



Screening tools for artificial electron transfer to P450 BM3

by

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Abstract

Cytochrome P450 monooxygenases catalyze a variety of reactions, but biotechnological approaches with isolated enzymes are still rare. One of the main challenges is their cofactor dependency hampering use in industrial processes. A promising possibility is the substitution of the cofactor by electrochemical means, which includes the use of soluble redox mediators, shuttling electrons between an electrode and the enzyme in solution, or the immobilization of the enzyme at the electrode surface for direct electron transfer. For both strategies a technology is needed to parallelize electroenzymatic experiments and to accelerate research efforts.

An electrochemical microtiter plate (eMTP), which is described in the first part of this work, enables parallelized electrochemical experiments with simultaneous optical data acquisition in up to eight wells of a microtiter plate. This is the first example for parallel spectroelectrochemistry in commercially available microtiter plates, wherein each well contains a set of independent electrodes. The eMTP allows for the fast screening of reaction conditions and its use was exemplified for mediated electron transfer. The well known redox probe potassium ferrocyanide and the mediator ABTS were characterized in the eMTP to proof the functionality and reliability of the system. Furthermore a mutant library of P450 BM3, a well studied fatty acid monooxygenase with high catalytic activity, was screened for its performance in the surrogate pNCA assay via the mediator cobalt sepulchrate. The eMTP allowed a fast comparison of P450 BM3 mutants in terms of reaction rates. In addition several reaction conditions were varied and the influence on product formation rates was determined. The eMTP was used to find optimized reaction conditions for the electroenzymatic process. For example some mutants were found that exhibited a different potential optimum or displayed an altered mediator concentration dependency.

The second part of the thesis focused on the conducting polymer polypyrrole, which is often used as immobilization matrix for redox molecules in biosensor applications. Due to its biocompatibility, conductivity and structural versatility polypyrrole provides all requirements needed for the efficient immobilization and electrochemical actuation of redox molecules at electrodes. Direct electron transfer between redox enzymes and electrodes is of high interest, since it represents an approach for heterogeneous process design with integrated enzyme retention and simultaneous control of the redox state of the enzyme's active site. Pyrrole and two carboxylic acid modified derivatives were introduced as flexible immobilization matrices. A set of redox active iron compounds and small heme type redox proteins was screened towards improved direct electron transfer via the conducting polymer matrix. Based on the commercially available monomers several immobilization methods were employed. Besides the entrapment of redox molecules into the growing polymer film, the covalent attachment to the functionalized polymer surfaces was tested. In addition the carboxylic acid modified polymer surface was used to immobilize metal cations and subsequently a His-tagged P450 BM3 via affinity interactions. The methods of the immobilization "tool box" were applied to P450 BM3, but due to the deeply buried heme moiety of the enzyme and the inherent properties of the employed polypyrrole immobilization matrix, no direct electron transfer was achieved.

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List of abbreviations

4-AAP	4-aminoantipyrine
12-pNCA	<i>p</i> -nitrophenoxydodecanoic acid
ABTS	2,2'-azino-bis-(3-ethyl benzothiazoline-6-sulfonic acid)
Ac ⁻	deprotonated acid
a.u.	absorbance unit
BMP	heme domain of P450 BM3
BMR	NADPH-cytochrome P450 reductase domain of P450 BM3
CoSep	cobalt sepulchrate
CST-MALM	Cystamine-N-succinimidyl 3-maleimidopropionate
CV	cyclic voltammogram
DCC	N,N'-dicyclohexylcarbodiimide
DDAB	didodecyldimethylammonium bromide
DET	direct electron transfer
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ΔE _p	peak potential separation
E	potential
<i>E. coli</i>	<i>Escherichia coli</i>
EDC	N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide
ELISA	enzyme-linked immunosorbent assay
Emp	midpoint potential
eMTP	electrochemical microtiter plate
E _{pa}	anodic peak potential
E _{pc}	cathodic peak potential
EQCM	electrochemical quartz crystal microbalance
F	Faraday constant
FAD	flavin adenine dinucleotide
fc	potassium ferrocyanide
Fe-N ⁺	5,10,15,20-tetrakis-(N-methyl-4-pyridyl)-porphyrin-Fe(III)
Fe-SO ₃ ⁻	5,10,15,20-tetrakis-(4-sulfonatophenyl)-porphyrin-Fe(III)
f _s	serial resonance frequency
HAc	protonated acid
His	histidine
IMAC	immobilized metal ion affinity chromatography
IR drop	voltage drop across solution
IR spectroscopy	infrared spectroscopy

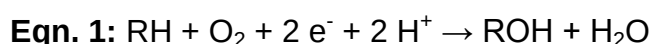
ITO	indium tin oxide
k_{cat}	turnover number [s^{-1}]
K_{M}	Michaelis constant
Mb	myoglobin
MES	2-(<i>N</i> -morpholino)ethanesulfonic acid
MET	mediated electron transfer
M_{ox}	oxidized mediator molecule
M_{red}	reduced mediator molecule
NADPH	β -nicotinamide adenine dinucleotide phosphate (reduced)
NADH	β -nicotinamide adenine dinucleotide (reduced)
NHE	normal hydrogen electrode
P450	cytochrome P450 monooxygenase
PBA	4-(3-pyrrolyl)butyric acid
PDDA	polydiallyldimethylammonium chloride
PEI	polyethylenimine
pNCA	<i>p</i> -nitrophenoxycarboxylic acid
PPA	1- <i>H</i> -pyrrole-1-propionic acid
PPy	polypyrrole
PSS	polystyrenesulfonate
Py	pyrrole
SAM	self-assembled monolayer
SCE	saturated calomel electrode
SD	standard deviation
SDS	sodium dodecyl sulfate
SEC	spectroelectrochemical cell
SPFR	specific product formation rate [$\text{U} / \mu\text{mol P450} = 60 \cdot k_{\text{cat}}$]
SPFR_{cor}	specific product formation rate, corrected for absorbance decrease
Sulfo-NHS	<i>N</i> -hydroxysulfosuccinimide
TBAP	tetrabutylammonium hexafluorophosphate
TMAFM	tapping mode atomic force microscopy
Tris	tris(hydroxymethyl)aminomethane
U	unit [$\mu\text{mol product} / \text{min}$]
UV/Vis spectroscopy	ultraviolet–visible spectroscopy
WT	P450 BM3 wild type

(all other P450 BM3 mutant abbreviations are listed in section 2.1.1.)

1. Introduction

1.1. Cytochrome P450 monooxygenases

Cytochrome P450 monooxygenases are a heme containing enzyme family with over 4500 members and can be found in almost all living organisms [1]. They are involved in biotransformation of xenobiotics and drugs, degradation of herbicides and insecticides and biosynthesis of a diverse range of physiologically important compounds, including steroids and fatty acids [2]. Thereby P450s can convert a variety of substrates like alkanes, terpenes and aromatic compounds [2]. The most prominent reaction catalyzed by P450s is the hydroxylation of unactivated C-H bonds in the form of equation 1, which cannot be achieved easily by chemical means [3]. In brief P450 catalyzed reactions feature the reductive activation of molecular oxygen and the concomitant consumption of two electrons and protons. Thereby, as the name monooxygenase implies, P450s transfer one atom of oxygen to the substrate and the second oxygen atom is consumed for water formation. Electrons are provided by the cofactor NAD(P)H. Besides alkane hydroxylations P450s catalyze several other reactions like heteroatom oxidations, dealkylations, aromatic hydroxylations, reductions and dehalogenations [2].



All P450s contain heme, or respectively iron protoporphyrin IX, as prosthetic group in their active site [4]. The heme iron is axially ligated by a thiolate of a cysteine, which is highly conserved throughout the enzyme family. The name of cytochrome P450 monooxygenases is derived from their heme protein properties, manifested in reddish color ("P" for pigment) and unusual spectral behavior. Reduced P450s exhibit a characteristic absorption band at 450 nm when carbon monoxide is bound to the iron of the active site. This spectral property is also used for P450 concentration determination [5]. The inactive P450 form, due to distortion of the heme-thiolate bond, can be seen at 420 nm giving it the name P420 [6].

Cytochrome P450 monooxygenases need electron transfer partner proteins, which transport electrons from their natural cofactor NAD(P)H to the active site of the P450 domain. Since electron transfer partner proteins differ significantly in between enzymes, P450s were divided in several classes according to the nature of their protein partners [7]. Most prominent are eukaryotic P450s of class II, which are membrane associated and need a NADPH-cytochrome P450 reductase, containing the two flavins flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), for electron transfer from NADPH. For example class II P450s can be found in the human liver where they take part in phase I metabolism of drugs. In comparison soluble bacterial P450s like P450cam use a FAD-containing reductase for interaction with NADPH and a ferredoxin, which shuttles electrons between the reductase and the P450cam heme domain.

1.1.1. Catalytic cycle

Substrate conversion by P450s follows a general catalytic cycle, which is described in the following (figure 1) [8]. In the resting state water is ligated to the heme distal site (1). Upon substrate binding water is expelled and the iron spin-state equilibrium shifts from a six coordinate low-spin to a five coordinate high-spin state. This is accompanied by a positive shift in the iron redox potential enabling electrons to be transferred. When no substrate is bound this “thermodynamic switch” prevents uncoupled electron transfer, which would result in energy waste by unproductive consumption of NAD(P)H and enzyme damage due to formation of reactive oxygen species [9]. After the first electron is transferred via the corresponding redox partner, the iron is reduced to its ferrous (+II) form (3). Next in the reaction cycle molecular oxygen is bound (4) to yield a ferrous-oxy intermediate. Now the second electron is transferred reducing the ferrous iron and yielding the ferric peroxy state (5), which is rapidly protonated to give compound 0 (6), a ferric hydroperoxo form. After O₂ bond cleavage due to a second protonation, resulting in the formation of water, compound I (7) is obtained. Compound I is a ferryl-oxo species. Concomitantly a radical porphyrin π cation is formed (7), which is supposed to be responsible for substrate oxygenation. When the hydroxylated substrate (8) is released, water is bound to yield again the resting

state (1). Besides the described reaction cycle several side reactions can occur [8]. The ferrous-oxy intermediate (4) may decompose to the ferric form (2), accompanied by the production of superoxide (a). If the substrate is resistant to the oxidative attack or not appropriately positioned in the binding pocket, side reaction (c) may happen with concomitant release of water. This can also be the case when electrons or protons are not delivered in time. Furthermore in (b) compound 0 may decompose by releasing hydrogen peroxide. In the reversed way this reaction is known as peroxide shunt pathway. Here compound 0 may be formed from the substrate bound ferric form (2) in presence of hydrogen peroxide without the need of steps (3) to (5) of the reaction cycle, including electron transfer from the redox partner [8].

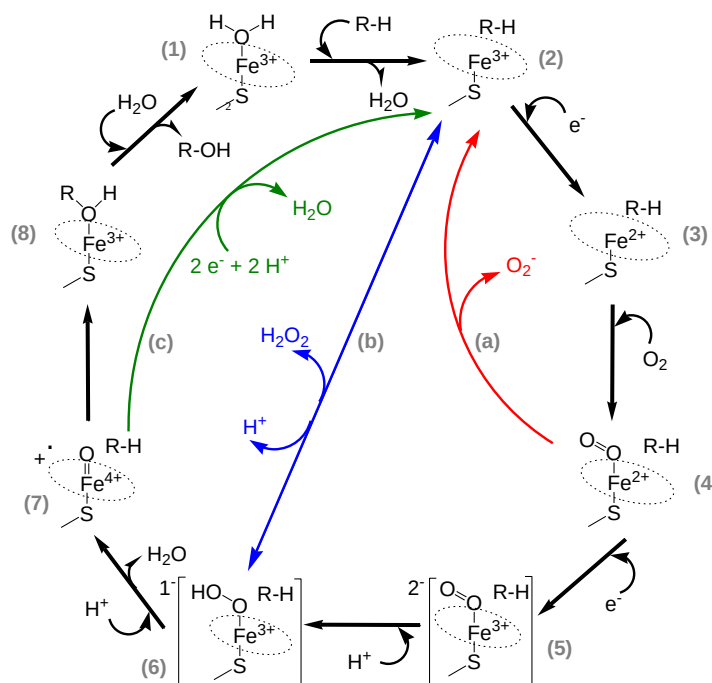


Figure 1: Scheme of the P450 catalytic cycle [8]. Dashed line represents the porphyrin system surrounding the heme iron, which is fixed to the protein backbone by a proximal thiolate ligand from a cysteine.

1.1.2. Cofactor dependency

Despite their versatility biotechnological approaches including P450s are still rare [2]. Besides low stability, activity and the need for redox partner proteins, mainly their dependency on the expensive cofactor NAD(P)H hampers use in industrial processes. NAD(P)H has to be supplemented in equimolar concentrations to

sustain reactions and is thus by far too expensive for industrial applications [10]. To circumvent the problem of cofactor dependency several approaches were investigated including *in vivo* and *in vitro* methodologies [10]. In whole cell systems the cofactor is provided by the cellular metabolism, but poor substrate uptake, product degradation and/ or toxicity and product purification are complicating the overall process [1]. Further approaches include chemical substitution, enzymatic cofactor regeneration and cofactor substitution, or respectively regeneration, by electrochemical methods [10]. Thereby electrochemistry has gained a lot of attention during the last decades since it was also used to characterize electron transfer properties and catalytic behavior of P450s [6, 10]. Moreover interest in creating electrochemical biosensors and bioreactors for environmental monitoring and for pharmaceutical industry is high [6]. Providing electrons via an electrode to drive a P450 catalyzed reaction, instead of using the expensive cofactor NAD(P)H, would reduce process costs drastically [11]. Furthermore electrochemical reactions can be operated at conditions suitable for enzymatic reactions, like ambient temperature and atmospheric pressure, and are regarded as zero effluent technology [12]. Normally product recovery is quiet simple since electrolyte composition is known and contains only few components. Electrochemical attenuation of enzymes can generally be achieved in two modes, direct or via a small redox mediator shuttling electrons between the electrode and the enzyme (figure 2). Both approaches were already employed successfully to drive P450 reactions [10].

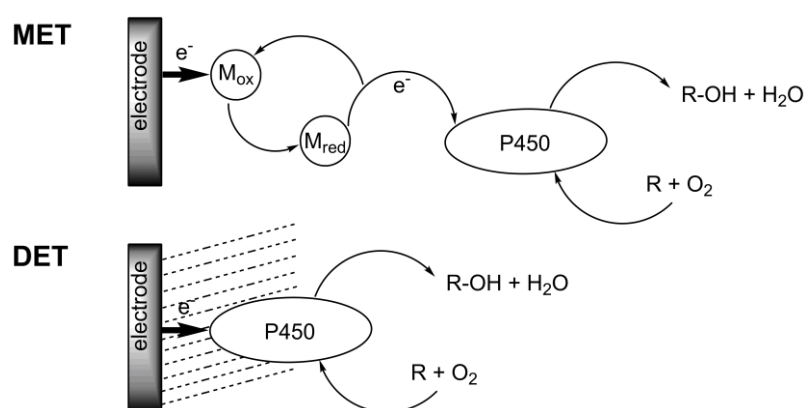


Figure 2: Scheme for mediated electron transfer (MET) and direct electron transfer (DET).
MET: Electrons are provided by an electrode to reduce a small redox active molecule, the so called mediator (M), which subsequently shuttles electrons to P450 in solution.
DET: Electrons are delivered directly to the immobilized P450 via the electrode.

1.2. The fatty acid monooxygenase P450 BM3

P450 BM3 (CYP102A1) from *Bacillus megaterium* is one of the most studied P450s and exhibits the highest catalytic activity so far reported for any P450 [9]. It naturally catalyzes the sub-terminal hydroxylation of medium and long chain fatty acids as well as fatty amides and alcohols [13]. As a fatty acid monooxygenase it needs the cofactor NADPH as electron donor for substrate hydroxylation in the form of equation 1 [8]. P450 BM3 is a self-sufficient catalytic entity of 119 kDa on one single polypeptide chain [7]. Its heme domain (BMP) is fused via a short peptide linker to its NADPH-cytochrome P450 reductase domain (BMR), which contains one FAD and one FMN as prosthetic groups [14]. Even though P450 BM3 is a soluble bacterial system, it shows high sequence homology with microsomal liver P450s of class II [7]. Therefore P450 BM3 is regarded as a model system for human P450s, notably since it can be expressed heterologously in *E. coli* and exhibits higher reaction rates than liver P450s [13]. Furthermore P450 BM3 was adapted to a variety of non-natural substrates [15]. Several amino acid positions influencing e.g. substrate specificity, binding of oxygen or FMN, influencing electron transport or respectively the state of the heme are known [9, 16-19]. For example the replacement of phenylalanine at position 87 with alanine, yielding mutant F87A, not only changes the substrate profile, but also greatly increases the hydrogen peroxide driven substrate hydroxylation of P450 BM3 and its stability towards hydrogen peroxide [20].

Hence P450 BM3 is not only interesting for biotechnological product generation but also to gain insight into general aspects of P450 function [14]. This is even more supported by the fact that P450 BM3 displays a well regulated multidomain structure, of which single domains can be expressed separately allowing to study their function [21]. In addition analytical protocols assaying the activity of the holoprotein and its single domains are known, which include reduction of cytochrome *c* by the reductase domain under NADPH consumption [22].

1.2.1. Domain organization and structural properties of P450 BM3

Substrate hydroxylation by P450 BM3 follows the general P450 catalytic cycle depicted in figure 1 [8]. Electron transfer occurs via NADPH to FAD. From FAD electrons are transferred one by one via FMN to the heme active site (figure 3) [23]. Thereby electron transfer via the reductase domain is highly efficient and does not limit the overall reaction [23]. Interestingly it was shown that P450 BM3 exists in a dimeric form in solution and that inter-subunit electron transfer in between reductase and heme domain (FMN₁-to-heme₂) takes place in fatty acid hydroxylation by the dimer [24].

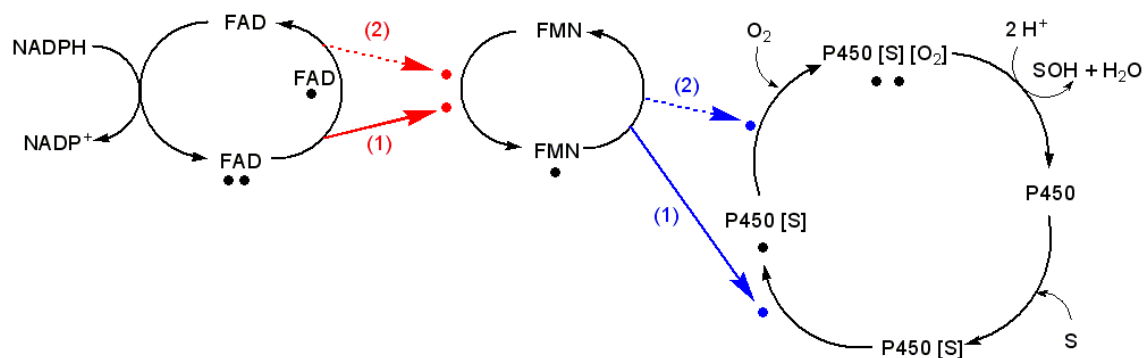


Figure 3: Scheme of electron transfer in P450 BM3 during catalysis [23]. Electrons are derived from NADPH (hydride transfer) to FAD. From FAD electrons are delivered to FMN (red), which transports electrons one by one to the heme active site (blue). (1) and (2) thereby indicates first and second electron transfer.

The midpoint redox potential for the FAD domain is -303 mV versus a normal hydrogen electrode (NHE), and therefore in the range for electron transfer from NADPH (-320 mV), for the FMN domain it is -192 mV [25]. According to the “thermodynamic switch” described above the midpoint reduction potential of the first electron transfer to heme in P450 BM3, in absence of substrate, equals -368 mV and is thus lower than the corresponding diflavin domain potentials and therefore prevents electron transfer from the reductase to take place [25]. Upon substrate binding the redox potential of the heme shifts to higher values, e.g. -265 mV for palmitate, enabling electron transfer from the reductase to occur [25]. Thereby the shift in potential upon substrate binding is dependent on the nature of the substrate [25]. Nevertheless it has been shown that the shift in spin-state is

presumably the trigger for electron transfer from the reductase to occur, rather than the positive shift in the heme reduction potential [16]. In presence of high NADPH amounts a decrease in electron transfer rates is seen [9, 23]. Like the “thermodynamic switch” this effect is related to prevention of futile electron cycling, which would result in production of reactive oxygen species damaging the enzyme and waste of energy [9]. The observed inhibition of substrate conversion at high NADPH concentrations is assumed to be related to an over-reduction of the FMN component, which might be inefficient in supplying electrons to the heme or might induce a conformational change of the protein [17].

Furthermore it is assumed that electron transfer from the flavin reductase domain to the heme domain is accompanied by a hinge bending motion [9]. A linker region in between the FAD and FMN domain enables the FMN domain to move in between FAD and heme as to reduce distances for electron transfer, which may also serve as a timing mechanism [9]. Thereby the FMN binding domain is located at the proximal side of the heme, which is closest to the surface [26], and orientated in direction of the heme cysteinate ligand [9, 27]. Also some conformational change upon reduction, moving the substrate in closer proximity to the heme, is known to occur.

The atomic structure of the P450 BM3 holoprotein is still not completely resolved [14, 17]. Nevertheless some information was obtained for BMP and BMP combined to FMN. Thereby the heme domain represents a triangular prism shape with the heme moiety embedded in the hydrophobic pocket inaccessible from the bulk solvent [26]. Modeling of the heme domain of P450 BM3 F87A revealed that the protein is slightly negatively charged at pH 7, due to its pI of 5.4. In addition at this pH the protein exposes a small positive patch close to the reductase binding site, which is able to influence binding of the protein to negatively charged surfaces [28]. With increasing pH the overall charge is further decreasing, with concomitant extinction of the positive patch. In contrast for more acidic pHs the overall charge is increasing to neutral [28].

1.3. P450 BM3 as model system for bioelectrochemical studies

Like for other P450s interest in regenerating or substituting the natural cofactor NADPH to reduce costs for enzymatic processes with P450 BM3 is high. Liberation from the need for NADPH in P450 BM3 reactions was already successfully achieved by establishing whole cell processes, chemical reduction of the P450 [29] or enzymatic cofactor regeneration methods [30]. Also the peroxide shunt pathway was used to drive fatty acid hydroxylation with P450 BM3, but the idea suffered from low rates and protein denaturation [28, 31, 32]. Hence electrochemical methods for artificial electron transfer to P450 BM3 are of high interest, since current is a rather low cost driving force compared to the natural cofactor NADPH [11]. Furthermore reaction needs are less complex than in whole cell approaches or in case of a coupled secondary enzyme reaction for cofactor regeneration. Therefore approaches in which P450 BM3 was electrochemically addressed are presented below.

1.3.1. Mediated electron transfer

Artificial electron transfer to P450s can be achieved via an indirect way using small redox molecules, so called mediators, shuttling electrons between the electrode and the enzyme [33]. This has been successfully applied to ω (-) - hydroxylations of lauric acid via the mediator cobalt sepulchrate (CoSep) in an electrochemical cell. CoSep exhibits a midpoint potential of -350 mV vs. NHE and is reduced by the electrode from its Co^{3+} to its Co^{2+} form [33]. Under control of the oxygen level and with a polarizing potential of around -453 mV vs. NHE in an electrolysis experiment a conversion rate of 110 nmol per min and per nmol P450 was obtained, which was around 12 % of the rate achieved with NADPH [34, 35]. It was also shown that by using CoSep in an electrolysis experiment electrons were transferred to the heme domain of P450 BM3 in the absence of substrate, which is not observed in the natural electron transfer pathway starting from NADPH. Furthermore electron transfer to the heme domain was achieved resulting in substrate conversion [36]. The resulting product profiles did not differ significantly in between use of the holoprotein or heme domain. Furthermore

1,1'-dicarboxycobaltocene was employed, which showed higher water solubility than CoSep [36]. In experiments with lauric acid hydroxylation rates and total turnover were lower than with CoSep, but again electron transfer to the heme domain as well as the holoprotein was possible resulting in product formation. The authors also determined coupling efficiencies between charge consumed and product formed to be less than 2 %, which is related to the high oxygen sensitivity of the mediator. The reduced mediator is oxidized by interaction with molecular oxygen and hydrogen peroxide is produced as byproduct, reducing the overall current yield. To prevent product formation by the hydrogen peroxide shunt pathway the authors used catalase in the electrolysis system.

Substrate hydroxylation via CoSep was also shown for P450 BM3 without the use of an electrode as driving force. The zinc driven pNCA assay with P450 BM3 establishes the mediator cobalt sepulchrate in combination with zinc dust as electron source [37]. The pNCA assay is a photometrical assay determining activity of P450 BM3, where the enzyme catalyses the conversion of *p*-nitrophenoxydodecanoic acid (12-pNCA) to ω -hydroxydodecanoic acid and the chromophore *p*-nitrophenolate [38]. P450 BM3 F87A achieved 20 % of the activity it exhibits with NADPH when operated with the zinc/ CoSep system and shows a turnover of 125 nmol substrate per nmol P450 and per min [37].

Based on the zinc/ CoSep driven pNCA assay a medium-throughput screening in a 96 well plate was developed [39]. By screening a saturation mutagenesis library at position R47 mutants of P450 BM3 F87A with increased catalytic efficiencies with the artificial electron donor system compared to NADPH were identified [39]. Also a directed evolution experiment was conducted with this medium throughput screening to engineer P450 BM3 towards cost-effective process conditions *in vitro* [40]. As starting point mutant P450 BM3 F87A was used and a mutant was found with improved kinetic parameters in the zinc CoSep assay. In this regard also heme domains were tested, which are not active with NADPH. Experiments confirmed that the reductase domain in P450 BM3 is not needed for electron transfer from CoSep, but values for k_{cat} and the catalytic efficiency were slightly reduced compared to the holoprotein.

For the bacterial P450cam it was shown that its electron transfer partner protein putidaredoxin can serve as natural mediator [41]. Putidaredoxin was reduced at an electrode surface and shuttled electrons to P450cam. The same cannot be expected for P450 BM3. Besides structural differences in the electron transfer partner proteins, the reductase and heme domain of P450 BM3 do not show high affinity for each other when expressed separately [17].

1.3.2. Direct electron transfer

Often direct electron transfer is sought to be established preventing the use of additional molecules in solution, which are often heavy metal complexes of certain toxicity and make product purification more complex. Since the heme of P450s is usually deeply buried inside the insulating protein shell and naturally futile electron cycling is prevented by several control mechanisms, it is not easy to achieve direct electron transfer [33]. In addition often proteins are denatured rather fast at metal electrodes making a more biocompatible surface modification necessary [33]. To be successful in achieving direct electron transfer, orientation of the protein is one key issues of immobilization. For direct electron transfer the protein has to be located close to the electrode surface while substrate access and product release should not be hindered by the immobilization [42]. Protein conformation should not be altered and some residual flexibility is needed. At best the interaction site for electron transfer of the protein should be orientated towards the electrode. The direct electron transfer process is theoretically described by Marcus theory, which emphasizes the importance of the distance between the involved redox centers, the reorganization energy and the driving force, namely the applied potential [43].

Several immobilization matrices were used to fulfill these requirements (figure 4 and table 1). Prominent examples are surfactant films, which consist of an ionic headgroup and a non-charged, hydrophobic tail [44]. Surfactants are naturally non-water soluble and form ordered multilayer films on the electrode, which are similar to biological membranes [45]. Another option for surface modification are polyelectrolytes, which are electrostatically adsorbed to surfaces due to their charged polymer moieties [46]. A common tool for electrode coatings are

self-assembled monolayers (SAM). The most prominent example are probably alkanethiols, which strongly chemisorb on gold surfaces forming a monolayer coverage [47]. In addition conductive polymers like polypyrrole are often introduced for biomolecule immobilization, since they can be electropolymerized in their conductive state directly on the electrode surface [48-50].

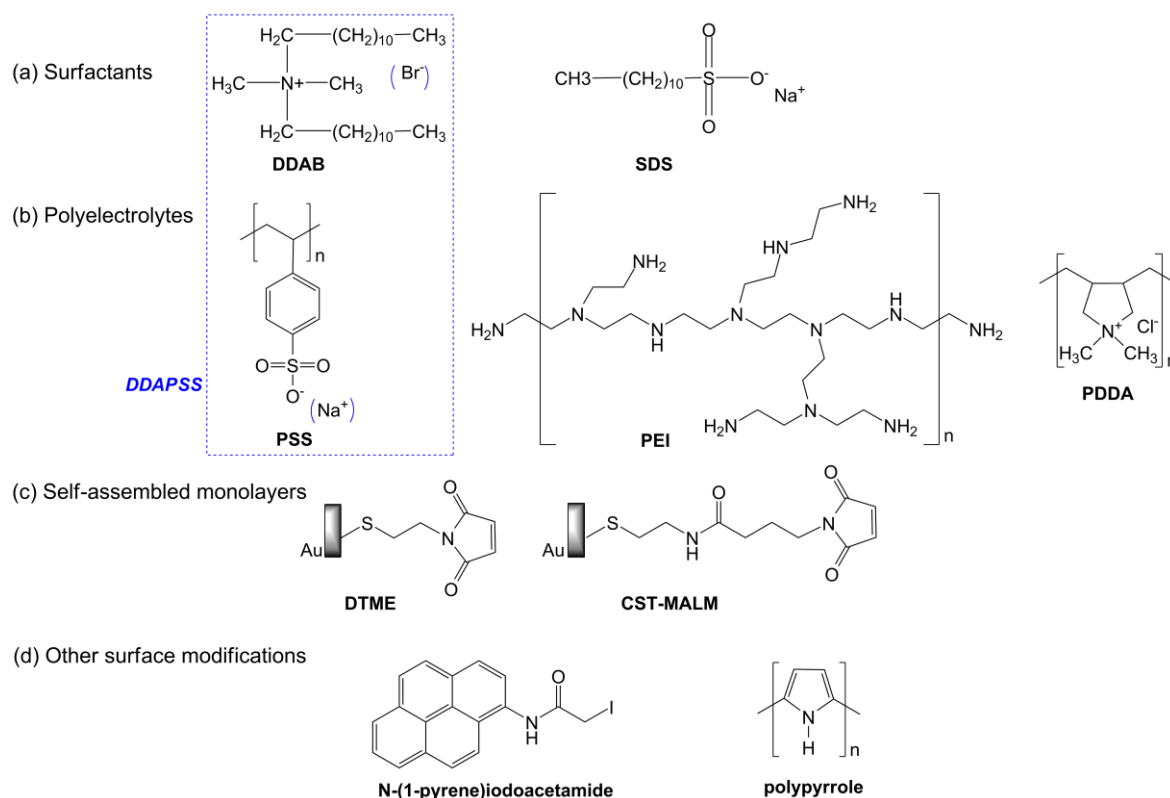


Figure 4: Electrode surface modifications used for the immobilization of P450 BM3. DDAB: didodecyldimethylammonium bromide [51, 52]; SDS: sodium dodecyl sulfate [53]; PSS: polystyrenesulfonate; DDAPSS: DDAB in combination with PSS [52]; PEI: polyethylenimine [45]; PDDA: polydiallyldimethylammonium chloride [6, 54]; DTME: dithio-bismaleimidoethane [55]; CST-MALM: cystamine-N-succinimidyl 3-maleimidopropionate [55]. The self-assembled monolayer systems are displayed as final assembly on the gold electrode, since they are produced in a multi-step procedure via several intermediate stages (not shown).

1.3.2.1. Surfactant films

One of the first reports on direct electron transfer of P450 BM3 was reported by Fleming et al. [51]. The surfactant didodecyldimethylammonium bromide (DDAB) (figure 4) was casted on edge plane graphite electrodes and afterwards the modified electrode was incubated with P450 BM3 or respectively its heme or reductase domain. With this method it was possible to electrochemically contact both, the reductase and the heme domain, directly via the modified electrode (table 1). Immobilization of the single domains showed that midpoint peak potentials did not shift strongly when domains were tested separately. In cyclic voltammograms the heme domain redox couple showed reversible behavior with current depending linear on the scan rate. The amount of electroactive heme domain was found to be in the nmol per cm² range. The authors stated that electron transfer rates were fast and comparable to the natural system with NADPH (table 1). The midpoint potential exhibited a pH dependency and a positive shift in potential was obtained upon CO binding. Interestingly several substrates were tested but no peak shift was found upon substrate binding. In addition the heme redox peaks were sensitive to oxygen.

Even though by immobilizing P450s in surfactants high electron transfer rates were achieved, catalytic turnover was mostly poor [56]. In addition redox potential shifts upon CO binding were rather small [56]. Udit et al. showed that these effects were most probably related to a perturbed protein conformation in the film. The authors assumed that protein structure around the heme was prone to collapse. In the surfactant film dehydration of the heme occurs with concomitant tightening of the hydrogen bonding network, resulting in increased amounts of the biologically inactive P420 form [56].

Another surfactant tested for the immobilization of the P450 BM3 heme domain (BMP) was sodium dodecyl sulfate (SDS) (figure 4), which was applied on basal-plane graphite electrodes [53]. Reversible redox peaks were obtained in cyclic voltammetry experiments (table 1). The surface coverage of electroactive BMP was found to be 2×10^{-10} mol cm⁻², which relates to 33 % of the total amount

of protein used for immobilization. Furthermore, catalytic reduction of molecular oxygen was monitored. Electrolysis experiments with SDS immobilized BM3 holoprotein were performed with the substrates lauric acid and styrene, but no substrate conversion was detected. Since SDS itself can serve as substrate for BM3 and binding to the heme was shown, it was assumed that SDS might be blocking the heme. But nevertheless, also no SDS oxidation product was detected. Finally the authors concluded that polarizing the negatively charged SDS film at a negative voltage led to complete film desorption.

1.3.2.2. Surfactant / polyelectrolyte combination

Heme domains of wild type P450 BM3 and a mutant named 1-12G were incorporated in the surfactant polyelectrolyte film didodecyldimethylammonium polystyrenesulfonate (DDAPSS) (figure 4) on basal plane graphite electrodes [52]. In cyclic voltammetry reversible redox peaks were obtained (table 1), which displayed characteristics of thin-film voltammetry at varied scan rates. Both proteins differed in the electron transfer rates by a factor of eight, even though midpoint potentials were similar. The authors related this effect to different heme environments, for which they also saw indications in the absorption spectra of the two proteins. Since DDAPSS films possess mechanical stability the authors used a rotating disc electrode for electrolysis experiments reducing molecular oxygen. Thereby both mutants showed differences in their reaction rate around a factor of 14 [52].

Table 1: Electrochemical studies with P450 BM3, which was in solution or immobilized at the electrode.

P450 BM3 domain		E _{mp} vs. NHE [mV]	electron transfer rate [s ⁻¹]	test for catalytic activity	Ref.	
P450 BM3 in solution						
	diflavin FAD	-332			[25]	
	diflavin FMN	-203				
	BMP	-368				
	BMP + S _{palmitate}	-265				
	BMP + S _{arachidonate}	-239				
	holoprotein + S _{myristate}		223		[23]	
	holoprotein + S _{laurate}		130			
Immobilized P450 BM3						
Immobilization matrix						
DDAB	Surfactant film	holoprotein	-147* and -9*	138	[51]	
		BMP	-3*	221		
		BMP + CO	+47*			
		BMP + S _{lauric acid, palmitic acid or octane}	-3*			
		BMR	-164*			
DDAB	Surfactant film	BMP	-19*		[56]	
		BMP + imidazole	-6*			
SDS		BMP	-133**	10	dioxygen reduction	[53]
		holoprotein			No conversion with lauric acid + styrene. No SDS oxidation.	
DDAPSS		BMP	-0.195	250	dioxygen reduction	[52]
		BMP of mutant 1-12G	-0.202	30		
PEI	Polyelectrolyte	n.d.	-269*		dioxygen reduction	[45]
PDDA		BMP	-167	> 221		[54]
		BMP + CO	-97			
		BMP + S _{p-nitrophenol}	-167		p-nitrophenol to p-nitrocatechol	
		flavodoxin + BMP	-376 and -130	<< BMP rate		
		flavodoxin + BMP + CO	-376 and -80			
	flavodoxin + BMP + S _{p-nitrophenol}	-376 and -130		p-nitrophenol to p-nitrocatechol		
pyrene		BMP	-143**	650 ± 50	dioxygen reduction	[57]
		BMP + CO	-108**			
polypyrrole		holoprotein	n.d.	n.d.	12-pNCA to p-nitrophenolate	[58]

For comparison potentials were converted for reference electrodes other than NHE [59]: * original data were given vs. SCE; ** original data were given vs. Ag/AgCl. DDAPSS is a combination of surfactant film and polyelectrolyte technology; pyrene moieties are directly grafted on a carbon electrode and polypyrrole belongs to the group of conductive polymers. Abbreviations are explained in the text.

1.3.2.3. Polyelectrolytes

Besides surfactants also the polyelectrolyte polyethyleneimine (PEI) (figure 4) was used for a layer-by-layer immobilization of P450 BM3 [45]. Thereby the positively charged PEI was adsorbed to the electrode followed by the adsorption of the negatively charged protein [45]. Film deposition was followed by scanning electron microscopy. In cyclic voltammetry experiments redox peaks were obtained (table 1). Thereby only 5 % of the protein consumed for film deposition showed electrochemical activity. Furthermore catalytic current for oxygen reduction was obtained.

In another study PEI was used to immobilize BMP on tin-oxide electrodes for cyclic voltammetry and concomitant optical measurements [6]. In addition to PEI also the polycation polydiallyldimethylammonium chloride (PDDA) was used (figure 4). For both films electron transfer reducing the heme iron was achieved, since a spectral shift upon CO binding was observed. Thereby for the PDDA film the absorbance maximum recorded at 450 nm represented active P450. For the PEI film an absorbance maximum at 420 nm was obtained, which indicated the formation of catalytically inactive P450. The inactivation of P450 was supposed to be due to dehydration of the substrate binding cavity, decrease of the electron donor strength of the heme axial ligand or by interference of the H-bonding network [6]. The authors assumed that the hydrogen bonding network of the protein is affected in PEI films, since PEI contains primary and internal amino groups that are capable of forming H-bonds with the protein. In contrast PDDA possesses quaternary ammonium groups which are positively charged throughout a wide pH range and cannot form hydrogen bonds with the protein. Kinetics of electron transfer depended on the applied potential and increased with an increase in reductive current. From cyclic voltammetry experiments the authors concluded that film properties were influencing the redox potential, but not the nature of the underlying metal electrode. In combination with optical measurements the authors could show that obtained reduction peaks can be a mixture of two contributions, namely the catalytically active P450 and the inactive P420 form.

In another approach P450 BM3 components were used to build artificial multi-domain redox proteins with tailored redox properties [27, 60, 61]. The authors call this approach “molecular Lego”. BMP was fused to the flavodoxin of *Desulfovibrio vulgaris*, which is homologue to the FMN binding domain of P450 BM3 [61]. Functionality of the fusion protein was proven and it showed improved electron transfer properties when compared to the natural BMP [27]. Furthermore BMP was immobilized at a glassy carbon electrode via the polyelectrolyte PDDA (figure 4) [54]. A reversible redox couple was obtained and electron transfer was assumed to be fast (table 1). The midpoint potential shifted in presence of CO, while upon substrate binding no peak shift was observed. The experiment was repeated for the flavodoxin BMP fusion protein. In cyclic voltammograms two redox peaks were obtained (table 1). Both peaks exhibited low electron transfer rates compared to BMP alone. By addition of the substrate *p*-nitrophenol no peak shifts were seen. Upon CO binding the second redox couple shifted in the positive direction (table 1). Furthermore for the first time electrochemically driven substrate conversion was shown for the immobilized BMP flavodoxin fusion, namely the conversion of *p*-nitrophenol to *p*-nitrocatechol. Interestingly for BMP alone six times less product was formed, even though the electron transfer rate constant was high.

1.3.2.4. Entrapment in conductive polymers

The entrapment of P450 BM3 in the conductive polymer polypyrrole (figure 4) during electropolymerization was shown [58]. Electropolymerization conditions applied led to a polymer film of 0.5 μm thickness. The embedded enzyme proved to be active in the pNCA assay with NADPH. Furthermore in the absence of NADPH, when cycling the potential in between -600 to +600 mV vs. Ag/AgCl at a scan rate of 100 mV/s, product formation was seen. Thereby reaction rates depended on the electrode material used. While for a platinum electrode with a P450 BM3/ polypyrrole layer electrochemical reaction conditions yielded a product formation rate of $0.66 \mu\text{M h}^{-1} \text{cm}^{-2}$, for glassy carbon it was only $0.13 \mu\text{M h}^{-1} \text{cm}^{-2}$ (table 1). The authors assumed that the difference may be due to different morphologies of the polymer film. Furthermore the protein may interact with

functional groups on the surface of glassy carbon or molecular oxygen may be reduced to hydrogen peroxide, which is more likely to happen at glassy carbon than platinum. In general product formation rates with NADPH were higher than with the electrochemically driven system ($3.25 \mu\text{M h}^{-1} \text{cm}^{-2}$ for platinum and $0.15 \mu\text{M h}^{-1} \text{cm}^{-2}$ for the glassy carbon electrode).

