane). Numbers on both sides represent the molecular weight of the protein bands (kDa).

RESULTS AND DISCUSSION

Characterization of the *E. coli* lysate containing P450 BM3 and GDH

As already mentioned, P450s are enzymes that require a cofactor to perform the redox reactions they catalyse. One of the strategies widely used for overcoming the disadvantages of co-factor dependency is the use of an enzyme-coupled reaction for the regeneration of the expensive NADPH. In this work, GDH (EC 1.1.1.47) from the archaea *Thermoplasma acidophilum* was used as NADPH-recycling enzyme using glucose as sacrificial substrate (Fig. 1). This robust and stable tetrameric protein is reported to be an efficient enzyme for NADPH regeneration.^{34,35,41}

In the present work, GDH was co-expressed with P450 BM3 in *E. coli* and both enzymes were present in the cell lysates utilized.

The characterization of the lysate was performed regarding protein content, enzyme content (SDS-PAGE, Fig. 2), activity and P450 BM3 active form concentration and the results are shown in Table 1.

The P450 BM3 was expressed at a higher level than the GDH, however, under the conditions in which the activity tests were

performed, GDH gave higher specific activity. Table 1, also shows the correlation between activity units (Us) and concentration (nmols) of active enzyme; for the P450 BM3 case, 2.85 U nmol^{-1} .

Screening of supports for the immobilization of P450 BM3 – Purolite®

The main objective of the present work was to obtain a bi-functional self-sufficient oxidative biocatalyst by co-immobilization of P450 BM3 and GDH. To accomplish this, supports and immobilization conditions that were found to be most suitable for the P450 BM3 enzyme were prioritized, since cytochrome is the key enzyme performing the target reaction and it has been described as an unfavourable enzyme for immobilizing. GDH, on the contrary, has been described as a robust and more stable enzyme so difficulties in its immobilization were not anticipated.⁴¹ In fact, successful immobilization of GDH on different kinds of support has been reported by several authors.⁴² The results obtained for P450 BM3 immobilization, the materials tested and its features are summarized in Table 2. The supports purchased in the screening kits encompass a range of different particle sizes (45 to $710 \mu\text{m}$), matrices (methacrylate, styrene and agarose), functional groups (amino, epoxy and aldehyde), enzyme-support interaction (covalent binding or hydrophobic adsorption) and linker lengths (2 to 18 carbon atoms). All of these properties have been identified as critical for successful enzyme immobilization.¹²

As shown in Table 2, the immobilization yields obtained were higher than 90% in all cases, except for the supports that interact with the enzyme by hydrophobic adsorption: ECR1061M and ECR1030M. In both cases the immobilization yields only reached $21 \pm 6\%$ and $33 \pm 1\%$ respectively. On the other hand, no retained activities or very low values ($<20\%$) were observed in all tested supports, except for Praesto 45, 65 and 90, all epoxy-agarose supports (Fig. 3(A)). According to the titration performed, Praesto 45, 65 and 90 contained an epoxide concentration of 9, 6 and $8 \mu\text{mol}$ per gram of support respectively and resulted in retained activities of 60–68% after 4 h incubation. The maximum loading capacities of these epoxy-agarose resins ranged from 18 to 20 U g^{-1} and 7 to $8 \text{ mg protein g}^{-1}$ of support. The immobilization profiles were very similar among the three supports and they did not show any significant correlation with the particle size or the initial epoxide concentration (Fig. 3(A)).

Regarding the methacrylate and styrene matrices, even though different ionic strengths and pH values were assessed, none of the tested supports showed retained activities above 20%. P450 BM3 had adhered to the matrices in most cases, but did not remain active. Two methacrylate-based materials harbouring amino groups on six carbon chain linkers (ECR8409F and the ECR8415F) gave retained activities of $24 \pm 4\%$ and $22 \pm 3\%$ respectively when the enzyme was adsorbed onto the resins solely by ionic interactions, however once carbodiimide was added, to promote covalent bond formation, the activity was reduced to $6 \pm 3\%$ and $18 \pm 1\%$ respectively. Furthermore, following the supplier's protocol, the four amino methacrylate based supports were further functionalized with glutaraldehyde prior to enzyme binding, but none resulted in successful immobilization either.

