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# Immobilization of P450 BM-3 monooxygenase on mesoporous molecular sieves with different pore diameters

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ABSTRACT

The immobilization of the isolated heme domain of P450 BM-3 (BM3H_F87A) on two mesoporous molecular sieves, MCM-41 (pore diameter 25 Å) and SBA-15 (pore diameter 60 Å and 133 Å) was examined systematically, and the activity of the immobilized enzyme toward *para*-nitrophenoxydodecanoic acid (12-*p*NCA) and *n*-octane was determined. Hydrogen peroxide was utilized as source of electrons and oxygen to support the monooxygenase activity of BM3H_F87A. The mesoporous materials were characterized by X-ray diffraction and nitrogen adsorption analyses before and after immobilization. The results revealed that the immobilization efficiency of MCM-41 and SBA-15 after single immersion was strongly affected by the pH value of the enzyme solution, initial enzyme concentration and agitation conditions. By modelling the 3D structure *in silico* and performing electrostatic potential calculations, the pH-dependence of the enzyme immobilization could be explained and a possible orientation of the protein on mesoporous materials was predicted. The oxidizing activity of the immobilized enzyme was found to depend on pore diameter and accessibility of the substrate for the enzyme. The highest activity toward 12-*p*NCA of 830 nmol product/mg P450/min was observed with BM3H_F87A immobilized on SBA-15 with pore diameter 133 Å. Enzyme activity toward *n*-octane was similar for the enzyme immobilized on SBA-15 of 60 Å and 133 Å, and was at least two-fold higher as compared to a system with free enzyme.



## 1. Introduction

In general, immobilization with respect to enzyme stabilization refers to associating an enzyme with an insoluble matrix, so that it can be reused under stabilized conditions. Immobilized biocatalysts offer several other advantages, like improved enzyme storage and operational stability, resistance to elevated temperatures and organic (co)-solvents, the possibility for continuous processes and greater control over enzymatic reactions. Although immobilization to solid carriers is perhaps the most frequently used strategy to improve the stability of enzymes, only a few reports of successful cytochrome P450 immobilization can be found in literature. Cytochrome P450 enzymes (P450s) are heme containing proteins that catalyze oxygenation of a vast variety of organic molecules. One of the problems regarding the use and immobilization of P450 enzymes is their dependency on the pyridine co-factors NADH and NADPH and the need for the corresponding reductases, which transfer electrons from NAD(P)H to the heme group. The first example on immobilization of P450s dates back to 1988 when Wiseman and co-workers [1] immobilized purified P450s from *Saccharomyces cerevisiae* along with the corresponding reductase by entrapment in calcium alginate or in polyacrylamide, or by adsorption on cyanogen bromide-activated sepharose. A decade later, the plant CYP71B1, fused to a P450 reductase, was immobilized onto colloidal liquid aphrons [2]. Kelly and co-workers reported the co-immobilization of prokaryotic CYP105D1 with a ferredoxin onto the ionic exchange resin DE52-72 [3]. However, most of these systems suffered from the leaching of the enzyme activity from the support.

Previously we have reported the immobilization of the P450 BM-3 from *Bacillus megaterium* (CYP102A1) and some of its mutants on different supports [4]. P450 BM-3 monooxygenase is a self-sufficient natural fusion flavocytochrome (119 kDa) consisting of a heme domain and a diflavin reductase domain [5]. The enzyme was unable to adsorb neither on celite, a porous silicate matrix derived from diatomaceous soil, nor on Eupergit C via covalent

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binding. Negative results obtained with polypropylene derivatives and phenyl-, octyl- and butylsepharose showed that procedures based on hydrophobic interactions were also unsuitable for the efficient immobilization of P450 BM-3. P450 BM-3 binds to anion exchangers such as DEAE and SuperQ [4]. However, the most substrates and products of the oxidation reaction also adsorbed on these matrices. In addition, enzyme leaching from the carrier occurs in buffers with high ionic strength. Furthermore, the purified P450 BM-3 A74G/F87V/L188Q mutant was successfully encapsulated in a sol–gel matrix derived from tetraethoxyorthosilicate (TEOS) upon polymerization. The entrapment of P450 BM-3 in this sol–gel-type material resulted in a very high long-term storage stability of the enzyme at different temperatures. A half-life of 29 days was measured at 25 °C for immobilized P450 BM-3 and only 2 days for the free enzyme. A sol–gel immobilized P450 BM-3 mutant was able to oxidize substrates of diverse substance classes such as terpenoids, polyaromatic hydrocarbons, *n*-alkanes, and fatty acid analogs with high activity [4]. However, since the entrapment of P450 BM-3 was performed during polymerization of TEOS, we were not able to control pore diameter and pore geometry properly. The use of ordered mesoporous silicates, synthesized using surfactant templating routes and therefore having defined pore diameter and pore geometry, would clarify these aspects. Since the first report by Diaz and Balkus in 1996 [6] various commercially available enzymes such as cytochrome *c* [7], lysozyme [8], lipase [9,10] and albumin [11] have successfully been immobilized on ordered mesoporous materials like MCM-41, MCM-48 (Mobil Composition of Matter) SBA-15 or SBA-16 (Santa Barbara). In some cases proteins were adsorbed only on the external surface area of mesoporous materials, in the others – immobilized within their pores. In the meantime only two reports dedicated to P450 monooxygenases were published [12,13]. Both reports describe immobilization of rabbit CYP2C9 and human CYP2B4 on aluminum-substituted MCM-41 containing aluminum ions at different ratios. Interestingly, catalytic activity of immobilized CYP2C9 and CYP2B4 was observed even in the absence of the cytochrome P450 reductase, which is necessary for electron transfer from NADPH to the heme group. The authors suggested that electron transfer to the immobilized P450s can occur through the Lewis acid, i.e., the Al-centers in the silicate walls [12].