1.3.2.5. Covalent binding to self-assembled monolayer (SAM)

A self-assembled monolayer system displaying maleimide as reactive head group was used by Ferrero et al. [55]. The heme domain of P450 BM3 was engineered to expose one of two distinct surface cysteines (C62 or C156), which can bind covalently to the maleimide head group due to their thiol moiety [55]. The SAMs established were cystamine-N-succinimidyl 3-maleimidopropionate (CST-MALM) and dithio-bismaleimidoethane (DTME) and differ in their length as well as thiol binding site to the gold electrode (figure 4). By tapping mode atomic force microscopy (TMAFM) authors showed that for the unmodified electrode the protein was strongly interacting with the surface, which resulted in conformational changes and most probably partial unfolding. Besides covalent attachment also electrostatic interactions seemed to bind the protein. By using the monolayer the protein was protected from direct contact with the electrode and furthermore enough flexibility for P450 was left for a correct protein fold. For BMP without exposed cysteines no binding was seen to both SAM surfaces, also indicating that the SAM prevents non specific interactions with the gold surface. In cyclic voltammetry at bare gold electrodes for both mutants no electrochemical response was obtained. On a CST-MALM and DTME modified electrodes redox couples for WT and C156S were obtained (table 1). Peak current was linearly increasing with scan rate, which is typical for a surface confined process. In addition peak separation was independent of scan-rate, but values indicated slow electron transfer.

1.3.2.6. Covalent attachment via pyrene moieties

The heme domain of P450 BM3 was covalently attached to a basal plane graphite electrode via pyrene (figure 4) [57]. The pyrene moiety was bound to a certain surface exposed amino acid resulting in a defined protein orientation. The side of the heme domain normally connected to the reductase unit faced the electrode, while the substrate access channel was facing away to the bulk solution. In cyclic voltammetry experiments a reversible redox peak was obtained (table 1). Similar to the DDAB immobilization a positive shift in potential was seen compared to the enzyme in solution, which the authors related to local electrostatic effects on the protein upon immobilization. The midpoint potential was shifting with pH, indicating proton coupled electron transfer. It was confirmed that a one electron transfer takes place and a surface confined species was present, since plots of peak current versus scan rate were linear. The authors achieved submonolayer coverage (44 %), which was determined by integrating peaks in cyclic voltammetry and was further supported by atomic force microscopy images. The electron transfer rate constant was higher than for P450 BM3 immobilized via DDAB (table 1). Furthermore in electrolysis experiments at -500 mV the authors showed that dioxygen was efficiently reduced by the immobilized heme domain, yielding predominantly water and not hydrogen peroxide.

1.3.3. P450 BM3 fusion proteins

Further interesting approaches include the covalent attachment of photoactivable ruthenium groups to the heme domain of P450 BM3 [26]. By chemical modification of the protein with electroactive relay groups the insulating properties of the protein shell can be altered [33]. By modifying P450 BM3 in this way light induced electron transfer from the ruthenium group to the active site iron was obtained in the presence and absence of substrate.

In another study the subdomains of P450 BM3 were genetically fused to a self-assembling protein, named HaloTag, by Erkelenz et al. [22]. After expression the BM3 domains were covalently bound to a DNA oligonucleotide via the

HaloTag, which resulted in reduced enzymatic activity. Nevertheless out of both domains functional holoprotein could be assembled by DNA hybridization, as proven by the pNCA assay in presence of NADPH. Even though no electrochemical experiment was conducted the approach is of interest, since several examples for modification of electrodes with DNA exist.

1.4. Thesis outline and objectives

1.4.1. Development of an electrochemical microtiter plate for P450 screening

Time-to-market pressure in the chemical and pharmaceutical industries forces engineers to develop productive tools for synthesis and process development. Combinatorial screening and high throughput technologies are considered as core technologies in all major pharmaceutical companies [62]. The majority of high throughput concepts were developed in the pharmaceutical and medical field, while it is now extending to material sciences [63]. Basic interests focus on miniaturization, automation and parallel processing of small amounts of material. Most frequently used screening technologies comprise optical and mass spectrometry methodologies [62]. Signal-to-noise ratio, cross-talking and accuracy should be as low as possible, thereby it is annotated that generally deviations below 10 % are sufficiently low for screening experiments [62]. Also work flow should be standardized and scientific data management systems are needed. In traditional approaches a linear and sequential proceeding was followed. But with increasing complexity and multi-dimensional problems this methodology is not feasible anymore, giving rise to combinatorial approaches [63]. The advantage of electrochemistry is that it can perform both, synthesis of material as well as its characterization [63]. Material science approaches in combinatorial electrochemistry focus on miniaturization combined with material analysis techniques like mass spectrometry, nuclear magnetic resonance spectroscopy, gas chromatography or several scanning probe techniques [63]. Miniaturization is for example achieved by creating spatially addressable (micro)arrays [64], screen printed electrodes and microfluidic systems. While optical approaches like fluorescence [65] and IR spectroscopy can be found at times, high throughput electrochemistry is rarely combined with UV/Vis spectroscopy [63]. The reason might be that information gained include absorbance properties and molecule conjugation [63], which both do not contribute significantly to material characterization. In contrast electrochemistry in microtiter plates, which can be combined with parallel optical measurements, would be of high interest for bioelectrochemistry.

1.4.1.1. State of the art: Electrochemistry in microtiter plates

A first parallelized system for electrosynthesis has been developed by Siu et al. [66-69]. The system consists of 4 x 4 wells each equipped with a counter and working electrode in which the latter were connected parallel. Siu et al. used their set-up in galvanostatic mode for the parallel anodic oxidation of carbamates, amides and sulfonamides [67] as well as for the evaluation of the catalytic properties of synthesized bithiophene and pyrrole based copolymers [66]. Commercially available microtiter plates were used for several material screening approaches by electrochemical means, mainly using electrode bundles which are moved from well to well by a robotic system. Examples are the screening of quarter-3-arylthiophenes [70], ruthenium complexes for their use as hydrogenation catalysts [71], the electrosynthesis of libraries of iminoquinol ethers and triazolo-pyridinium ions [72] or the electroensing of ascorbic acid [73]. One example for this approach is the fully automatic electrochemical robotic system designed for the screening of a library of electrodeposition paints towards their use as immobilization matrix in an amperometric glucose biosensor and for the screening of a ruthenium complex library [74, 75]. The system consists of a height adjustable three electrode bundle which can be inserted in one well of a 24 well microtiter plate by moving the plate underneath the electrode holder. In addition the automatic system allows for stirring, the addition of reagents, removing of solutions and maintenance of inert gas atmosphere while several electroanalytical techniques can be applied. Furthermore microtiter plates in which each well represents its own set of electrodes are known. Reiter et al. [76] used a three-electrode-system in which all counter and reference electrodes were short connected to the potentiostat for the optimization of glucose dehydrogenase and glucose oxidase labeling with ruthenium complexes [76]. The system enabled sequential-parallel electroanalysis, e.g. via cyclic voltammetry and differential pulse voltammetry. Tang et al. employed an electrochemical multichannel detector in which eight two-electrode-systems were positioned in one row of a microtiter plate for parallel amperometric detection in an immunoassay [77]. Sparkes et al. [78] described a sensor array for measuring 48 samples simultaneously in an amperometrical experiment with a two electrode system per well. Electrodes,

which were attached to a circuit board, were immersed into a commercially available microtiter plate from the top and data were recorded via a high-speed acquisition system [78]. Also in 48 wells a two electrode biamperometric system was established for amperometric or coulometric analyte detection, in which the electrodes were immersed from the top as well [79]. The system was used for the quantification of oxidizable species like ascorbic acid and enzymatic assays, including glucose sensing by glucose oxidase [79]. An immunosensor array, based on a 96 well plate equipped with screen printed electrodes, was used for the detection of aflatoxin B₁ in combination with ELISA tests [80]. Recently a 96 well microtiter plate based electrochemical system for the detection of drug binding constants to human P450s was developed [81]. P450s were covalently bound via a self-assembled monolayer to the gold working electrode, which was positioned at the bottom of the well. Besides the working electrode each well contained a carbon counter and silver/silver chloride working electrode connected to an eight channel multi-potentiostat. The authors claim that their disposable array should cost less than 10 US\$ per unit, and therefore electrodes were constructed by sputtering and screen printing methodologies. The system was used to determine K_M values for certain drugs by measuring catalytic currents at different drug concentrations. Electrode arrays in microtiter plates were furthermore used to sense the influence of toxicants on cell growth, e.g. by impedance measurements [82]. Similarly the influence of antimicrobial agents on bacteria in clinical samples or polyphenols on cancerous cells was tested with an electrochemical 96 well system named DOX-96 [83, 84]. The DOX-96 system allows monitoring of cell viability by measuring the consumption of molecular oxygen due to cell respiration and consists of three disposable electrodes on the bottom of each well of a 96 well microtiter plate. The DOX-96 system was also used for bacteria detection and classification [85, 86].

1.4.1.2. Need for an electrochemical microtiter plate (eMTP)

Nevertheless approaches combining electrochemical measurements in microtiter plates with simultaneous photometrical measurements have not been described in literature yet. Optical methods are of main importance in medicine and biotechnology since they are used to detect e.g. enzyme activity, production of biomass or redox states of mediators. Bioelectrochemistry itself is of growing interest and examples for its industrial applications in chemical synthesis are the regeneration and substitution of expensive cofactors [87-90] as well as in bioelectrocatalysis for the synthesis of fine and bulk chemicals [91-93]. For the further development of bioelectrochemical processes towards industrial applications a technology is needed to screen in parallel reaction parameters. Acceleration of research can be achieved by running parallel and combinatorial experiments [63]. Starting in the field of chemistry and material sciences combinatorial screening approaches are recognized to have potential use for biomaterial discovery [94]. Such a technology could be used to find suitable mediators for efficient electron transfer to enzymes, to optimize the direct electron transfer between an electrode and an enzyme or to find optimal cofactor regeneration conditions. Furthermore combinatorial approaches in enzyme engineering rely on a screening strategy that is able to select mutants with desired properties from a large library in an efficient and easy way [60]. Thereby the eMTP allows a combinatorial approach, which means several reaction parameters can be screened in parallel, and is of high interest for enzyme screening, since enzyme behavior in an electrochemically driven reaction cannot be predicted by theoretical means alone [64]. The eMTP may therefore be helpful towards the engineering of P450 mutants with improved electronic actuation properties like it was shown for the zinc CoSep system.

In this work an electrochemical microtiter plate should be developed, which enables parallel electrochemistry in a conventional microtiter plate with simultaneous optical data acquisition. The functionality of the developed plate was tested in combination with several well-known redox active species, like potassium ferrocyanide and ABTS. In terms of combinatorial chemistry the eMTP serves as

secondary screening tool, enabling medium-throughput evaluation on the basis of a rational approach along defined parameters [95]. Thereby target of secondary screening is the optimization of already existing processes, while primary screening focuses on the discovery of new materials in high-throughput [95]. Accordingly a P450 BM3 mutant library, which was optimized towards the zinc driven pNCA assay via the mediator cobalt sepulchrane, was tested in the eMTP towards its performance in an electrochemically driven reaction.

1.4.2. Direct electron transfer to P450 BM3: Development of a screening protocol based on polypyrrole

For biocatalytic applications the immobilization of the biocatalyst offers several advantages, which include reusability, improvement of enzyme stability, possible exposure to a range of reaction media and conditions as well as high substrate concentrations [96, 97]. In addition for bioelectrochemical process design it would be of high interest to spare the use of soluble mediators, which are often compounds of certain toxicity and complicate downstream processing. An alternative to mediated electron transfer is the direct electron transfer from the electrode to the enzyme active site. As described by electron transfer theory the electron transfer rate is mainly dependent on the distance between redox sites involved [43]. Further influencing factors are the structural rigidity of the involved compounds as well as the difference in potential between both redox sites [98]. Due to slow protein diffusion and low electron transfer rates direct protein electrochemistry at unmodified electrode surfaces is rather hard to achieve [99]. Furthermore exposing proteins directly to unmodified electrode surfaces often leads to adsorption and protein denaturation, which can be prevented by modifying the electrode surface with a biocompatible matrix [99]. Over the years many strategies for electrode surface modifications for direct contacting of the biocatalyst were developed [100-102], mainly in the field of biosensors.

1.4.2.1. The conductive polymer polypyrrole in bioelectrochemistry

Polypyrrole belongs to the group of conductive polymers. Conductive polymers are of interest since they combine properties of organic polymers with the conductivity of semiconductors [103, 104], which also brought them the designation as “soft metals”. Besides being of low cost also their easy processing makes them attractive materials for a vast area of applications [105]. Compared to other conductive polymers polypyrrole is of special interest since its monomer is easily oxidized, polymerization can be done in aqueous media and the monomer and several derivatives thereof are commercially available. In addition polypyrrole is stable in air [106] and regarded as biocompatible [104, 107, 108]. Furthermore

polypyrrole exposes good redox properties with conductivities around 10^4 S m^{-1} . Since polypyrrole can be reversibly switched between different redox states the polymer is an interesting material for bioelectrochemistry [109]. For example polypyrrole is applied as drug delivery system and as transducer in electrochemical biosensors, where it also serves as immobilization matrix for the sensing biomolecules. In amperometric biosensors polypyrrole was used for the immobilization of glucose oxidase, cholesterol oxidase, horseradish peroxidase, hemoglobin, lactate oxidase and urease as well as DNA for the sensing of hybridization processes [48, 50, 98, 109-111]. In most approaches the biomolecule is added to the polymerization solution resulting in entrapment of the protein in the growing polymer film in random orientation. In addition several other techniques were developed, e.g. for the covalent attachment of proteins to the polymer surface [48]. Direct electron transfer to entrapped biomolecules via polypyrrole was already shown for several redox proteins like cytochrome c [112], glucose oxidase [113], hemoglobin [114], NADH dehydrogenase [115] and quinohemoprotein alcohol dehydrogenase [116].

For further information on polypyrrole, its polymerization process and electronic properties see the interposed box below.

Infobox**A short introduction to the conductive polymer polypyrrole**

Besides polypyrrole other prominent conductive polymers are polyacetylene, polyaniline and polythiophene (figure 5). Conductive polymers find applications as corrosion inhibitors, compact capacitors, antistatic coatings, electromagnetic shielding of computers as well as transistors, light-emitting diodes and solar cells [105, 106].

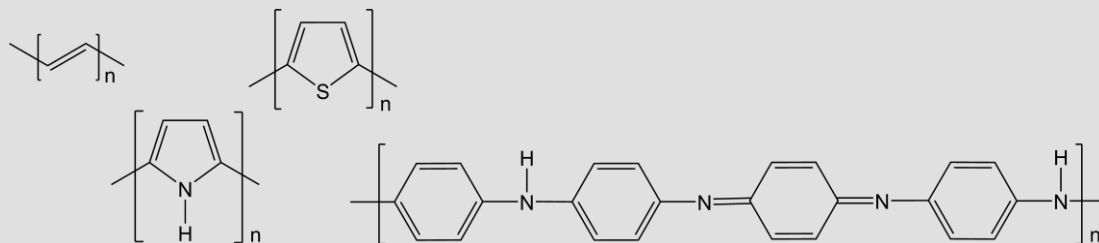


Figure 5: Chemical structures of conductive polymers (from left to right): polyacetylene, polypyrrole, polythiophene and polyaniline [104].

Polypyrrole can be formed by chemical or electrochemical polymerization of its monomer pyrrole (figure 6). Electropolymerization yields an inherently conductive polymer film which directly forms at the electrode surface. Thereby polymer properties like morphology, conductivity and mechanic stability strongly depend on the polymerization conditions chosen [104]. Like in other conductive polymers, conductivity of polypyrrole is based on the conjugated π -electron system in the polymer backbone. By electrochemical oxidation of the monomer a positive charge is introduced at every third to fourth pyrrole unit of the resulting polymer [103], named as “hole” in reference to semiconductors [106]. The polymer is in its conductive p-doped state (figure 7). The positive charge carriers in form of a radical cation are called polaron, or bipolaron if a dication was produced. The hole can be filled by electrons provided by the delocalized π -electron system creating a new hole and so on, resulting in charge transport over long distances [106]. Thereby charges are transported along one polymer chain and in between adjacent chains through an electron hopping mechanism. Charging of the polymer is closely related with the incorporation of counter ions from solution, since the overall charge of the polymer is always zero. To counterbalance its charge the polymer in- or excludes ions from the surrounding electrolyte (figure 7), which in turn results in swelling or reduction of polymer volume [103]. Incorporated anions can make up around 35 % of the total polymer weight [103]. This property made polypyrrole an attractive material for e.g. the development of artificial muscles.

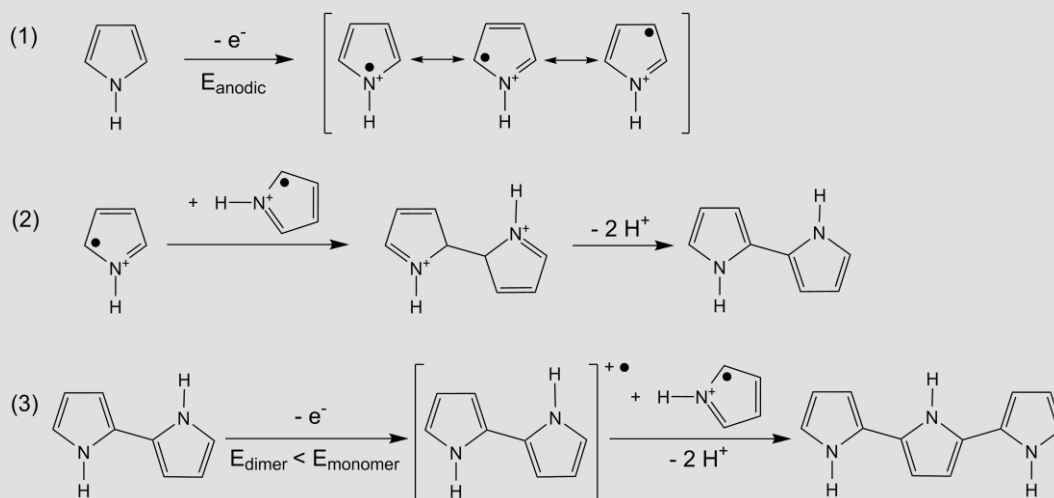


Figure 6: Scheme of pyrrole electropolymerization according to the mechanism proposed by Diaz [103]. (1) Pyrrole oxidation yielding a resonance stabilized radical cation. (2) The radical cation reacts with another radical resulting in dimer formation with concomitant loss of two hydrogen atoms. (3) Chain growth. The dimer is oxidized to a radical cation, which in turn reacts with another radical yielding the trimer, and so on.

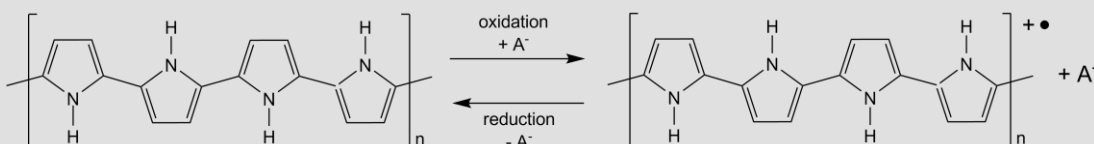


Figure 7: The neutral, non-conducting polypyrrole (left) is oxidized to its conducting, p-doped state (right), which is the actual form obtained during electropolymerization [103]. Anions (A^-) from solution are incorporated to counterbalance charges [103].

1.4.2.2. Polypyrrole as versatile immobilization matrix: development of a screening tool box

In this project the ability of the conductive polymer polypyrrole as electron delivering immobilization matrix for redox proteins should be investigated. Since electron transfer properties depend strongly on the nature of the protein the idea was to develop a universally applicable screening protocol with polypyrrole as immobilization matrix, which would enable the screening of a set of enzymes, e.g. in the eMTP (figure 8).

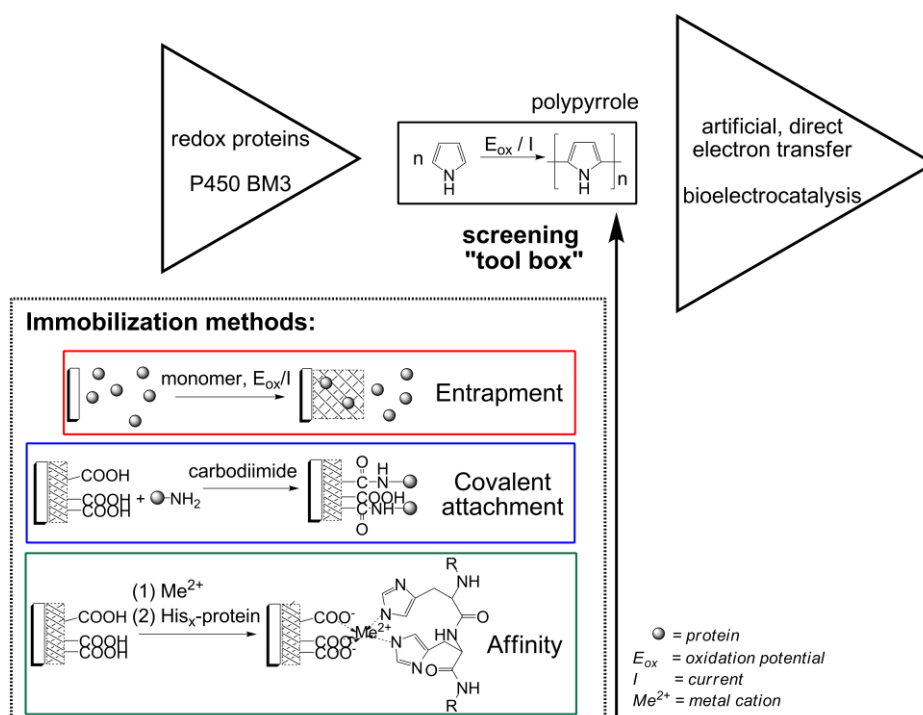


Figure 8: Scheme representing the idea of a screening “tool box” based on polypyrrole, which should help to find optimized conditions for immobilization and direct electron transfer for a range of proteins.

As described before polypyrrole seems to be a promising immobilization matrix, in particular since it offers high structural versatility by changing e.g. polymerization conditions, which is a prerequisite for tailor-made electrode surfaces needed for efficient electron transfer. In the end an immobilization “tool box” should be found, which allows for the screening of a set of enzymes by several immobilization methods based on only a small number of commercially available, pyrrole based monomers.

In literature entrapment methods are most common for the immobilization of biomolecules in polypyrrole. One of the main reasons is the ease of preparation, since it is a one pot reaction, and the virtual increase in electrode surface due to the porous polymer structure, which allows the electrochemical contacting of enzymes even in greater distances to the electrode surface [117]. For entrapment the biomolecule is added to the polymerization solution and embedded in the growing polymer network during polymerization. Thereby the protein is exposed to polymerization conditions including high currents and potential. Most of the introduced protein is lost, since only small percentages are incorporated in the polymer film. Also polymers often possess hydrophobic properties, which may influence folding of the entrapped protein, and substrate and product of the enzymatic reaction have to diffuse through the film [104]. However, some examples show that proteins can deal with this immobilization method resulting in electrochemically accessible assemblies.

Since possible drawbacks are evident, two other immobilization techniques should be tested in addition (figure 8). They are based on two carboxylic acid derivatives of pyrrole, namely 1-H-pyrrole-1-propionic acid (PPA) and 4-(3-pyrrolyl) butyric acid (PBA) (figure 9). Due to the carboxylic acid modification of the resulting polymer it is possible to attach biomolecules covalently via carbodiimide coupling or via affinity interactions. Compared to the entrapment method polymerization and binding event are separated. Since the biomolecule is bound at the polymer surface diffusion limitations are reduced as well as constraints in protein flexibility, even though the covalent attachment method may result in protein multipoint attachments. In addition the affinity based immobilization allows for reversible and specific binding of histidine-tagged proteins in uniform direction.

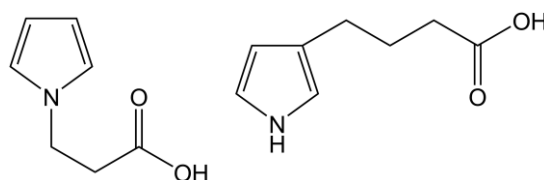


Figure 9: Chemical structures of the pyrrole derivatives 1-H-pyrrole-1-propionic acid (PPA), left, and 4-(3-pyrrolyl) butyric acid (PBA), right.

1.4.2.3. Transfer to P450 BM3

In general it proves difficult to achieve direct electron transfer to P450 monooxygenases, since the heme prosthetic group is deeply buried within the insulating protein shell [27]. But direct electron transfer to P450 BM3 was shown several times for various electrode surface modifications (see chapter 1.3.2.). The conductive polymer polypyrrole was successfully established as immobilization matrix for a variety of proteins, mainly in amperometric biosensors. Even though polypyrrole seems to be a promising immobilization matrix only few articles exist in combination with P450 monooxygenases. Polypyrrole was successfully applied for the immobilization and direct electrochemical attenuation of P450cam by Sugihara et al. [118]. In addition electrochemically driven product formation was also shown for P450 BM3 entrapped in polypyrrole, but with slow reaction rates and low immobilization efficiency [58].

In this project also the ability of the conductive polymer polypyrrole as electron delivering immobilization matrix for P450 BM3 should be investigated. It was shown that P450 BM3 can be evolved towards improved performance with the mediator cobalt sepulchrate and zinc as electron source [39, 40]. In analogy an electrochemical screening system with an established immobilization protocol could be helpful in identifying proteins with improved performance for direct artificial electron transfer via the conductive polymer polypyrrole.

In summary, starting with small redox active molecules and proteins a “tool box” including a set of pyrrole based and commercially available monomers, diverse polymerization techniques and several immobilization methods should be developed, which allows for a fast screening of proteins towards direct artificial electron transfer. In the end the idea is to see if the tool box methodology can be transferred to P450 BM3 to find conditions enabling artificial electron transfer to a deeply buried heme active site.

2. Electrochemical microtiter plate (eMTP)

Approaches combining parallel electrochemical measurements in conventional microtiter plates with simultaneous photometrical measurements in all wells have not been described in literature yet. For the further development of bioelectrochemical processes towards industrial applications a technology is needed to screen reaction parameters in parallel. Such a technology could be used to find suitable mediators for efficient electron transfer to enzymes, to optimize the direct electron transfer between an electrode and an enzyme or to find optimal cofactor regeneration conditions.

In this chapter the development of an electrochemical microtiter plate (eMTP) is described based on standard laboratory equipment, e.g. commercial microtiter plates and respectively a microtiter plate reader. The eMTP should feature easy implementation and handling. The technical set-up as well as the preparation of miniaturized reference electrodes is described. In first experiments the eMTP was tested with the well-known redox probe potassium ferrocyanide to gain insight into its performance (e.g. reproducibility). As a first screening application the oxidation of the mediator 2,2'-azino-bis(3-ethyl benzothiazoline-6-sulfonic acid) (ABTS) was tested at several reaction conditions. Finally the eMTP was applied to an enzyme screening issue. A P450 BM3 mutant library, created by Schwaneberg group at RWTH Aachen, was investigated with respect to its behavior in an electrochemically driven pNCA assay with the mediator cobalt sepulchrate (CoSep). Besides determining reaction rates also the influence of single reaction parameters on P450 BM3 performance was evaluated.

2.1. Material and Methods

2.1.1. Material

Electrodes and electrode material for the eMTP

platinum wire	d = 0.5 mm	Goodfellow GmbH, Bad Nauheim, Germany
platinum sheet	d = 0.1 mm	Goodfellow GmbH, Bad Nauheim, Germany
gold wire	d = 0.5 mm	Goodfellow GmbH, Bad Nauheim, Germany
gold sheet	d = 0.1 mm	Goodfellow GmbH, Bad Nauheim, Germany
silver wire	d = 0.5 mm	Goodfellow GmbH, Bad Nauheim, Germany
silicon tube	d = 0.5 mm	Carl Roth, Karlsruhe, Germany
Tygon tube	R3607, blue/green, d _{inner} = 0.5 mm, d _{outer} = 2.5 mm	Saint Gobain Performance Plastics, via VWR International GmbH, Darmstadt, Germany
magnesia rod	d = 1.5 mm, l = 140 mm	Carl Roth, Karlsruhe, Germany

Other electrodes and electrode material:

SE 21 Ag/AgCl reference electrode	l = 120 mm, ceramic diaphragm	Sensortechnik Meinsberg GmbH, Ziegra-Knobelsdorf, Germany
Luggin capillary	l = 25 cm, d = 1.5 cm, ceramic frit (d = 2 mm)	manufactured by gaffer

Measuring cells

24well plate	polystyrene, F-bottom	TPP Techno Plastic Products AG, Trasadingen, Switzerland
spectroelectrochemical cell	SEC-C Thin Layer Quartz Glass Spectroelectrochemical Cell Kit with Pt counter and Au gauze working electrode	ALS, via C3 Prozess- und Analystechnik GmbH, Haar, Germany

Instrumentation

Infintite M200 microtiter plate reader with software Magellan	Tecan, Crailsheim, Germany
Electrochemical multiplexer ECM8	Gamry Instruments, via C3 Prozess- und Analystechnik GmbH, Haar, Germany
Potentiostat PCI4 300 with software Gamry Framework 5.50 (2008)	Gamry Instruments, via C3 Prozess- und Analystechnik GmbH, Haar, Germany
Potentiostat PGSTAT12 with software Nova 1.6	Metrohm, Filderstadt, Germany
AvaLight-DHS-DeepUV + AvaSpec-2048	Avantes Ltd., Leatherhead, UK
Lambda 25 UV/VIS Spectrometer	Perkin Elmer, Waltham, USA

Chemicals and proteins

2,2'-azino-bis(3-ethyl benzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (≥ 98 %)	Sigma-Aldrich, Taufkirchen, Germany
catalase from bovine liver (295200 U/ mL)	Fluka Biochimica
cobalt (III) sepulchrate trihydrate (95 %)	Sigma-Aldrich, Taufkirchen, Germany
NADPH (≥ 95 %)	Sigma-Aldrich, Taufkirchen, Germany
p-nitrophenoxydodecanoic acid (12-pNCA)	Schwaneberg group, Institute of Biotechnology, RWTH Aachen University, Aachen, Germany
potassium hexacyanoferrate (II) trihydrate (≥ 99 %)	Fluka, Buchs, Switzerland
All other chemicals were of analytical grade or higher quality and purchased from Fluka (Buchs, Switzerland), Sigma-Aldrich (Taufkirchen, Germany) or Carl Roth (Karlsruhe, Germany).	

P450 BM3 mutants in the eMTP screening**Abbreviation**

P450 BM3 wild type ¹	WT
P450 BM3 F87A ¹	F87A
P450 BM3 R47F F87A M354S D363H ²	M3
P450 BM3 R47F F87A M354S D363H R471C ²	M3 R471C
P450 BM3 R47F F87A M354S D363H N543S ²	M3 N543S
P450 BM3 R47F F87A M354S D363H R471C N543S ²	M3 DM (double mutation)
P450 BM3 R47F F87A M354S D363H R471C N543S I401D ²	M3 DM I401D
P450 BM3 R47F F87A M354S D363H R471C N543S I401M ²	M3 DM I401M
P450 BM3 R47F F87A M354S D363H R471C N543S I401V ²	M3 DM I401V
P450 BM3 R47F F87A M354S D363H R471C N543S + unknown mutation ²	M3 DM nd

¹Strain collection of biochemical engineering group, DECHEMA Research Institute, Frankfurt am Main, Germany.

²Mutants were obtained from A. J. Ruff, Schwaneberg group, Institute of Biotechnology, RWTH Aachen University, Aachen, Germany.

All genes were in the pET28a vector and expressed in *E. coli* BL21 (for further details see section 2.1.2.1.).

2.1.2. Methods – Protein expression and characterization**2.1.2.1. Protein expression**

Strains of *E. coli* were stored as glycerol stocks at -80 °C, which were prepared from fresh overnight cultures by addition of 15 % (v/v) glycerol. Cultures of *E. coli* BL21 with the pET28a+ vector (Novagen, Darmstadt, Germany) containing genes for P450 BM3 variants were grown under control of the T7 promoter in 500 mL flasks containing 50 mL terrific broth (TB) supplemented by 35 µg kanamycin mL⁻¹ at 37°C and 180 rpm. One litre of the TB medium contained 12 g tryptone, 24 g yeast extract, 4 mL glycerol, 0.17 M KH₂PO₄ and 0.72 M K₂HPO₄. The salts were added after autoclaving. At an optical density (600 nm) of 0.8-1, the expression was induced by 0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG). Expression was carried out at 25°C and 180 rpm overnight. Cells were harvested by centrifugation at 3220 rcf and 4 °C for 20 min and resuspended in 50 mM potassium phosphate buffer (pH 7.5). The procedure was repeated twice to eliminate traces of TB medium. Cells were disrupted by sonication on ice (2 times, 2 min total sonication time (1 s sonication, 2 s wait time), amplitude 35%, Branson sonifier, Dietzenbach, Germany). Cell debris was removed by centrifugation at 3220 rcf and 4 °C for 30 min. The supernatant was filtrated by 0.24 µm filters to remove any residual cells and cell debris and stored at 4°C until use.

2.1.2.2. Protein characterization

P450 content was estimated via CO difference spectra as described by Omura and Sato [5] with an extinction coefficient of $91 \text{ mM}^{-1} \text{ cm}^{-1}$ for the reduced, CO bound heme complex at 450 nm.

P450 activity was determined by the pNCA assay with NADPH [38]. Therefore 10 to 100 μL of crude cell extract were added to potassium phosphate buffer (50 mM, pH 8.2). After addition of 8 μL 12-pNCA (15 mM in DMSO) the sample was incubated for 5 minutes. The reaction was started by addition of 20 μL NADPH (5 mM in dd. H_2O) and product formation was followed photometrically at 410 nm.

2.1.3. Methods – Electrochemical microtiter plate

Details of the set-up and evaluation of the electrochemical microtiter plate can also be found in a recently published manuscript by Ley et al. [119].

2.1.3.1. Electrode cleaning

In between all measurements working and counter electrodes were cleaned by immersing the electrodes in freshly prepared Piranha solution (H_2SO_4 conc./ H_2O_2 30% 1:1, on ice) for several seconds (gold) or some minutes (platinum). The electrodes were rinsed with dd. H_2O and sonicated for 10 minutes in dd. H_2O . The procedure was repeated three times.

2.1.3.2. Working and counter electrode preparation

Electrodes in the size of 1.5 cm x 0.8 cm and with 0.1 mm thickness were manually cut out of metal sheets. A metal wire of 1.5 cm length and 0.5 mm diameter was fused to the longitudinal side of the electrode by spot-welding [15 ms, 900 W, 4 V, tungsten electrode with graphite].

2.1.3.3. Preparation of miniaturized silver/ silver chloride electrodes

Silver wire of 1.5 cm length and 0.5 mm diameter was incubated over night in saturated FeCl_3 solution acidified with HCl [120]. After rinsing with water the wire was introduced into a Tygon tube of 1.2 cm length capped with a magnesia frit and filled with 3 molar KCl saturated with AgCl [76]. For further details see figure 10. Reference electrodes were stored in 3 M KCl when not in use. After three to four days of use the electrode housing was prepared freshly and the silver wire reincubated in acidified FeCl_3 over night.

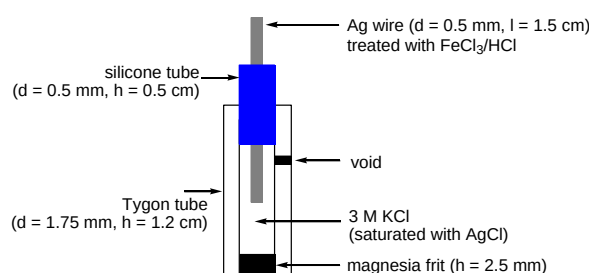


Figure 10: Scheme of a miniaturized Ag/AgCl reference electrode built for the eMTP (side view)

2.1.3.4. General eMTP set-up

The electrochemical microtiter plate (eMTP) was developed based on a flat bottomed 24 well plate (figure 11). A circuit board served as lid to which three electrodes per well were connected reversibly via plug connections (figure 12, left). The circuit board had holes for keeping the light path free and the electrodes were arranged correspondingly at the edge of the wells. Each well contained three electrodes, a working, a counter and a reference electrode. As working electrode material platinum or gold was used (figure 12, right). The counter electrode was made of platinum in all cases. Self-made miniaturized Ag/AgCl electrodes ($\text{Ag/AgCl}_{\text{eMTP}}$) or platinum wire could be used as reference electrode. The working electrode surface to volume ratio was $1.27 \pm 0.24 \text{ cm}^2 \text{ cm}^{-3}$ at a fill level of 0.497 cm corresponding to a total volume of 1 mL per well. The eMTP was included in a standard microtiter plate reader (Tecan Infinite M200) without further modification of the instrument. The electrochemical instrumentation was a

multiplexed potentiostat (Electrochemical Multiplexer ECM8 and potentiostat PCI4 300, Gamry Instruments) with 8 independent channels. Connection to the circuit board was carried out via insulated cables in position C3, C4 and D1 to D6 of the 24 well plate.

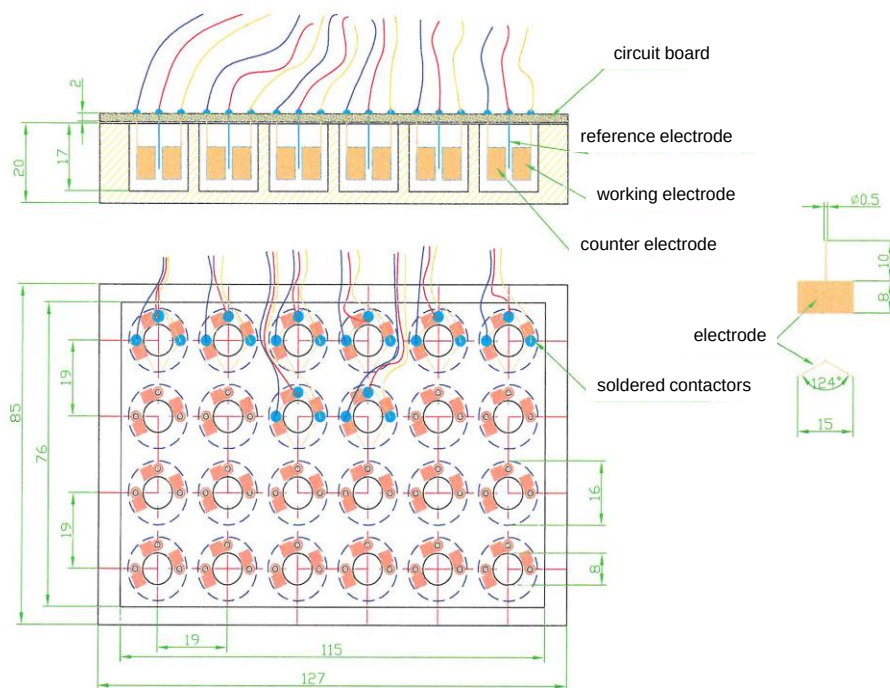


Figure 11: Technical scheme of the eMTP [119]. Top: side view; bottom: top view.

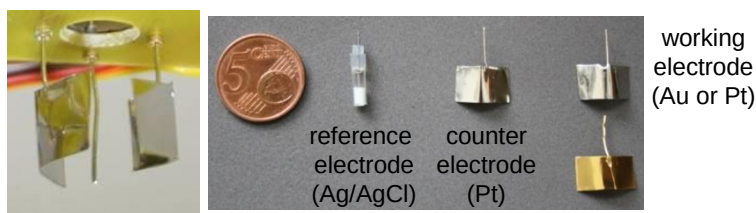


Figure 12: Photographs of the eMTP electrodes. Left – platinum working, counter and platinum wire reference electrode connected to the lid (side view). Right - Overview of electrodes used within the eMTP (coin is given for size comparison).

2.1.3.5. Software and data acquisition

The multiplexer ECM8 and the potentiostat PCI4 300 were controlled via the software Gamry Framework Version 5.50. Parallel potentiostatic experiments were done with the multiplexed corrosion software package. For parallel potentiostatic experiments a two step program was established, starting with two minutes at zero potential and then switching to the potential of interest, which allowed the manual triggering with the microtiter plate reader.

Potentiodynamic measurements can be done sequentially. Measurement protocols for sequential experiments were written with the sequence wizard tool offered by the Framework program.

For simultaneous optical data acquisition via the Tecan microtiter plate reader, controlled by the software Magellan, two modes were used: a wavelength scan or an absorbance measurement at a certain wavelength. In both modes a kinetic cycle of 30 minutes was applied including a mixing phase of 30 to 60 seconds after each measurement round [orbital shaking, 3 mm amplitude, 44.3 rpm].