It should also be mentioned that methacrylic matrices with the same functional groups as the epoxy-agarose support (ECR8204F, ECR8215F and ECR8285) did not lead to actively immobilized enzyme, as can be seen by the low retained activities obtained (0% , $2 \pm 1\%$ and $3 \pm 2\%$ respectively). These results could indicate that

Table 1. Characterization of the cell-free extract containing P450 BM3 and glucose dehydrogenase

Enzyme	[Protein] (mg protein/ mL lysate)	Specific activity (U/mg protein)	Enzyme ^a (mg enzyme/ mg protein)	[Enzyme] (mg enzyme/ mL lysate)	P450 BM3 active form concentration ^b ($\mu\text{mol L}^{-1}$)
P450 BM3	45.4 $\pm$ 0.8	2.8 $\pm$ 0.6	0.52 $\pm$ 0.01	23.8 $\pm$ 0.6	44.9 $\pm$ 2.1
GDH		3.6 $\pm$ 0.4	0.14 $\pm$ 0.01	6.64 $\pm$ 0.16	–

^a Data obtained from SDS-PAGE analysis.
^b Data obtained from CO difference spectrum assay.

Table 2. Main characteristics of the matrices tested and their results regarding immobilization and retained activity of P450 BM3

Code	Functional group (Linker)	Matrix	Interaction	Pore diameter ($\text{\AA}$)	Particle size (μm)	Immobilization yield (%)	Retained activity (%)
ECR8204F	Epoxy	Methacrylate	Covalent	300–600	150–300	92 $\pm$ 4	0
ECR8215F	Epoxy	Methacrylate	Covalent	1200–1800	150–300	99 $\pm$ 1	2 $\pm$ 1
ECR8309F	Amino (C2)	Methacrylate	Ionic/Covalent	600–1200	150–300	95 $\pm$ 5	3 $\pm$ 2
ECR8315F	Amino (C2)	Methacrylate	Ionic/Covalent	1200–1800	150–300	97 $\pm$ 2	4 $\pm$ 2
ECR8409F	Amino (C6)	Methacrylate	Ionic/Covalent	600–1200	150–300	100	6 $\pm$ 3
ECR8415F	Amino (C6)	Methacrylate	Ionic/Covalent	1200–1800	150–300	98 $\pm$ 1	18 $\pm$ 1
ECR8285	Epoxy (C4)	Methacrylate	Ionic/Covalent	400–600	300–710	100	3 $\pm$ 2
ECR8806F	None (C18)	Methacrylate	Hydrophobic	500–700	150–300	93 $\pm$ 2	0
ECR1061M	None	Styrene/Methacrylic	Hydrophobic	600–750	300–710	21 $\pm$ 6	0
ECR1030M	None	Styrene/Methacrylic	Hydrophobic	200–300	300–710	33 $\pm$ 1	7 $\pm$ 14
Praesto 45	Epoxy	Agarose	Covalent	ND	45	92 $\pm$ 6	63 $\pm$ 1
Praesto 65	Epoxy	Agarose	Covalent	ND	65	95 $\pm$ 1	68 $\pm$ 3
Praesto 90	Epoxy	Agarose	Covalent	ND	90	95 $\pm$ 1	60 $\pm$ 11

The criterion chosen to select a support for further studies sets a minimum for immobilization at 90% and 25% for retained activity.

for P450 immobilization the hydrophilicity of the matrix is a key factor. The higher hydrophobicity of the methacrylate/styrene matrices, together with the flexible nature of the enzyme and the presence of two active sites, are probably the causes of the deactivation of the biocatalyst.⁴³ This hypothesis would also be in accordance with the low immobilization yields and retained activities obtained with the styrene matrices (ECR1061M and ECR1030M). The immobilization mechanism of these matrices is also based on hydrophobic adsorption and both the immobilization yield and retained activity are low. Other authors have also reported unsuccessful attempts to immobilize P450s by hydrophobic adsorption. Maurer *et al.* attempted to immobilize the P450 BM3 on hydrophobic supports such as EP100, MP1000, phenylsepharose, octylsepharose and nultylsepharose but none of them presented final retained activity.¹³

Taking into account the results obtained in the screening which showed a strong effect of the hydrophilicity of the matrix on P450 immobilization, agarose was chosen to perform further studies.