In the present study the immobilization of the isolated heme domain of P450 BM-3 (without the reductase domain) on two mesoporous ordered materials, MCM-41 (pore diameter 25 Å) and SBA-15 (pore diameter 60 Å and 133 Å), was systematically examined. For comparison, a commercial silica gel with a broad pore diameter distribution was included into this study, too. The aim of this study was to develop an effective immobilization procedure and to investigate the effect of the pore diameter on the loading capacity of both materials and oxygenase activity of the immobilized P450 BM-3 heme domain.

## 2. Experimental

### 2.1. Materials

All chemicals reagents were of analytical grade purity and purchased from Roth (Karlsruhe, Germany) and Fluka (Steinheim, Germany). $H_2O_2$ was purchased as a 30 wt.% solution from Fluka. The stock solution was prepared freshly in 50 mM potassium phosphate buffer, pH 7.5. Silica gel-Type 62 and sodium water glass (25.5–28.5 wt.% $SiO_2$, 7.5–8.5 wt.% $Na_2O$, rest: water) were obtained from Merck (Germany). 12-*para*-nitrophenoxydodecanoic acid (12-*p*NCA) was synthesized as described elsewhere [14] and dissolved in dimethyl sulfoxide (DMSO).

### 2.2. Preparation and mutagenesis of the P450 BM-3 heme domain

In our previous work the gene CYP102A1 encoding the cytochrome P450 BM-3 has been amplified from genomic DNA of *B. megaterium* ATCC 14581 and cloned into the pET28a(+)-vector yielding the pET-28a_BM-3 construct [15]. The gene fragment coding for the P450 BM-3 heme domain was amplified from pET-28a_BM-3 using the following primers: 5′-3′: GCGGATCCATGACAATTAAAGAAATGCCTCAGC; 5′-3′: GCGAATTCTTAGCGTACTTTTTTAGCAGACTGTTC. The primer for the 3′-end of the gene contains an additional stop codon. The amplified gene as well as the pET28a(+)-vector were cut using the endonucleases *BamH*I and *Eco*RI and then ligated together by T4-DNA ligase. The replacement of the phenylalanine at position 87 by smaller alanine was performed using the Quick-Change Kit (Stratagene). The primers were as follows: 5′-3′: gcaggagacgggttagctacaagctggacgc and 5′-3′: gcgtccagcttgtagctaacccgtctcctgc. PCR was carried out following the manufacturer's protocol. The correct gene insertion and mutation were checked by sequencing. The His6-tagged P450 BM-3 F87A heme domain (further referred to as BM3H_F87A) was expressed in *Escherichia coli* BL21(DE3) and purified on Ni-NTA sepharose as described previously for the holoprotein [4]. The P450 concentrations were quantified from the CO-binding difference spectra of the reduced form as described elsewhere [16]. The extinction coefficient of 91 $mM^{-1}$ $cm^{-1}$ was used for calculations.

### 2.3. Immobilization of the P450 F87A heme domain on mesoporous materials

If not stated otherwise 1.5 mL of purified BM3H_F87A with a final concentration of 15–150 μM was added to 20 mg of a mesoporous material. The immobilization procedure was optimized upon different agitation conditions such as stirring, slow rotation at 15 rpm or intensive mixing. Experiments with stirring were carried out with a magnetic stirrer in a covered beaker. Experiments with rotation were carried out in a Rotamix (RM-1, ELMI, Latvia). Intensive mixing was performed in a Beadmill (MM2000, Retsch, Haan, Germany). Immobilization was performed during 1–24 h at 10 °C. The solids with the immobilized enzyme were recovered by centrifugation (10 min, 2000 g, 4 °C). The supernatant was used for estimation of the concentration of non-immobilized active P450 by measuring CO-difference spectra. The recovered solid fractions were washed four times with 50 mM potassium phosphate buffer, pH 7.5, and were stored then at −20 °C before use. CO-difference spectra measured with washing solutions were used for estimation of enzyme leaching. For sequential immersion the already one or more time loaded portion of a mesoporous material was reloaded again under equal conditions. The recovered solid was washed four times with 50 mM potassium phosphate buffer, pH 7.5 between reloading steps.

### 2.4. Activity toward p-nitrophenoxydodecanoic acid (12-pNCA)

P450 BM-3 activity assays were performed using the *p*NCA assay [14]. The reaction was carried out at room temperature in a final volume of 1.0 mL containing 50 mM potassium phosphate buffer, pH 8.1, 200 μM 12-*p*NCA dissolved in DMSO (final concentration 1%), and the corresponding amount of the purified enzyme. The reaction was started by adding 10 mM $H_2O_2$. Formation of *p*-nitrophenolate was followed at 410 nm on an Ultraspec 3000 photometer (Pharmacia Biotech, Uppsala, Sweden) and calculated using extinction coefficient of 13.2 $mM^{-1}$ $cm^{-1}$. Activity measurements with immobilized BM3H_F87A (20 mg for each experiment) were carried out under stirring in a flow-through cuvette at room temperature. Formation of *p*-nitrophenolate was followed with a Nicolet evolution 1000 photometer (Thermo Electron Corpo-