2.1.3.6. Cyclic voltammetry experiments with mediators

In potentiodynamic measurements without simultaneous optical data acquisition the potentiostat PGSTAT12 (Metrohm) was used. The potentiostat was connected to the electrodes of one eMTP well after the other manually.

The cyclic voltammetry experiment was started at a potential close to the open circuit value. The potential was scanned in positive (anodic) direction at a defined scan rate v [V/s] until the first switching potential was reached. Then the sign of the scan rate is inverted and the potential changed in negative (cathodic) direction. When the second, cathodic switching potential was reached the potential was returning to the starting value and the first cyclic voltammogram was completed. While changing the potential linearly over time the corresponding current was recorded. All further details (switching potentials, electrolyte and scan rate) are stated in the respective cyclic voltammograms shown in the experimental section.

2.1.3.7. pNCA assay with P450 BM3 and cobalt sepulchrate

If not stated otherwise all experiments were done at 30°C and standard conditions, which include:

- 837 μL potassium phosphate buffer (50 mM, pH 8.2)
- + 100 μL crude cell extract (in 50 mM potassium phosphate buffer, pH 7.5)
- + 8 μL *p*-nitrophenoxydodecanoic acid (12-pNCA; 15 mM in DMSO)
- 5 min incubation*
- + 5 μL catalase (295200 U/mL)
- + 50 μL cobalt sepulchrate (0.1 M in dd. H₂O)

The reaction was started by applying a potential of -750 mV vs. Ag/AgCl_{eMTP}.

Since the crude cell extract contained different concentrations of the P450 BM3 mutants throughout the experiments several dilutions were used. Concentration of P450 BM3 is given for the single experiments, but the total volume of pH 7.5 buffer is always 100 μL . The buffer mixture gives a starting pH of 8 for the reaction solution. Optical data were obtained by wavelength scans from 600 to 300 nm or by absorbance measurements at 410 nm.

Specific product formation rate

Specific product formation rates were calculated from the slope of the absorbance increase at 410 nm with an extinction coefficient for *p*-nitrophenolate of $\epsilon = 0.0132 \text{ L } \mu\text{mol}^{-1} \text{ cm}^{-1}$ [38] and a light path length of 0.497 cm (which corresponds to a fill level of 1 mL per well). The specific product formation rate is given as U/ μmol P450 with $U = \mu\text{mol}_{\text{product}} \text{ min}^{-1}$. The specific product formation rate divided by 60 corresponds to $k_{\text{cat}} [\text{s}^{-1}]$.

Coupling efficiency

Electrochemical data, e.g. measured currents, were related to the amount of product formed by calculating the coupling efficiency [36]. Therefore the amount of product p-nitrophenolate obtained at the end of the linear increase in absorbance at 410 nm, namely when the slope approximates zero, was determined. The charge passed during the corresponding time interval was obtained via integration of the I vs. t plots or by multiplying the average current by the time. From the charge the number of electrons was determined using the Faraday constant ($F = 96487 \text{ As mol}^{-1}$). The mols of electrons passed were related to the number of product produced in the corresponding time interval with the assumption that two electrons are needed for the formation of one molecule of product. The total mols of electrons passed during the time interval were set to 100 % and the coupling efficiency was calculated as the percentage of mols consumed for product formation.

In the results section all coupling efficiencies shown were calculated from the charge obtained by multiplying the average current by time. Coupling efficiencies calculated from the charge obtained from integration of the I vs. t graph differ by less than 8 % from the presented data. Corresponding average currents, charge values, time intervals and product concentrations are displayed in the appendix.

2.2. Results

2.2.1. Evaluation of reference electrodes: Pt wire and miniaturized Ag/AgCl

Platinum wire was tested as pseudo reference electrode as well as self-made miniaturized Ag/AgCl reference electrodes ($\text{Ag/AgCl}_{\text{eMTP}}$). Cyclic voltammograms of potassium ferrocyanide were recorded with both electrode types and compared to a commercially available large-scale Ag/AgCl reference electrode (figure 13). For the platinum wire a shift in the measured potential of the ferrocyanide redox pair to lower potentials was observed with a midpoint potential of 33 mV vs. Pt and 252 mV vs. Ag/AgCl (table 2). Thereby the midpoint potential E_{mp} is defined as the potential midway in between anodic and cathodic peak and normally resembles the formal potential of the redox reaction [121]. The $\text{Ag/AgCl}_{\text{eMTP}}$ midpoint potential was 235 mV and differed from a commercially available Ag/AgCl by 6.4 %.

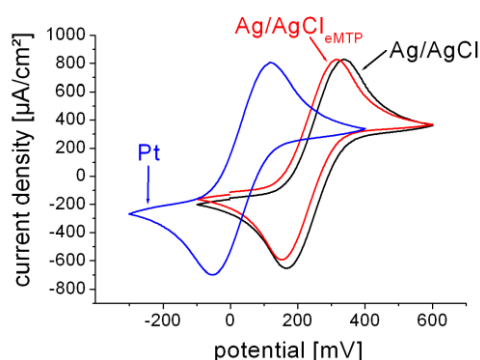


Figure 13: Cyclic voltammetric characterization of 15 mM potassium ferrocyanide in 0.1 M KCl at 10 mV/s. Pt working and counter electrode. Investigated reference electrodes: platinum wire (Pt), commercially available Ag/AgCl reference electrode (Ag/AgCl), and a miniaturized Ag/AgCl reference electrode made for the eMTP ($\text{Ag/AgCl}_{\text{eMTP}}$).

Further characteristics like the separation between the anodic and cathodic peak (ΔE_p) and the current ratio in between maximal peak current densities of the anodic and cathodic peak ($i_{\text{pa}}/i_{\text{pc}}$) were determined and did not differ significantly in between the different reference electrodes employed.

Table 2: Midpoint potential E_{mp} , peak potential separation ΔE_p and current ratio from voltammograms in figure 13.

	E_{mp} [mV]	ΔE_p [mV]	i_{pa} / i_{pc} [1]
Pt	33	172	1.02
Ag/AgCl	252	170	1
Ag/AgCl_{eMTP}	235	163	0.98

2.2.2. eMTP evaluation

2.2.2.1. Characterization of the redox probe potassium ferrocyanide

To evaluate well-to-well reproducibility cyclic voltammetry experiments were done sequentially in 8 wells (figure 14). A midpoint potential of 221.7 ± 7.5 mV vs. Ag/AgCl_{eMTP} was determined for the potassium ferrocyanide redox couple at a scan rate of 100 mV/s (table 3). It corresponded to a relative standard deviation of 3.4 %. An anodic peak current of 1951 ± 255 mA and a cathodic peak current of 2173 ± 280 mA were obtained which related to an error of 13.1 and 15.8 % respectively.

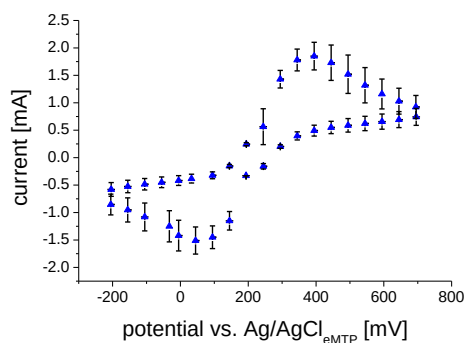


Figure 14: Cyclovoltammetric characterization of 15 mM potassium ferrocyanide in 0.1 M KCl at 100 mV/s. Au working, Pt counter and Ag/AgCl_{eMTP} reference electrode. The average CV out of 8 wells is shown with the corresponding standard deviation of the current and potential.

Table 3: Cyclovoltammetric characterization of 15 mM potassium ferrocyanide in 0.1 M KCl at 100 mV/s (see also figure 14). Sequential measurement in 8 wells. As average values are shown: anodic and cathodic peak potentials (E_{pa} and E_{pc}), anodic and cathodic peak currents (I_{pa} and I_{pc}) and the corresponding standard deviations. Derived from these results were the average midpoint potential E_{mp} , the peak potential separation ΔE_p and the current ratio I_{pa}/I_{pc} .

	Average	SD	RSD [%]
E_{pa} [mV]	383.4	22.4	5.8
E_{pc} [mV]	59.9	15.7	26.3
I_{pa} [mA]	1951.3	254.7	13.1
I_{pc} [mA]	1770.2	280.0	15.8
E_{mp} [mV]	221.7	7.5	3.4
ΔE_p [mV]	323.5	35.7	11.0
I_{pa}/I_{pc} [1]	1.11	0.06	5.74

Cyclic voltammograms for potassium ferrocyanide were recorded at different scan rates (figure 15, left). The current ratio between anodic and cathodic peak was greater than 1 in all cases. Peak separation was high already at low scan rates. Furthermore peak separation increased from 244 mV to 331 mV when the scan rate v was raised from 25 to 100 mV/s. For comparison the same experiment was repeated with a commercially available spectroelectrochemical cell (SEC) (figure 15, right). The peak potential separation increased from 212 mV at 25 mV/s to 292 mV at 100 mV/s and the current ratios remained close to 1 at all scan rates (0.99 ± 0.03). With SEC a midpoint potential of 257 ± 2 mV vs. Ag/AgCl was determined. For the eMTP a midpoint potential of 219 ± 3 mV was obtained for the ferrocyanide redox pair, which corresponded to a relative deviation of 15 %. It was not necessary to determine the surface area of the Au gauze working electrode in SEC since current ratios were calculated (table 4). Therefore absolute current values for eMTP and SEC in figure 15 cannot be compared directly.

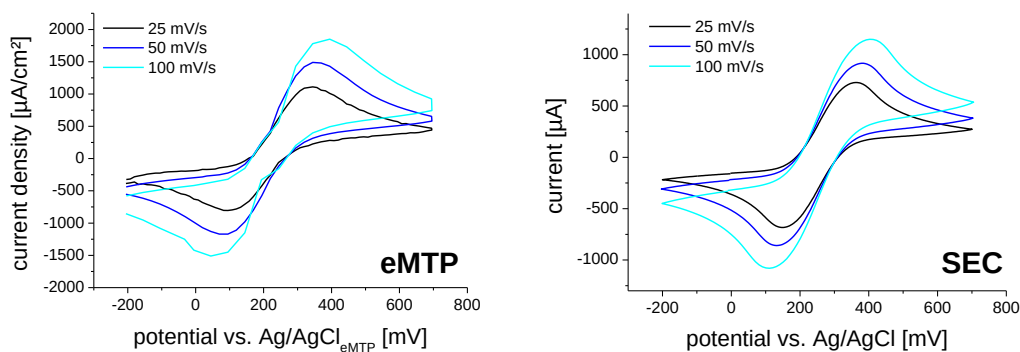


Figure 15: Cyclovoltammetric characterization of 10 mM potassium ferrocyanide in 0.1 M potassium phosphate buffer (pH 7) and 0.1 M KCl at three different scan rates. Left: CVs obtained with the eMTP. Average CV out of 8 wells is shown. Au working, Pt counter and Ag/AgCl_{eMTP} reference electrode. Right: CVs obtained in a commercially available low volume spectroelectrochemical cell (SEC). Au gauze working, Pt counter and Ag/AgCl reference electrode connected to the cell via a Luggin capillary filled with 0.5 M Na₂SO₄.

Table 4: Peak potentials of the anodic (E_{pa}) and cathodic (E_{pc}) peak, midpoint potentials (E_{mp}), peak potential separation (ΔE_p) and current ratios obtained from the CVs in figure 15.

	v [mV/s]	E_{pa} [mV]	E_{pc} [mV]	E_{mp} [mV]	ΔE_p [mV]	current ratio [1]
eMTP	25	339	95	217	244	i_{pa} / i_{pc} 1.21
	50	353	82	218	271	1.13
	100	387	56	221	331	1.12
SEC	25	362	151	256	212	i_{pa} / i_{pc} 0.97
	50	383	135	259	248	0.98
	100	401	110	255	292	1.03

The electrochemical set-up was combined with the electrochemical unit and the oxidation of potassium ferrocyanide was monitored photometrically via a microtiter plate reader in a chronoamperometry experiment (figure 16). An increase in absorbance at 420 nm due to ferrocyanide oxidation was seen only in case when potential was applied. Oxidation rates were determined from the absorbance values with a relative standard deviation of 8.5 %.

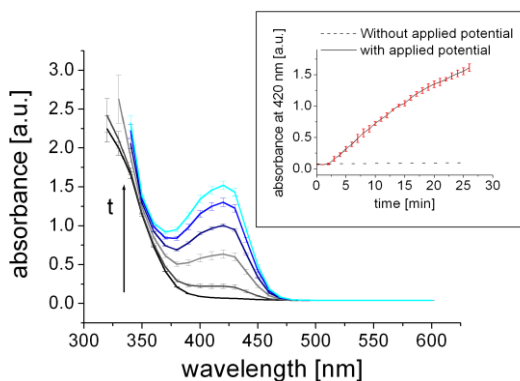


Figure 16: 5 mM potassium ferrocyanide in 0.1 M KCl. Application of a constant potential (+300 mV vs. Pt) in 3 wells parallel. Pt working and counter electrode. Simultaneous measurement of wavelength scans by the microtiter plate reader (scans are shown in 5 min intervals). Error bars correspond to the standard deviation between 3 wells. Inset: Absorbance at 420 nm over time.

2.2.2.2. Screening for optimized ABTS oxidation conditions

CVs of ABTS were recorded in 8 wells revealing a midpoint potential of 488 ± 9 mV/s, which kept constant at all scan rates tested (figure 17). In addition CVs in the commercially available SEC were recorded for comparison (table 5).

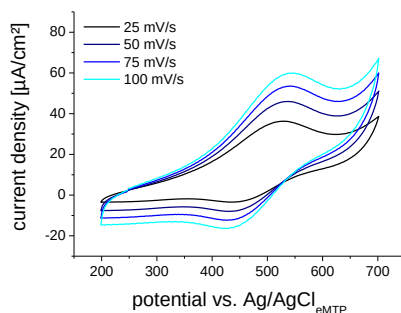


Figure 17: 0.2 mM ABTS in 50 mM TrisHCl buffer (pH 8). Au working and Pt counter electrode. Average cyclic voltammogram out of 8 wells at four different scan rates.

Table 5: Characteristics of CVs obtained for 0.2 mM ABTS in 50 mM TrisHCl buffer (pH 8). eMTP data were recorded in 8 wells sequentially (standard deviation in between wells is indicated). Au working, Pt counter and Ag/AgCl_{eMTP} reference electrode. For comparison the experiment was additionally performed in SEC with a commercially available Ag/AgCl.

	v [mV/s]	E_{pa} [mV]	E_{pc} [mV]	E_{mp} [mV]	ΔE_p [mV]	current ratio [1]
eMTP	25	528 ± 8	444 ± 9	486 ± 8	84 ± 5	i_{pa} / i_{pc} 3 ± 0.8
	50	535 ± 8	440 ± 11	488 ± 9	95 ± 5	2.6 ± 0.8
	75	539 ± 9	435 ± 11	487 ± 10	104 ± 5	2.4 ± 0.6
	100	543 ± 9	433 ± 10	488 ± 9	111 ± 5	2.5 ± 0.7
SEC	25	576	446	511	130	i_{pa} / i_{pc} 3.7
	50	568	457	513	111	3.9
	75	554	460	507	94	3.6
	100	561	458	509	103	4.8

Furthermore in a first screening approach different conditions for ABTS oxidation were tested to find best conditions including pH, buffer concentration and the applied potential. At 420 nm oxidation rates were highest at pH 7.5 and a medium buffer concentration of 50 mM (figure 18). The highest potential tested was +300 mV vs. Pt and also showed the highest oxidation rate for ABTS. For the oxidation of ABTS at standard conditions (pH 8, +250 mV vs. Pt and 50 mM TrisHCl) the relative standard deviation is approximately 20 %.

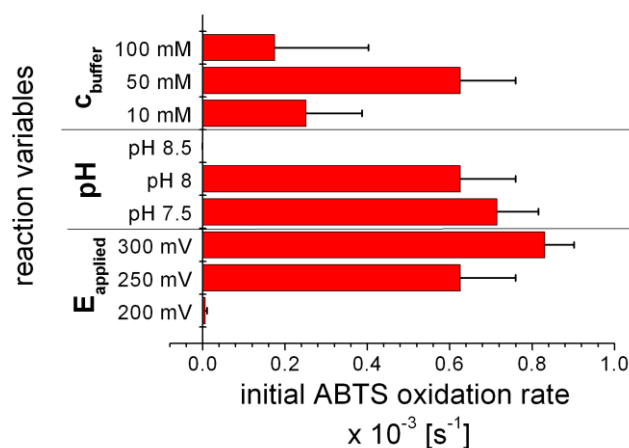


Figure 18: Screening of reaction variables to find optimal ABTS oxidation conditions with the eMTP set-up. Oxidation rate for ABTS was determined by increase in absorbance at 420 nm. Standard deviations are given in s⁻¹ and standard conditions comprised: 50 mM TrisHCl at pH 8 and +250 mV vs. Pt.

2.2.3. Electrochemically driven pNCA assay with the mediator cobalt sepulchrate: Screening of a P450 BM3 mutant library in the eMTP

2.2.3.1. Electrochemical characterization of the mediator cobalt sepulchrate

The electrochemical behavior of the mediator cobalt sepulchrate was tested in 50 mM potassium phosphate buffer at pH 8 (figure 19). CVs revealed a midpoint potential of -662 ± 3 mV vs. $\text{Ag}/\text{AgCl}_{\text{eMTP}}$ at a scan rate of 25 mV/s which remained constant (± 3 mV) at all scan rates tested. The reduction peak potential E_{pc} was at -760 mV vs $\text{Ag}/\text{AgCl}_{\text{eMTP}}$ at 25 mV/s but decreased to -812 mV at a scan rate of 100 mV/s. This finding was consistent with increasing peak potential separations at higher scan rates.

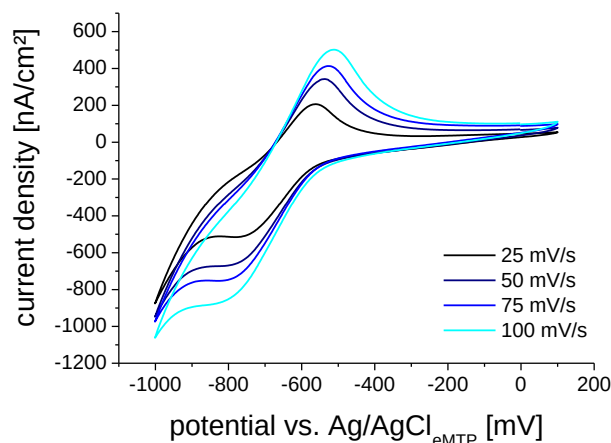


Figure 19: Cyclic voltammograms of cobalt sepulchrate at pNCA assay conditions (50 mM potassium phosphate buffer at pH 8, 5 mM CoSep, 1476 U catalase) without the substrate 12-pNCA (instead 8 μL DMSO were added). Last CV of 3 is shown. Switching potentials were at 100 and -1000 mV vs. $\text{Ag}/\text{AgCl}_{\text{eMTP}}$. An Au working and a Pt counter electrode were used.

Table 6: Anodic (E_{pa}) and cathodic (E_{pc}) peak potentials, midpoint potential (E_{mp}) and peak potential separation (ΔE_{p}) for CoSep (derived from CVs in figure 19).

v [mV/s]	E_{pa} [mV]	E_{pc} [mV]	E_{mp} [mV]	ΔE_{p} [mV]
25	-564	-760	-662	196
50	-539	-774	-657	235
75	-529	-798	-664	269
100	-513	-812	-663	299

2.2.3.2. Proof of concept: Electrochemically driven reaction in the eMTP

The pNCA assay was performed in the eMTP with simultaneous optical data acquisition. The reaction was driven electrochemically (-750 mV vs. $\text{Ag}/\text{AgCl}_{\text{eMTP}}$) in the presence of CoSep and crude cell extract containing P450 BM3 F87A. At 410 nm absorbance increased over time due to product formation for the first 15 minutes, after which the rate of absorbance increase was leveling off. Without enzyme or without mediator CoSep no product formation was detected (figure 20).

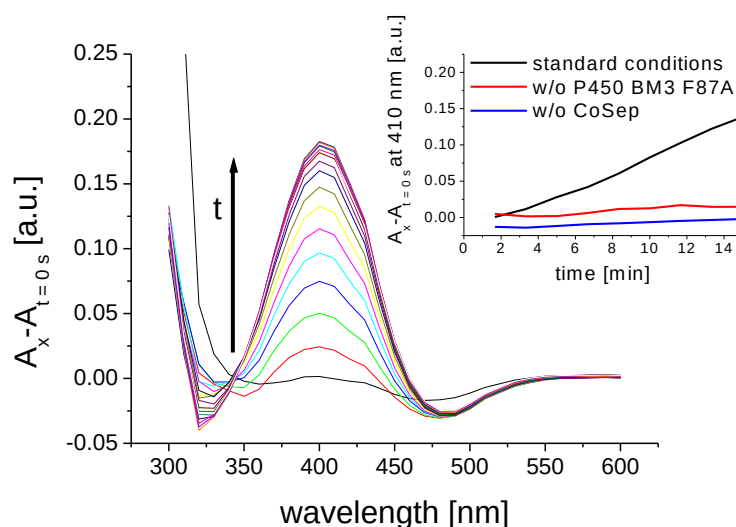


Figure 20: Electrochemically driven pNCA assay with P450 BM3 F87A [$1 \mu\text{M}$]. Wavelength scan over time (every two minutes a wavelength scan was taken). Inset: Absorbance increase at 410 nm in the first 15 minutes under standard conditions (black), without P450 BM3 F87A (red) and without CoSep (blue).

During application of a constant potential, resulting currents were recorded by the electrochemical unit. Signals were dominated by the shaking process which manifested in distinct current spikes (figure 21). In an experiment without CoSep smaller reduction currents were obtained than with CoSep, but the difference in currents between the assay with and without enzyme is negligible (table 7).

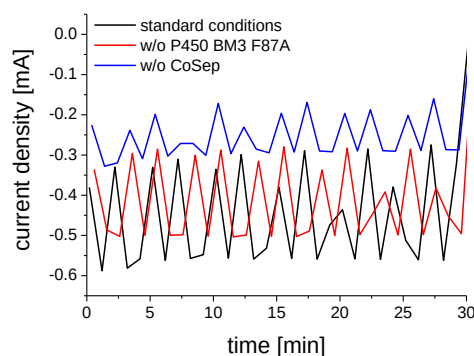


Figure 21: Data recorded by the electrochemical unit during the electrochemically driven pNCA assay shown in figure 20.

Table 7: Values for the calculation of the coupling efficiency between passed charge and product formed during 20 minutes of reaction for P450 BM3 F87A in the electrochemically driven pNCA assay with CoSep. The deviation in current resulted from disturbance of the signal by the shaking process.

	n_{product} [$\mu\text{mol/L}$]	Average current [μA]	charge [C]	coupling efficiency [%]
standard conditions	27.6	-825 ± 205	1.072 ± 0.266	0.25 ± 0.08
w/o P450 BM3 F87A		-760 ± 173	0.987 ± 0.225	
w/o CoSep		-388 ± 75	0.505 ± 0.097	

In addition several control reactions were performed to evaluate the influence of reaction conditions on P450 BM3 WT activity in terms of stability or respectively denaturation. The pNCA assay was performed with NADPH as electron source with fresh crude cell extract and with a reaction solution which had been under electrochemical pNCA assay reaction conditions for 30 minutes. After 30 minutes at eMTP reaction conditions the specific product formation rate of P450 BM3 WT in the NADPH assay was reduced by approximately 26 % compared to fresh cell extract (table 8). In comparison fresh crude cell extract, which was incubated at 30°C for 30 minutes, did not show a decrease in its specific product formation rate (table 8). Furthermore samples were pretreated at electrochemically driven pNCA assay conditions but without applied potential, without substrate or without catalase (table 8). Of these samples only the exclusion of catalase had a significant effect reducing WT specific product formation rate by approximately 40 % (compared to standard conditions).

Table 8: Specific product formation rates for P450 BM3 WT in the pNCA assay with NADPH. Samples of WT have been treated differently prior to NADPH measurement (details of pretreatment are given). 5 minutes prior to NADPH addition fresh 12-pNCA substrate was provided to samples.

pretreatment	SPFR with NADPH [U/ μ mol P450]	rel. dev. [%]
crude cell extract		
no pretreatment	131.4 $\pm$ 4.8	3.7
pNCA assay with CoSep		
pNCA assay with CoSep at standard conditions	97.2 $\pm$ 0.7	0.7
without E	94.7 $\pm$ 2.8	3.0
without 12-pNCA	92.2 $\pm$ 5.9	6.4
without catalase	58.6 $\pm$ 0.6	1.0
crude cell extract (no pretreatment)	147.8 $\pm$ 25	16.9
crude cell extract (30 minutes at 30°C)	155.2 $\pm$ 23	14.8

2.2.3.3. Stability of *p*-nitrophenolate at assay conditions

When examining the optical data obtained during the experiments it was striking that with increasing potential a decrease in absorbance was seen at the end of the reaction. This phenomenon was monitored for all mutants, but is exemplified here for F87A (figure 22). While at -550 and -650 mV absorbance was increasing or respectively reached a plateau after 30 minutes, at more negative potentials a decrease in absorbance occurred. The decrease rate was the higher the more negative potentials were applied. Furthermore the observable absorbance decrease started earlier when more negative potentials were applied. Correspondingly absolute absorbance values reached decreased with increasing reductive power.

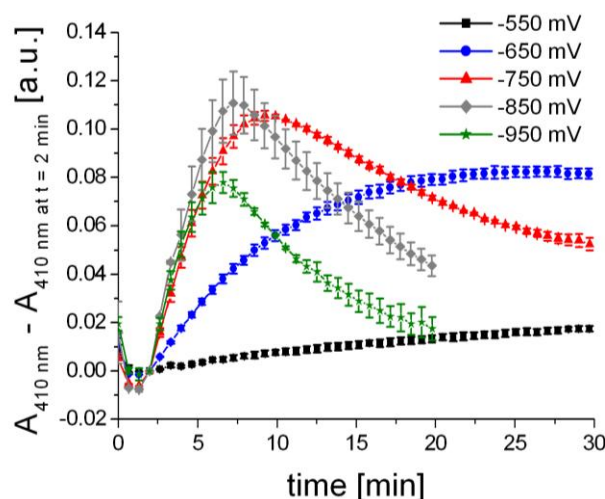


Figure 22: Absorbance at 410 nm over time for the mutant F87A at different applied potentials (vs. Ag/AgCl_{eMTP}). Experiments were performed in duplicate.

To elucidate this phenomenon the stability of *p*-nitrophenolate was tested at assay conditions with and without the presence of CoSep. *P*-nitrophenolate was stable in the reaction solution without applied potential since no absorbance decrease was observed during 30 minutes at 30°C (figure 23, left). When a potential of -750 mV was applied a decrease in absorbance was seen regardless of CoSep being present or not. When the amount of catalase was increased 10fold only a slight decrease in absorbance was obtained although potential was applied. Reduction of the applied potential to -850 and -950 mV increased the rate of absorbance decrease slightly (figure 23, right).

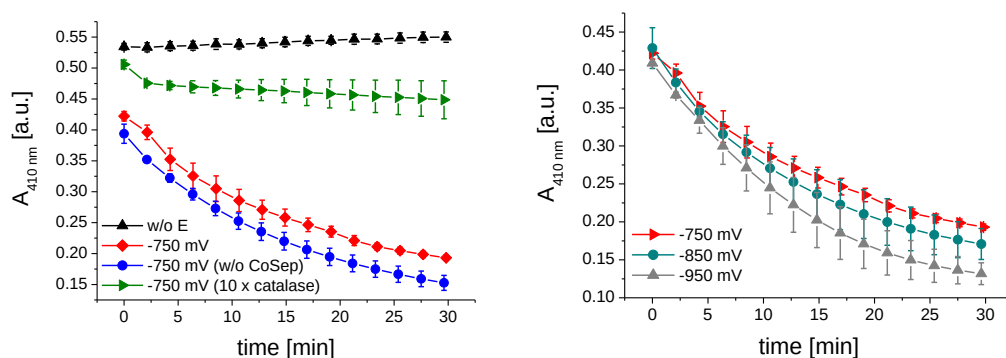


Figure 23: Stability of *p*-nitrophenolate (7.5 μM) in absorbance over time plots. Left (in all cases without P450): at pNCA assay standard conditions (red), without applied potential (black), without CoSep (blue) and with a 10 fold increased volume of catalase (green). Right: Stability in assay buffer with CoSep and catalase at different potentials.

One reason for this behavior may be a pH change of the reaction solution due to water electrolysis, which would lead to product protonation resulting in loss of its yellow color. Another possibility may be the formation of hydrogen peroxide as intermediate product in the reduction of oxygen at the counter electrode. Hydrogen peroxide as strong oxidizing agent may interact with the product. To evaluate the possible influence of a pH shift it was assumed that the total charge passed during the experiment was consumed for water electrolysis according to the following equation: $6 \text{ H}_2\text{O} \rightarrow \text{O}_2 + 4 \text{ H}_3\text{O}^+ + 4 \text{ e}^-$ [122]. The total charge passed was obtained from integration of the current versus time plots. Via the Faraday constant the amount of electrons passed was calculated and related to the theoretical concentration of H_3O^+ ions ($n_{\text{e}^-} = n_{\text{H}_3\text{O}^+}$). Subsequently the actual pH at each point of the current versus time plots was calculated via the Henderson-Hasselbalch equation with the following determinations for the phosphate buffered system: apparent pK_a at $23^\circ\text{C} = 6.83$ [123]; $c_0(\text{HAc}) = 2.56 \text{ mmol/L}$ and $c_0(\text{Ac}^-) = 44.19 \text{ mmol/L}$ [123]; $c_{\text{buffer}} = 46.8 \text{ mM}$; $\text{pH}_0 = 8.07$. As it can be seen in figure 24 the theoretical pH change correlated well with the decrease in absorbance at 410 nm. Exemplary the theoretically expected maximal pH changes for pNCA assay experiments with F87A were calculated like described before (table 9). As expected at lower potentials the theoretical decrease in pH would be higher, which corresponds well to the potential dependent decrease in absorbance observed in figure 22.

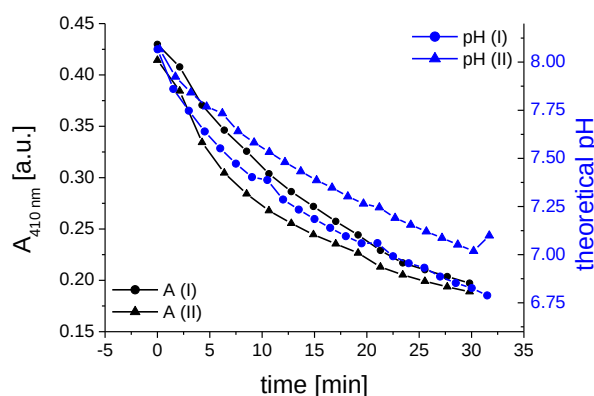


Figure 24: Correlation of absorbance decrease at 410 nm (left ordinate, black) and the theoretical pH change (right ordinate, blue) for samples at -750 mV comprising *p*-nitrophenol, CoSep and catalase.

Table 9: Total charge passed after 30 minutes of pNCA assay reaction with CoSep and F87A at the given potential. Calculated theoretical pH at the end of 30 minutes if all passed charge was consumed for water electrolysis.

	Total charge [C]	theoretical pH
-550 mV	0.598 ± 0.055	7.47 ± 0.03
-650 mV	1.052 ± 0.025	7.22 ± 0.01
-750 mV	1.858 ± 0.127	6.89 ± 0.05
-850 mV	1.424 ± 0.046	7.06 ± 0.02
-950 mV	1.667 ± 0.044	6.96 ± 0.02

Furthermore the theoretically calculated pH after 10 minutes at -750 mV is around 7.5, which corresponds to a value of 7.5 measured with a pH electrode after 10.8 minutes of applied potential (-750 mV) in a solution containing phosphate buffer, CoSep, pNCA and catalase. Based on the Henderson-Hasselbalch equation and a pK of 7.1 for *p*-nitrophenol [124], after 10 minutes around 25 % of the colored *p*-nitrophenolate would be protonated to the non-colored *p*-nitrophenol. This is in accordance with the measured absorbance upon application of a constant potential. Here the initial absorbance value is reduced by approximately 30 % after 10 minutes of applied potential.

In the following experiments a mathematical correction of the absorbance decrease was undertaken to estimate the influence of this effect on the obtained data. For each measurement the rate of absorbance decrease was independently determined as the slope of the linear decrease range directly after the absorbance maximum was reached (figure 22 and table 10). The decrease rate was regarded as being constant from the beginning of the measurement. Since the decrease in absorbance is not truly linear this is only an approximation to give an impression of error introduced to calculation of reaction rates.

Table 10: Rates of absorbance decrease at 410 nm for *p*-nitrophenolate in assay buffer (without P450, figure 23) and for assays with P450 BM3 F87A derived from figure 22. Specific product formation rates obtained from figure 22 (SPFR) are compared to specific product formation rates which were corrected for the absorbance decrease (SPFR_{cor}).

	absorbance decrease rate [1/s]	SPFR [U/μmol P450]	SPFR _{cor} [U/μmol P450]	SPFR deviation [%]
Without P450				
-750 mV	$-2.61 \pm 0.36 \times 10^{-4}$			
-850 mV	$-2.98 \pm 0.95 \times 10^{-4}$			
-950 mV	$-2.84 \pm 0.67 \times 10^{-4}$			
With P450 BM3 F87A				
-750 mV	$-0.60 \pm 0.01 \times 10^{-4}$	6.56 ± 0.60	7.55 ± 0.62	15.1
-850 mV	$-1.14 \pm 0.11 \times 10^{-4}$	9.05 ± 0.19	10.93 ± 0.02	20.8
-950 mV	$-1.21 \pm 0.18 \times 10^{-4}$	7.81 ± 0.80	9.79 ± 1.10	25.4

The correction of the absorbance decrease resulted in higher product formation rates and the introduced error becomes more pronounced at lower potentials (table 10). For F87A at standard conditions a specific product formation rate of 6.6 ± 0.6 was obtained and with correction of the absorbance decrease it was 7.6 ± 0.6 (table 10). Both values differ by 15 %. The absorbance decrease corrected values for F87A are displayed in figure 22. In general no absorbance decrease correction was applied to measurements at -550 or -650 mV, since no decrease in absorbance was seen at those potentials. As it can be seen in the following chapters influence of absorbance decrease was evaluated with this method and SPFR_{cor} values are given in addition to the apparent SPFR values if an absorbance decrease at 410 nm was obtained. The SPFR_{cor} values are only an approximation, but as it can be seen in table 10 even though SPFR and SPFR_{cor} differ in their absolute values the relation to each other is not changed. With and without absorbance decrease correction maximal rates were achieved at -850 mV and lowest SPFR as well as SPFR_{cor} was obtained at -750 mV (table 10).

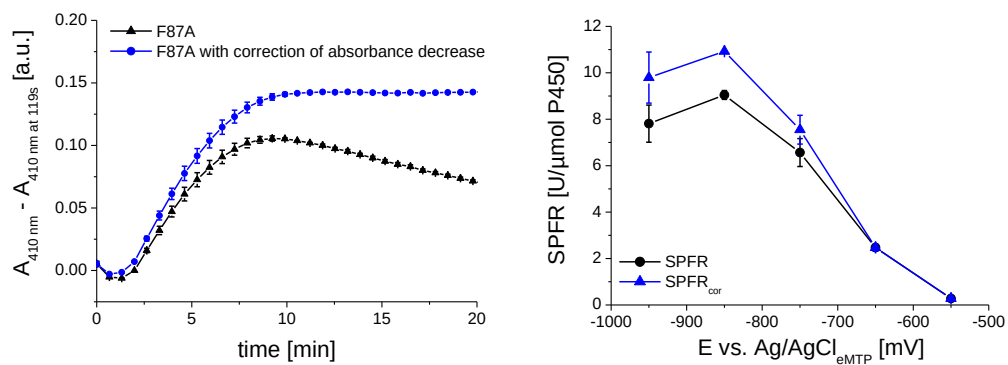


Figure 25: Example of absorbance decrease correction applied to P450 BM3 F87A at -750 mV (left). Slope of absorbance decrease was calculated for the linear range after the absorbance maximum was reached. Right: Comparison of specific product formation rates calculated from the absorbance increase at 410 nm with (SPFR_{cor}) and without absorbance decrease correction (SPFR).

2.2.3.4. P450 BM3 mutant screening

A mutant library of P450 BM3 was screened in the eMTP for its performance in an electrochemically driven pNCA assay with the mediator CoSep (figure 26). Furthermore crude cell extract of *E.coli* BL21 cells containing the empty vector pET28a was tested and no product formation was detected.

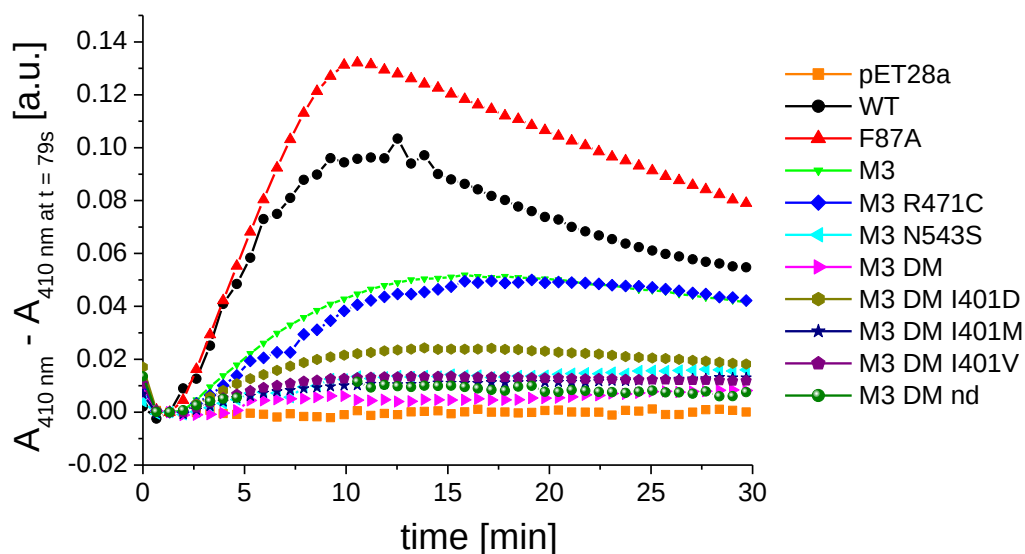


Figure 26: Electrochemically driven pNCA assay with CoSep at standard conditions (120 μ M 12-pNCA, 1476 U/mL catalase and 5 mM CoSep in 50 mM potassium phosphate buffer at pH 8, $V_{\text{end}} = 1$ mL) with 0.38 μ M P450 at 30°C. After 2 minutes at 0 V a potential of -750 mV vs. Ag/AgCl_{eMTP} was applied for 28 minutes. Average of two tests per mutant is shown.

Specific product formation rates were determined based on the increase of absorbance at 410 nm over time (figure 27). P450 BM3 WT and F87A displayed the highest specific product formation rates with CoSep at the selected reaction conditions, with 7 ± 0.1 and respectively 7.9 ± 0.8 U/ μ mol P450. Both are followed by M3, which showed a more than two times lower SPFR of 2.9 ± 0.3 U/ μ mol P450. Also noticeable were the specific product formation rates of M3 R471C, M3 DM I401D and M3 DM N543S with 2.4 ± 0.03 , 1.27 ± 0.09 and 1.11 ± 0.3 U/ μ mol P450. All other mutants showed specific product formation rates below 1 U/ μ mol P450.

As it can be seen in figure 26, absorbance values decreased after 10 to 15 minutes. This decrease was most pronounced for F87A and WT, which also comprised the highest absolute absorbance values of all mutants. Also for M3, M3 R471C and M3 DM I401D this effect can be observed and very low decrease rates were still visible for M3 DM I401V and M3 DM nd. For pET28a, M3 N543S, M3 DM and M3 DM I401M no absorbance decrease was detected and no mathematical correction was conducted for those mutants (figure 27).