Immobilization of P450 BM3 onto different functionalized agaroses

In addition to the epoxy resins already tested (Praesto 45, 65 and 90), two other agarose matrices with free epoxy groups and different epoxide concentrations were studied: a commercial lyophilized epoxy-agarose resin from Sigma-Aldrich® (epoxy-agarose-SIGMA) and an in-house functionalized agarose resin (epoxy-agarose-UAB) prepared as shown in the experimental

section. In addition, an agarose based matrices containing aldehyde (GLYOXYL-agarose) and primary amine (AMINO-agarose) functional groups were tested.

The epoxy-agarose-SIGMA resin, according to the titration performed, contained an epoxide concentration of $110 \pm 7 \mu\text{mol g}^{-1}$ of support and gave an immobilization yield greater than 95% after 0.5 h (Fig. 3(B)). However, a rapid decrease in activity of the suspended biocatalyst was noted during the immobilization procedure, indicating strong deactivation of the enzyme after immobilization. Thus, after just half an hour, low retained activities ($35 \pm 8\%$) were observed from this support. These results could indicate that the utilization of an epoxy-agarose with high epoxide concentration, such as the epoxy-agarose-SIGMA, results in a progressive loss of activity over time due to the multipoint attachment of the enzyme to the support.⁴⁴ The explanation can be found in the reactivity of the epoxy groups which differs over time: first, reacting with the amines present on the surface of the enzyme and second reacting with the carboxyl and thiol groups.⁴⁵ At the end, if the incubation time is long enough, the protein can be attached at too many points and lose its whole catalytic activity. This issue can be stopped by adding a blocking agent that removes the unreacted epoxy groups once the protein is immobilized. In Fig. 3(B), β -mercaptoethanol (0.2 mol L^{-1} final concentration) was added after 0.5 h and the suspension was incubated for 3.5 h at 25°C . No significant loss of activity was observed during this period which validates the hypothesis of multipoint attachment as the main reason for enzyme deactivation. However, due to its elevated cost (118 € g^{-1}) and low retained activities, compared with the

Figure 3. P450 BM3 immobilization onto: (A) Praesto 45 [discontinued line (—)], 65 [dotted line (····)] and 90 [combined dotted and discontinued line (— · — · —)] [sodium phosphate buffer 1 mol L⁻¹, pH 8]; (B) epoxy-agarose-Sigma [sodium phosphate buffer 1 mol L⁻¹, pH 8]; (I) the enzyme is bound to the support (II) β -mercaptoethanol 0.2 mol L⁻¹ final concentration is added to eliminate the unreacted epoxy groups; (C) epoxy-agarose-UAB [sodium phosphate buffer 1 mol L⁻¹, pH 8]; and (D) AMINO-agarose [sodium phosphate buffer 0.05 mol L⁻¹, pH 6]; (I) adsorption of the enzyme to the support (II) incubation with certain amount of carbodiimide (EDC) (III) incubation with 0.5 M NaCl and desorption of the unattached protein. The activity offered always ranged from 3.5 to 6 U g⁻¹ of resin. The activities of a blank (squares), the supernatant (rhombus) and the suspension (triangle) were continuously analysed. Error bars correspond to standard deviation (n = 2).

Praesto supports, the epoxy-agarose-SIGMA was discarded from further studies.