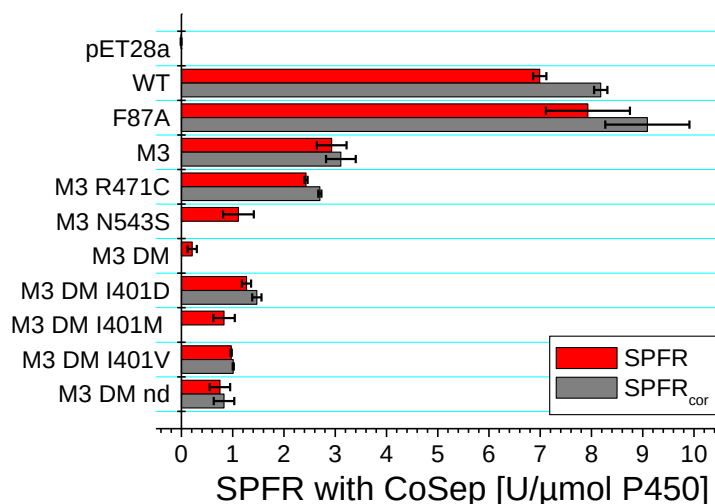


Figure 27: Specific product formation rates (red, SPFR) calculated from the slope of the absorbance increase at 410 nm shown in figure 26. Each mutant was tested in duplicate. Mathematical correction of the absorbance decrease at -750 mV yields slightly higher SPFR values (gray, SPFR_{cor}).

The experiment was repeated with NADPH as electron source (figure 28) and the assay was measured in cuvette format in an UV/Vis photometer. The same reaction conditions were applied as for the CoSep assay (except the exclusion of catalase and CoSep). For WT the specific product formation rate with NADPH was approximately a factor of 30 higher than with CoSep. With NADPH WT had a specific product formation rate of 188 U/μmol P450 and F87A of 444 U/μmol P450. The difference between both displayed with NADPH could not be seen as distinctive in the assay with CoSep. Furthermore the mutant M3 DM nd exceeded the mutant M3 R471C in its specific product formation rate when NADPH was used and had a three times higher specific product formation rate than M3 N543S. In contrast M3 DM nd showed an approximately 30 % reduced rate compared to

M3 N543S when CoSep was used as electron source. Moreover M3 DM nd had almost the same specific product formation rate than WT when NADPH was used (175 U/ μ mol P450 M3 DM nd and 188 U/ μ mol P450 BM3 WT), but with CoSep the specific product formation rate did not approximate the values of WT. M3 seemed to have an improved performance with CoSep, since it had the highest specific product formation rate after WT and F87A. Its specific product formation rate with NADPH was at one level with M3 DM I401V and less than for M3 DM I401D, which were both exceeded by M3 when CoSep was used. M3 R471C exposed the fourth highest specific product formation rate after WT, F87A and M3. This mutant also performed well with NADPH with a specific product formation rate of 123 U/ μ mol P450 (fourth highest SPFR after F87A, WT and M3 DM nd).

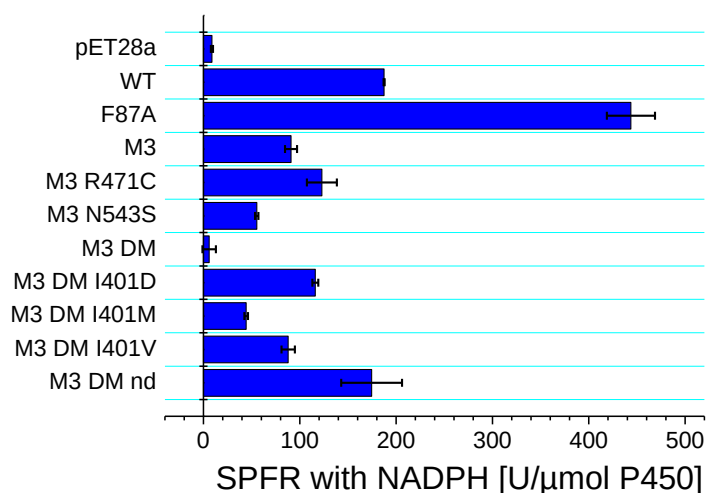


Figure 28: pNCA assay with NADPH at standard conditions (120 μ M 12-pNCA in 50 mM potassium phosphate buffer at pH 8, $V_{\text{end}} = 1$ mL, at 30°C) and a P450 concentration of 0.38 μ M. The reaction was started by addition of 0.1 mM NADPH. All experiments were done in duplicate.

For the electrochemically driven 12-pNCA conversion with CoSep the resulting current was recorded by the electrochemical unit. Since the signal was disturbed regularly by the shaking process the average current value of single experiments was obtained with a deviation of ± 8 %. The highest current values were obtained for WT, F87A, M3, M3 R471C and M3 DM I401D when compared to pET28a (figure 29), but in most cases high error bars between measurements in duplicate prevent further differentiation. In addition the behavior of M3 DM nd is noticeable,

which was the only mutant that gave significantly lower current values than the empty vector pET28a.

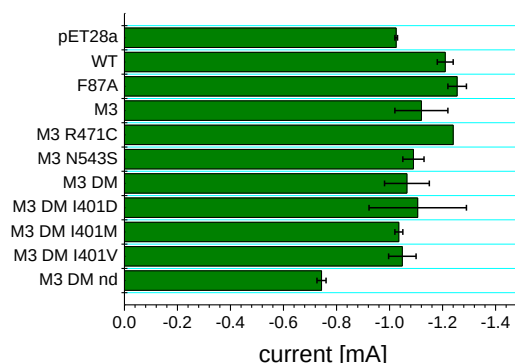


Figure 29: Average current recorded at 30 minutes of pNCA assay with CoSep at -750 mV. Deviation between average currents of two independent experiments per mutant is shown.

Nevertheless the average current was related to the amount of product that was formed until the rate of absorbance increase at 410 nm over time approached zero. The amount of product, corresponding to this point in time, is displayed in figure 30. It has to be noted that for mutant M3 DM during 28 minutes of reaction this point in time was not attained since the linear increase in absorbance did not stop until 28 minutes were reached. In this case the values at 28 minutes were taken to calculate product concentration as well as charge passed.

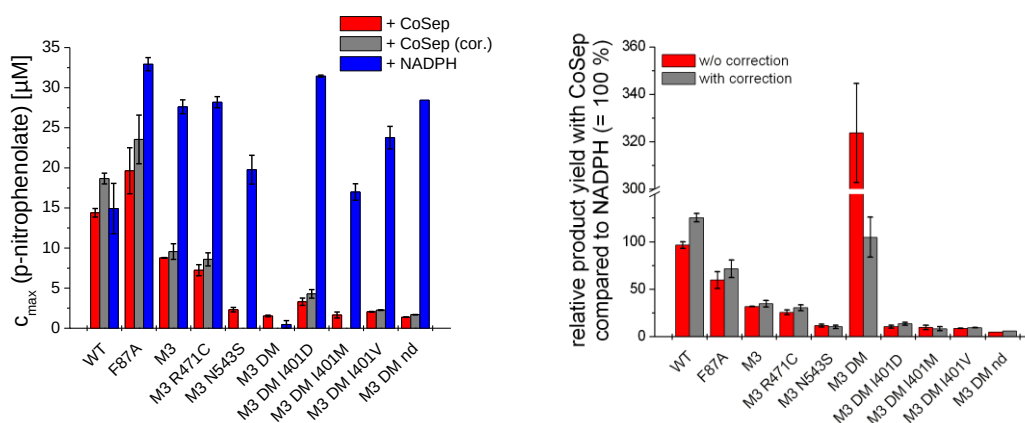


Figure 30: Left – Product concentration reached when the rate of absorbance increase at 410 nm approached zero. Right – Product concentration obtained with NADPH was set to 100 % and compared to values obtained with CoSep. Calculations were conducted also for values which were corrected for the absorbance decrease at 410 nm (cor. or respectively correction).

WT produced the same amount of product with both electron sources, NADPH and CoSep. In all other cases during 28 minutes of reaction time the values obtained with NADPH were not reached. F87A showed approximately 40 % reduced yields compared to NADPH while all other mutants showed relative yields below 50 % of the NADPH value. Only M3 DM showed improved relative yields compared to NADPH (around 320 %), but in regard of the actual product concentration (figure 30, left) values for NADPH and CoSep were comparable and at a low level.

The product concentration obtained when the rate of absorbance increase at 410 nm approached zero was related to the charge passed in the same time interval to yield the coupling efficiency (figure 31). Coupling efficiency was highest for F87A (0.58 %) followed by WT (0.40 %), M3 (0.19 %) and M3 R471C (0.14 %). All other mutants showed coupling efficiencies below 0.1 %. Since average currents did not differ significantly (figure 29), higher coupling efficiencies corresponded to higher product yields.

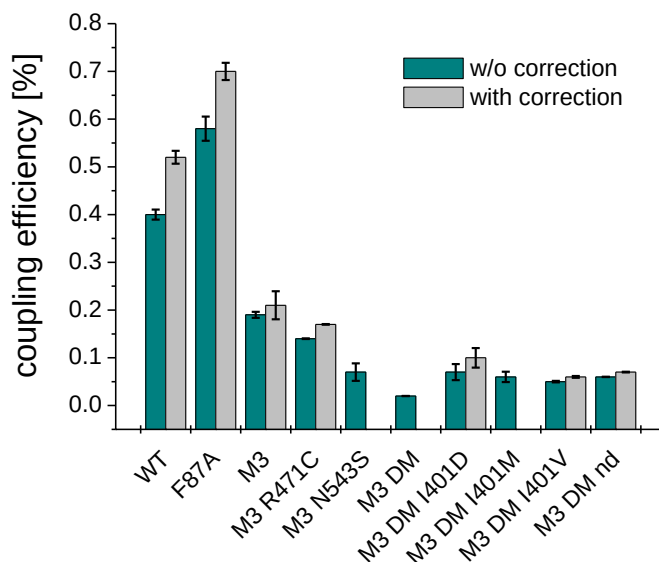


Figure 31: Coupling efficiency between passed charge and amount of product formed in a certain time interval (“correction” regards to values which have been corrected for the absorbance decrease at 410 nm).

2.2.3.5. Variation of reaction conditions I: Enzyme concentration

In the following experiment P450 concentration was varied for WT and M3. All other reaction conditions were kept constant at standard conditions for the electrochemically driven pNCA assay with CoSep.

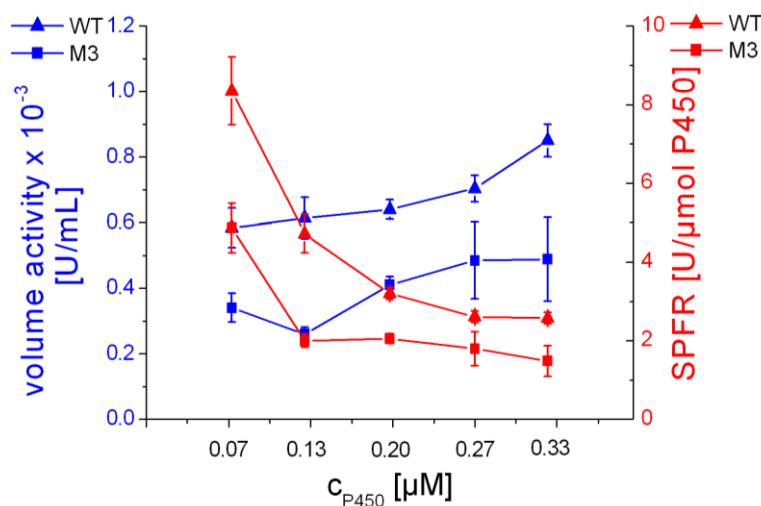


Figure 32: pNCA assay with CoSep at standard conditions with different concentrations of P450 BM3 WT (triangles) and M3 (rectangles). Volume activities (blue) and corresponding specific product formation rates (SPFR, red) are spotted versus P450 concentration. All experiments were done in duplicate.

At low enzyme concentrations of 0.07 to 0.2 μ M P450 a volume activity of around 0.6×10^{-3} U/mL was obtained for WT, which started to increase at a higher rate up to 0.85×10^{-3} U/mL when enzyme concentration was further increased (figure 32, blue). This lead to high specific product formation rates at low enzyme concentrations of 8.6 U/ μ mol P450 BM3 WT (figure 32, red), which decreased when enzyme concentration was varied from 0.07 μ M to 0.2 μ M to a specific product formation rate of 3.2 U/ μ mol P450 BM3 WT. With a further increase of enzyme concentration from 0.2 to 0.33 μ M WT, the specific product formation rate remained constant at approximately 2.6 U/ μ mol P450 BM3 WT.

For M3 also the highest specific product formation rates were obtained at low enzyme concentrations with 4.9 U/ μ mol M3 at an enzyme concentration of 0.07 μ M M3 (figure 32). In between enzyme concentrations of 0.13 to 0.2 μ M

specific product formation rates seemed to be constant. A slight decrease in specific product formation rate was seen at enzyme concentrations higher than $0.2 \mu\text{M}$ M3. In comparison to WT volume activities, M3 volume activity values approached a plateau at enzyme concentrations in between 0.27 and $0.33 \mu\text{M}$, while WT showed an increase in volume activity at these concentrations.

Furthermore for each measurement coupling efficiencies between the passed charge and product formed were calculated (figure 33). While for WT coupling efficiency was increasing with enzyme concentration, for M3 coupling efficiencies remained constant for the whole concentration range tested.

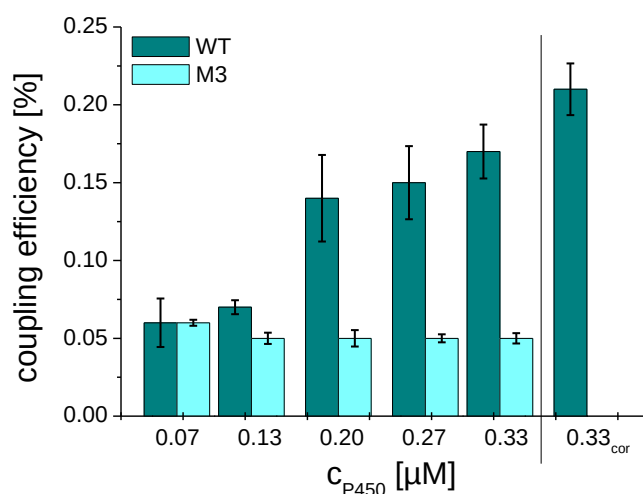


Figure 33: Coupling efficiencies between passed charge and the amount of product formed at different enzyme concentrations.

2.2.3.6. Variation of reaction conditions II: Mediator concentration

The electrochemically driven pNCA conversion was performed at different mediator concentrations with WT, M3 and M3 R471C. Specific product formation rates for WT increased almost linearly with mediator concentration (figure 34). Maximum SPFR was 5.3 U/ μ mol WT at 10 mM CoSep. At CoSep concentrations higher than 10 mM white precipitate was obtained whose amount increased with increasing CoSep concentration. For M3 the maximal specific product formation rate was achieved already at 7.5 mM CoSep. With 3.5 U/ μ mol P450 it was close to the value obtained for the WT at the same mediator concentration (3.8 U/ μ mol P450). M3 R471C behaved similar to M3. At 2.5 mM CoSep both mutants had the same specific product formation rate of 0.93 U/ μ mol P450. At higher CoSep concentrations M3 R471C displayed the lowest specific product formation rates, but like M3 its maximum was reached at 7.5 mM CoSep. A further increase in mediator concentration did not improve specific product formation rates of M3 and M3 R471C.

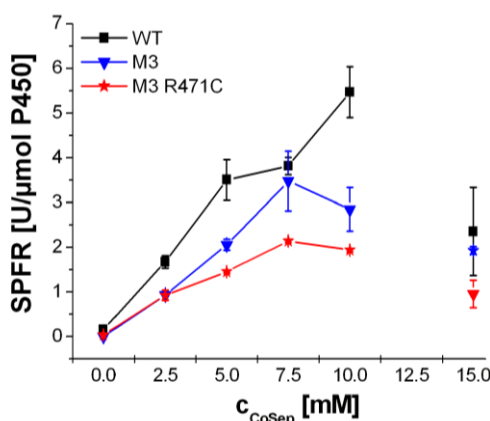


Figure 34: Electrochemically driven pNCA assay with three P450 BM3 mutants at different CoSep concentrations. P450 concentration was 0.2 μ M. All tests were performed in duplicate.

Coupling efficiencies between passed charge and product formed increased for WT with increasing CoSep concentration and a maximal coupling efficiency of 0.16 % was obtained at 10 mM CoSep (figure 35). For M3 the maximal coupling efficiency was reached at 7.5 mM CoSep with 0.1 % and decreased to 0.09 % at a higher CoSep concentration of 10 mM. Nevertheless M3 showed a similar

coupling efficiency to WT at 7.5 mM CoSep, while at a higher CoSep concentration of 10 mM it exhibited an almost 50 % reduced coupling efficiency compared to WT. At 2.5 mM CoSep both mutants, WT and M3, had coupling efficiencies around 0.06 %. For M3 R471C coupling efficiencies were constantly around 0.05 % in between CoSep concentrations of 2.5 to 10 mM.

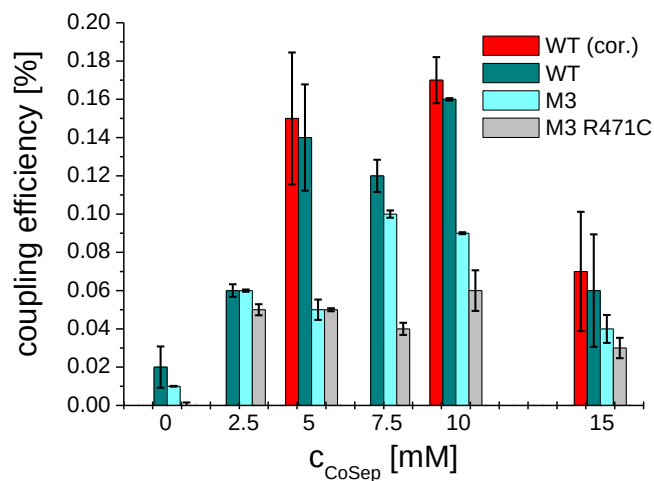


Figure 35: Coupling efficiency (between passed charge and the amount of product formed during a certain time interval) at different mediator concentrations.

2.2.3.7. Variation of reaction conditions III: Applied potential

The pNCA assay with CoSep was performed at different potentials vs. $\text{Ag}/\text{AgCl}_{\text{eMTP}}$ for 7 mutants, which had shown the highest specific product formation rates in the mutant screening (see chapter 2.2.3.4.). At -550 mV SPFR for all mutants were low and did not differ significantly (figure 36). At lower potentials F87A showed always the highest SPFR followed by WT. Both mutants showed their maximal SPFR of $6.8 \pm 1.2 \text{ U}/\mu\text{mol WT}$ and $9.1 \pm 0.2 \text{ U}/\mu\text{mol F87A}$ at -850 mV. At this potential all other mutants also showed their maximal SPFRs values which were around $2 \text{ U}/\mu\text{mol P450}$. For M3, M3 R471C and M3 N543S SPFR values obtained at -850 mV resembled values at -950 mV. In contrast SPFR of F87A, M3 DM I401D and M3 DM nd displayed a decrease in SPFR at -950 mV compared to values at -850 mV. This could also be seen to less extent for WT. Furthermore the mutant M3 DM nd was the only mutant which exhibited already at -750 mV (from its point of view) a high SPFR value of $1.8 \pm 0.3 \text{ U}/\mu\text{mol P450}$. This value differed only slightly from its maximal SPFR at -850 mV ($2 \pm 0.4 \text{ U}/\mu\text{mol P450}$).

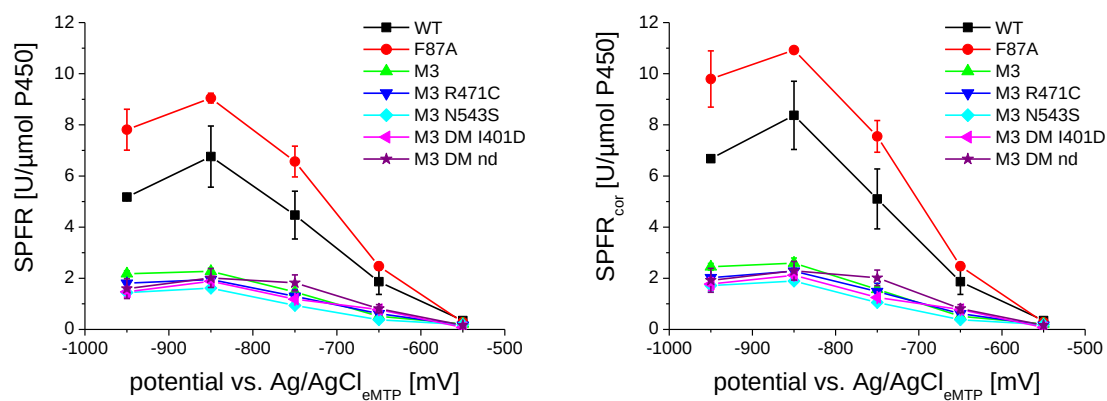


Figure 36: Electrochemically driven pNCA assay with CoSep and several P450 BM3 mutants. Different potentials were applied for 30 minutes respectively. Specific product formation rates obtained at each potential are shown on the left (each experiment was done in the eMTP in duplicate) and SPFR_{cor} values with mathematical correction of the absorbance decrease are displayed on the right. $C_{\text{P450}} = 0.45 \mu\text{M}$.

Since absolute SPFR values differ strongly they were set in relation to their maximal value at -850 mV, which was set to 100 % (figure 37). It can be seen that for example M3 was invariant in its SPFR in between -850 and -950 mV while SPFR for WT was reduced by around 20 % compared to the value at -850 mV. Again the already high rate for M3 DM nd at -750 mV was striking.

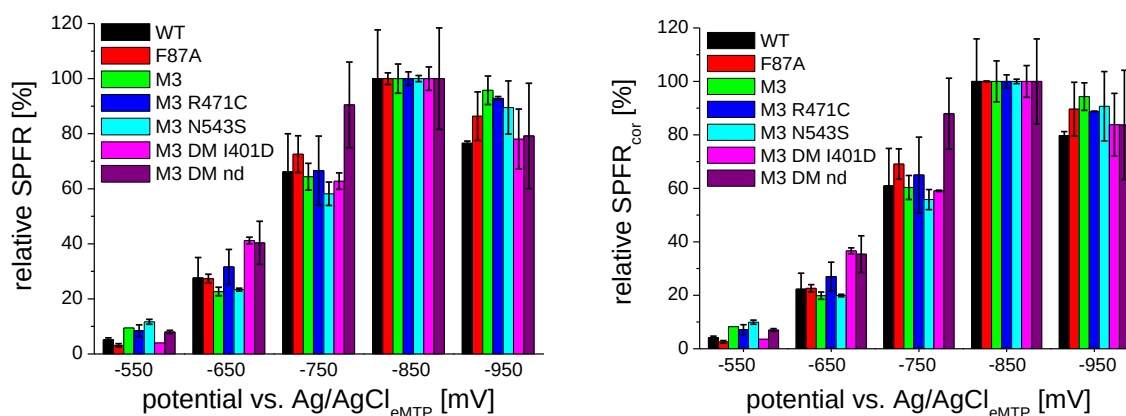


Figure 37: Since absolute SPFR values differ strongly the maximum SPFR value of each mutant, which was reached at -850 mV in all cases, was set to 100 % (left). The same was done for SPFR_{cor} values (right).

As already described in the beginning the optical data obtained during the experiment showed a decrease in absorbance after an initial increase at 410 nm. This phenomenon could be seen for all mutants, but not at -550 and -650 mV. At lower potentials the decrease in absorbance occurred and was especially pronounced for samples containing WT and F87A (figure 38).

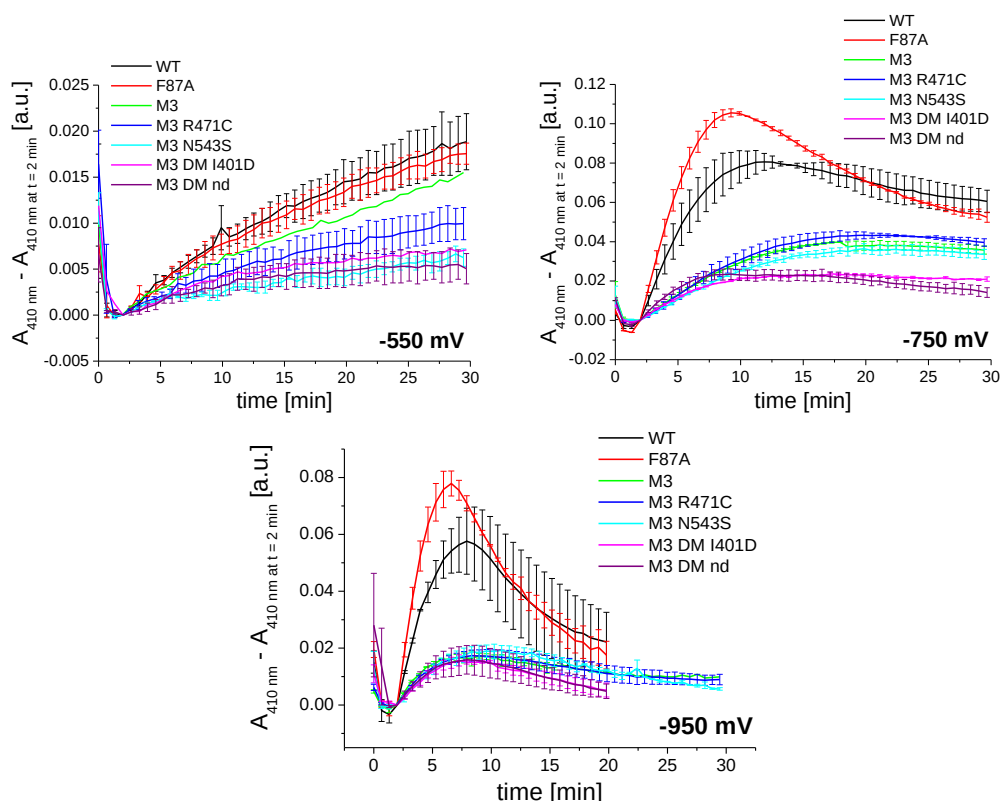


Figure 38: Absorbance at 410 nm over time for the tested mutants at -550 mV (upper row, left), -750 mV (upper row, right) and -950 mV (lower row). Potentials were applied vs. $\text{Ag}/\text{AgCl}_{\text{eMTP}}$ at standard conditions.

In addition coupling efficiencies were calculated (figure 39). Highest coupling efficiencies were obtained for F87A and WT at -850 mV, which correlated to the potential at which all mutants also showed their highest specific product formation rates. For F87A a coupling efficiency of almost 1 % was reached for the absorbance decrease corrected values.

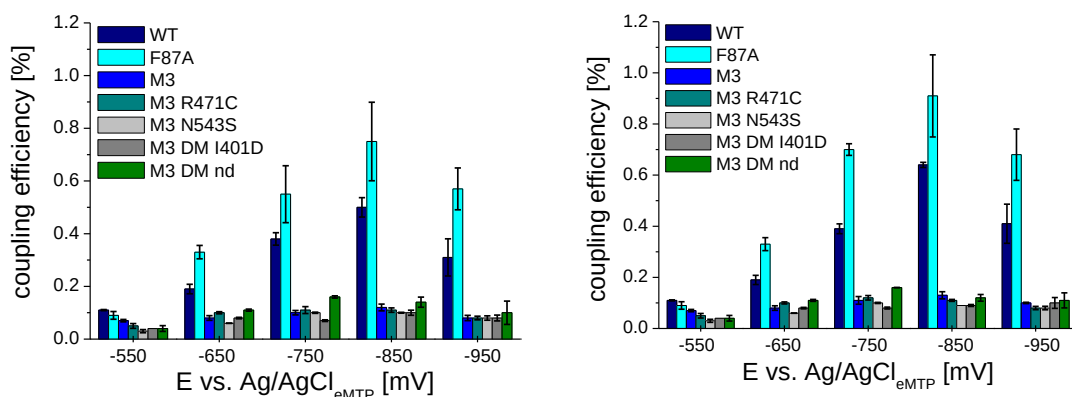


Figure 39: Left - Coupling efficiencies for mutants at different applied potentials. Right - Coupling efficiencies based on absorbance decrease corrected values.

2.3. Discussion

2.3.1. Evaluation of reference electrodes

For the eMTP easy to handle and reliable reference electrodes are needed. Platinum wire was the first material chosen for investigation since it is easily implemented. Since platinum does not have a fixed redox potential it is regarded as pseudo reference electrode. Potentials measured versus a platinum pseudo reference electrode have to be compared to a reference electrode with fixed potential by measuring a well known redox species, e.g. potassium ferrocyanide. In the eMTP experiments Pt performed well at positive potentials in between 0 to 500 mV, the potential did not shift and remained constant for several measurements. But at negative potentials a strong shifting of the potential was seen (data not shown). This may be attributed to a charging process of the Pt wire. For example the redox potentials of mediators like CoSep, which have a negative formal potential, could not be detected with a Pt reference electrode (data not shown). To overcome this limitation miniaturized silver/ silver chloride reference electrodes were built. Silver/ silver chloride reference electrodes are standard reference electrodes generally used in aqueous medium. Unlike Pt their half-cell potential is constant and does not depend on the reaction solution composition. Compared to a commercially available silver/silver chloride reference electrode the miniaturized Ag/AgCl reference electrodes for the eMTP performed well and showed only slight deviation in measured redox potential of the well-know redox active species potassium ferrocyanide. For example in literature a value of 415 mV vs. NHE [125], or respectively 205 mV vs. Ag/AgCl, is given for the ferrocyanide midpoint potential. In comparison measurements with the Ag/AgCl_{eMTP} in the eMTP set-up yielded a midpoint potential of around 220 mV, which corresponds to a relative standard deviation of 7 %.

Measured potentials were stable in between sets of electrodes as can be seen by measurements of potassium ferrocyanide or ABTS in 8 wells sequentially which comprise eight individually prepared reference electrodes. By preparing the Ag/AgCl_{eMTP} in a Tygon tube chemical inertness is given. It has to be noted that

neither leak rate nor the electrical resistance of the magnesia junction were measured. Contamination of the measured solution with the filling of the reference electrode comprising high chloride concentration or silver ions cannot be excluded. But since the magnesia junction was prepared freshly in certain time intervals no visible leakage was obtained.

In conclusion the $\text{Ag}/\text{AgCl}_{\text{eMTP}}$ reference electrode is suitable for the eMTP application, since reference electrodes are easily prepared and implemented. Furthermore prepared electrodes performed reliable and comparable to commercially available reference electrodes.

2.3.2. eMTP evaluation

2.3.2.1. Reliability

Performance of the eMTP set-up including self-made reference electrodes, the arrangement of electrodes in the well and the connection to the multiplexed potentiostat were evaluated by measuring well-known redox active species. First the redox probe potassium ferrocyanide was measured in several electrolytes and compared with a commercially available low volume spectroelectrochemical cell (SEC) with a defined reaction cell set-up. In general peak broadening is more pronounced in the eMTP set-up (around 11 % increased peak separation compared to SEC). This is most probably related to uncompensated IR drop effects which will be found less influencing in the low volume SEC reaction cell. Furthermore midpoint potentials determined with the eMTP and SEC differ by 15 %, with the eMTP value being closer to literature values for potassium ferrocyanide. Also current ratios are slightly higher than 1 in the eMTP set-up compared to SEC, where current ratios were close to 1 at all scan rates.

Secondly the mediator ABTS was characterized in both reaction cell set-ups. The midpoint potential determined for ABTS (488 ± 9 vs. $\text{Ag}/\text{AgCl}_{\text{eMTP}}$) differs only by 5 % from values obtained with the SEC system comprising a commercially available Ag/AgCl (510 ± 3 mV). This value is consistent with literature values

which give a midpoint potential of 485 mV for the $\text{ABTS}^{2-}/\text{ABTS}^{\bullet-}$ redox pair in pH 9 carbonate buffer [126]. As described before the same holds true for potassium ferrocyanide, whose midpoint potential was determined in accordance with literature values as well. The rather irreversible performance of ABTS in regard to its current ratio is seen in both cases (eMTP and SEC) and related to gold as material for the working electrode and not to inherent properties of the reaction cell set-up.

In ABTS as well as potassium ferrocyanide measurements deviations in current are quite high (around 15 %), which is likely related to the surface area of the working electrode. Since electrodes were cut manually an average surface area of $1.27 \pm 0.24 \text{ cm}^2 \text{ cm}^{-3}$ was obtained which corresponds to a relative deviation of 18.9 % in size. Correction of surface differences can be done electrochemically by measuring a defined volume and concentration of a redox active substance and relate the measured differences in current to size. Theoretically this correction has to be done before each measurement, but to save time in P450 screening this method was abandoned. Furthermore the surface correction methodology was in particular avoided to prevent contamination of the reaction solution in P450 screening. By choosing another method of electrode preparation, e.g. laser cutting, here is a starting point for further improvement of the eMTP.

In conclusion the three electrode arrangement established in the eMTP worked properly since no significant difference to SEC measurements were obtained for potassium ferrocyanide as well as ABTS. Deviations in potential and current were generally less than 15 %.

2.3.2.2. Reproducibility

Well-to well reproducibility was investigated revealing small deviations in potential with 3.4 % for potassium ferrocyanide and 1.8 % for ABTS when determining the midpoint potential. Since each well comprised its own set of electrodes manufacturing and incorporation of $\text{Ag}/\text{AgCl}_{\text{eMTP}}$ as well as working and counter electrode is satisfactory.

2.3.2.3. Combination with the optical unit

The combination of the eMTP, connected to the multiplexed potentiostat, to the Tecan reader was established and the oxidation rate of potassium ferrocyanide was determined. Standard deviation of reaction rates in between 3 wells was lower than 10 % which means the error introduced by manual triggering of both instruments is acceptable. Furthermore it has to be noticed that the cover panel of the Tecan is open during measurement. But as it can be seen by the low relative standard deviations obtained sensitivity is not significantly disturbed.

2.3.2.4. Test screening with ABTS

Since ABTS did not show reversible behavior at gold electrodes it is rather hard to determine optimized oxidation conditions in our set-up. Nevertheless it has been possible to determine the influence of reaction conditions on ABTS oxidation rate. Several effects can be distinguished including pH and buffer composition within a narrow range (e.g. one pH unit), even though relative standard deviation is rather high (around 20 %).

2.3.3. P450 BM3 mutant screening in the eMTP

2.3.3.1. Proof of concept

pNCA conversion could be driven electrochemically in the eMTP via the mediator CoSep and with several P450 BM3 variants. Reduction of the mediator was achieved at -760 mV (at 25 mV/s, vs. Ag/AgCl_{eMTP}) and a reduction potential of -750 mV was set as standard potential for this assay. With increasing scan rate reduction potential was increasing, but to minimize side effects prone to high driving forces a potential of -750 mV was regarded as sufficient for standard conditions. Furthermore no oxidation of 12-pNCA to the product *p*-nitrophenolate was seen when one of the reaction components (P450, CoSep and potential) was missing. In wavelength scans obtained during the assay product formation was monitored at 410 nm [38]. In addition CoSep reduction is possible in the eMTP set-up, even though work is done under oxygen atmosphere. Due to the presence of oxygen a high percentage of the reduced mediator will be reoxidized by air during the time course of the experiment [36] with concomitant production of hydrogen peroxide. Interestingly negative controls show that application of a reduction potential of -750 mV for half an hour at 30°C did not harm the enzyme. Only if addition of catalase was avoided activity of the enzyme decreased significantly (by approximately 40 %), indicating that hydrogen peroxide was formed during the reaction. Hydrogen peroxide formation may be due to oxygen reduction at either counter or working electrode [125] or by uncoupling of the mediator with dissolved molecular oxygen (figure 40). Since catalase was present hydrogen peroxide was decomposed to water and oxygen, which should prevent enzyme degradation as well as oxygen depletion [34]. Since no activity decrease was seen for P450 F87A at standard conditions with and without applied potential, the amount of catalase present seemed to be adequate to decompose all evolving hydrogen peroxide. Nevertheless negative controls prepared in the eMTP show 25 % reduced specific product formation rates when compared with fresh lysate. Most probably a systematic error is introduced here since this effect was observed for all negative controls. Anyway the reduction of the overall specific product

formation rates was not a result of temperature effects as well as adsorption of protein to the electrodes or respectively electrode fouling (table 8).

As indicated in figure 40 several diffusion effects have to be taken into account. To avoid the development of diffusion profiles at the electrode surface a mixing protocol was established. Nevertheless mixing had to be stopped during the optical data acquisition and measured current profiles indicate a diffusion limited current evolving in between (figure 21).

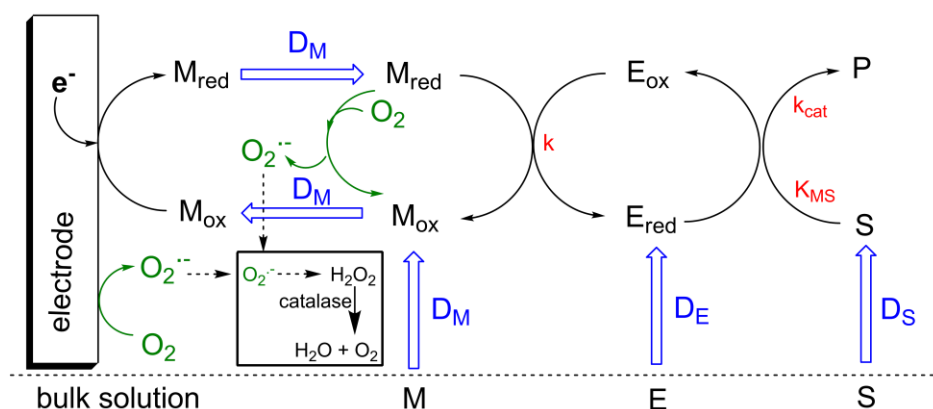


Figure 40: Reaction scheme for the electrochemically driven pNCA assay via the mediator CoSep (derived from [127] and [125]). M = mediator (CoSep); E = enzyme (P450 BM3); S = substrate (12-pNCA); P = product (*p*-nitrophenolate). In red: enzyme characteristics. In blue: diffusion coefficients D . In green: Side reactions producing superoxide – (a) Oxygen is reduced at the electrode (e.g. working or counter), (b) oxygen reacts with the reduced mediator. Inset: Superoxide decomposes yielding hydrogen peroxide, which is converted by catalase to give water and oxygen.

2.3.3.2. Stability of *p*-nitrophenolate at assay conditions

An absorbance decrease at 410 nm over time was observed in experiments at -750 mV or at lower potentials. The rate of absorbance decrease was increasing with increasing reductive power. Concomitantly maximal amounts of product reached decreased. Most probably a decrease in pH over time due to water electrolysis took place and resulted in protonation of the product *p*-nitrophenolate, which lead to loss of its yellow color [38]. Besides product protonation a decrease in pH might also be influencing enzyme activity itself. For a BM3 variant it was

shown that relative activity remained almost constant in a pH range in between pH 7 and 8 [128], consequently pH effects on enzyme activity were neglected in determination of initial rates. Furthermore it cannot be excluded that either product and/ or the substrate are decomposed electrochemically, which would both influence measured reaction rates. It was already shown that in the CoSep assay with zinc the product *p*-nitrophenolate was prone to reduction when operating in Tris buffer [37]. Nevertheless with WT 100 % relative yield of product were obtained with CoSep compared to NADPH, although at a lower specific product formation rate (figure 30). Most probably discussed parameters will influence more strongly reaction rates of “slow” mutants, since a pH effect as well as product or substrate degradation will be more pronounced with increasing reaction time. In accordance those mutants exhibit a low product yield compared to mutants with high SPFR.

In addition a beneficial influence of increased catalase amounts on product stability was seen (figure 23). As described before the amount of catalase present was sufficient to decompose hydrogen peroxide evolving from oxygen reduction of molecular oxygen, either by the electrode or uncoupling with CoSep, since no activity decrease for P450 F87A was seen at standard conditions. Furthermore absorbance decrease at 410 nm is independent of CoSep being present or not (figure 23), which further confirms that hydrogen peroxide formation was not the reason for product instability. Beyond that it was shown for the zinc driven assay that a hydrogen peroxide concentration up to 400 mM did not alter the optical properties of *p*-nitrophenolate [37]. This finding supports the assumption that loss in absorbance over time will be due to a pH shift of the reaction solution rather than hydrogen peroxide attacking the product. Probably the high catalase amount changed viscosity and conductivity of the reaction solution, so that less current is generated which would result in a lower theoretical pH change. Also organic materials may adsorb to the electrode blocking its surface.

2.3.3.3. Evaluation of electrochemical data

With the present set-up the reaction was driven by applying a constant reduction potential over time. As a consequence a concentration profile will develop at the electrode surface and regular shaking of the plate is needed for prevention. The shaking process in turn resulted in high noise of the current signal (figure 21). Only an average current was assumed during the whole time interval and distinct current behavior could not be seen with this method. Nevertheless current data were used to calculate coupling efficiencies, which relate consumed charge to the amount of product formed [36]. In all cases coupling efficiencies were low ($< 1\%$) and mostly influenced by the amount of product formed (which differed between mutants) and not current, which is comparable and in high excess in all experiments. As a consequence differences in current and respectively charge do not impair strongly on the calculation of coupling efficiencies. Most of the charge will be consumed for mediator reduction, which was reoxidized by uncoupling with oxygen, resulting in the high background current observed (compare values for pET28a in figure 29). It has to be remembered that the area of working electrodes is differing by 18 %. Despite that in the mutant screening low standard deviations were obtained (figure 27), for well performing mutants like WT and F87A even below 10 %. This finding again demonstrates that the recorded current is much higher than needed for product formation, and differences in electrode size did not influence the measured reaction rates significantly. This is also demonstrated by the fact that coupling efficiencies correspond to specific product formation rates. High SPFR gave high absolute product concentrations and therefore resulted in high coupling efficiencies. Nevertheless it is interesting to note at which point (at equal values of current) product formation is increasing. In general coupling efficiencies obtained are in the range of literature values. A coupling efficiency of 2 % was obtained for P450 BM3 converting lauric acid in an electrochemically driven reaction via the mediator cobaltocene [36]. The authors argument, that the low coupling efficiency is due to the mediator reacting with molecular oxygen, which will also be the case for CoSep in the eMTP. Taking into account the higher amount of CoSep in the present experiment (5 mM vs. 1 mM for cobaltocene [36])

it is not surprising that coupling efficiencies are even worse (around 0.6 % for F87A).

With another electrochemical method the disadvantages of the chronoexperiment may be prevented. By using a pulse method, which would allow regeneration of concentration profiles at the electrode, or cyclic voltammetry the shaking process might not be necessary anymore. Furthermore effects of electrolysis (e.g. a pH change of the solution) would be reduced or circumvented. But in those cases mediator reoxidation by oxygen may be even more pronounced, since a permanent driving force for mediator reduction is missing.

2.3.3.4. Mutant screening

P450 BM3 is known to convert substrates by the aid of CoSep in electrolysis experiments. Estabrook et al. obtained a specific product formation rate of $110 \text{ nmol min}^{-1} \text{ nmol}^{-1} \text{ P450}$ for the electrochemically driven $\omega(-)$ -hydroxylations of lauric acid [34, 35], which was 12 % of the rate the authors obtained with NADPH. In case of the eMTP for P450 BM3 WT a rate of $7 \text{ U}/\mu\text{mol P450}$ was obtained with the substrate 12-pNCA, which equals $7 \text{ nmol min}^{-1} \text{ nmol}^{-1} \text{ P450}$. This value corresponded to 3.7 % of the rate obtained with NADPH. Rates obtained with the eMTP cannot be compared directly to Estabrook et al., since reaction parameters were not identical. Differences in procedure for Estabrook et al. include the use of purified protein, electrode material, temperature, buffer system as well as reactant concentrations. One main reason for the good performance of the Estabrook system will be the sophisticated cell set-up including a counter electrode separated from the reaction solution by a frit and the control of the oxygen level. Both factors will influence the appearance of side reactions, but cannot be accomplished in an eMTP set-up. The eMTP therefore serves mainly as a prescreening to choose promising enzyme candidates or determine the influence of certain reaction parameters like mediator concentration or applied potential. Also Estabrook et al. showed that the reaction was kept running for 2 hours without destruction of P450. In case of the eMTP experiment it is clear that only initial rates can be determined since at elongated times side reactions (pH shift,

oxygen depletion) will be dominating and eventually reduce reaction rates to zero (figure 38). This was seen especially for mutants which exhibit low reaction rates and did not reach absolute product yields achieved with NADPH (figure 30).

Generally SPFR rates determined in this work will not represent real reaction rates of the mutants. Product formation is possibly accompanied by a pH change, hydrogen peroxide formation, substrate or product decomposition or oxygen depletion due to uncoupling of CoSep or oxygen reduction at the counter electrode. All processes will overlap giving the apparent product formation rate measured. A correction of the absorbance decrease observed was applied to estimate the error introduced by a pH change of the solution due to electrolysis (see chapter 2.2.3.3.). Besides interaction with the mediator also inherent mutant properties like pH stability, hydrogen peroxide tolerance or usage for the shunt pathway, as well as the substrate affinity and turnover will contribute to the apparent reaction rate (compare figure 40). Nevertheless mutants can be differentiated and SPFR are determined with high reproducibility and low error (around 10%) at standard conditions. The coefficient of variation is in accordance with other screening approaches including P450 BM3 and the pNCA assay [129], also with the zinc CoSep system [39]. It has to be mentioned that relative standard deviations were increased for mutants which display already low specific product formation rates like M3 DM and M3 DM nd (figure 27). Error is higher in experiments where the potential was varied since with increasing reductive power side reactions are more prone to occur (figure 37).

The mutant screening with the eMTP revealed that in terms of specific product formation rates F87A and WT performed best independently of the electron source used (NADPH or CoSep). In general order of performance with CoSep is similar to performance with NADPH (figure 41).

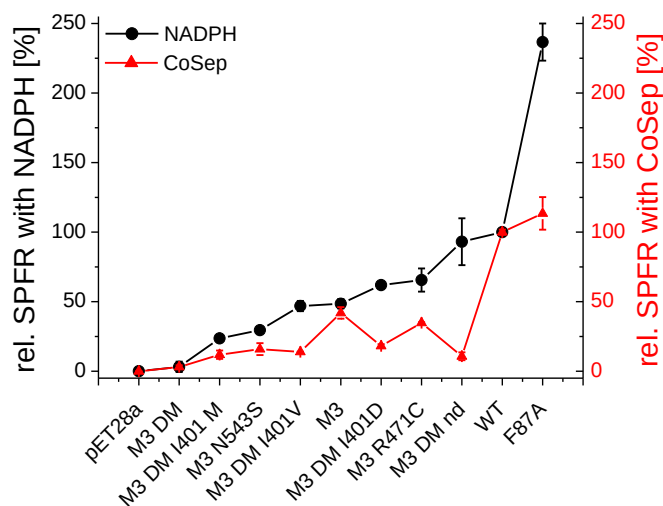


Figure 41: Comparison of relative SPFR from low to high values. WT SPFR was set to 100 % for both ordinates.

M3 seems to have an improved performance with CoSep compared to NADPH measurements, since it exhibits higher relative SPFR than the circumjacent mutants. Accordingly it is striking that M3 DM nd and F87A perform worse with CoSep compared to their SPFR obtained with NADPH (figure 41). As it can be seen in figure 41 mutants can be clearly differentiated from each other and the error obtained with the eMTP does not differ significantly from error resulting from the standard pNCA assay with NADPH. Generally high product formation rates yielded also the highest product concentrations. One reason might be that for mutants with lower SPFR during the time course of the reaction influences of pH (or generally side reactions due to electrochemistry) might be more influencing. However, after a certain reaction time the reaction solution may be depleted from oxygen, which can be reduced by the electrode or by uncoupling with the mediator CoSep. Nevertheless oxygen depletion is unlikely to happen in the first minutes of the reaction in which SPFR is determined, since for WT with CoSep the same amount of product was formed than with NADPH (even though at a lower rate). One interesting exception is F87A. It displayed a high SPFR and also a high amount of product was obtained. But compared to WT, F87A did not reach the amount of product formed with NADPH when switching to CoSep as electron source (figure 30). With CoSep F87A yields only around 60 % of the value obtained with NADPH. One might assume that either the destruction of

p-nitrophenolate (electrochemically or by protonation due to the pH decrease) is more pronounced the more product is formed, or that some other unknown limitation occurs here. The same is valid for SPFR. While with NADPH F87A converts the substrate 2.4 times faster than WT, with CoSep SPFR values were almost the same for both mutants. Interestingly, in terms of product formed until $A_{\max}$ is reached, with NADPH WT produced 45 % of the F87A value. Instead with CoSep it reached already 74 % of the product amount F87A produced. Highest coupling efficiencies were obtained for mutants which also have high SPFR, as expected. One exception is P450 BM3 M3 nd, which displayed both, low coupling efficiency and low SPFR. It has to be noted that mutants were compared in their performance at one substrate concentration only. It is known that BM3 mutants evolved for mediated electron transfer display differences in their kinetic parameters [39, 40]. From this it follows that chosen substrate concentration might not be in the saturating region for all mutants. Mutants that exhibit low SPFR might show improved performance when other substrate concentrations are tested.

2.3.3.5. Enzyme concentration

It is known that in electrolysis experiments the rate of substrate conversion is dependent on P450 and mediator concentration [125]. Volume activity is a direct measure of activity found per well while SPFR relates the found activity to the amount of enzyme present. With increasing enzyme concentration in tendency higher volume activities and a rather constant specific product formation rate were obtained for both proteins tested, namely M3 and WT (figure 32). It is somewhat surprising that at low enzyme concentrations of 0.07 μM already high SPFR values were obtained for both proteins, which decreased when enzyme concentration was doubled. This behavior may be attributed to a changed relation of enzyme to substrate or enzyme to mediator (figure 40). Furthermore enzyme aggregation is known to occur [37] and may display a concentration dependency. At higher enzyme concentrations around 0.27 μM SPFR values remained almost constant. It has to be noted that for an enzyme concentration of 0.33 μM a lower SPFR is obtained than during the mutant screening (which provided an enzyme concentration of 0.38 μM). This is the case for WT and M3, which were reduced in

SPFR by the same factor, and the decrease in SPFR is related to instability of enzyme over time. Nevertheless the ratio between both protein SPFRs remained constant.

One interesting finding is that coupling efficiency for M3 is constant at all enzyme concentrations tested, while for WT coupling efficiency is increasing with increasing enzyme concentration (figure 33). This finding corresponds to the maximal amount of product formed (see appendix), which is increasing for WT and is constant for M3 in the enzyme concentration range tested. As expected charge consumed is constant for M3, but differed for WT. In WT measurements charge consumed was decreasing with increasing enzyme concentration magnifying the observed effect of increased coupling efficiency.

2.3.3.6. Mediator concentration

In case of the eMTP experiments with P450 BM3 WT a strong increase in reaction rates is seen with CoSep concentrations up to 10 mM. For WT SPFR was limited by the precipitation phenomenon. Until 10 mM CoSep SPFR is increasing almost linearly with mediator concentration. If no precipitation would take place probably a further increase of WT SPFR could be expected. Interestingly for M3 and M3 R471C the highest SPFR is already obtained at 7.5 mM CoSep, after which M3 R471C reaches a plateau and M3 already showed a decrease in SPFR. Optimal CoSep concentrations differ between mutants (figure 34). It also interesting to note that at low CoSep concentrations of 2.5 mM M3 and M3 R471C exhibit the same SPFR while at 5 mM CoSep they differ by approximately 30 %. M3 seems to tune its activity more sensitive to CoSep concentration than M3 R471C. Astonishing is furthermore that coupling efficiencies for all three mutants are equal at 2.5 mM CoSep, while at 5 mM CoSep WT exceeds M3 and M3 R471C already by a factor of 3 (figure 35). At 7.5 mM CoSep M3 approaches the value of WT while M3 R471C did not differ in its coupling efficiency value.

In addition mediator concentration had an impact on measured currents (see appendix for current data and figure 21). With increasing mediator concentration

increased currents were obtained. Since also SPFR was increasing with mediator concentration both observations are an indication that the surface area of the electrode is not a limiting factor for mediator reduction. Of course also the higher SPFR may be related to an effect of CoSep interacting with the enzyme and triggering its activity [39]. If only that case would apply, coupling efficiencies would be expected to be constant or reduced at higher mediator concentrations, since additionally reduced mediator does not benefit the reaction. This is not the case and therefore increase in SPFR will most probably be related to increased electron transfer via the reduced mediator. In this regard it has to be emphasized that in this work CoSep is used in five to ten fold excess compared to literature values [37, 39]. Interestingly Schwaneberg et al. noticed precipitation at CoSep concentrations higher than 5 mM [37]. In addition F87A showed highest conversion rate at 0.5 to 1 mM CoSep in the zinc driven assay, while conversion rate was constant in between 2 and 5 mM CoSep [37]. Both effects were not observed in the eMTP screening. Precipitation of mediator occurred at concentrations higher than 10 mM, which may be due to the different buffer system used. Furthermore a clear increase in SPFR was obtained when changing CoSep concentrations from 2.5 to 10 mM CoSep, at least for WT. Probably in the zinc CoSep system mediator reduction at concentrations higher than 2 mM is limited by the amount of zinc present. As it was shown before CoSep reduction is not limited in case of the eMTP, explaining the observed increase in reaction rate with mediator concentration. In contrast Estabrook et al. did only see a slight increase in reaction rate for the hydroxylation of progesterone with their electrode driven system when changing from 1 to 1.5 mM CoSep [34]. Besides using another P450 this effect may be attributed to the reaction cell set-up. In the eMTP screening is done under oxygen atmosphere while most literature data are based on cell set-ups with controlled low-oxygen level. Most of the mediator reduced in the eMTP will be reoxidized by oxygen. This uncoupling keeps the amount of reduced mediator, which is capable to interact with P450, lower than the total amount of mediator present. With regard to this eMTP results can be set in relation to literature data and lower optimal mediator concentrations obtained within literature can be explained.

2.3.3.7. Applied potential

Tested BM3 mutants exhibited a potential dependency of their SPFR. Influence of the applied potential becomes noticeable as the rate of mediator reduction as well as the occurrence of side reactions. Generally at the lowest potential tested, -550 mV vs. Ag/AgCl_{eMTP}, for all mutants almost no activity was seen. At -650 mV still SPFR values were low, but WT and F87A showed a more than 2 fold increased SPFR than other mutants. In all cases maximal SPFR was obtained at -850 mV (figure 36). In literature a potential of -650 mV vs. Ag/AgCl is given for optimal reaction rates with CoSep and P450 [34, 125]. Stronger reducing potentials were not tested in most of the corresponding literature [34, 35, 125], but can also be found in a study by Udit et al., where a potential of -900 mV was applied for an electrolysis experiment [36]. At a potential of -850 mV vs. Ag/AgCl_{eMTP}, WT and F87A exceeded all other mutants in their absolute SPFR by a factor of more than 3. The most interesting observation is that one of the mutants showed a shift in its optimal potential to -750 mV, namely M3 DM nd. Moreover SPFR of the mutant M3 remained constant when reducing the potential further to -950 mV. In contrast other mutants like WT showed a decrease in SPFR at -950 mV when compared to their optimal value at -850 mV (figure 37). The lower the potential the more side reactions are favored, which include pH change or oxygen reduction. As a result for mutants with high SPFR probably no further increase in absorbance can be seen, even though the real rate may be higher. In terms of coupling efficiencies it is remarkable that F87A exhibited a value of almost 1 % at -850 mV, which is close to literature values [36]. Again coupling efficiencies correlate with SPFR. In general it has to be noted that error bars are quite high (up to 20 %) in this experiment, which is probably due to an increased appearance of side reactions due to application of high reductive power.

3. Development of a screening tool box based on polypyrrole: Towards direct electron transfer to P450 BM3

Based on polypyrrole and two derivatives thereof several immobilization procedures for redox proteins were investigated, which should finally lead to a method suitable for immobilization of P450 BM3 and direct electron transfer to its heme active site.

One of the most common methods described in literature for biomolecule immobilization via polypyrrole is the entrapment in the growing polymer film. Therefore the biomolecule is added to the polymerization solution and incorporated in the polymer during electropolymerization. First the redox active molecules potassium ferrocyanide and two differently charged porphyrin systems, serving as heme analogs, were tested for entrapment. Furthermore the small heme protein myoglobin was incorporated in polypyrrole, since its heme active site is located close to the protein surface, unlike in P450 BM3 [130]. Also the influence of polymerization conditions on polymer growth and P450 BM3 activity were assessed.

As alternative to the entrapment method, which may possess several drawbacks like its influence on protein folding or diffusion limitations of substrate and/ or product through the film, two additional immobilization strategies were investigated, which result in biomolecule immobilization on the surface of the polymer.

First the polymerization behavior of two pyrrole derivatives, which expose carboxylic acid functionalities, was tested in aqueous and organic electrolyte. The latter is possible since the immobilization at the surface of the polymer leads to a separation of the polymerization and immobilization step. The resulting carboxylic acid containing polymers were assessed for their stability and nickel binding abilities. Via nickel chelation His-tagged proteins may be immobilized reversibly at

the polymer in analogy to the IMAC (immobilized metal affinity chromatography) principle. The immobilization of His-tagged P450 BM3 was followed by a quartz crystal microbalance study.

In addition a covalent attachment method was tested based on the same functionalized polymers. Via carbodiimide coupling the redox active molecule aminoferrocene was bound to the polymer surface. Secondly the approach was tested with cytochrome *c*, which is known to possess surface exposed lysine residues close to its heme active site [131]. They may serve as anchor points for the carbodiimide coupling reaction to the carboxylic acid groups of the polymer resulting in formation of a stable peptide bond between both.

3.1. Material and Methods

3.1.1. Material

Electrodes and electrode material

platinum sheet	d = 0.1 mm	Goodfellow GmbH, Bad Nauheim, Germany
gold sheet	d = 0.1 mm	Goodfellow GmbH, Bad Nauheim, Germany
SE 21 Ag/AgCl reference electrode	l = 120 mm, ceramic diaphragm	Sensortechnik Meinsberg GmbH, Ziegra-Knobelsdorf, Germany
Luggin capillary	l = 25 cm, d = 1.5 cm, ceramic frit (d = 2 mm)	manufactured by gaffer
Quartz crystal Au	disk/disk, polished, bonded, 10 MHz	Elchema, Potsdam, USA

Measuring cells

Glass reactor with four necks		manufactured by gaffer
EQCM Flow Cell Kit	with RE-3V Ag/AgCl reference electrode	ALS, via C3 Prozess- und Analystechnik GmbH, Haar, Germany

Instrumentation

Potentiostat PCI4 300 with software Gamry Framework 5.50 (2008)	Gamry Instruments, via C3 Prozess- und Analystechnik GmbH, Haar, Germany
Potentiostat PGSTAT12 with software Nova 1.6	Metrohm, Filderstadt, Germany
eQCM 10 M	Gamry Instruments, via C3 Prozess- und Analystechnik GmbH, Haar, Germany
Lambda 25 UV/VIS Spectrometer	Perkin Elmer, Waltham, USA

Chemicals and proteins

1-H-pyrrole-1-propionic acid (97 %)	Sigma-Aldrich, Taufkirchen, Germany
4-(3-pyrrolyl)butyric acid (95 %)	Sigma-Aldrich, Taufkirchen, Germany
5,10,15,20-tetrakis-(N-methyl-4-pyridyl)-porphyrin-Fe(III) pentachloride	Porphyrin Systems GbR, Lübeck, Germany
5,10,15,20-tetrakis-(4-sulfonatophenyl)-porphyrin-Fe(III) chloride	Porphyrin Systems GbR, Lübeck, Germany
aminoferrocene (> 96 %)	TCI Europe, Zwijndrecht, Belgium
cytochrome c from bovine heart (≥ 95 %)	Sigma-Aldrich, Taufkirchen, Germany
His Trap HP column (5 mL)	GE Healthcare Life Sciences, Freiburg, Germany
myoglobin from equine heart (≥ 95 %)	Sigma-Aldrich, Taufkirchen, Germany
NADPH (≥ 95 %)	Sigma-Aldrich, Taufkirchen, Germany
N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (BioXtra)	Sigma-Aldrich, Taufkirchen, Germany
N-Hydroxysulfosuccinimide sodium salt (≥ 98.5 %)	Sigma-Aldrich, Taufkirchen, Germany
Ni Sepharose 6 Fast Flow	GE Healthcare Life Sciences, Freiburg, Germany
N,N'-dicyclohexylcarbodiimide	Sigma-Aldrich, Taufkirchen, Germany
p-nitrophenoxydodecanoic acid (12-pNCA)	Schwaneberg group, Institute of Biotechnology, RWTH Aachen University, Aachen, Germany
potassium hexacyanoferrate (II) trihydrate (≥ 99 %)	Fluka, Buchs, Switzerland
pyrrole (98 %)	Sigma-Aldrich, Taufkirchen, Germany
Silica 60 (0.02-0.045 mm)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
tetrabutylammonium hexafluorophosphate (≥ 99 %)	Sigma-Aldrich, Taufkirchen, Germany
All other chemicals were of analytical grade or higher quality and purchased from Fluka (Buchs, Switzerland), Sigma-Aldrich (Taufkirchen, Germany) or Carl Roth (Karlsruhe, Germany).	

P450 BM3 mutants

P450 BM3 V26T R47F A74G F87V L188K + N.-term. His-tag¹ (quintuple mutant, abbreviated as QM)
P450 BM3 F87A + N.-term. His₆-tag¹
P450 BM3 Y51F T577G²

¹Strain collection of biochemical engineering group, DECHEMA Research Institute, Frankfurt am Main, Germany.

²The mutant P450 BM3 Y51F T577G was obtained as lyophilisate from A. J. Ruff, Schwaneberg group, Institute of Biotechnology, RWTH Aachen University, Aachen, Germany.

All genes were in the pET28a vector and expressed in *E. coli* BL21.

3.1.2. Methods – Protein expression and purification

All P450 BM3 mutants were expressed and characterized as described in section 2.1.2..

3.1.2.1. Affinity purification of His-tagged P450 BM3

Purification with HIS-Select Spin Columns (for P450 BM3 QM)

Purification was achieved with HIS-Select Spin columns (Sigma). To the spin column 600 μ L equilibration buffer [50 mM Na_xPO_4 , 0.3 M NaCl, pH 8] was added, centrifuged (2100 rcf, 23°C, 2 min) and the flow through discarded. 600 μ L of cell extract were added, centrifuged and the flow through was discarded. Next 600 μ L of wash buffer [50 mM Na_xPO_4 , 0.3 M NaCl, 5 mM imidazole, pH 8] was added and centrifuged. The wash procedure was repeated and then the His-tagged protein was eluted in a new collection tube by adding 500 μ L elution buffer [50 mM Na_xPO_4 , 0.3 M NaCl, 250 mM imidazole, pH 8].

Purification with His Trap HP 5 mL column (for P450 BM3 F87A)

The column was fixed to a stand and the mobile phase was applied manually via a syringe (Luer Lock system) with a flow rate of 5 mL min⁻¹. The flow through was collected in 15 mL Falcon tubes. After washing with 50 mM dd. H₂O the column was equilibrated with 50 mL binding buffer [20 mM Na_xPO_4 , 0.5 M NaCl, 40 mM imidazole, pH 7.4]. After application of the sample (cells were disrupted in binding buffer) the column was treated with 100 mL of binding buffer for elution of unspecifically bound protein. Elution was achieved by adding 25 mL of elution buffer [20 mM Na_xPO_4 , 0.5 M NaCl, 500 mM imidazole, pH 7.4].

Buffer exchange and analysis of collected fractions

After elution buffer was exchanged to 50 mM K_xPO_4 (pH 7.5) by the use of centrifugal filter units (Amicon Ultra, Millipore) with a molecular weight cut off of 50 kDa. The eluate was added to the membrane unit and 5 mL of K_xPO_4 buffer were added. The filter unit was centrifuged at 3220 rcf for 30 min at 4°C. The procedure was repeated three times to remove all imidazole. Retentate volume of the filter unit is 200 μ L. The obtained fractions and the retentate were checked by the BCA assay (Pierce BCA Protein Assay Kit, Thermo Scientific) for their protein content and compared to the P450 content, determined by CO difference spectra (see appendix A.3.). Also activity of the purified P450 was tested by the pNCA assay to exclude enzyme deactivation by imidazole.

3.1.2.2. Purification of P450 BM3 by ion exchange chromatography

Ion exchange purification of P450 BM3 was obtained with an Äkta explorer system. The column used was a HiPrep 16/10 DEAE FF (1.6 x 10 cm, Amersham Biosciences) of 20 mL bed volume. The column was equilibrated with buffer A (0.1 M TrisHCl buffer, pH 7.8). After application of the crude cell extract to the column elution was started by addition of 3 % of buffer B (0.1 M TrisHCl, 2 M NaCl, pH 7.8). After three column volumes of 3 % buffer B a gradient was applied, which increased the amount of buffer B to 20 % within ten column volumes. Purification progress was followed by absorbance detection at 280 nm, 400 nm and 417 nm (see appendix A.3. for the chromatogram). Collected fractions, which showed increased absorbance at 417 nm, were controlled by SDS-PAGE analysis [132] , CO difference spectra and the pNCA assay. Buffer was exchanged against 0.1 M TrisHCl (pH 7.5) like described in section A.1.2.2., but with a centrifugal filter unit of 30 kDa cut off.

3.1.3. Methods – Development of a screening tool box based on polypyrrole

3.1.3.1. General procedure for electrochemical measurements

Reaction cell set-up

If not stated otherwise all electrochemical experiments were performed in a glass reactor with four necks, which allows for introduction of electrodes and maintenance of nitrogen atmosphere (figure 42). In all cases a three electrode configuration was used. Platinum was used as working and counter electrode with an Ag/AgCl reference electrode connected to the reactor via a Luggin capillary filled with 0.5 M Na₂SO₄. Platinum working electrodes had a surface area of 2 cm². In cases where this working electrode was used only “currents” are given instead of current densities. Current densities are given when the surface area deviated from the case described above. Generally 3 cyclic voltammograms were applied, which was mostly sufficient to obtain a stable signal. Therefore only the last CV is shown in the graphs.

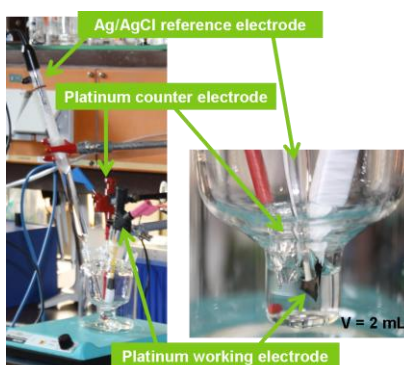


Figure 42: Set-up for electrochemical experiments. Left – reactor with connected electrodes; Right – zoom into reaction chamber.

Cleaning of platinum electrodes

Platinum electrodes were cleaned by repeated immersion in freshly prepared Piranha solution (30% H₂O₂ and concentrated sulfuric acid 1:1) and sonication in bidistilled water. To proof the cleanness of the electrodes electropolishing in 0.5 M sulfuric acid (250 CVs, -200 to +1300 mV vs. Ag/AgCl, 1 V/s) was performed afterwards.

Pyrrole purification

Pyrrole was purified if the stock solution started to turn yellow. Purification was done via silica column chromatography without an additional solvent. A 20 cm column capped with a ceramic frit was filled with silica. The collecting vessel was fused beneath the frit via a joint connection with an additional vent. The whole system was degassed with nitrogen. Then pyrrole was added on top of the column and flushed through the silica by the nitrogen stream. Contaminants (mainly polymerized pyrrole) stuck to the silica while pure pyrrole monomer was collected in the vessel. Purified pyrrole was stored in brown glass vials under nitrogen at 4°C until use.

The pyrrole derivatives 1-H-pyrrole-propionic acid and 4-(3-pyrrolyl)butyric acid were used as received and stored under nitrogen at room temperature (1-H-pyrrole-propionic acid) and -20°C (4-(3-pyrrolyl)butyric acid)).

3.1.3.2. Entrapment in the growing polymer film*Entrapment of charged species in polypyrrole during electropolymerization – potentiodynamic coating procedure*

The reaction cell was equipped with all electrodes and degassed with nitrogen. In the meantime the polymerization solution was prepared as follows: 2.5 mL 0.1 M potassium phosphate buffer (pH 7) and corresponding salt components (e.g. potassium chloride, potassium ferrocyanide or any porphyrin system) were introduced to a 4.5 mL brown glass vial with a cap containing a septum. The buffer solution was degassed with nitrogen prior to the addition of pyrrole. Then 0.1 M pyrrole was added and the reaction mixture was sonicated for several minutes. The reaction solution was transferred to the degassed reaction chamber via a syringe and the polymerization procedure was started. Electropolymerization was done potentiodynamically from -100 to +900 mV vs. Ag/AgCl. Scan rate was either 10 mV/s (potassium ferrocyanide and myoglobin) or 50 mV/s (porphyrins) and starting potential was 0 V at all times. For potassium ferrocyanide entrapment in

one experiment a polypyrrole prelayer was formed on the electrode prior to cyanoferrate entrapment. Conditions for prelayer formation were as follows: 3 CVs, 10 mV/s, -100 to +900 mV, 0.1 M pyrrole in 0.1 M potassium phosphate buffer (pH 7) and 0.1 M potassium chloride.

Galvanostatic polymerization of pyrrole in presence of myoglobin

Sample preparation procedure was the same than for the potentiodynamic coating described above. All steps were done under nitrogen atmosphere. The polymerization solution contained 20 mg/mL myoglobin and 0.5 M pyrrole in 0.1 M potassium phosphate buffer (pH 7) and 0.1 M potassium chloride. 30 or 60 mA were applied for time intervals in between 2 to 8 s. Electrode areas were in the range of 2 cm². Exact electrode areas were geometrically determined for each experiment and found expression as current densities. All coatings described in the results section were performed out of one reaction solution. Characterization of the coated electrodes was done by cyclic voltammetry in 0.1 M potassium phosphate buffer (pH 7) and 0.1 M potassium chloride under nitrogen atmosphere.

Stability of P450 BM3 under electropolymerization conditions

P450 BM3 WT was purified before by ion exchange chromatography. All tests were performed in 0.1 M TrisHCl buffer at pH 7.5. The purified enzyme (31 μ M) was incubated with several concentrations of pyrrole and potassium chloride or for several time intervals. Therefore samples of 500 μ L purified enzyme and 500 μ L TrisHCl buffer were prepared in Eppendorf tubes and kept on ice. To each Eppendorf tube the corresponding amount of pyrrole or potassium chloride was added. After incubation of one minute one ice 5 μ L of the sample were transferred to a cuvette filled with 962 μ L TrisHCl (0.1 M, pH 7.5). 8 μ L of 15 mM pNCA (in DMSO) were added. After 5 minutes 5 μ L catalase were added and the reaction was started by addition of 20 μ L NADPH (5 mM in H₂O dd.). The reaction was followed photometrically at 410 nm. For time dependent tests incubation times on ice in presence of 0.1 M pyrrole or 0.1 M potassium chloride were elongated accordingly. For tests of residual activity after application of electrochemistry

500 μL purified enzyme and 500 μL TrisHCl buffer (0.1 M, pH 7.5) were added to wells of 24 well microtiter plate. In each well a pin shaped glassy carbon working and platinum counter electrode was introduced ($A = 0.16 \text{ cm}^2$). Platinum wire served as pseudo reference electrode. To the well either 5 cyclic voltammograms from -600 to +900 mV vs. Pt at 50 or 100 mV/s were applied. After each cycle samples (10 μL) were taken for the pNCA assay as described above. In another experiment constant potential was applied to the wells. 0.1, 0.25, 0.5 and 0.75 mA were applied for 120 seconds. Then a sample for the pNCA assay was taken (10 μL) and again current was applied for 120s, and so on.

Entrapment of P450 BM3 in polypyrrole

The enzyme used in this experiment was provided by Schwaneberg group (Institute of Biotechnology, RWTH Aachen) as lyophilized powder of P450 BM3 Y51F T577G, which was purified via ion exchange chromatography before and therefore contains high salt concentrations. The lyophilized protein was resuspended in H_2O dd. and yielded a dark red solution. To the resuspended P450 0.5 M pyrrole was added and polymerization was done potentiodynamically (5 CVs, -100 to +900 mV vs. Ag/AgCl, 10 mV/s) on a platinum working electrode under nitrogen atmosphere. For comparison another electrode was coated with polypyrrole with the same polymerization procedure. Therefore 0.5 M pyrrole was added to 50 mM sodium phosphate buffer (pH 7) and 0.1 M sodium chloride. Characterization of the electrodes was done in 0.1 M potassium phosphate buffer with 0.1 M potassium chloride under nitrogen atmosphere. Electrochemically driven substrate conversion was tested as follows: 2 mL 50 mM potassium phosphate buffer (pH 7.5) were added to the standard glass reactor. 20 μL of 3-phenoxytoluene (90 mM in DMSO) were added to the reactor and the solution was kept stirring throughout the reaction. 2000 CVs at 50 mV/s from -700 to +600 mV vs. Ag/AgCl were applied. At the end samples were taken and analyzed according to the 4-AAP assay protocol [133].

3.1.3.3. Immobilized metal cation affinity based immobilization at polypyrrole derivatives

Stability and activity of P450 BM3 F87A + N-terminal His₆-tag immobilized at commercially available nickel loaded IMAC material

100 mg of Ni Sepharose 6 Fast Flow material was weighted in Eppendorf tubes and equilibrated with 50 mM potassium phosphate buffer (pH 7.5). Therefore buffer was added and the tube was vortexed, centrifuged and the supernatant discarded. The procedure was repeated three times. 500 μ L *E. coli* crude cell extract containing P450 BM3 F87A + N-terminal His₆-tag or buffer (for the negative controls) was added. P450 content in the crude cell extract was estimated as 21.5 ± 1.7 μ M by CO difference spectra. After mixing tubes were centrifuged and the supernatant was discarded. The Sepharose material was washed four times with 1 mL of 50 mM potassium phosphate buffer at pH 7.5. Samples of all fractions were taken and analyzed by CO difference spectra and the pNCA assay with NADPH. The activity of P450 bound to the Sepharose material was tested as end point measurement. To the P450 loaded Sepharose 973 μ L of 50 mM phosphate buffer (pH 7.5) and 7.5 μ L pNCA (6 mM in DMSO) was added. The tube was vortexed and incubated for 5 minutes. Then 20 μ L NADPH (5 mM in H₂O dd.) was added and the tube was mixed for one minute at 1000 rpm. After centrifugation the supernatant was transferred to a cuvette and in a spectrophotometer a wavelength scan was performed from 600 to 280 nm to detect product formation.

Polymerization of pyrrole and pyrrole derivatives in methanol [134]

Polymerization was performed in the standard reaction cell described above. To methanol 1 M conc. H₂SO₄ and 0.1 M monomer were added. The reaction solution was sonicated and added to the reaction chamber. Polymerization was performed in presence of an Ag/AgCl reference electrode and in air.

Nickel binding to polypyrrole derivatives

Polymer films were incubated with 0.02 M nickel sulfate in H₂O dd.. Optionally the electrode can be polarized at -600 mV for acceleration of nickel adsorption. After incubation time electrodes were rinsed and transferred to 0.1 M NaOH. Cyclic voltammograms at 100 mV/s from -800 to +1000 mV vs. Ag/AgCl were applied. With each cycle a reduction in the initial background charge was obtained. CVs were applied until no further decrease in background charge was seen.

Electrochemical quartz crystal microbalance (EQCM)

With the EQCM it is possible to detect mass changes at an electrode in nanogram scale during an electrochemical experiment. The core of the system is an AT cut quartz crystal which exposes two circular gold electrodes on both sides. Via the gold electrodes the microbalance device applies an alternating current voltage exciting the crystal to oscillate at its resonant frequency due to a reverse piezoelectric effect. At the same time one of the gold electrodes serves as working electrode for a conventional three electrode system and is connected to a potentiostat. If mass is deposited on the quartz gold electrode the resonant frequency is decreasing. The decrease in frequency can be related to mass increase for thin films via the Sauerbrey equation:

$$\text{Eqn. 2: } \Delta f = -2 f_0^2 m n / (\rho \mu)^{1/2}$$

Thereby the constants are characteristics of the quartz (f_0 = resonant frequency, n = harmonic number of the oscillation, μ = shear modulus of the quartz, ρ = density of the quartz) and are generally combined to one constant called the sensitivity factor C_f [59].

$$\text{Eqn. 3: } \Delta f = - C_f m$$

Set-up of the EQCM

The EQCM measuring cell represents a standard three electrode electrochemical cell. The gold coated quartz (figure 43, left (3)) was the working electrode and in addition a reference electrode (2) and a counter electrode (1) were integrated. The counter electrode is inserted into a lid, as well as the reference electrode (figure 43, center). The whole system was placed in a Faraday cage during measurements.

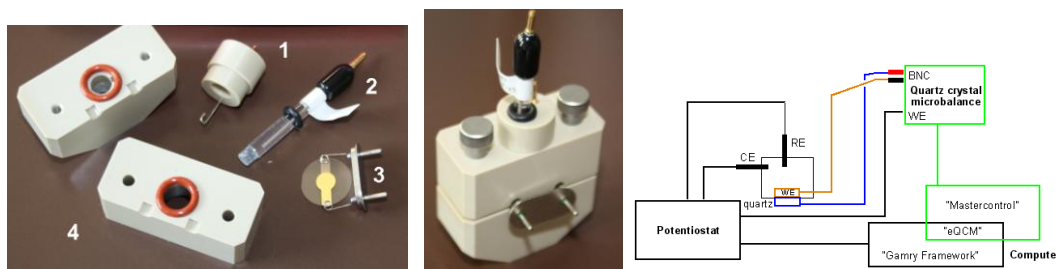


Figure 43: Left – Components of the EQCM reaction cell. (1) Platinum counter electrode in the lid, (2) Ag/AgCl reference electrode, (3) quartz crystal with gold electrode, (4) upper and lower part of the measuring cell, the quartz is screwed in between two silicon rings (red). Center: The assembled measuring cell, ready to use. Right – Connection of the measuring cell to the quartz crystal microbalance and the potentiostat (CE = counter electrode, RE = reference electrode). The working electrode (WE) is the gold coated quartz side directed towards the solution. Both instruments can be controlled independently or via a triggered software called “eQCM”.

EQCM Calibration: Determination of the sensitivity factor C_f

For calibration of the EQCM system the sensitivity factor was determined by electrochemical deposition of copper at the gold electrode. Therefore 250 μL of 0.1 M copper sulfate in 1 M sulfuric acid were added to a static three electrode EQCM cell. A platinum wire and an Ag/AgCl electrode served as counter and reference electrode. Cyclic voltammetry experiments from -600 to +800 mV at 50 mV/s were performed (figure 44). The resulting charge was plotted versus the change in frequency (figure 45). From the slope the sensitivity factor can be determined using the Faraday law. The procedure is exemplified in the following with a 10 MHz quartz crystal.

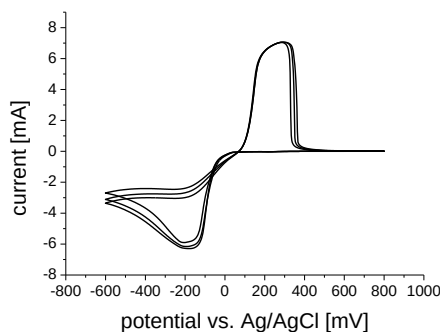


Figure 44: Calibration of the EQCM system by potentiodynamic copper reduction and oxidation. Three cyclic voltammograms were applied (-600 to +800 mV, 50 mV/s) with a starting potential of +800 mV.

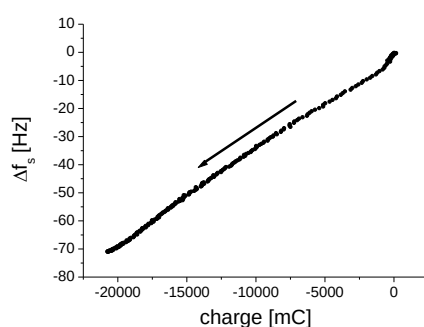


Figure 45: Course of frequency change vs. charge during the first reductive scan starting at 0 V to -600 mV vs. Ag/AgCl. Direction of applied potential is indicated.

The slope determined from figure 45 was 297.3 Hz mC^{-1} . Thereby 1 mC corresponded to $0.329 \text{ } \mu\text{g}$ of copper as defined by Faraday law:

$$\text{Eqn. 4: } m_{\text{Cu}} = (v_{\text{Cu}} M_{\text{Cu}}) / (v_e F) * Q$$

Values and constants were as follows: $v_{\text{Cu}} = 1$; $M_{\text{Cu}} = 63.55 \text{ g mol}^{-1}$; $v_e = 2$; $F = 96485 \text{ C mol}^{-1}$. Including the surface area of the gold electrode ($A = 0.196 \text{ cm}^2$) yields the sensitivity factor C_f of $177.3 \text{ Hz cm}^2 \mu\text{g}^{-1}$. Sensitivity factors were determined for several quartzes of different resonant frequency and suppliers and are listed in table 11. The determined sensitivity factors were in the range of literature values, e.g. for 5 MHz quartzes in aqueous solution sensitivity factors are typically around $42 \text{ Hz cm}^2 \mu\text{g}^{-1}$ [59].

Table 11: Determination of sensitivity factor C_f for several quartz types. The non-bonded quartz was fixed to the holder by two-component adhesive prior to measurement.