On the other hand, the epoxy-agarose-UAB displayed an epoxide concentration of $30 \pm 3 \mu\text{mol g}^{-1}$ of support and gave more than 95% immobilization yield and $83 \pm 8\%$ retained activity after 0.5 h (Fig. 3(C)), with a maximum loading capacity of $30 \pm 2 \text{ U g}^{-1}$ of support ($7.5 \pm 0.8 \text{ mg protein g}^{-1}$ of support). A decrease in retained activity can also be seen, reaching $50 \pm 5\%$ after 4 h of incubation probably due to the excessive number of covalent bindings. In this case, as well as for Praesto supports (Fig. 3(A)), the agarose contains a lower epoxide concentration compared with epoxy-agarose-SIGMA. Therefore, the activity loss due to multi-point attachment is lower for the epoxy-agarose-UAB or even negligible for the Praesto compared with the epoxy-agarose-SIGMA. Thus, when choosing the right support, a consensus should be found. Higher epoxide concentration may imply higher loading capacity as is seen when comparing Praesto (18 to 20 U g^{-1} of support) and epoxy-agarose-UAB ($30 \pm 2 \text{ U g}^{-1}$ of support); however, at the same time, higher epoxide concentration entails higher deactivation of the enzyme over time as is the case comparing all three epoxy-agaroses: Praesto (32–40% activity lost), epoxy-agarose-SIGMA (75% activity lost) and epoxy-agarose-UAB

(50% activity lost). In this sense, due to the loading capacity, the high retained activities reached and the low cost (2.5 € g^{-1}), the epoxy-agarose-UAB was considered the best candidate for the immobilization of P450 BM3 among epoxy-agaroses. This study represents a deeper insight into the work of Axarli *et al.*¹⁴ in the understanding of the immobilization of P450 BM3 on epoxy-agaroses and sets the basis for an optimal process.

As mentioned before, other agarose-based supports with different functional groups were also tested such as GLYOXYL-agarose and AMINO-agarose. The GLYOXYL-agarose (aldehyde activated) immobilization must be pursued in alkali media,⁴⁶ however, P450 BM3 is not stable above pH 8.5 (data not shown). After screening a range of immobilization pHs (6.5–8.5), 7.5 was the best performing one resulting in $50 \pm 2\%$ retained activity after 6 h incubation. For the reduction of the Schiff base formed between the aldehyde of the resin and the amine of the enzyme, two reducing agents were tested: (i) sodium borohydride (1 mg mL^{-1}) which is added at the end of the incubation; and (ii) sodium cyanoborohydride (0.05 mg mL^{-1}) which has slower kinetics and can be added at the beginning of the immobilization. Sodium borohydride resulted in complete deactivation of the enzyme while the sodium cyanoborohydride strategy resulted in $36 \pm 0.1\%$ of

retained activity. Thus, sodium cyanoborohydride was selected as the most suitable reducing agent. Regarding the studies on maximum loading capacity, the values previously obtained in the characterization ($36 \pm 0.1\%$ retained activity) at low enzyme loadings were not extrapolated and a major inactivation occurred. This support has proved suitable for the immobilization of P450 BM3, nevertheless, since the covalent immobilization using high loading was not successful, it was discarded from further studies.

Regarding AMINO-agarose, as for the other tested supports functionalized by amino groups (i.e. ECR8309F, ECR8315F, ECR8409F and ECR8415F) the immobilization is based on a three-stage process. In the first stage, the enzyme is adsorbed onto the support surface by ionic interaction of the amino groups of the support and the carboxyl groups of the enzyme, thus an ionic adsorption occurs. In the second stage, carbodiimide is added to the medium aiming to activate the carboxyl groups present on the enzyme making covalent bonding possible. Solutions with high ionic strength such as sodium chloride (0.5 mol L^{-1}) can be finally added to desorb all the protein attached non-covalently (third stage). Ionic adsorption on AMINO-agarose was performed at pH 6 and gave $58 \pm 6\%$ retained activity (Fig. 3(D) phase I). This value was 2.4–2.6 fold higher than the retained activities obtained during the ionic adsorption for the methacrylate based materials ECR8409F and ECR8415F harbouring the same functional group ($24 \pm 4\%$ and $22 \pm 3\%$), as previously discussed. The maximum loading capacity achieved was $112 \pm 2 \text{ U g}^{-1}$ of support ($50 \pm 4 \text{ mg protein g}^{-1}$ of support) which represents the highest value among all the resins tested. Covalent binding of the amine and the carboxyl groups was carried out by adding EDC following ionic adsorption phase (Fig. 3(D) phase II). Since carbodiimide can lead to enzyme deactivation, different concentrations and incubation times were studied to determine the optimal conditions for the covalent binding phase. These studies showed optimal values of 3 mmol L^{-1} EDC and 30 min reaction time respectively. Following covalent attachment, the biocatalyst was incubated with 0.5 mol L^{-1} NaCl for 2 h (Fig. 3(D), phase III) in order to increase the ionic strength of the media and release all the protein not adhered covalently. $28 \pm 1\%$ of the initial activity offered was covalently bound to the agarose, which represents 30% less activity than the value obtained for the ionic adsorption phase (Fig. 3(D), phase I). On the other hand, compared with the methacrylate-based supports, the retained activity was 4.7-fold and 1.6-fold higher than that obtained with ECR8409F and ECR8415F, respectively. Regarding the maximum load, $53 \pm 2 \text{ U g}^{-1}$ of resin was reached ($47 \pm 3 \text{ mg protein g}^{-1}$ of support), representing a 52% decrease in immobilized activity compared with that obtained by ionic adsorption, likely due to enzyme deactivation caused by the carbodiimide. When testing for the maximum loading capacity the concentration of EDC was further optimized since more enzyme was added (Fig. 4). It resulted in an optimal concentration between 6 and 12 mmol L^{-1} . This second value was chosen due to the higher immobilization yield that it entailed. This support represents a step forward in comparison with the other tested agaroses and it is the first work reporting a successful covalent immobilization of P450 BM3 in an amino-based matrix.