Supplier	Quartz type	f_0 [MHz]	C_f [Hz cm ² μg ⁻¹]
Elchema	polished, non-bonded	10 MHz	171
Elchema	unpolished, bonded	10 MHz	200 ± 4
ICM	polished, bonded	10 MHz	176 ± 1.3
ICM	polished, bonded	5 MHz	35.3

Immobilization of His-tagged P450 BM3 at a PBA coated electrode

In between all electrode modification steps the quartz was removed from the measuring cell and both, quartz and cell components, were carefully rinsed. After each modification step a measurement in potassium phosphate buffer (25 mM, pH 7.5) followed. Measurements include measuring of the resonant frequency (Mastercontrol software) while applying an electrochemical measurement (Gamry Framework software), namely cyclic voltammetry. After determination of the resonant frequency of the uncoated quartz in buffer the gold electrode was coated with a polymer of the pyrrole derivative 4-(3-pyrrolyl)butyric acid (PBA). Thereby 0.1 M PBA monomer was added to 0.25 M H₂SO₄ and 0.05 M tetrabutylammonium hexafluorophosphate (TBAP) in methanol. The electrolyte was degassed with nitrogen and filled in the EQCM measuring cell. For polymerization +780 mV vs. Ag/AgCl were applied for 42 s. After characterization of the coated electrode in buffer the electrode was exposed to nickel solution (0.02 M in H₂O dd.), characterized in buffer and finally incubated with N-terminally His₆-tagged P450 BM3 V26T R47F A74G F87V L188K (named in the following as quintuple mutant “QM”). The His-tagged P450 BM3 QM was purified before via affinity chromatography and imidazole was removed by buffer exchange. After characterization in buffer the electrode was removed from the cell and used for a pNCA assay with NADPH.

3.1.3.4. Covalent attachment

Carbodiimide coupling of aminoferrocene to PPA

Polymerization of PPA and PPy was performed potentiodynamically in acetonitrile with 0.1 M TBAP under nitrogen atmosphere onto platinum working electrodes ($A = 2 \text{ cm}^2$). Platinum counter and Ag/AgCl reference electrodes were used. Polymerization conditions were as follows with a platinum counter and Ag/AgCl reference electrode were as follows:

- (1) PPy: 6 CVs from -100 to +900 mV at 10 mV/s;
- (2) PPA (I): 6 CVs from -100 to +900 mV at 10 mV/s, then in addition 3 CVs from -100 to +1200 mV at 10 mV/s were applied;
- (3) PPA (II): 3 CVs from -100 to +1100 mV at 10 mV/s, then in addition 3 CVs from -100 to +1100 mV were applied (since PPA formation was hardly visible in comparison to PPy additional CVs were applied).

In all cases monomer concentration was 0.1 M. Coated electrodes were characterized in monomer free acetonitrile with 0.1 M TBAP (3 CVs, -600 to +600 mV, 25, 50 and 100 mV/s) and under nitrogen atmosphere. In between and after characterization electrodes were rinsed and stored in acetonitrile with 0.1 M TBAP. At the end electrodes were rinsed with pure acetonitrile and DMSO.

24 mg aminoferrocene were dissolved in 8 mL DMSO. For PPy and PPA (I) 250 mg N',N-dicyclohexylcarbodiimide were added to 2 mL of the DMSO aminoferrocene solution. Electrodes were incubated for 5 hours and finally rinsed with DMSO and acetonitrile. Electrodes were electrochemically characterized in acetonitrile with 0.1 M TBAP as described above. After characterization electrodes were rinsed with (a) pure acetonitrile, (b) double distilled water and (c) 0.1 M potassium phosphate buffer (pH 7) with 0.1 M potassium chloride. Electrodes were then characterized by cyclic voltammetry in phosphate buffer (0.1 M, pH 7) with 0.1 M potassium chloride.

Carbodiimide coupling of cytochrome c to PBA

The experiment was performed in the eMTP, but only the electrochemical instrumentation was used. Gold served as working, platinum as counter and pseudo reference electrode. Potentials were corrected by addition of +220 mV to yield values vs. Ag/AgCl. Electrodes were coated galvanostatically to produce three different film thicknesses. 0.164 mA were applied for either 10s (E1), 15s (E2) or 20s (E3). The electrolyte consisted of 1 M sulfuric acid in methanol with 0.2 M TBAP and 0.2 M PBA monomer. Polymerization as well as characterization were performed in deaerated solutions. Characterization of the coated electrodes before and after carbodiimide coupling was done by cyclic voltammetry in 50 mM potassium phosphate buffer (pH 7.5) from -380 to +420 mV vs. Ag/AgCl at 5, 10, 25, 50 and 100 mV/s.

Coated gold electrodes were incubated in 1 mL of 25 mM EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) and 62.5 mM Sulfo-NHS (N-hydroxysulfosuccinimide) in 0.1 M MES buffer with 0.5 M sodium chloride at pH 6. Solutions were prepared freshly before use and chemicals were stored under nitrogen at -20°C (EDC) or 4°C (Sulfo-NHS). After 20 minutes of incubation electrodes were rinsed with phosphate buffer (50 mM, pH 7.5) and incubated with 5 mg/ mL cytochrome c in 50 mM potassium phosphate buffer (pH 7.5) for two hours at room temperature. After two hours electrodes were rinsed with double distilled water and characterized by cyclic voltammetry in 50 mM potassium phosphate buffer (pH 7.5).

3.2. Results

3.2.1. Entrapment in the growing polymer film

3.2.1.1. Entrapment of charged species in polypyrrole during electropolymerization

Electropolymerization of pyrrole was tested in the presence of three electro active species of different charge and size (figure 46). The low molecular weight complex potassium ferrocyanide was used since its redox behavior is well known. As heme analogs furthermore two ferric porphyrin complexes of opposite charge, namely 5,10,15,20-tetrakis-(4-sulfonatophenyl)-porphyrin-Fe(III) chloride (abbreviated as Fe-SO_3^-) and 5,10,15,20-tetrakis-(N-methyl-4-pyridyl)-porphyrin-Fe(III) pentachloride (abbreviated as Fe-N^+), were tested in combination with pyrrole polymerization. Since it is known that electropolymerization of polypyrrole proceeds via a radical cation mechanism yielding a doped polymer which is positively charged, film formation is influenced by charge carrying species in solution [135].

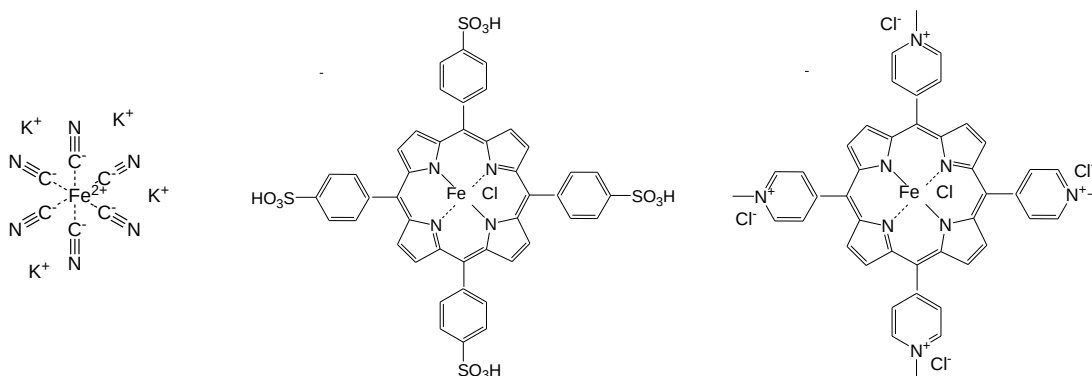


Figure 46: Structures of potassium ferrocyanide (left), 5,10,15,20-tetrakis-(4-sulfonatophenyl)-porphyrin-Fe(III) chloride (center) and 5,10,15,20-tetrakis-(N-methyl-4-pyridyl)-porphyrin-Fe(III) pentachloride (right).

Entrapment of potassium ferrocyanide

Polypyrrole films were polymerized on a polypyrrole prelayer in presence and absence of potassium ferrocyanide. By characterizing both electrodes in buffer under anaerobic conditions a clear difference could be seen in cyclic

voltammograms in between -100 to +500 mV vs. Ag/AgCl for scan rates from 5 to 100 mV/s. The background current due to polypyrrole was different for both electrodes, for the ferrocyanide containing electrode it was increased around 50 % (figure 47, left). In addition the plot of maximal peak currents versus scan rate yielded an almost linear relationship, which is indicative of a surface confined process (figure 47, right). Furthermore for polypyrrole at higher scan rates than 10 mV/s no peaks in the cathodic range were obtained. Interestingly when cycling with an uncoated electrode in the characterization solution of the Ppy/ ferrocyanide electrode, redox peaks for ferrocyanide were obtained, although with four times lower currents (data not shown). This exemplifies the ion exchange properties of polypyrrole, which loses incorporated ions during its redox cycle to the surrounding solution by exchange with anions like chloride. To see if this competing anion already influences incorporation of potassium ferrocyanide during the polymerization process 0.1 M potassium chloride were added to the polymerization solution. The resulting film exhibited around factor 2 higher currents and two redox peaks were still visible (data not shown). Unlike the film polymerized in absence of KCl these peaks vanished at higher scan rates than 25 mV/s, starting with the cathodic peak. At 100 mV/s also the anodic peak was almost non visible as well.

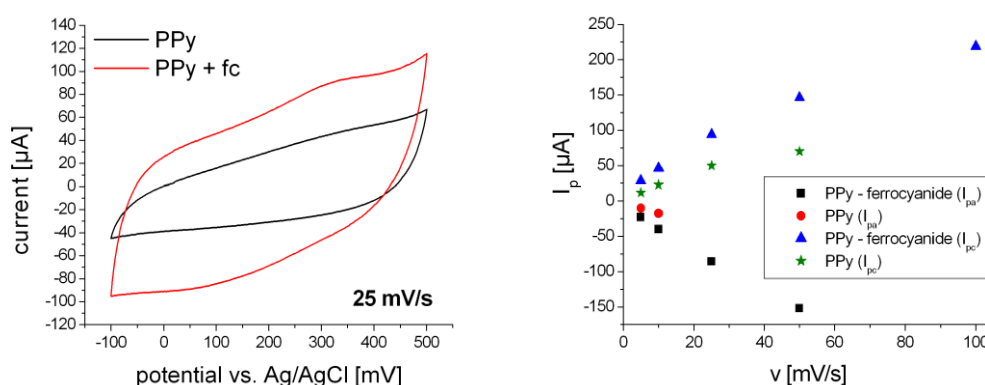


Figure 47: Left - Polypyrrole (Ppy) and a polypyrrole film polymerized in presence of 10 mM potassium ferrocyanide (Ppy + fc), which were both polymerized on a polypyrrole prelayer. Characterization was done in potassium phosphate buffer (pH 7) and 0.1 M potassium chloride under anaerobic conditions. Last CV of three is shown. Right: Plot of maximal anodic and cathodic peak currents vs. scan rate.

In another experiment concentration of potassium ferrocyanide was varied to determine detection limits for cyclic voltammetry experiments (figure 48). In this case films were prepared directly, without previous deposition of a polypyrrole (pre)layer, to reduce the overall polypyrrole film thickness. In the polymer film characterization for 1 mM and 0.1 mM ferrocyanide only weak pronounced redox peaks in the cyclic voltammograms were obtained. Maximal peak currents decreased with decreasing ferrocyanide concentration (figure 48, right). Also the anodic peak currents were smaller than the corresponding cathodic peak currents.

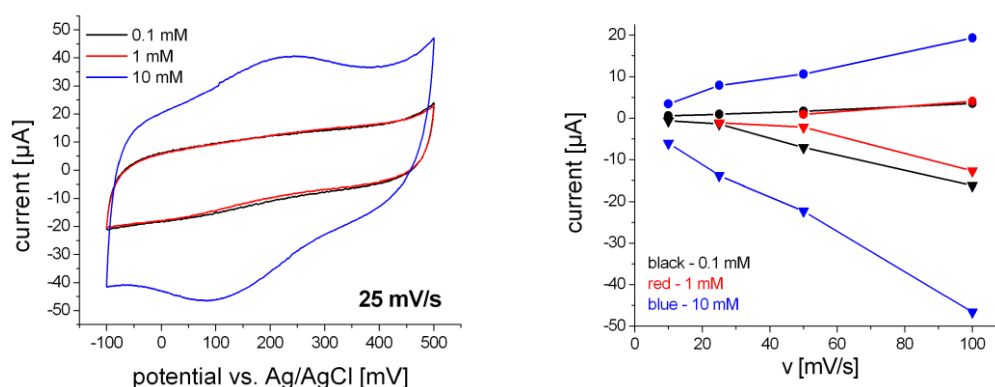


Figure 48: Left - Characterization of polypyrrole films polymerized in presence of three different ferrocyanide concentrations (as indicated). Cyclic voltammograms were recorded at 25 mV/s in potassium phosphate buffer (pH 7) and 0.1 M potassium chloride under anaerobic conditions. Right - Maximal peak currents versus scan rate. Dots represent the anodic peak currents and triangles the cathodic peak currents.

Entrapment of ferric porphyrin complexes

Polypyrrole was polymerized in presence of Fe-SO_3^- or potassium chloride. Polymerization in presence of Fe-SO_3^- yielded higher polymer background currents than in case of solely potassium chloride (figure 49). In addition no distinct redox peaks were seen in either case, but for Fe-SO_3^- a broad anodic wave was visible at around -200 mV. Similar for the cathodic scan a broad reductive peak was visible shortly before the switching potential.

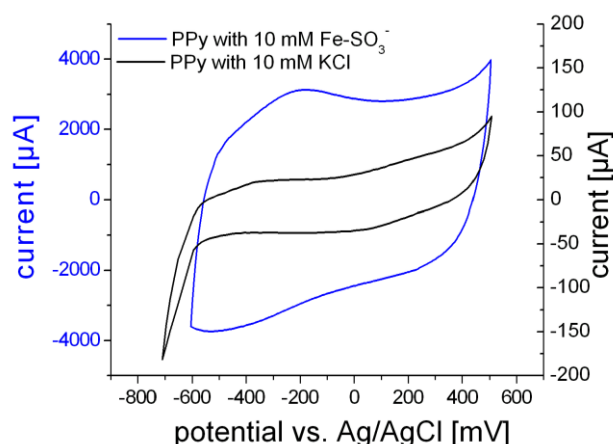


Figure 49: Characterization of polypyrrole films which were polymerized in presence 10 mM Fe-SO_3^- or 10 mM KCl. In both cases electrode size is 2 cm^2 . Characterization was done in 0.1 M potassium phosphate buffer (pH 7) with 0.1 M KCl under nitrogen atmosphere. 50 mV/s, last CV of three is shown.

When varying the Fe-SO_3^- concentration in the polymerization solution the polymer response in cyclic voltammetry was drastically changed (figure 50). Polymer background currents were strongly reduced and several broad peaks with low peak maxima (compared to the background charge) were seen.

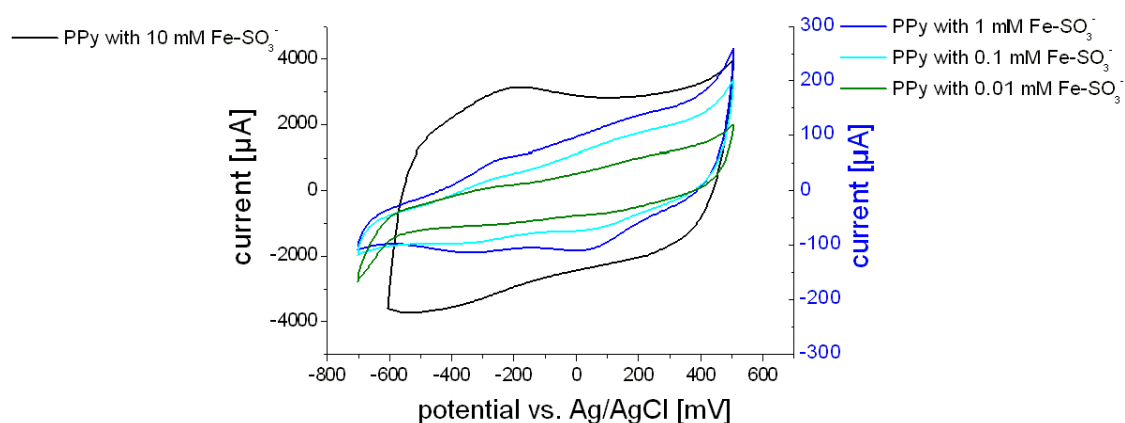


Figure 50: Characterization of polypyrrole films grown from solutions with different Fe-SO_3^- concentrations (as indicated). Characterization was done in potassium phosphate buffer (0.1 M, pH 7) and 0.1 M KCl at 10 mV/s. Nitrogen atmosphere, 50 mV/s, last CV of three is shown. Electrode area is 2 cm^2 in all cases.

In addition polypyrrole was polymerized in presence of several potassium chloride concentrations. The resulting voltammograms were compared to the polypyrrole /

Fe-SO_3^- voltammograms of figure 50 in terms of their peak potentials (E_p) (figure 51). Thereby two anodic (E_{pa1} and E_{pa2}) and two cathodic peaks (E_{pc1} and E_{pc2}) were identified and numbered after their appearance in the CV, starting in both cases from the negative potential on the left of the x-axis. From figure 51 it can be seen that peak E_{pa1} and E_{pc2} were found in all CVs, independent of the counter ion present during the polymerization process. Furthermore peak position was almost independent of the concentration of the introduced anion. Peak E_{pa2} and peak E_{pc1} were exclusively found for polymers which were formed in presence of Fe-SO_3^- . Thereby peak position was strongly influenced by the Fe-SO_3^- concentration. For both peaks E_{pa2} and E_{pc1} peak potentials shifted towards more negative potentials with increasing Fe-SO_3^- concentrations. For 10 mM Fe-SO_3^- E_{pa2} could not be detected anymore. Plotting the maximal peak currents of E_{pa2} and E_{pc1} versus the scan rate yields a linear relation for E_{pc1} , while for E_{pa2} the peak current is fluctuating (figure 52). This was related to the broadness of the peak (figure 50), which makes exact determination of the peak characteristics hard to achieve.

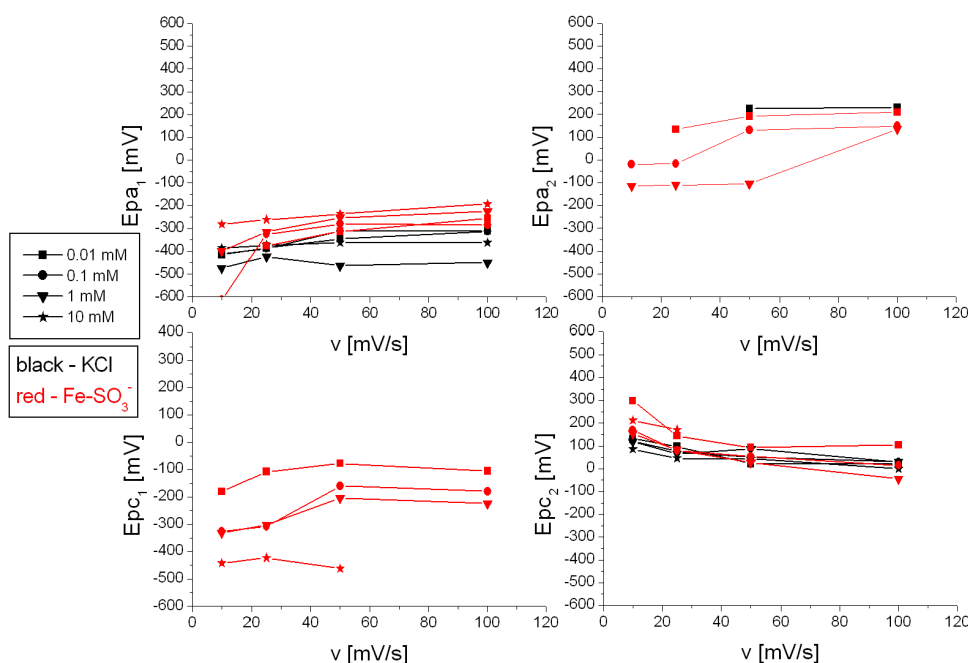


Figure 51: Comparison of peak potentials (y-axis) at different scan rates (x-axis) for polypyrrole polymerized at different concentrations (as indicated by the symbols) of either KCl (black) or Fe-SO_3^- (red). In general four peaks, two anodic (upper row) and two cathodic peaks (lower row) were identified (peak numbering was done with view to the corresponding CV in figure 50 according to their appearance, starting from low potential values left on the x-axis).

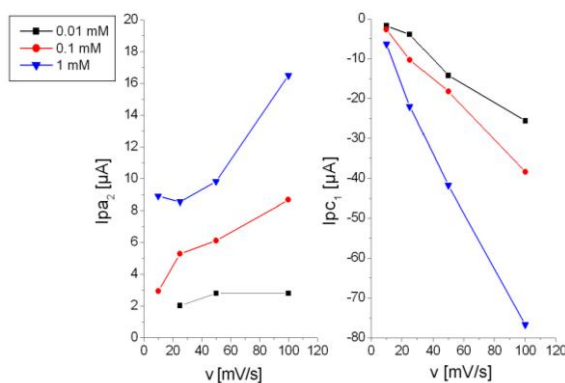


Figure 52: Plot of maximal peak currents (I_p) versus scan rates for E_{pa_2} and E_{pc_1} of figure 51.

When comparing polymers obtained in presence of Fe-SO_3^- with polymers obtained with KCl or Fe-N^+ , far lower background currents were obtained for the latter (figure 53). Also when plotting for example one peak found for Fe-SO_3^- as well as potassium chloride, namely E_{pc_2} , versus scan rate, the differences in maximal peak currents are obvious (figure 53, right). Furthermore in all cases the relation between peak current and scan rate was linear, indicating a surface confined process.

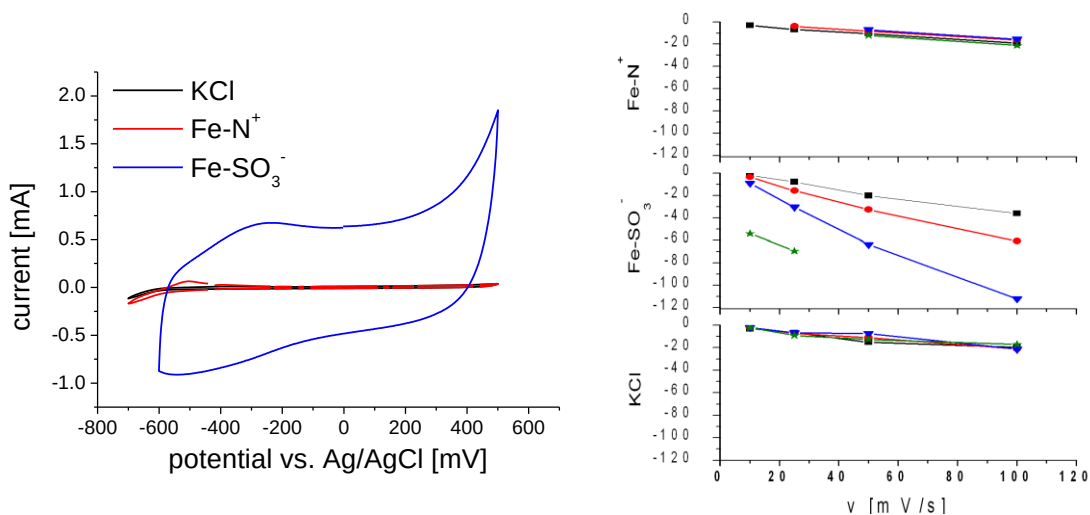


Figure 53: Polymerization of polypyrrole in presence of either 10 mM KCl, Fe-N^+ or Fe-SO_3^- . Polymerization conditions further include: 0.1 M pyrrole in 0.1 M potassium phosphate buffer (pH 7), 5 CVs from -100 to +900 mV vs. Ag/AgCl, nitrogen atmosphere. Pt working ($A = 2 \text{ cm}^2$) and Pt counter electrode. Left: Cyclic voltammetric characterization was done in potassium phosphate buffer (0.1 M, pH 7) and 0.1 M KCl under nitrogen atmosphere, last CV of three is shown. Right: Plot of maximal peak currents of E_{pc_2} (as defined above) versus scan rate.

One polypyrrole coated electrode, which was polymerized in presence of 10 mM Fe-SO_3^- , was exposed to oxygen and hydrogen peroxide to see if the presence of either of the two molecules may alter the current detected during cyclic voltammetry (figure 54). After introduction of oxygen a small decrease in the overall current response was monitored, which did not change when hydrogen peroxide was added.

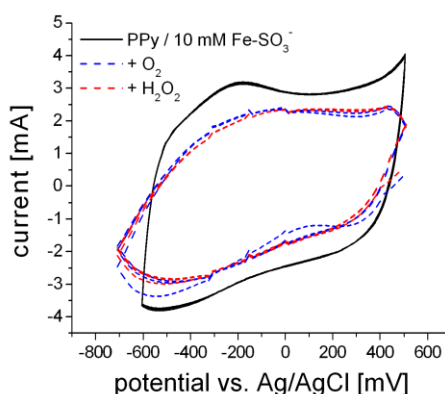


Figure 54: An electrode coated with PPy in presence of 10 mM Fe-SO_3^- was characterized in 0.1 M potassium phosphate buffer (pH 7) and 0.1 M KCl under nitrogen atmosphere (black, straight line), oxygen atmosphere (blue, dotted line) and under oxygen atmosphere with the addition of hydrogen peroxide to a final concentration of 0.06 mM (red, dotted line).

3.2.1.2. Entrapment of myoglobin in polypyrrole during electropolymerization

To proof electropolymerization of polypyrrole in presence of a protein myoglobin was chosen. Myoglobin is a small protein compared to P450 BM3 and has its heme active center close to the surface. Furthermore it is commercially available in high purity. Potentiodynamic coating methods, like described above, did only yield a polymer film when potassium chloride was present in the polymerization solution. When only myoglobin in buffer was present no polymer formed on the electrode. In cyclic voltammetry experiments the CV was equal to one obtained with an uncoated electrode (data not shown). In presence of potassium chloride a polymer film was obtained exposing an anodic and cathodic redox peak at 195 and -58 mV vs. Ag/AgCl at 25 mV/s (figure 55). Upon increase of the scan rate peak broadening was observed. At 100 mV/s the peaks were obtained at 219 and -131 mV vs. Ag/AgCl.

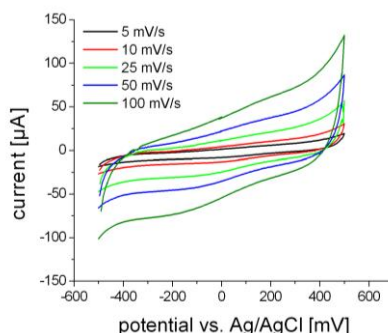


Figure 55: The electrode was coated potentiodynamically with polypyrrole in presence of 20 mg/ mL myoglobin. The figure shows the characterization of the coated electrode, which was performed at several scan rates in monomer and myoglobin free electrolyte (namely 0.1 M potassium phosphate buffer (pH 7) and 0.1 M potassium chloride under nitrogen atmosphere).

Furthermore a galvanostatic polymerization method was tested, which was originally applied to the entrapment of cytochrome c in polypyrrole [112]. Thereby film thickness, as displayed by the recorded background charge in cyclic voltammetry, was influenced by the time of current application as well as the height of current (figure 56). But not only the background charge is altered by a change of polymerization conditions, also the shape of the CV was changing as it can be seen on the right in figure 56. Nevertheless polymerization of polypyrrole in presence of the protein myoglobin is highly reproducible (figure 56, left) and film thickness can be controlled.

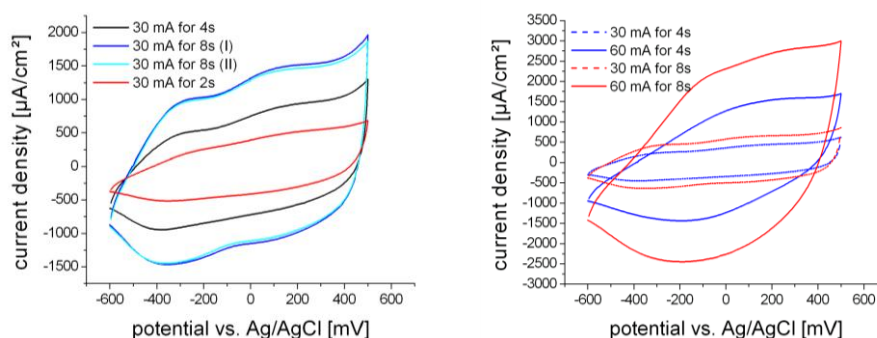


Figure 56: Influence of the galvanostatic coating procedure on film performance. Characterization of the films displayed here was performed in 0.1 M potassium phosphate buffer (pH 7) and 0.1 M potassium chloride. CVs were recorded at 50 mV/s. Polymerization conditions were as indicated. The polymerization solution contained myoglobin (20 mg/mL) and 0.5 M monomer in 0.1 M potassium phosphate buffer (pH 7) and 0.1 M potassium chloride, under nitrogen atmosphere. Left: Different time intervals for polymerization. Right: Different currents for polymerization.

In addition the same coating procedures were applied to samples without protein. The corresponding polypyrrole films generally showed higher background currents than the polymer films polymerized under the same conditions, but in presence of myoglobin (figure 57).

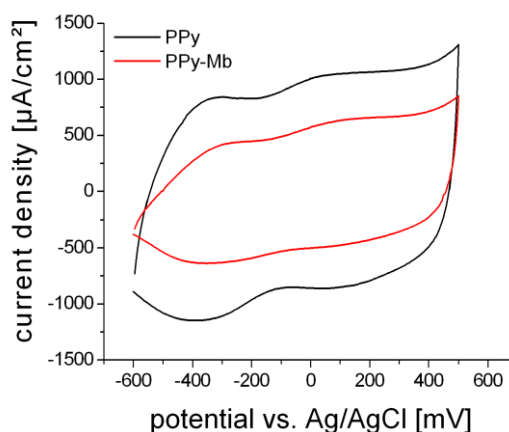


Figure 57: Characterization of a PPy coated electrode in 0.1 M potassium phosphate buffer (pH 7) and 0.1 M KCl under nitrogen atmosphere at a scan rate of 50 mV/s. Polymerization was done galvanostatically (30 mA for 8 s) in absence (PPy) and presence of 20 mg/ mL myoglobin (PPy-Mb).

Both polymer films, with and without myoglobin, displayed distinct redox peaks. In a plot of maximal peak current density versus scan rate for three different film thicknesses and in absence and presence of myoglobin in the polymerization solution a linear relationship was obtained (figure 58). Furthermore, as described above, maximal peak current densities were higher for polypyrrole films which were polymerized in absence of myoglobin.

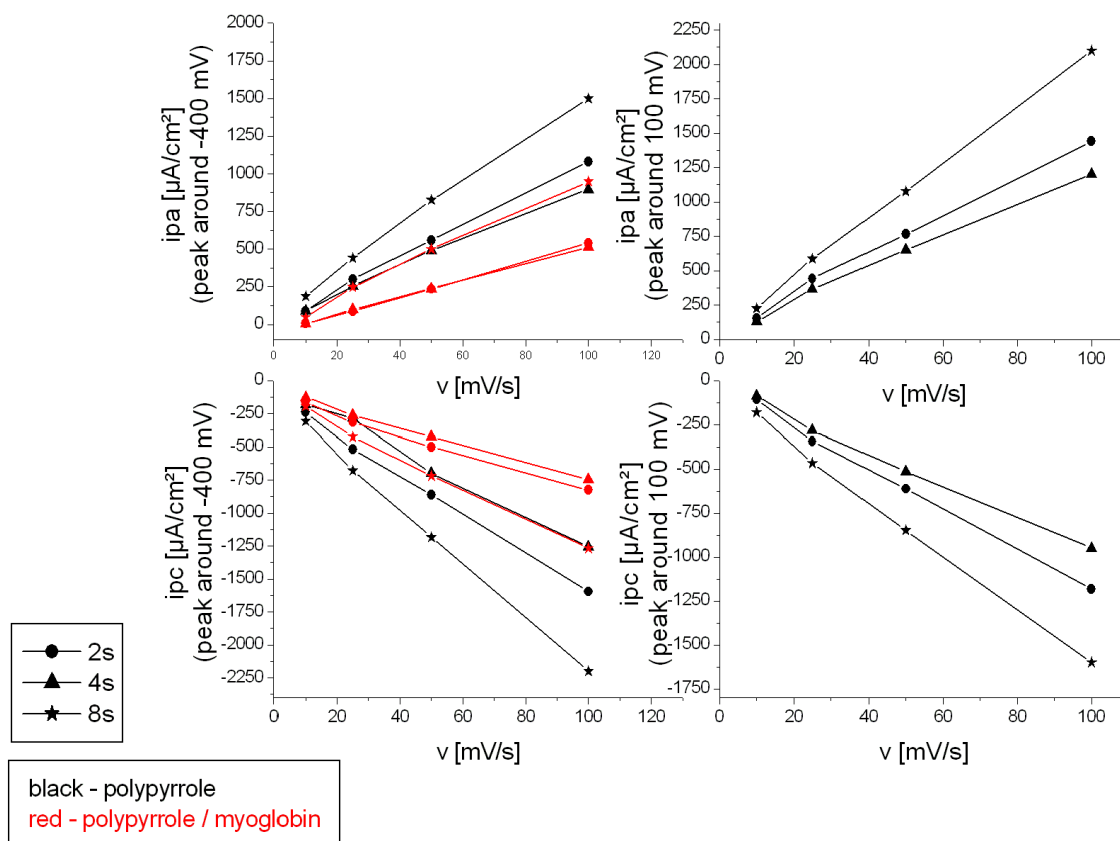


Figure 58: Plots of maximal peak current densities versus scan rate for polypyrrole films polymerized at 30 mA for duration as indicated. Films were polymerized in presence (red) and absence (black) of myoglobin. The upper row represents anodic peak current densities and the lower row the cathodic peak current densities. The peak around 100 mV vs. Ag/AgCl (compare figure 57) was only evaluated for polypyrrole, since no distinct peaks at this position were observed for polypyrrole and myoglobin.

Electrodes, which were coated at 30 mA for 8s, were exposed to hydrogen peroxide. Myoglobin is known to possess a peroxidase activity. Therefore cyclic voltammetry experiments were performed in presence of hydrogen peroxide with polypyrrole and polypyrrole / myoglobin coated electrodes (figure 59). Both electrodes did not differ significantly in their response to hydrogen peroxide. The polypyrrole electrode even showed higher currents than the polypyrrole / myoglobin electrode.

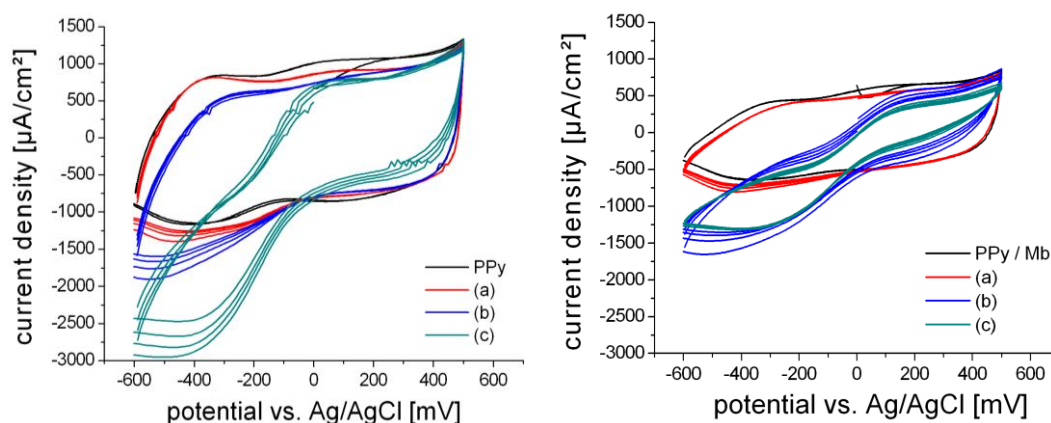


Figure 59: Test for hydrogen peroxide decomposition. Several amounts of 3 % hydrogen peroxide were added to the electrode system in 50 mM potassium phosphate (pH 7) and 0.1 M KCl: (a) 2.5 μL , (b) 10 μL and (c) 50 μL . After each hydrogen peroxide addition 4 CVs at 50 mV/s were recorded.

3.2.1.3. Stability of P450 BM3 at electropolymerization conditions

P450 BM3 WT, which was purified by ion exchange chromatography, was exposed to reaction conditions needed for pyrrole polymerization. The protein was exposed to high salt concentrations, which are eminent for high conductivity of the electrolyte, and different monomer concentrations (figure 60). After exposure residual activity of P450 BM3 WT was tested by the pNCA assay with NADPH. Therefore the specific product formation rate obtained in 0.1 M TrisHCl buffer at pH 7.5 was set to 100 %. Furthermore influence of electrochemistry on the protein was tested. Since several electropolymerization methods for pyrrole are known, the protein was exposed to potentiodynamic, or respectively cyclic voltammetry, and galvanostatic conditions (figure 61).

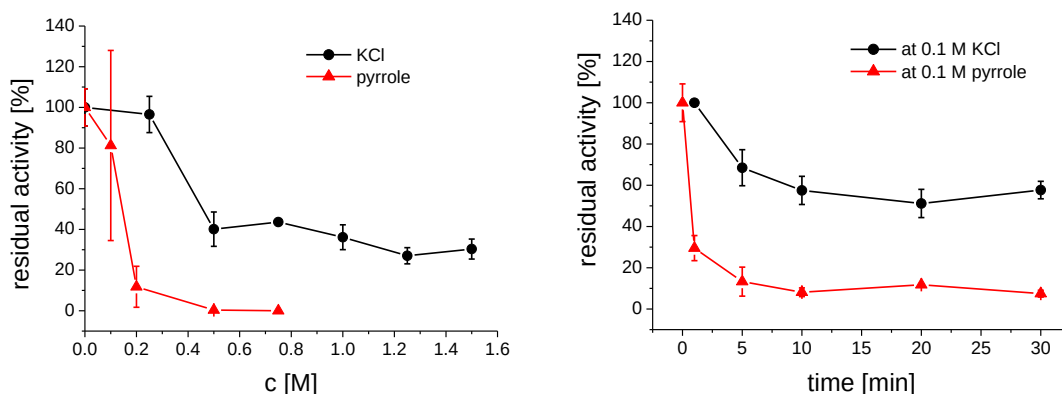


Figure 60: Residual activity of P450 BM3 WT, which was exposed to different salt and pyrrole monomer concentrations (left). Right: Residual activity over time at a salt and monomer concentration of 0.1 M.

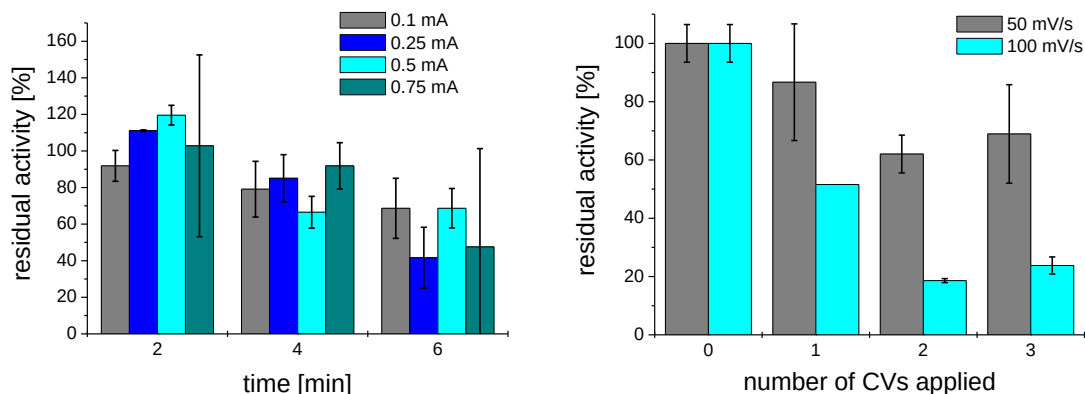


Figure 61: Residual activity of P450 BM3 WT exposed to galvanostatic conditions (left), which included four different currents, and cyclic voltammetry (right) at two different scan rates. Conditions for cyclic voltammetry include: 3 cyclic voltammograms (CV) from -600 to +900 mV vs. Ag/AgCl with a starting potential of 0 V.

As it can be seen increasing salt and especially monomer concentrations diminish P450 BM3 WT activity. Furthermore decrease in activity is depending on incubation times. After 2 to 4 minutes under constant current activity is decreased around 20 %. Furthermore a higher scan rate in cyclic voltammetry experiments seems to be more damaging to enzyme activity.

3.2.1.4. Entrapment of P450 BM3 in polypyrrole

Pyrrole was polymerized in presence of P450 BM3 Y51F T577G, which was obtained from Schwaneberg group (RWTH Aachen) as lyophilized powder. The protein was purified before by ion exchange chromatography. Polymerization was performed under nitrogen atmosphere in a potentiodynamic manner, to see the growth of the polymer film directly while polymerizing. The same coating procedure was performed for polypyrrole without protein. Resulting polymer films were characterized by cyclic voltammetry (figure 62). As already seen for polymerization with myoglobin, the polymer background current was reduced in presence of protein. The shape of the polymer response in cyclic voltammetry was similar, but the redox peaks obtained for polypyrrole at around -500 and +300 mV were less well defined in case of polypyrrole which was polymerized in presence of protein.