Other authors have worked with P450s and agarose based matrices before. In 1988 King *et al.* obtained 100% immobilization yield and 66% retained activity using a cyanogen bromide-sepharose 4BCL and a P450 from *Saccharomyces cerevisiae*. Another example is the aforementioned case of Axarli *et al.*, who immobilized

Figure 4. Optimization of the EDC concentration in AMINO-agarose experiments using high loads. The immobilization was carried out using sodium phosphate buffer 50 mmol L^{-1} pH 6 and offering 92 U g^{-1} of resin. Retained activity (black dots) and immobilization yield (grey bars) are represented. Error bars correspond to standard deviation ($n = 2$).

the P450 BM3 on epoxy-agarose and obtained 81% retained activity.¹⁴

Co-immobilization of P450 BM3 and glucose dehydrogenase

As has already been mentioned, for co-immobilization of both GDH and P450 BM3, it was decided to prioritize the supports and immobilization conditions that were found to be suitable for P450 BM3, i.e. epoxy-agarose-UAB and AMINO-agarose.

The immobilization of GDH using epoxy-agarose-UAB resulted in poor affinity of the protein for the support with immobilization yields below 20%. However, of the enzyme that did adhere, a slight over-activation was observed compared with the same quantity of free enzyme. At the end of the incubation (4 h), retained activity of $20 \pm 5\%$ and a maximum loading capacity of $3 \pm 0.1 \text{ U g}^{-1}$ of support were achieved. Since the conditions were exactly the same as reported above for P450 BM3, the activity for the cytochrome was comparable and resulted in $50 \pm 5\%$ retained activity and $30 \pm 2 \text{ U g}^{-1}$ ($7.5 \pm 0.8 \text{ mg protein g}^{-1}$ of support).

With AMINO-agarose, GDH presented high affinity for the support and it also showed an over-activation effect when adhered to the matrix. It had better tolerance of the presence of EDC compared with P450 BM3, with 10 mmol L^{-1} being the optimal concentration for this enzyme ($57 \pm 2\%$ retained activity) in the characterization phase. Co-immobilization of both enzymes, initially using a low carbodiimide concentration of 3 mmol L^{-1} , resulted in $25 \pm 4\%$ retained activity for GDH and $28 \pm 1\%$ for P450 BM3. Using a higher loading of EDC (12 mmol L^{-1}) resulted in a loading capacity of $18 \pm 1 \text{ U g}^{-1}$ of support for GDH and $53 \pm 2 \text{ U g}^{-1}$ of support for P450 BM3. In summary, a self-sufficient oxidative biocatalyst was obtained.