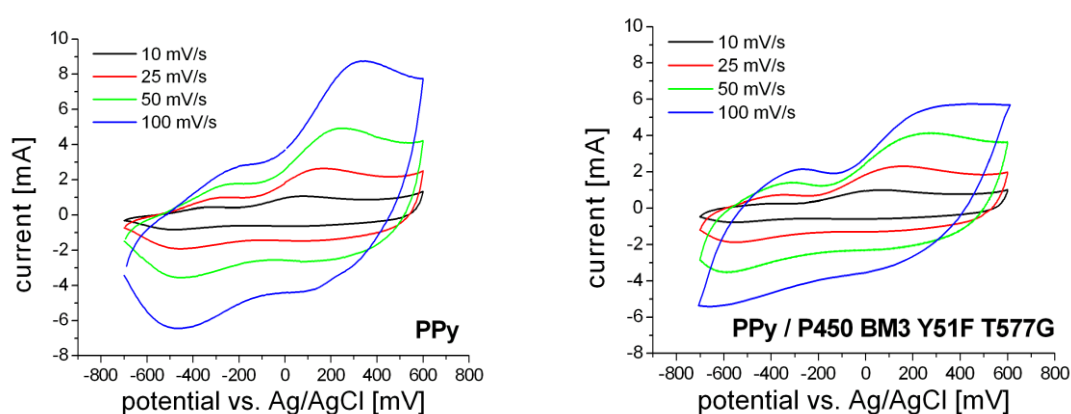


Figure 62: Characterization of polypyrrole, which was polymerized potentiodynamically in absence (left) and in presence (right) of P450 BM3 Y51F T577G. Characterization was done in 0.1 M potassium phosphate buffer (pH 7) and 0.1 M potassium chloride under nitrogen atmosphere.

In addition for the polypyrrole / P450 BM3 electrode electrochemically driven substrate conversion of 3-phenoxytoluene via the 4-AAP assay [133] was tested. A potentiodynamic method was applied for 42 hours with a scan rate of 50 mV/s from -700 to +600 mV vs. Ag/AgCl. After 42 hours no reaction product was detected.

3.2.2. Immobilized metal cation affinity based immobilization at polypyrrole derivatives

As alternative immobilization strategy for P450 immobilization an affinity based method based on the IMAC (immobilized metal cation affinity chromatography) principle was chosen. Compared to entrapment methods the enzyme is not exposed to harsh polymerization conditions and sterical constraints should be reduced, since the enzyme is bound to the polymer surface instead being entrapped inside the polymer matrix. Furthermore substrate access and product release should be eased. Since the immobilization method is basically reversible the polymer surface can be regenerated.

3.2.2.1. Stability and activity of P450 BM3 F87A + N-terminal His₆-tag immobilized at commercially available nickel loaded IMAC material

The stability of the immobilization of P450 BM3 F87A with an N-terminal His₆-tag upon immobilization on a conventional nickel loaded IMAC material was tested. Therefore a defined amount of IMAC material was incubated with crude cell extract containing the P450. Afterwards the material was washed several times and collected fractions were tested on their P450 content (figure 63, left). As it can be seen immobilization was stable and during four washing steps, including centrifugation, no P450 was lost. Furthermore the bound P450 was active in the pNCA assay with NADPH (trace at 340 nm) while the IMAC material itself and the last wash fraction did not show any absorbance increase at 410 nm due to *p*-nitrophenolate product formation (figure 63, right).

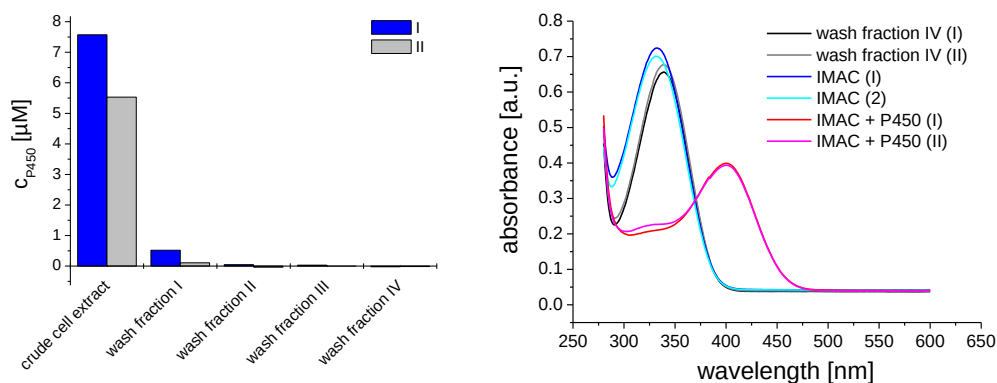


Figure 63: Left - Immobilization of P450 BM3 F87A + N-term. His-tag at commercially available nickel loaded IMAC material (in duplicate). Collected fractions were tested for their P450 content by CO difference spectra. Right – pNCA assay with NADPH conducted as endpoint measurement for wash fraction IV, nickel loaded IMAC material and IMAC material to which P450 F87A + N-term. His-tag was bound.

3.2.2.2. Affinity immobilization at polymer modified electrodes

It is known from literature that derivatives of pyrrole, which display carboxylic acid groups, are capable of metal cation complexation [134, 136, 137]. In this study two commercially available pyrrole derivatives, namely 1H-pyrrole-1-propionic acid (PPA) and 4-(3-pyrrolyl)butyric acid (PBA), were used.

Polymerization of pyrrole and its carboxylic acid derivatives

Polymerization of pyrrole for entrapment experiments is limited to aqueous electrolyte solutions around neutral pH. Since for the affinity based immobilization the polymerization process is separated from the biomolecule immobilization also organic electrolytes can be used. For pyrrole and its carboxylic acid derivatives the polymerization in methanol acidified with sulfuric acid was established (figure 64) [134]. It can be seen from the cyclic voltammograms in phosphate buffer that PPy displayed the highest current densities followed by a polymer of PBA. In contrast the PPA polymer performed in the range of the uncoated electrode.

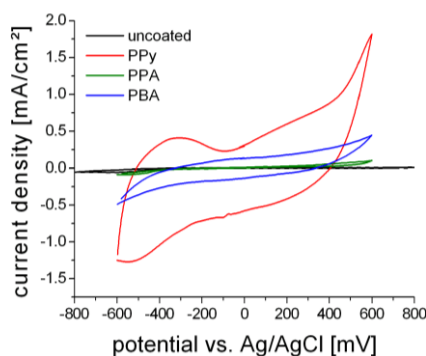


Figure 64: Characterization of an uncoated (black) and three coated glassy carbon electrodes in 50 mM potassium phosphate buffer (pH 7.5) at a scan rate of 50 mV/s. PPy – polypyrrole (red), PPA – poly(1-H-pyrrole-1-propionic acid) (green), PBA – poly(4-(3-pyrrolyl)butyric acid)) (blue). Electrodes were coated galvanostatically (2.6 mA for 150 s) with 0.1 M monomer, 0.1 M tetrabutylammonium hexafluorophosphate and 1 M sulfuric acid in methanol.

Thereby the nature of the electrode material did not significantly influence polymer formation when using a galvanostatic method (figure 65).

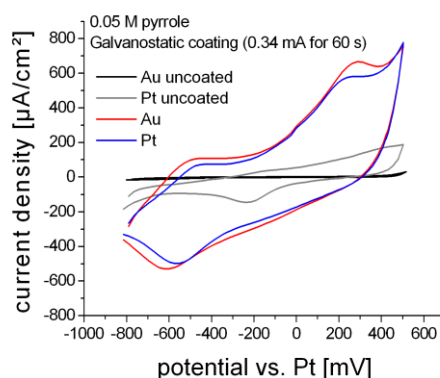


Figure 65: Cyclic voltammograms at 50 mV/s in 50 mM potassium phosphate buffer (pH 7.5) under nitrogen atmosphere. Polypyrrole was polymerized galvanostatically on two different working electrode materials. Polymerization solution: 0.05 M monomer, 0.1 M TBAP and 0.01 M H₂SO₄ in methanol.

Polymer characteristics were strongly influenced by the polymerization process applied. In galvanostatic polymerization procedures longer polymerization times yielded higher current densities in the resulting polymer film (figure 66). As already seen in figure 64, the PBA polymer showed smaller current densities than Ppy, independent of the applied polymerization time (figure 66, right). In addition it has to be noted that for conservation of the more labile PBA polymer switching potentials were chosen in a more narrow range than for PPy.

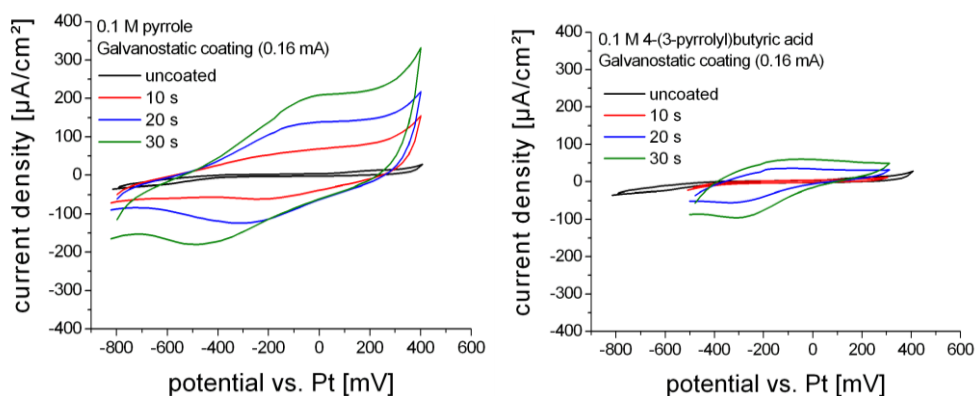


Figure 66: Characterization of polymers on gold electrodes in 50 mM potassium phosphate buffer (pH 7.5) under nitrogen atmosphere. 3 cyclic voltammograms (last one shown) at 50 mV/s. Polymerization conditions were varied as indicated: Nature of the monomer (center vs. right) and duration of the galvanostatic method (given in each figure).

Besides galvanostatic also potentiostatic or potentiodynamic methods can be used for electropolymerization. But compared to potentiodynamic methods galvanostatic methods are more suitable for production of thin films, as it can be seen in figure 67. This differs from the findings for polypyrrole polymerization in aqueous electrolytes (compare chapter 3.2.1.2.), where potentiodynamic coating processes yielded only polymers with low current densities. Nevertheless, the galvanostatic methods provided more reproducible films.

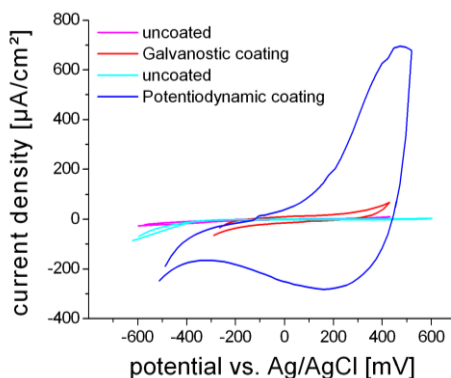


Figure 67: Characterization of PBA polymer coated gold electrodes in 50 mM potassium phosphate buffer (pH 7.5) at 50 mV/s. Polymerization solution contained 0.2 M monomer, 0.2 M TBAP and 1 M H₂SO₄ in methanol. Galvanostatic coating (red): 0.164 mA for 20 s (Pt reference electrode). The cyclic voltammogram of the galvanostatically coated electrode was originally measured vs. Pt. Values were corrected for Ag/AgCl by addition of +219 mV (as determined by potassium ferrocyanide measurement). Potentiodynamic coating (blue): 3 cyclic voltammograms, 50 mV/s, -100 to +800 mV vs. Ag/AgCl.

Nickel binding to PPA and PBA

Since nickel ions are not electroactive cyclic voltammetry experiments were performed in sodium hydroxide solution. At basic pH nickel hydroxide is forming and its redox peaks can be detected electrochemically. In addition conductive pyrrole type polymers are not stable at high pH since the conjugated backbone is prone to nucleophilic attack e.g. by hydroxide molecules. Due to the loss of the polymer backbone conductivity redox peaks for nickel hydroxide can be seen even at low concentrations. When comparing PBA and PPA films it is noticeable that PBA displayed the higher polymer background charge (figure 68, left). PPA behaved like the uncoated electrode in terms of residual current density. This was already the case after polymerization (figure 64) and cycling in NaOH did not affect the polymer response strongly. In case of PBA cycling in NaOH did not overoxidize the polymer completely. Instead several cycles had to be performed to reduce the initial background current (data not shown). Therefore it was probably not possible to see any nickel response. For the PPA polymer with a low background current a nickel hydroxide redox peak was found at a midpoint potential of 467 mV vs. Ag/AgCl. The nickel hydroxide peaks vanished after treatment in hydrochloric acid since the acid groups were protonated, which resulted in loss of their metal binding capability and elution of nickel from the polymer.

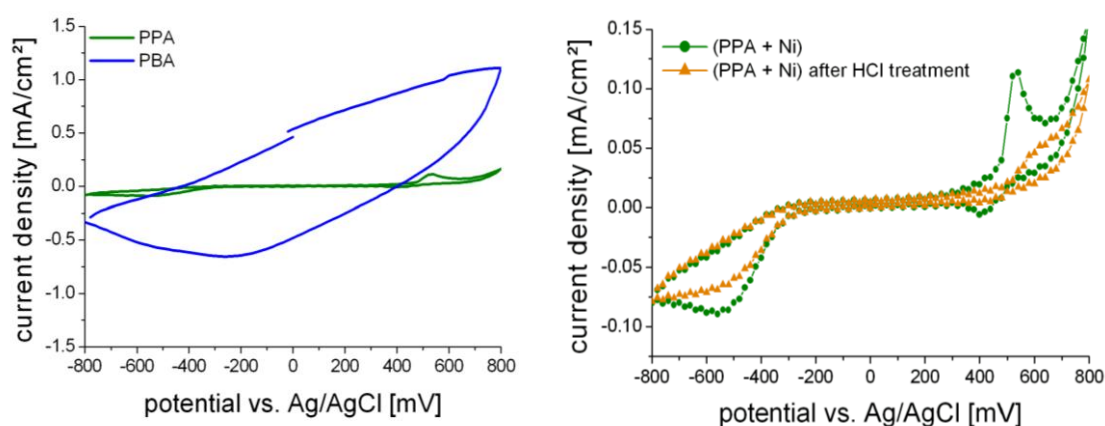


Figure 68: Cyclic voltammograms in 0.1 M NaOH at 50 mV/s. Left: PPA and PBA coated electrodes were incubated in 0.02 M nickel sulfate for one hour by polarizing at -600 mV vs. Ag/AgCl before measurement in NaOH. Right: PPA coated and nickel incubated electrode (green) and the same electrode after treatment with hydrochloric acid (orange).

Immobilization of His-tagged P450 BM3 at a PBA coated electrode

Due to the higher stability of the PBA polymer in aqueous electrolytes compared to a PPA polymer immobilization experiments were performed with the PBA polymer. The immobilization process was followed by an electrochemical quartz crystal microbalance (EQCM). For the 10 MHz quartz used here the sensitivity factor was determined by electrochemical copper deposition (for details see material and methods) as $171 \text{ Hz cm}^2 \mu\text{g}^{-1}$. The gold quartz was coated with PBA and subsequently exposed to nickel solution and His-tagged P450 BM3 QM in buffer. To compare the results after each incubation step the electrode was measured in 25 mM potassium phosphate buffer (pH 7.5) (figure 69). In all cases after incubation the resonant frequency was decreased reflecting mass increase at the electrode. The reversible fluctuation seen in the resonant frequency corresponds to the cyclic voltammetry experiments applied and is due to in- and exclusion of ions by the polymer film upon changing its oxidation state. CVs recorded showed no significant differences (figure 69, right). A well defined reductive peak was obtained at -510 mV for all surface modifications. Also for the uncoated electrode a peak is visible at -390 mV. Furthermore the background current of the PBA polymer is slightly decreased in the following modification steps.

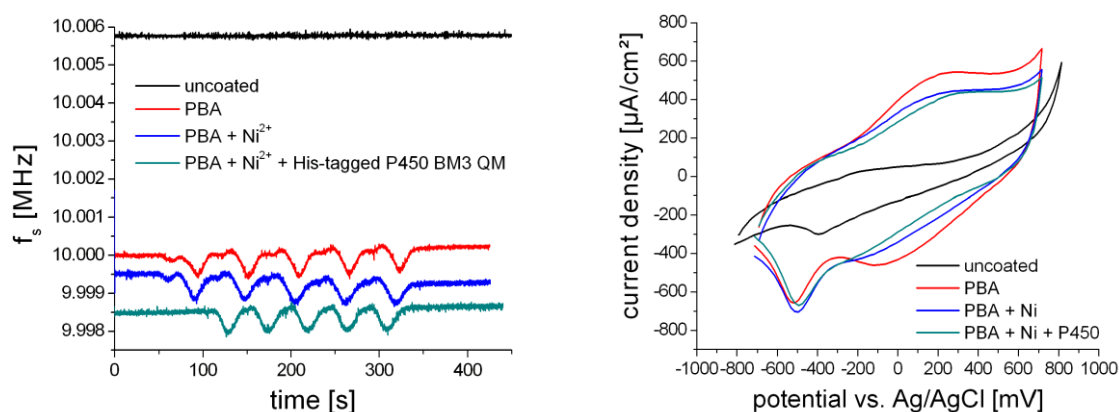


Figure 69: Left - Decrease in resonant frequency f_s during the electrode modification process. First the uncoated electrode was coated with PBA, exposed to nickel and finally His-tagged P450 BM3 QM. All measurements were performed in 25 mM potassium phosphate buffer (pH 7.5). Right – Cyclic voltammograms recorded during the measurement of resonant frequency (on the left). Conditions: 5 CVs (last one shown), -700 to +700 mV at 50 mV/s.

Taking into account the sensitivity factor and the molecular masses of the PBA monomer (153.2 g/mol), nickel (58.7 g/mol) and P450 BM3 (119060 g/mol) the molar amount of the substances at the electrode can be estimated (table 12).

Table 12: Resonant frequency changes obtained with the EQCM upon surface modification. From the change in frequency mass loading and molar amount of the substances deposited on the electrode can be calculated.

Electrode modification step	Δf_s [Hz]	m [$\mu\text{g}/\text{cm}^2$]	n [nmol/cm^2]
PBA	5800	33.9	224.356
PBA + Ni	740	4.3	73.735
PBA + Ni + P450	770	4.5	0.038

The ratio between PBA monomer and nickel cations bound at the electrode surface is 3.04, which is expected since each PBA monomer exposes one carboxylic acid group and three carboxylic acid groups are needed for chelation of one nickel cation.

The molecular dimension of the P450 BM3 holoprotein is not known, but a theoretical approximation for a diameter can be gained by the following method [138, 139]. The radius of a globular protein can be related to its mass by equation 5 [138]:

$$\text{Eqn. 5: } R = ((3 \cdot M \cdot v_2) / (4 \cdot \pi \cdot N_A))^{1/3}$$

Thereby M represents the molecular weight of the protein, which is 119061 g mol⁻¹ for a 119.5 kDa protein. N_A stands for the Avogadro constant of 6.02 x 10²³ mol⁻¹ and v₂ reflects the partial specific protein volume which is given as 0.73 cm³ g⁻¹ [138] or 1.01 cm³ g⁻¹ for a hydrated protein [139]. For BM3 this results in a theoretical radius of 3.3 nm to 3.7 nm, depending on the partial specific protein volume used. This results in a circular area of 3.4 x 10⁻¹³ cm², or respectively 4.3 x 10⁻¹³ cm² for the hydrated protein. For simplification a rectangular cross section with the circle diameter as side length was assumed for calculation of the maximal surface coverage corresponding to a monolayer coverage. For the hydrated protein and an quartz electrode surface area of 0.196 cm² a maximal

monolayer surface coverage of 3.8 pmol cm^{-2} was calculated (or respectively 3.1 pmol cm^{-2} for a partial specific protein volume of $0.73 \text{ cm}^3 \text{ g}^{-1}$). This value roughly corresponds to the value found for the immobilization of His-tagged P450 BM3 at a nickel loaded PBA polymer film via the EQCM (table 12). Both, the theoretical assumption and the mass increase upon P450 incubation, were in the pmol per cm^2 range, but the measured mass increase is higher than the theoretical monolayer coverage by a factor of 10. Several reasons come in consideration, first the theoretical monolayer coverage calculated here does not equal the real dimension of the protein, and secondly also some unspecific protein adsorption to the polymer surface may occur giving higher values than for a monolayer coverage.

The coated and P450 loaded electrode was exposed to pNCA assay conditions with the natural cofactor NADPH, but after 15 minutes of incubation time no product was detected.

3.2.3. Covalent attachment

One of the main methods to covalently attach proteins at surfaces is carbodiimide based coupling (figure 70), which results in a peptide bond formation between amine groups of the polymer and carboxylic acid groups on the surface, or vice versa.

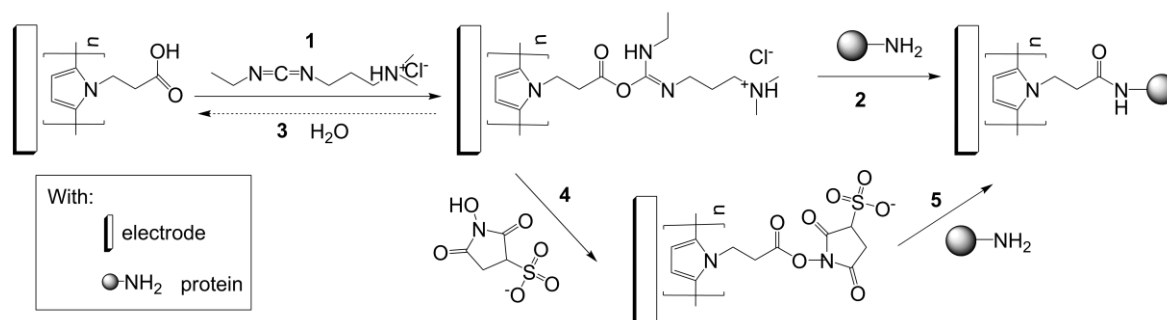


Figure 70: Reaction scheme for the coupling of a protein to a carboxylic acid modified electrode surface via carbodiimide. As electrode modification exemplarily a polymer consisting of the pyrrole derivative PPA is spotted, but can be substituted by any other carboxylic acid bearing moiety. (1) The electrode is incubated with N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC). The resulting ester then reacts with an amine group displayed at the surface of the protein (2). Since the ester is prone to hydrolysis (3) in addition N-hydroxysulfosuccinimide (Sulfo-NHS) can be added (4), which results in the formation of a stabilized intermediate able to react with the amine functionality of the protein (5).

3.2.3.1. Covalent attachment of aminoferrocene to PPA

Aminoferrocene is a redox active organometallic compound with iron as central metal atom, which is bound to two cyclopentadienyl rings of which one exposes an amine functionality (figure 71). Aminoferrocene was bound via carbodiimie chemistry to a PPA polymer. Since it is known that the PPA polymer is not stable in aqueous electrolytes (see chapter 3.3.2.2.) a carbodiimide coupling procedure in DMSO was chosen, which is also compatible with aminoferrocene.

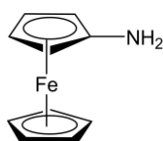


Figure 71: Aminoferrocene

After polymerization in acetonitrile the PPA films exposed strikingly high currents at potentials in between 400 and 600 mV vs. Ag/AgCl (figure 72, left), which strongly distinguishes PPA polymer from PPy. Furthermore a peak shoulder is obtained at 200 to 300 mV, which also cannot be found for PPy. After incubation in DMSO the difference between PPy and PPA films vanished, since the current spikes at high potentials were lost (figure 72, center). Nevertheless, PPA still exposed higher background currents compared to PPy. For the coupling of aminoferrocene no significant difference was seen for PPA electrodes with and without addition of the coupling reagent DCC. Instead the PPA electrode, which was not treated with DCC, exposed slightly higher background currents and narrow reductive redox waves around 50 and 300 mV (figure 72, center). When electrodes were transferred to an aqueous buffer system redox behavior of the polymer films inverted. PPy exposed higher background currents than both PPA films and a well defined reductive peak was obtained at 131 mV (figure 72, right). A broad anodic wave was obtained at 344 nm. In contrast PPA films perform similar to an uncoated electrode and only a weak reductive wave around 223 mV can be seen in case of the PPA electrode incubated with aminoferrocene in absence of DCC.

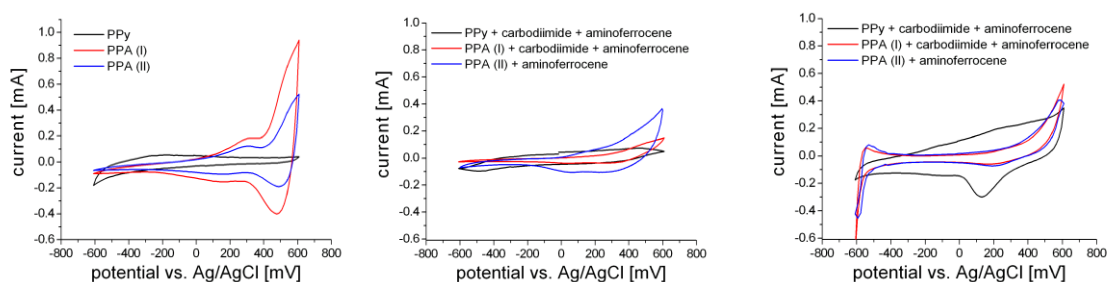


Figure 72: Characterization of coated electrodes in monomer free acetonitrile with 0.1 M TBAP (left), after incubation with the coupling reagent DCC (carbodiimide) in acetonitrile and 0.1 M TBAP (center) and after coupling in phosphate buffer (right). All CVs were performed at 50 mV/s.

3.2.3.2. Covalent attachment of cytochrome c to PBA

It was shown that PBA is stable in aqueous electrolytes. Therefore a covalent attachment method with the water soluble carbodiimide EDC in combination with Sulfo-NHS was chosen. As test molecule for immobilization cytochrome c was chosen, since it is commercially available and is known to have a surface exposed heme with exposed lysines close by. Each step of the surface modification was followed by cyclic voltammetry (figure 73). Polymerization yields a conductive polymer film, as it can be seen from the increase in current compared to the uncoated electrode. The coupling procedure with Sulfo-NHS/EDC and cytochrome c altered the current profile over potential. Increased currents were obtained in the range of 200 to 400 mV. A midpoint peak potential of 254 mV vs. Ag/AgCl was determined. The observed redox peak could not be detected again in cyclic voltammetry when the electrode was stored over night in buffer with cytochrome c.

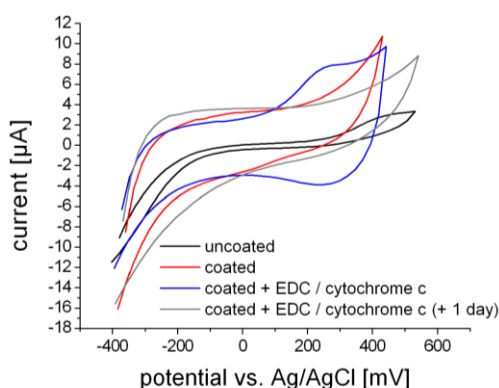


Figure 73: All intermediate steps in cytochrome c attachment to a PBA polymer were characterized by cyclic voltammetry in phosphate buffer at 50 mV/s. Black – uncoated electrode; Red – electrode coated with PBA (0.164 mA for 20s); Blue – after incubation with Sulfo-NHS and EDC and subsequent incubation with cytochrome c; Grey – The electrode was stored over night in buffer / cytochrome c at 4°C and characterized again at the next day.

In addition electrodes with thinner polymer films (as introduced by reduced galvanostatic coating times) were treated the same way. For all tested electrodes the treatment with EDC and cytochrome c induced a change in the polymer films as expressed by a difference in the voltammogram before and after incubation

(figure 74). Resulting midpoint potentials were 285 mV (10s coating) and 305 mV (15s coating). Furthermore a clear dependency of the current in the peak range of 200 to 400 mV on film thickness is seen. In all cases the peaks vanished after storage of the electrodes over night.

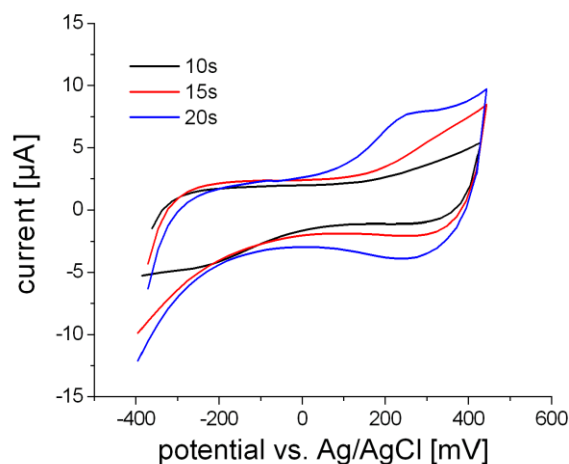


Figure 74: Electrodes were coated galvanostatically (0.164 mA) for different times, as indicated. Displayed are cyclic voltammograms at 50 mV/s in phosphate buffer after treatment of the PBA coated electrodes with EDC, Sulfo-NHS and cytochrome c.

3.3. Discussion

3.3.1. Entrapment in polypyrrole

For all entrapment experiments the ion added to the polymerization solution did affect polymer properties, as it can be seen from cyclic voltammograms in absence and presence of the respective ion. For potassium ferrocyanide it was shown that the ion was incorporated into the polymer film, since after cycling the film in ferrocyanide free buffer the ion could be detected with an uncoated electrode in the electrolyte afterwards. Most probably during polymer reduction the negatively charged ferrocyanide is expelled from the polymer [112, 140]. For the characterization of polypyrrole, which was polymerized in presence of 10 mM potassium ferrocyanide onto a polypyrrole prelayer, broad redox waves with a midpoint potential of 217 mV were obtained in cyclic voltammograms at a scan rate of 25 mV/s. This value is in the range of a literature value of around 230 mV vs. Ag/AgCl, which was reported for galvanostatic incorporation of ferricyanide into polypyrrole [141]. When changing to a thinner film, which was prepared without a polypyrrole prelayer, the midpoint potential of the redox waves was shifted to 183 mV vs. Ag/AgCl. Due to the similarity in midpoint potential, also with the solution species which exposes a midpoint potential of 220 mV vs. Ag/AgCl_{eMTP} (compare eMTP chapter), the difference in background charge to a polypyrrole film polymerized without ferrocyanide and the detection of the ion in the supporting electrolyte after polymer characterization the conclusion can be drawn that potassium ferrocyanide was entrapped in the polymer film. Nevertheless some doubts remain about the direct electrochemical oxidation and reduction of the incorporated ferrocyanide, as it will be discussed in the following.

In case of Fe-SO₃⁻ incorporation is also quiet clear, since polymer films which were grown in presence of Fe-SO₃⁻ exhibit far higher background currents than polymer films grown with solely potassium chloride. For Fe-SO₃⁻ it was already shown in literature, that addition to the pyrrole polymerization solution during electropolymerization lead to irreversible incorporation, but no well defined redox peaks were obtained [142]. The authors argued that peaks were not clearly

defined due to the high background current of polypyrrole. Also they could not show catalytic response towards NO [142]. In another study a steep increase in current was seen upon exposition of Fe-SO_3^- to molecular oxygen, while under nitrogen atmosphere no distinct redox wave was obtained [143]. This is in line with the results obtained in this work. For the incorporation of Fe-SO_3^- in polypyrrole no well defined redox peaks were found, independent of the concentration of Fe-SO_3^- present during electropolymerization. Nevertheless several broad redox waves were obtained. When comparing the position of the broad redox waves with literature values the peak origin cannot be identified directly (table 13). The first observed redox pair with a midpoint potential of -323 mV can also be found for polypyrrole which was polymerized in absence of Fe-SO_3^- , even though the peak potential is close to the formal potential of Fe-SO_3^- in solution of -367 mV. The second redox pair observed ($E_{\text{mp}} = -146$ mV) is in the range determined in literature for Fe-SO_3^- entrapped in polypyrrole (-171 mV). But in addition no increased electroactivity of a Fe-SO_3^- polypyrrole film in presence of oxygen was observed. All in all incorporation of Fe-SO_3^- in polypyrrole presumably occurred, since an effect on polypyrrole background charge in cyclic voltammograms is seen. But direct electrochemical communication between polypyrrole and (eventually) incorporated Fe-SO_3^- could not be ambiguously proven, since no clear redox peaks were detected and no catalytic interaction upon oxygen exposure was obtained.

Table 13: Midpoint potentials given in literature for the Fe-SO_3^- redox couple, spotted for comparison with data obtained in this work. *: Values were converted versus an Ag/AgCl reference electrode.

	c (Fe-SO_3^-)	Polypyrrole coating procedure		E_{mp} [mV] vs. Ag/AgCl
		Method	Electrolyte	
This work	1 mM	Potentiodynamic	0.1 M phosphate (pH 7)	(1) -323 (2) -146 at 25 mV/s
Reference [143]	1 mM	Galvanostatic	methanol	-171*
Reference [143]	<i>In solution</i> 1 mM			-367*
Reference [112]	0	Potentiostatic	0.1 M phosphate (pH 7) and 0.1 M KCl	(1) -315* (2) -5*

In comparison to Fe-SO_3^- the results obtained for the positively charged Fe-N^+ are even harder to interpret, since the porphyrin complex is positively charged and will not serve as adequate counter ion in electropolymerization. Nevertheless incorporation of Fe-N^+ may occur since also chloride and phosphate ions are present during electropolymerization, which can counterbalance the positive polypyrrole backbone charge and Fe-N^+ may be entrapped due to its presence at the polymerization site [48, 50]. Background currents obtained with Fe-N^+ were far lower than for Fe-SO_3^- and in the range of polymers which were grown in presence of solely chloride. Hence the addition of the positively charged molecule Fe-N^+ did not boost polymer formation or conductivity strongly, and values were comparable to the standard polypyrrole produced in presence of potassium chloride in phosphate buffer.

In summary in all entrapment experiments it is not clear whether the observed broad redox waves stem from the incorporated ion or polypyrrole itself, which may alter its properties e.g. with film thickness as function of the counter ion. The observed background current obtained for every polypyrrole film is due to the oxidation and reduction of several polymer sites, leading to a non-specific, broad redox wave [144]. Thereby it has to be noted that an observed change in polymer background current may be related to a change in polymer film thickness or a change in polymer conductivity, or essentially both. During the study of entrapment of charged species into polypyrrole in all cases the electrolyte additionally contained potassium phosphate and potassium chloride. Agostiano et al. [112] used the same electrolyte system and characterized the redox behavior of the resulting polypyrrole films. They determined two anodic peaks at -260 and -50 mV vs. SCE [112], which equals approximately -220 and -10 mV vs. Ag/AgCl [59]. The corresponding reductive peaks were obtained at -450 and -40 mV vs. SCE [112] or respectively -410 and 0 mV vs. Ag/AgCl. The authors related the first redox pair of -220/ -410 mV to an interaction between polypyrrole and phosphate, while the second redox pair of -10/ 0 mV vs. Ag/AgCl was attributed to a polypyrrole interaction with chloride ions. Thereby, depending on the supporting electrolyte and film treatment, the polymer properties as expressed by the shape of the CV did alter [112]. For example the authors also obtained a film which exposed only

one redox wave at -460 (anodic) and -360 mV (cathodic) vs. Ag/AgCl. In conclusion in the entrapment experiments all detected peaks, besides the potassium ferrocyanide experiments, were positioned in this potential range and may also stem from polypyrrole itself and not the entrapped redox molecule.

This problem is especially seen for the entrapment of myoglobin in polypyrrole. When comparing the obtained redox waves at one scan rate the anodic wave shifts in between 30 to -290 mV and the cathodic wave in between -180 to -400 mV vs. Ag/AgCl, depending on the film preparation procedure or respectively film thickness. In addition CVs obtained for two identically prepared polymer films, except one was polymerized in presence and one in absence of myoglobin, led to different shapes in the voltammogram. Thereby films grown in presence of myoglobin exhibit lower background currents, and it is not clear if the obtained redox peaks come from the myoglobin active site being reduced or oxidized or if polymer properties are changed yielding different redox behavior of the film. It is also known that proteinaceous compounds adsorb to electrode surfaces [145], which would hinder polymer film growth and lead to decreased background conductivity. But on the other hand a high load of protein in the polymer would also alter polymer structure and lead to decreased conductivity. When looking on the midpoint potential obtained for myoglobin/ polypyrrole electrodes at 50 mV/s, they were around -330 mV vs. Ag/AgCl. The corresponding polypyrrole redox pair gave a midpoint potential of around -360 mV vs. Ag/AgCl. In literature values for immobilized myoglobin are for example around -330 mV at pH 7 for myoglobin immobilized on a gold nanoparticle modified ITO electrode [146]. Since the midpoint potential determined for polypyrrole and polypyrrole/ myoglobin was quiet similar in addition hydrogen peroxide was added, which normally can be reduced by the heme active site [147]. This would result in a so called “catalytic current” in the voltammogram with an increase in the reductive peak current and concomitant vanishing of the oxidation peak [147]. But upon addition of hydrogen peroxide no catalytic current was obtained. In case of myoglobin polymer shape in the voltammogram was changing, but the same was the case for pure polypyrrole, indicating possible destruction of the polymer or conversion of hydrogen peroxide at the electrode surface by diffusion through the film.

The same holds true for the entrapment of P450 BM3. The cyclic voltammogram of the polymer looked essentially the same than its P450 free counterpart and no substrate conversion was detected, even after 42 hours of potentiodynamic cycling. This is not surprising, since compatibility experiments showed that enzyme activity was strongly decreased in presence of pyrrole monomer. Thereby it is interesting to note that P450 BM3 can convert substances like aniline and indole [14], which have some structural similarity to the pyrrole monomer (figure 75). Furthermore it is known that heterocycles containing nitrogen, for example imidazole and pyridine (figure 75), can ligate to the iron of heme. For example it was shown that these compounds reversibly inhibit the human liver P450 3A4 by interaction with the P450 heme [148]. Also imidazoles and derivatives thereof are known as inhibitors for P450 BM3 [149, 150]. This may indicate a possible blocking of the heme active site by the pyrrole monomer towards the substrate pNCA in the stability tests. In addition potential cycling did affect enzyme activity as well as application of current and presence of high salt concentrations. Inactivation may also be related to protein denaturation at the unmodified electrode surface or hydrogen peroxide evolution, which in turn destroys the enzyme.

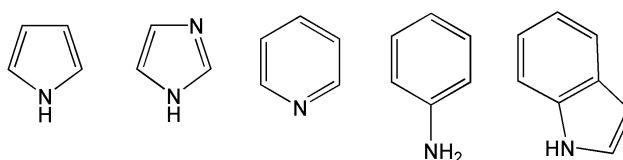


Figure 75: Pyrrole, imidazole, pyridine, aniline and indole (from left to right).

Nevertheless it was reported that electrochemically driven substrate conversion via entrapment in polypyrrole was observed for P450cam and P450 BM3 [58, 118]. Since P450 BM3 is negatively charged around a pH of 7 to 8 [30], incorporation in the growing polypyrrole network should principally be feasible. Results showed that around neutral pH polypyrrole can be polymerized, whereas a more acidic pH improves film formation. But in both cases described in literature no direct electrochemically communication between the polymer and the enzyme was shown. Even though the direct electron transfer between polypyrrole and entrapped enzymes is often described in literature, the nature of the electron transfer process is still under discussion [50, 98, 117, 151]. For example

denatured protein could contribute to signals related to direct electron transfer [50, 151]. It is also argued that in some cases oligomeric fractions may serve as mediator between the electrode and the enzyme and no direct electron transfer process is taking place [117]. This finding would correlate with the observation that no direct electron transfer to the P450 BM3 heme was shown, but nevertheless electrochemically driven substrate conversion at slow rate was seen in literature [58]. On the other hand the lack of detectably of direct electron transfer to the P450 BM3 heme does not explicitly prove that no direct electron transfer is taking place. The polypyrrole background current may superimpose on any weak redox signal coming from the enzyme. Finally it is not surprising that the heme active site was not electrochemically accessible by cyclic voltammetry, since also use of isolated hemin like Fe-SO_3^- did not lead to clearly defined redox peaks and the heme of P450 BM3 is in addition surrounded by an insulating protein shell. Furthermore polypyrrole is known to be of hydrophobic nature, which may lead to slow substrate diffusion through the polymer [98] or probably adsorption of the resulting product, resulting in reduced amount of free product in solution accessible for analysis.