There are several examples in the literature of the immobilization of GDH.⁴² Regarding the immobilization on agarose based matrices, Anwar *et al.* immobilized a baker's yeast GDH on cyanogen bromide-sepharose obtaining a retained activity of 3%.⁴⁷ Persson *et al.* purified a GDH from *Bacillus subtilis* by immobilizing it on thiopropyl-sepharose and recovered up to 65% of the initial activity.⁴⁸ However, as far as the authors know, this is the first time that GDH has been covalently co-immobilized with P450 BM3.

Co-immobilization of P450 BM3 and GDH into polyvinyl alcohol particles – entrapment

Finally, as an alternative to immobilization by adsorption or by covalent attachment, an entrapment strategy was also investigated for co-immobilization. Both P450 BM3 and GDH were successfully embedded into Lentikats® (polyvinyl matrix) as described in the experimental section. The analysis undertaken, showed maximum retained activities of $100 \pm 0.1\%$ for both P450 BM3 and GDH.

In the sense of the loading capacity employed, the activity and concentration of protein in the polymer was $1.8 \pm 0.2 \text{ U g}^{-1}$ of P450 BM3, $0.8 \pm 0.1 \text{ U g}^{-1}$ of GDH and $1.1 \pm 0.1 \text{ mg protein g}^{-1}$ of support respectively. It was not further increased due to operational issues. The capacity is limited by an aggregation effect observed when charging high concentrations of lysate with which the lenses form a compact mass that cannot be used in our reactions. This is a serious drawback when looking to industrial feasibility because the enzyme must be previously purified or the specific activity of the support would remain too low.³¹ However, since the immobilization had been successful, albeit with low enzyme loading, and it is the first work that reports a successful entrapment of both P450 BM3 and GDH in Lentikats®, it was decided to continue working with this immobilized derivative.

Reusability of the biocatalyst using sodium laurate as substrate

From all the supports tested, AMINO-agarose, epoxy-agarose-UAB and Lentikats® were considered good candidates to be potential carriers in bio-catalysis applications. It is well-known that uncoupling effects on P450s produce reactive oxygen species that inactivate the enzyme (uncoupling).⁴⁹ This handicap is less relevant when working with native substrates such as sodium laureate and so this substrate was used as a model for studying the reusability of the self-sufficient biocatalyst. First, the coupling efficiency of P450 towards this substrate was determined and then reusability studies were performed using the self-sufficient P450/GDH biocatalysts obtained with the aforementioned carriers.

Reactions using sodium laureate (1.3 mmol L^{-1}) and NADPH (0.2 mmol L^{-1}) were performed, monitoring and comparing substrates consumption by means of GC-FID. A coupling efficiency of $90.4 \pm 8.3\%$ was observed, which means that, 90.4% of the NADPH consumed was employed for sodium laurate hydroxylation, with the remainder directed towards uncoupled reactions. This substrate was considered adequate to assess the operational stability of the enzymes in repeated reaction cycles.

Total turnover number (mols of substrate consumed/mols of P450 BM3) (TTN) was chosen as the metric to evaluate the yield of the self-sufficient biocatalyst in comparison with the soluble lysate. To assess it, the immobilized P450 BM3/GDH (epoxy-agarose-UAB, AMINO-agarose and Lentikats®) biocatalysts were tested in sodium laurate reaction cycles of 1 h each utilizing glucose as the sacrificial substrate and the cofactor in its oxidized form (NADP⁺) thus forcing the cofactor regeneration to start before the P450-catalysed reaction. Moreover, NADP⁺ starting concentration was 6.5-fold lower than the sodium laureate. The amount of P450 BM3 required to convert >90% of sodium laurate in 1 h was found to be $0.18\text{--}0.2 \text{ U mL}^{-1}$ ($0.063\text{--}0.07 \text{ nmol mL}^{-1}$). GDH on the other hand, was always ensured to be present in excess so that it did not limit the hydroxylation reaction. The conversions obtained in each cycle for the immobilized systems are presented in Fig. 5. The TTN for the soluble reaction was $21\,500 \pm 290$ mols of sodium laurate consumed/mol of P450 BM3 in 1 h of reaction.

The immobilized derivatives allowed the re-utilization of the self-sufficient biocatalyst boosting the TTN by 2.31-fold for the epoxy-agarose-UAB, 2.98-fold for AMINO-agarose and 2.3-fold for the enzymes immobilized into Lentikats®. As shown in Fig. 5, the conversion dropped in an exponential manner for epoxy-agarose (Fig. 5(A)). On the contrary, with AMINO-agarose and Lentikats® the decay was more linear (Fig. 5(B) and (C)). At the end, all three derivatives could be re-used successfully, thus demonstrating significant operational stability.

Some examples can be found in the literature about the reusability of P450s. Lee *et al.* immobilized a phasin fused P450 BM3 and re-used it four times with no loss of activity.¹⁷ Zhao *et al.* were able to use a P450 BM3 mutein together with catalase in 10 batch cycles with conversions up to 80%.¹⁶ Regarding GDH, different studies of re-usability have been reported. A recent example, Petrovicová *et al.* co-immobilized a ketoreductase and a GDH from *Bacillus megaterium* in polyvinyl alcohol particles and were able to use the derivative 18 times with minimal loss of activity.⁵⁰

CONCLUSIONS

P450 BM3 has gained much interest in recent years in biocatalysis for its oxy-functionalization characteristics, although, the implementation of this enzyme in industrial processes has lacked optimal metrics. Operational stability as well as its requirement for NADPH in stoichiometric amounts have been identified as the main bottlenecks. Aiming to overcome these two main drawbacks, P450 BM3 and GDH have been co-immobilized in the present work to obtain a robust bi-functional self-sufficient biocatalyst.

P450 BM3 and GDH were covalently co-immobilized on epoxy-functionalized agarose and amino-functionalized agarose. Both enzymes were also successfully co-immobilized by entrapment in a polyvinyl alcohol/polyethylen-glycol matrix named Lentikats®. From the five epoxy-agaroses tested, the optimal support (epoxy-agarose-UAB) presented an epoxide concentration of $30 \pm 3 \mu\text{mol g}^{-1}$ of support and retained activities of $83 \pm 8\%$ for P450 BM3 and $20 \pm 5\%$ for GDH. On the other hand, AMINO-agarose showed retained activities of $28 \pm 1\%$ for P450 and $25 \pm 4\%$ for GDH. Finally, the entrapment of both enzymes in Lentikats® led to P450 BM3 and GDH co-immobilization maintaining 100% of the activity initially loaded.

P450 BM3 was also immobilized on aldehyde-functionalized agarose and presented $36 \pm 0.1\%$ retained activity. However, this support did not allow high enzyme loads required for reaction testing, thus making this support unattractive for further studies.

The operational stability of the obtained bi-functional self-sufficient biocatalysts (epoxy-agarose-UAB, AMINO-agarose and Lentikats®) was analysed in terms of re-usability for sodium laureate oxidation. All three derivatives showed successful reuse over five cycles, entailing an increment in the TTN of 2.31-fold for the epoxy-agarose-UAB, 2.98-fold for the AMINO-agarose and 2.3-fold for the Lentikats® compared with the soluble enzymes.

The P450 BM3 wild type served as a model in the immobilization studies in this work. On the other hand, mutated P450 BM3s, usually required for oxidative reactions of non-natural substrates which entail high uncoupling effects, can follow the exact same principles reported here since the surface of the enzyme is usually unaffected.^{1,5,6,51} In this sense, immobilization can be an added value to the process, for these new enzymes, due to the many advantages that it confers.

Figure 5. Reaction cycles using both P450 BM3 and GDH immobilized onto: (A) epoxy-agarose-UAB (10 mg of agarose mL⁻¹); (B) AMINO-agarose (10 mg of agarose mL⁻¹); and (C) Lentikats® (50 mg of Lentikats mL⁻¹). Reactions were carried out at 5 mL scale adding 0.18–0.2 U of total activity and using sodium phosphate buffer 50 mmol L⁻¹ pH 7.5, 1.3 mmol L⁻¹ sodium laurate, 0.2 mmol L⁻¹ NADP⁺, 12.5 mmol L⁻¹ D-glucose, temperature control at 25°C and constant 1000 rpm agitation using a multi-therm H5000-HC-E thermos shaker. Error bars correspond to standard deviation (n = 2).

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