In summary the incorporation of all negatively charged ions into polypyrrole was achieved in high probability, since differences in polymer performance in cyclic voltammetry were obvious. Also in presence of the positively charged Fe-N^+ thin polypyrrole films, or respectively films of low conductivity, could be grown. But in most cases it is not clear whether the observed peaks stem from a redox reaction of the incorporated ion or polypyrrole itself. Only the incorporation of ferrocyanide led to polypyrrole films with redox peaks in a potential range where no peaks for the pure polymer were obtained. This may be related to the improved conductivity of polypyrrole in the oxidized state, while polypyrrole is non- or less conductive when reduced. For all other tested ions results suggest a redox reaction of polypyrrole, since e.g. peak potentials were in the range of polypyrrole and no catalytic activity could be detected, at least for Fe-SO_3^- and myoglobin. For P450 BM3 also no distinct redox peaks were obtained in cyclic voltammograms and in addition also no change in the voltammogram due to catalysis was seen, which was not surprising since already low amounts of the pyrrole monomer did inactivate the enzyme.

3.3.2. Immobilization via affinity interactions

Polymerization of the pyrrole derivatives 1-H-pyrrole-propanoic acid (PPA) and 3-(4-pyrrolyl)butyric acid (PBA) was achieved in acidified methanol [134]. When characterizing the polymer films by cyclic voltammetry in aqueous electrolyte, PBA exposed higher background currents while PPA performed in the range of an uncoated electrode or even resembled an insulating layer. Both polymers showed reduced background currents compared to polypyrrole. The reduced background currents obtained can be related either to a reduced amount of polymer formed at the electrode and/ or differences in polymer structure [152]. Due to the carboxylic acid functionality, connected to the pyrrole backbone via an alkyl linker, the planarity of the resulting polymer film will be reduced compared to polypyrrole, resulting in reduced conductivity [103, 153]. Furthermore by inductive effects due to the carboxylic acid group the pyrrole ring will show higher susceptibility for nucleophilic attack from e.g. hydroxide ions of the electrolyte, which will in turn result in disruption of the delocalized π electron system and reduced conductivities. Thereby N-substituted pyrroles like PPA are more affected than C-substituted pyrroles like PBA, which can also be seen by the difference in background current in between both when performing cyclic voltammetry in aqueous electrolyte. PPA was already used in literature for the immobilization of nickel cations and subsequent immobilization of polyhistidine and a histidine-tagged alkaline phosphatase [136, 154]. The immobilization of nickel could be shown by cycling the polymer in alkaline solution after nickel incubation. This results in polymer overoxidation and loss of its activity. Nickel hydroxide is forming, which can be detected electrochemically [154]. Nickel binding was shown for PPA resulting in the detection of nickel hydroxide peaks in cyclic voltammograms, which vanished after treatment with acid. Thereby, due to protonation of the carboxylic acid groups, nickel is eluted from the polymer. In contrast for PBA nickel binding could not be proven by this method. Since PBA displayed higher stability against alkaline conditions probably nickel could not be detected due to the still high polymer background current. Nevertheless it was shown that based on the carboxylic acid functionality the PBA polymer can be chemically modified with water soluble carbodiimide [134], which in turn indicates

accessibility of the carboxylic acid functionality towards solution species. Furthermore PBA displayed the higher conductivity and stability in aqueous electrolyte compared to PPA and was used in the following for a quartz crystal microbalance study.

Around 224 nmol per cm^{-2} PBA polymer were deposited on the quartz electrode, which related to a film thin enough for the application of the Sauerbrey equation [154]. Via the quartz crystal microbalance nickel binding to PBA could be shown as mass increase at the electrode. Thereby the ratio of one nickel per three PBA monomer molecules was obtained, which corresponds to three carboxylic acid groups chelating one metal cation. It was already shown that carboxylic acid groups bind to nickel cations in an unidentate way [154]. To obtain the ratio of three PBA monomers to one nickel cation all sites of the polymer need to have access to nickel in solution. In deed in literature it was shown that the structurally similar PPA film was porous and allowed the diffusion of nickel cations to the film backside [136]. Compared to the more hydrophobic polypyrrole, the modification with carboxylic acid groups increases film hydrophilicity and hence water content inside the polymer [155]. Nevertheless the obtained ratio of three to one will not represent the real value, since also additional water molecules are associated with nickel binding and carboxylic acid modified polymers are known to exhibit a behavior called self-doping. This means carboxylic acid groups may take part in counterbalancing the positive polymer background charge and hence reduce the amount of free binding sites available for cations. In fact for PPA a ratio of two carboxylic acid groups chelating one nickel cation was shown [136]. On the other hand for nitrilotriacetic acid modified surfaces three carboxylic acid groups are known to chelate one metal cation [156], which shows that the experimentally determined nickel loading capacity is in range of literature values.

Upon exposure of the nickel loaded PBA film to His-tagged P450 BM3 a mass increase of 38 pmol protein per cm^{-2} was obtained. Since the molecular dimension of the P450 BM3 holoprotein is not known, a theoretical cross sectional area of around $3 \text{ to } 4 \times 10^{-13} \text{ cm}^2$ was calculated based on the protein molecular weight and assuming a globular protein dimension [138, 139]. This estimate agrees in its

order of magnitude with values obtained for the P450 BM3 heme domain. The heme domain size is given as $70 \times 60 \times 60 \text{ \AA}$ [28], which results in a cross sectional area in the range of 10^{-13} cm^2 . From these assumptions a theoretical maximal monolayer surface coverage of around 3.5 pmol protein per cm^2 electrode area was calculated for the holoprotein. Via the EQCM measurement upon protein exposure a mass change was obtained which corresponded to a surface coverage of around 40 pmol His-tagged P450 BM3 per cm^2 electrode area and hence exceeded monolayer coverage. Most probably exposure of the PBA coated and nickel loaded quartz resulted in a mixture of directed immobilization via the His-tag and unspecific protein adsorption.

Some examples show that direct electron transfer to proteins can occur, when immobilized via a His-tag. But those examples are up to now limited to self-assembled monolayer modified surfaces and include the immobilization of photosynthetic reaction centers [157, 158], cytochrome c oxidase [159], thioredoxin, plastocyanin and P450c17 (the last three examples can be found in [160]). The latter example further supported the idea to achieve a direct electron transfer to P450 BM3 when immobilized via a His-tag to a polypyrrole coated electrode surface. But cyclic voltammetry characterization of the prepared assembly did not reveal any significant differences before and after nickel and P450 immobilization. The protein will probably be immobilized mainly at the outer layer of the polymer, which manifests in higher protein flexibility compared to entrapment methods and in good accessibility of the substrate. Nevertheless the His-tag immobilization procedure implies that the protein is separated from the conducting polymer backbone at least via the carboxylic acid bearing alkyl spacer, the metal cation and the His₆-tag. This may prevent any direct electron transfer, even though electrons are able to tunnel certain distances. Thereby the tunneling efficiency is reduced with increasing distance in between the involved redox centers. This effect is often encountered in literature, where the immobilization of His-tagged proteins to metal chelated electrode surfaces leads to successful immobilization, but no direct electrochemical communication with the protein is achieved [161]. The problem is often circumvented by adding soluble redox mediators, shuttling electrons between the electrode and the immobilized enzyme

[162-166]. But this approach is not applicable to the immobilization system used in this study, since polypyrrole coated electrodes cannot reduce soluble mediators with a highly negative reduction potential, as required for P450s, due their lacking conductivity in the completely reduced state [144]. A solution to this problem would be the introduction of redox active relays, to divide the initial electron transfer distance in several smaller distances [162]. Nevertheless this procedure is much more complex, starting with the synthesis of corresponding pyrrole monomers [167] or the binding of redox active molecules to the protein of interest [168], and not feasible for a screening application.

Beforehand it was shown that P450 BM3 is active when it is immobilized via the His-tag at nickel loaded surfaces. Also activity was seen when the protein was immobilized at a nickel loaded electrode surface modified with a self-assembled monolayer. When incubating the PBA coated, nickel loaded and P450 BM3 bound quartz with pNCA and NADPH no substrate conversion was seen photometrically. This can have several reasons. Most probably the amount of protein immobilized at the small quartz surface is too low for detection of the product. In addition non specific protein adsorption may reduce overall enzyme activity and low amounts of product may be adsorbed to the polymer surface.

3.3.3. Covalent attachment

Amide bond formation between carboxylic acid and amine groups via carbodiimide is a common method in protein sciences. Since a carboxylic acid functionalized polypyrrole surface was employed, covalent binding can take place with amine functionalities of the immobilization target. A general feature of the covalent attachment methods is the chemical modification of the target molecule with concomitant direct fixation to the surface, also via multipoint binding, leading to loss of conformational flexibility [96].

Since the PPA polymer did not show high conductivities in aqueous electrolyte the coupling of aminoferrocene was tested in DMSO and characterization of the polymer films was performed in acetonitrile. When characterizing polypyrrole and PPA polymer in acetonitrile, a higher background current was obtained for PPA compared to polypyrrole. Thereby the second PPA film was coated with a thinner polymer film. This can be seen by the reduced background current in cyclic voltammograms recorded in acetonitrile, when compared to the first PPA film. Nevertheless both PPA polymers exposed a dominant anodic and cathodic wave around 500 mV, independent of film thickness. These waves were also described for other N-substituted polypyrrole films, which exposed the polymer redox reaction at potentials more positive than polypyrrole [144]. After treatment with the coupling reagent to covalently attach aminoferrocene, the dominant redox waves of the PPA polymers vanished. No direct evidence was found for aminoferrocene coupling, since no distinct redox peak pairs were seen in cyclic voltammetry for PPA polymer samples with and without carbodiimide treatment and especially in comparison with polypyrrole. For the PPA polymer incubated with aminoferrocene broad and undefined cathodic waves were found at around 50 and 300 mV vs. Ag/AgCl, but no corresponding anodic peaks were obtained. Assuming adsorption of aminoferrocene, the same undefined wave should be found for the PPA polymer treated with the coupling reagent. But the PPA film treated with the coupling reagent did not yield a redox wave and behaved almost like polypyrrole in relation to the background charge. Since PPA films treated with and without carbodiimide behaved differently in cyclic voltammetry the coupling reagent

seemed to interact with the polymer, particularly because the thicker film exposed reduced background currents compared to its thinner counterpart and films were not affected in the same way by DMSO incubation.

Nevertheless, observed broad redox waves for the PPA polymer incubated with aminoferrocene cannot be attributed to aminoferrocene, since reversible electrochemistry of ferrocene at polypyrrole was shown in literature. For example it was reported that soluble ferrocene in acetonitrile can be detected as superimposed waves on a background of N-substituted polypyrrole coated electrodes at a formal potential of +470 mV vs. Ag/AgCl [144]. Furthermore a structural analog to aminoferrocene, which exposes an ethyl group as spacer between ferrocene and the amine group, was bound to a polymer of a 3-substituted pyrrole derivative, namely 3-acetic acid pyrrole, via carbodiimide chemistry [169]. The authors obtained almost reversible electrochemical behavior for the bound ferrocene in cyclic voltammetry with an oxidation peak at 363 mV and a reduction peak at 355 mV vs. Ag/AgCl. In addition the ferrocene ethylamine was covalently bound to an N-substituted pyrrole derivative, which exposed a C₁₁ spacer in between the pyrrole ring and the carboxylic acid function, and the assembly showed reversible electrochemistry [170]. It should also be noted that electrochemistry of ferrocene reported in literature is in the μ A range [169], while polymer films obtained in this study exposed higher background currents (by a factor of approximately 10). Probably thinner films would allow a clearer distinction of redox signals coming from the polymer or any bound ferrocene.

After characterization in acetonitrile the modified electrodes were transferred to an aqueous buffer system. As a result the background current of polypyrrole was boosted, probably due to swelling of the polymer upon water incorporation [171, 172], and a defined reduction wave was seen at 131 mV. Instead the background charge for PPA polymers remained at low level and no additional redox waves were detected.

In a second approach the covalent attachment of cytochrome *c* to a PBA polymer via the water soluble carbodiimide EDC was investigated, because PBA showed good redox stability in aqueous electrolyte. Cytochrome *c* is one of the most studied redox proteins since it exposes its heme partly to solution, which makes it an attractive model compound for bioelectrochemical studies [131]. Furthermore several lysine residues are positioned at the protein surface in close proximity to the heme [173]. This results in an almost uniform protein orientation at negatively charged electrode surfaces, with direction of heme towards the electrode when the protein is adsorbed [173]. Thereby the lysine residues may also serve as target for carbodiimide mediated amide bond formation between the carboxylic acid sites of the PBA polymer and cytochrome *c*. For polypyrrole it was shown that electrons can be transferred between a polymer coated electrode and cytochrome *c* in solution [174] and also the entrapment of cytochrome *c* in polypyrrole led to quasi-reversible redox behavior of the protein exposing peaks at 160 and -60 mV vs. Ag/AgCl [112]. Another study described redox peaks at 240 and 40 mV vs. Ag/AgCl for polypyrrole entrapped cytochrome *c*, which could also be detected after overoxidation of the polymer [175]. All studies showed that polypyrrole can mediate electron transfer between an electrode and cytochrome *c*, which can be either in solution or immobilized at the polymer site. Direct electron transfer to covalently immobilized cytochrome *c* was shown for several carboxylic acid modified surfaces, including self-assembled monolayer systems or diazonium grafting [176, 177]. For the latter a midpoint potential of approximately 140 mV vs. Ag/AgCl was obtained [177] and for self-assembled monolayer systems a formal potential of -50 to -60 mV was reported [176]. After the coupling reaction in between PBA polymer coated electrodes and cytochrome *c* via EDC, in cyclic voltammograms in aqueous buffer a broad anodic and cathodic redox wave with a midpoint potential around 300 mV vs. Ag/AgCl was seen. This wave was lost after storage of the electrode over night. Due to the difference in redox potential to reported values, which all exposed midpoint potentials around 100 mV vs. Ag/AgCl, it was concluded that the observed peaks did not reflect covalently immobilized cytochrome *c*. This is supported by the fact that the redox wave vanished when storing the electrode over night. For comparison it was reported that cytochrome *c* covalently bound to a self-assembled monolayer exhibited a

long-term stability of three weeks [176]. An adsorption of cytochrome c to PBA coated electrodes may be possible, since the polymer exposes carboxylic acid groups. Cytochrome c is known to display well behaved electrochemistry when adsorbed to negatively charged surfaces [178], but redox waves found for the immobilization at PBA are less well defined. Hence, it is more likely that the observed redox waves were related to the PBA polymer changing its redox behavior upon interaction with solution species. In addition the broadness of the redox wave is typically found for the underlying redox process in conducting polymers, namely oxidation and reduction of the polymer backbone.

In summary both immobilization strategies based on carbodiimide did not result in detectable direct electron transfer in cyclic voltammograms. For both polymer types it was already shown that carboxylic acid groups are accessible for solution species and also carbodiimide mediated modification [134, 154]. Although standard protocols for carbodiimide coupling were employed, no prove for the covalent attachment of the target molecules was obtained. Thereby it is not clear at which point the surface modification failed, either the reaction of carbodiimide with the carboxylic acid functionality or the concomitant amide bond formation, or if the deposited polymers were not able to transfer electrons to any bound substances. Since an effect on the polymer background current was seen when the polymers were treated with carbodiimide and in case of cytochrome c, which is known to show reversible electrochemistry even when adsorbed, no distinct redox peaks were obtained upon incubation with a PBA polymer, the conclusion is drawn that the produced polymers could not efficiently transport electrons to any adsorbed or bound substance. On the other hand it was shown in literature that polypyrrole can transfer electrons to amine functionalized ferrocene as well as cytochrome c, indicating failed immobilization of the target compounds. It is known that carbodiimide mediated covalent bonding occurs via an unstable intermediate, which is prone to hydrolysis and therefore efficiency of amide bond formation can be reduced [179, 180]. It can be assumed that a mixture of these effects occurred and low efficiency of immobilization in combination with sluggish electron transfer led to non-detectable signals in cyclic voltammetry.

4. Summary and conclusion

4.1 The eMTP as screening device for mediated electron transfer to P450 BM3

In this work an electrochemical microtiter plate (eMTP) was developed based on commercially available microtiter plates. The eMTP is characterized by its ease in operation and implementation and can be introduced in a standard microtiter plate reader, which makes the device an interesting option for laboratories working in the field of bioelectrochemistry. The unique feature of the eMTP is the simultaneous collection of optical data while performing parallelized electrochemical experiments in up to eight wells. Thereby each well comprises an independent three electrode configuration, which allows precise control over the electrochemical reaction parameters.

Here the eMTP was used to screen a P450 BM3 mutant library in the pNCA assay via the mediator cobalt sepulchrate. By variation of reaction conditions several parameters were found that influence specific product formation rates. All in all no mutant was found with improved rates compared to WT and F87A at the chosen reaction conditions. But a change in reaction conditions in some cases had an influence on specific product formation rates depending on the mutant tested. While the specific product formation rate of WT increased with increasing mediator concentration, for the enzyme variants M3 and M3 R471C the maximal specific product formation rate was already reached at lower mediator concentrations. Furthermore for the electrochemical reaction it is interesting to note that the reduction of the mediator did not seem to be the limiting factor of the reaction. The electrode area is large enough in the current reaction set-up, since an increase in mediator concentration did increase reaction rates in case of WT. This effect was also seen by variation of enzyme concentrations, since an increase in enzyme concentration did not result in decreased specific product formation rates, as it would be expected when mediator reduction would be the limiting factor. By variation of the applied potential it was shown that maximal specific product formation rates were achieved at -850 mV for most mutants. One mutant, namely

M3 DM, showed already a high reaction rate at -750 mV, which only differed slightly from its maximal specific product formation rate at -850 mV. Since with increasing reductive power more side reactions will occur, a less reductive working potential for maximal product formation would be beneficial. Another finding was that the mutants M3 and M3 R471C did show stable specific product formation rates at the lowest potential tested (-950 mV), which may indicate an increased mutant stability under electrochemical reaction conditions. This may include a higher pH stability or hydrogen peroxide tolerance. In summary the eMTP served as adequate prescreening tool to distinguish between mutants and find mutants with interesting properties for bioelectrochemical processes.

4.2. Development of a polypyrrole based immobilization “tool box” for direct electron transfer

Compared to many other immobilization techniques employed for the successful electrochemical attenuation of P450 BM3 polypyrrole offers high versatility, since several film thicknesses and surface modifications can be employed. While most other immobilization techniques used for P450 BM3 are based on creating a monolayer coverage, polypyrrole based methods would practically increase the electrode surface. Polypyrrole films on electrodes constitute a 3D assembly and theoretically a higher enzyme load can be achieved compared to monolayer methods. Thereby distant redox sites can still be actuated via the conducting polymer chains. But entrapment of the redox active test molecules into the growing polymer film during electropolymerization could not be proven. Redox waves obtained in cyclic voltammetry were always suspected to stem from the redox activity of the polypyrrole background itself. Polymer film thickness depended on the electrolyte used and therefore it was generally not possible to subtract an appropriate background current. A possible solution would be the incorporation of corresponding apoproteins as negative control, which would result in polymer films of similar thickness, morphology and conductivity. Consecutively distinction of redox waves would be eased. Nevertheless such an approach would not be feasible in any screening application aiming at medium throughput. In addition no catalytic currents were obtained in presence of substrates, which may also be

related to possible denaturation of the protein compound upon immobilization. Similar problems were observed for other electrode surface modification techniques like polyelectrolytes, and polypyrrole does not seem to be an alternative immobilization matrix with improved properties. Already short incubation times with low amounts of the pyrrole monomer led to strongly decreased activities of P450 BM3.

Hence, two other immobilization methods were tested, which separated the polymerization and immobilization processes. Therefore carboxylic acid derivatives of pyrrole were established as scaffold for affinity immobilization according to the IMAC (immobilized metal affinity chromatography) principle and carbodiimide mediated covalent attachment. In case of the affinity methodology hints for an immobilization of P450 BM3 were obtained, since mass increase at the electrode was observed via a quartz crystal microbalance study. In addition some unspecific adsorption seemed to occur and no electrochemical activity in cyclic voltammetry as well as activity with the natural cofactor NADPH was obtained. For the covalent attachment the polymer background currents, observed in cyclic voltammograms, differed upon treatment with the coupling reagent, but there was no evidence for direct electrical wiring of any of the test molecules and the procedure was not extended to P450 BM3.

In summary polypyrrole and its derivatives can be established as electrode modification matrix, but cyclic voltammograms were strongly dominated by the polymer background current. Also small redox active molecules could not be detected in cyclic voltammograms, indicating sluggish electron transfer. Since polymer properties strongly depend on the composition of the polymerization solution as well as the electrolyte used during characterization, correlation of recorded signals towards any immobilized component is hard to achieve. The problem of distinguishing between direct electron transfer to the biomolecules active site and the background signal is also discussed in literature [50, 181], since many publications claim direct electron transfer but did not prove catalytic activity or lack adequate control experiments. In consequence polypyrrole and its derivatives are not regarded as suitable immobilization matrix for P450 BM3 in a

screening application. To achieve direct electron transfer to P450 BM3 a more sophisticated experimental set-up would be required, including modification of monomers with electroactive relay groups, operation under nitrogen atmosphere and more sensitive analytical tools. All of these provisions cannot be employed in an application with the eMTP, when aiming at a screening technology of medium throughput.

Appendix

A.1. Specific product formation rates and product yields of the P450 BM3 mutant screening in the eMTP

Table A1: Specific product formation rates (SPFR) obtained for the tested mutants in the electrochemically driven pNCA assay with CoSep (left) and the pNCA assay with the natural cofactor NADPH (right). Each measurement was done in duplicate and the mean value with the corresponding deviation is given. Specific product formation rates based on the absorbance decrease corrected values are stated for comparison (SPFR_{cor}).

	CoSep				NADPH	
	SPFR [U/μmol P450]	rel. deviation [%]	<i>SPFR_{cor}</i> [U/μmol P450]	<i>rel. deviation</i> from SPFR [%]	SPFR [U/μmol P450]	rel. deviation [%]
pET28a	-0.0072 ± 0.0084	115.8			0.0009 ± 0.0001	12.5
WT	6.99 ± 0.13	1.9	8.18	17.0	187.5 ± 0.5	0.3
F87A	7.93 ± 0.82	10.3	9.09	14.6	443.7 ± 25.0	5.6
M3	2.93 ± 0.29	9.9	3.11	6.1	90.9 ± 6.3	6.9
M3 R471C	2.43 ± 0.03	1.2	2.70	11.1	122.9 ± 15.6	12.7
M3 N543S	1.11 ± 0.30	27.0			55.3 ± 1.7	3.1
M3 DM	0.21 ± 0.09	42.9			5.9 ± 7.0	118.9
M3 DM I401D	1.27 ± 0.09	7.1	1.47	15.7	116.1 ± 3.1	2.7
M3 DM I401M	0.83 ± 0.21	25.3			44.3 ± 1.9	4.2
M3 DM I401V	0.97 ± 0.01	1.03	1.01	4.1	87.9 ± 6.9	7.9
M3 DM nd	0.75 ± 0.20	26.7	0.83	10.7	174.6 ± 31.6	18.1

Table A2: Coupling efficiencies between passed charge and amount of product formed during a certain time interval (which started with application of potential and ended when the absorbance increase at 410 nm approached zero). (*In italics: Coupling efficiencies calculated for absorbance decrease corrected product concentrations.*)

	Cp-nitrophenolate (NADPH) [μM]	Δt [s]	Cp-nitrophenolate (CoSep) [μM]	Q [C]	coupling efficiency [%]
WT	14.9 ± 3.1	574 ± 20	14.4 ± 0.5 (18.7 ± 0.7)	0.694 ± 0.007	0.40 ± 0.01 (0.52 ± 0.01)
F87A	32.9 ± 0.8	515 ± 39	19.6 ± 2.9 (23.6 ± 3.0)	0.648 ± 0.067	0.58 ± 0.03 (0.70 ± 0.02)
M3	27.6 ± 0.9	792 ± 41	8.8 ± 0.04 (9.6 ± 1.0)	0.883 ± 0.033	0.19 ± 0.01 (0.21 ± 0.03)
M3 R471C	28.2 ± 0.7	790 ± 79	7.2 ± 0.7 (8.6 ± 0.8)	0.980 ± 0.098	0.14 ± 0.001 (0.17 ± 0.001)
M3 N543S	19.8 ± 1.8	634 ± 120	2.3 ± 0.3	0.686 ± 0.105	0.07 ± 0.02
M3 DM	0.5 ± 0.5	1664 ± 4	1.5 ± 0.1	1.773 ± 0.144	0.02 ± 0.0003
M3 DM I401D	31.4 ± 0.1	810 ± 59	3.3 ± 0.5 (4.3 ± 0.5)	0.885 ± 0.083	0.07 ± 0.02 (0.10 ± 0.02)
M3 DM I401M	17.0 ± 1.0	494 ± 20	1.7 ± 0.4	0.512 ± 0.028	0.06 ± 0.01
M3 DM I401V	23.8 ± 1.4	751 ± 0	2.1 ± 0.04 (2.3 ± 0.04)	0.787 ± 0.039	0.05 ± 0.002 (0.06 ± 0.002)
M3 DM nd	28.4 ± n.d.	632 ± 0	1.4 ± 0.02 (1.7 ± 0.02)	0.470 ± 0.011	0.06 ± 0.0004 (0.07 ± 0.001)

Table A3: Volume activities and specific product formation rates (SPFR) obtained at different concentrations of P450 BM3 WT and M3 in the electrochemically driven pNCA assay with CoSep. Only for WT at 0.33 μM a decrease in absorbance was seen and SPFR_{cor} was calculated.

C_{P450} [μM]	P450 BM3 WT			P450 BM3 M3		
	volume activity $\times 10^{-3}$ [U]	SPFR (SPFR_{cor}) [U/ μmol P450]	rel. deviation [%]	volume activity $\times 10^{-3}$ [U]	SPFR [U/ μmol P450]	rel. deviation [%]
0.07	0.58 ± 0.06	8.35 ± 0.86	10.3	0.34 ± 0.04	4.86 ± 0.63	13.0
0.13	0.61 ± 0.06	4.72 ± 0.49	10.4	0.26 ± 0.02	2.00 ± 0.16	8.0
0.20	0.64 ± 0.03	3.20 ± 0.15	4.7	0.41 ± 0.03	2.06 ± 0.12	5.8
0.27	0.70 ± 0.04	2.61 ± 0.15	5.7	0.49 ± 0.12	1.80 ± 0.43	23.9
0.33	0.85 ± 0.05	2.58 ± 0.15 (2.94 ± 0.15)	5.8 (5.1)	0.49 ± 0.13	1.48 ± 0.39	26.3

Table A4: Coupling efficiency and product concentration (at point in time when the rate of absorbance increase at 410 nm approached zero) for WT (left) and M3 (right) at different enzyme concentrations. (*In italics: absorbance decrease corrected values.*)

C_{WT} [μM]	coupling efficiency [%]	C_{M3} [μM]	coupling efficiency [%]
0.07	0.06 ± 0.016	0.07	0.06 ± 0.002
0.13	0.07 ± 0.005	0.13	0.05 ± 0.004
0.20	0.14 ± 0.028	0.20	0.05 ± 0.005
0.27	0.15 ± 0.024	0.27	0.05 ± 0.003
0.33	0.17 ± 0.017 (0.21 ± 0.017)	0.33	0.05 ± 0.003

Table A5: Specific product formation rates (SPFR) obtained for WT, M3 and M3 R471C at different CoSep concentrations. Experiments were done in duplicate. SPFR_{cor} was calculated for WT at c_{CoSep} of 5, 10 and 15 mM. In all other cases no absorbance decrease was observed.

C_{CoSep} [mM]	WT			M3		M3 R471C	
	SPFR [U/ μmol P450]	rel. dev. [%]	SPFR_{cor} [U/ μmol P450]	SPFR [U/ μmol P450]	rel. dev. [%]	SPFR [U/ μmol P450]	rel. dev. [%]
0	0.16 ± 0.03	18.8		0.01 ± 0.03	300	0.04 ± 0.00	0
2.5	1.68 ± 0.14	8.3		0.93 ± 0.02	2.2	0.93 ± 0.11	11.8
5	3.20 ± 0.15	4.7	3.51 ± 0.45	2.06 ± 0.12	5.8	1.46 ± 0.03	2.1
7.5	3.81 ± 0.19	5.0		3.48 ± 0.67	19.3	2.14 ± 0.04	1.9
10	5.27 ± 0.33	6.3	5.47 ± 0.57	2.85 ± 0.48	16.8	1.94 ± 0.04	2.1
15	1.83 ± 1.18	64.5	2.35 ± 0.98	0.96 ± 0.31	32.3	1.93 ± 0.09	4.7

Table A6: Coupling efficiencies [%] for WT, M3 and M3 R471C at different CoSep concentrations.

C _{CoSep} [mM]	WT	M3	M3 R471C
0	0.02 ± 0.011	0.01 ± 0.000	0.00 ± 0.002
2.5	0.06 ± 0.003	0.06 ± 0.001	0.05 ± 0.003
5	0.14 ± 0.028 (0.15 ± 0.035)	0.05 ± 0.005	0.05 ± 0.001
7.5	0.12 ± 0.008	0.10 ± 0.002	0.04 ± 0.003
10	0.16 ± 0.001 (0.17 ± 0.012)	0.09 ± 0.001	0.06 ± 0.011
15	0.06 ± 0.029 (0.07 ± 0.031)	0.04 ± 0.007	0.03 ± 0.005

Table A7: Influence of the applied potential on specific product formation rates (SPFR) [U/μmol P450] obtained in the pNCA assay with CoSep at standard conditions (*SPFR_{cor}* is given in italics). For -550 mV and -650 mV in all cases no absorbance decrease was monitored and no SPFR_{cor} was calculated.

E [mV]	WT	F87A	M3	M3 R471C	M3 N543S	M3 DM I401D	M3 DM nd
-550	0.35 ± 0.05	0.28 ± 0.06	0.21	0.16 ± 0.04	0.19 ± 0.01	0.08	0.16 ± 0.01
-650	1.87 ± 0.50	2.47 ± 0.15	0.51 ± 0.04	0.62 ± 0.12	0.38 ± 0.01	0.78 ± 0.02	0.81 ± 0.16
-750	4.47 ± 0.94 (5.10 ± 1.17)	6.56 ± 0.60 (7.55 ± 0.62)	1.46 ± 0.11 (1.57 ± 0.12)	1.30 ± 0.24 (1.48 ± 0.32)	0.94 ± 0.07 (1.06 ± 0.07)	1.18 ± 0.06 (1.25 ± 0.01)	1.82 ± 0.31 (2.02 ± 0.30)
-850	6.76 ± 1.20 (8.37 ± 1.33)	9.05 ± 0.19 (10.92 ± 0.02)	2.27 ± 0.12 (2.59 ± 0.20)	1.95 ± 0.05 (2.28 ± 0.06)	1.61 ± 0.02 (1.89 ± 0.02)	1.88 ± 0.08 (2.12 ± 0.13)	2.01 ± 0.37 (2.29 ± 0.37)
-950	5.17 ± 0.05 (6.68 ± 0.12)	7.81 ± 0.80 (9.79 ± 1.10)	2.18 ± 0.12 (2.45 ± 0.13)	1.81 ± 0.01 (2.02 ± 0.00)	1.44 ± 0.16 (1.71 ± 0.25)	1.47 ± 0.21 (1.77 ± 0.25)	1.59 ± 0.39 (1.92 ± 0.47)

Table A8: Coupling efficiencies between the amount of product formed and the average charge passed during the contemplated time interval. pNCA assay with CoSep was performed with several mutants at different potentials. (Calculations based on absorbance decrease corrected values are indicated in italics)

E [mV]	WT	F87A	M3	M3 R471C	M3 N543S	M3 DM I401D	M3 DM nd
-550	0.11 ± 0.002	0.09 ± 0.015	0.07 ± 0.005	0.05 ± 0.009	0.03 ± 0.006	0.04 ± n.d.	0.04 ± 0.011
-650	0.19 ± 0.018	0.33 ± 0.025	0.08 ± 0.009	0.10 ± 0.004	0.06 ± 0.001	0.08 ± 0.003	0.11 ± 0.004
-750	0.38 ± 0.024 (0.39 ± 0.019)	0.55 ± 0.108 (0.70 ± 0.023)	0.10 ± 0.008 (0.11 ± 0.015)	0.11 ± 0.013 (0.12 ± 0.009)	0.10 ± 0.002 (0.10 ± 0.002)	0.07 ± 0.003 (0.08 ± 0.004)	0.16 ± 0.004 (0.16 ± 0)
-850	0.50 ± 0.037 (0.64 ± 0.010)	0.75 ± 0.149 (0.91 ± 0.161)	0.12 ± 0.013 (0.13 ± 0.014)	0.11 ± 0.009 (0.11 ± 0.004)	0.10 ± 0.002 (0.09 ± 0)	0.10 ± 0.010 (0.09 ± 0.004)	0.14 ± 0.019 (0.12 ± 0.013)
-950	0.31 ± 0.071 (0.41 ± 0.077)	0.57 ± 0.079 (0.68 ± 0.100)	0.08 ± 0.010 (0.10 ± 0.003)	0.08 ± 0.007 (0.08 ± 0.007)	0.08 ± 0.008 (0.08 ± 0.007)	0.08 ± 0.011 (0.10 ± 0.021)	0.10 ± 0.044 (0.11 ± 0.029)

A.2. Data for calculation of coupling efficiencies in the P450 BM3 mutant screening via the eMTP

Enzyme concentration

Cp450 [μM]		Cp-nitrophenolate [μM]	mean [mM]	deviation [mM]	Δt [s]	average I [A]	charge Q [C]	n _{electrons} [μmol]	coupling efficiency via integration [%]	
WT									Mean	Deviation
0.07	I	5.61	5.73	0.12	1556	-0.00094	1.465	15.18	0.07	0.006
	II	5.85			1556	-0.00170	2.645	27.42		
0.13	I	5.89	5.90	0.01	1556	-0.00107	1.665	17.26	0.07	0.005
	II	5.91			1556	-0.00095	1.475	15.28		
0.20	I	7.40	6.82	0.58	908	-0.00092	0.837	8.67	0.14	0.027
	II	6.25			1297	-0.00081	1.047	10.85		
0.27	I	6.12	6.53	0.41	648	-0.00104	0.674	6.98	0.15	0.027
	II	6.95			1167	-0.00089	1.044	10.82		
0.33	I	6.93	6.74	0.19	648	-0.00109	0.706	7.32	0.17	0.027
	II	6.55			778	-0.00105	0.817	8.47		
0.33 _{cor}	I	8.21	8.15	0.06	648	-0.00109	0.706	7.32	0.20	0.029
	II	8.09			778	-0.00105	0.817	8.47		
M3										
0.07	I	4.55	4.90	0.35	1556	-0.00102	1.587	16.45	0.06	0.002
	II	5.26			1556	-0.00101	1.712	17.74		
0.13	I	3.98	3.93	0.05	1556	-0.00105	1.634	16.93	0.05	0.004
	II	3.88			1556	-0.00089	1.385	14.35		
0.20	I	3.91	4.01	0.09	1557	-0.00098	1.524	15.79	0.05	0.005
	II	4.10			1557	-0.00085	1.316	13.64		
0.27	I	4.02	4.46	0.43	1556	-0.00103	1.603	16.61	0.05	0.003
	II	4.89			1556	-0.00113	1.758	18.22		
0.33	I	3.66	4.03	0.37	1556	-0.00090	1.405	14.56	0.05	0.003
	II	4.40			1556	-0.00125	1.945	20.16		

Mediator concentration

C _{CoSep} [mM]		Cp-nitrophenolate [μM]	mean [mM]	deviation [mM]	Δt [s]	average I [A]	charge Q [C]	n _{electrons} [μmol]	coupling efficiency via integration [%]	
WT									Mean	Deviation
0	I	1.61	1.08	0.53	1557	-0.00059	0.919	9.53	0.02	0.012
	II	0.55			1557	-0.00055	0.855	8.86		
2.5	I	4.63	4.50	0.13	1557	-0.00085	1.317	13.65	0.06	0.003
	II	4.37			1557	-0.00089	1.378	14.28		
5	I	7.40	6.82	0.58	908	-0.00092	0.837	8.67	0.14	0.027
	II	6.25			1297	-0.00081	1.047	10.85		
5 _{cor}	I	9.40	7.82	1.58	1038	-0.00092	0.956	9.91		
	II	6.25			1297	-0.00081	1.047	10.85		
7.5	I	5.39	5.49	0.10	908	-0.00106	0.962	9.98	0.12	0.013
	II	5.59			778	-0.00111	0.864	8.95		
10	I	6.75	6.41	0.34	648	-0.00123	0.797	8.26	0.16	0.005
	II	6.06			519	-0.00137	0.711	7.37		
10 _{cor}	I	6.66	6.74	0.08	648	-0.00123	0.797	8.26		
	II	6.82			519	-0.00137	0.711	7.37		
15	I	0.56	2.22	1.66	259	-0.00149	0.386	4.00	0.06	0.030
	II	3.88			648	-0.00133	0.862	8.93		
15 _{cor}	I	2.00	3.28	1.29	648	-0.00149	0.966	10.01		
	II	4.57			648	-0.00133	0.862	8.93		
M3										
0	I	0.02	0.11	0.09	1557	-0.00003	0.052	0.54	0.00	0.002
	II	0.20			1557	-0.00048	0.743	7.70		
2.5	I	3.61	3.55	0.06	1557	-0.00079	1.224	12.69	0.06	0.001
	II	3.49			1557	-0.00078	1.210	12.54		
5	I	3.91	4.01	0.09	1557	-0.00098	1.524	15.79	0.05	0.005
	II	4.10			1557	-0.00085	1.316	13.64		
7.5	I	3.29	3.50	0.21	519	-0.00119	0.618	6.40	0.11	0.003
	II	3.70			519	-0.00139	0.721	7.48		
10	I	3.52	3.94	0.42	648	-0.00121	0.784	8.13	0.09	0.001
	II	4.36			648	-0.00148	0.959	9.94		
15	I	3.96	4.73	0.77	1556	-0.00148	2.303	23.87	0.04	0.007
	II	5.50			1556	-0.00143	2.225	23.06		

M3 R471C										
0	I	0.09	0.15	0.06	1557	-0.00058	0.901	9.33	0.00	0.002
	II	0.21			1557	-0.00053	0.820	8.50		
2.5	I	3.34	3.17	0.17	1557	-0.00081	1.265	13.11	0.05	0.003
	II	3.00			1557	-0.00083	1.285	13.31		
5	I	3.32	3.56	0.24	1557	-0.00089	1.391	14.42	0.05	0.001
	II	3.81			1557	-0.00099	1.542	15.98		
7.5	I	3.34	3.88	0.55	1168	-0.00133	1.553	16.10	0.04	0.003
	II	4.43			1557	-0.00115	1.791	18.56		
10	I	6.88	6.18	0.70	1557	-0.00130	2.024	20.98	0.06	0.011
	II	5.48			1557	-0.00153	2.382	24.69		
15	I	2.44	3.40	0.96	1297	-0.00170	2.205	22.85	0.03	0.005
	II	4.36			1557	-0.00169	2.631	27.27		

Variation of the applied potential

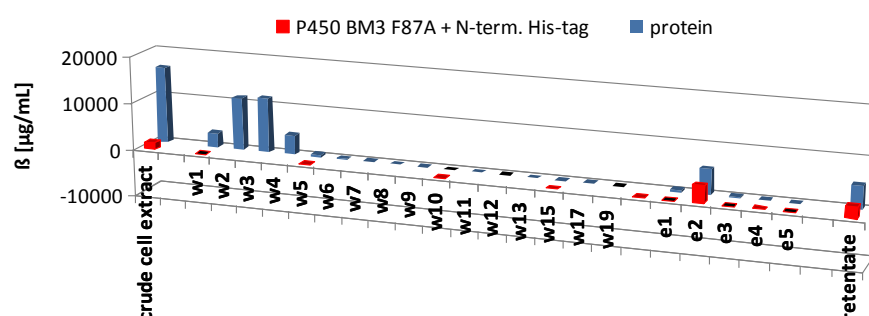
E [mV] vs. Ag/AgCl _{EMTP}		C _p -nitrophenolate [μM]	mean [mM]	deviation [mM]	Δt [s]	average I [A]	charge Q [C]	n _{electrons} [μmol]	coupling efficiency via integration [%]	
WT									Mean	Deviation
-550	I	3.34	2.87	0.46	1662	-0.00036	0.592	6.14	0.11	0.003
	II	2.41			1662	-0.00025	0.411	4.26		
-650	I	10.75	9.74	1.01	1662	-0.00074	1.229	12.74	0.19	0.018
	II	8.73			1662	-0.00050	0.823	8.53		
-750	I	13.15	12.66	0.49	475	-0.00133	0.632	6.55	0.40	0.028
	II	12.17			752	-0.00088	0.663	6.87		
-750 _{cor}	I	16.57	15.87	0.69	594	-0.00133	0.790	8.19	0.37	0.008
	II	15.18			910	-0.00088	0.800	8.29		
-850	I	15.08	15.03	0.05	355	-0.00153	0.543	5.63	0.49	0.030
	II	14.97			553	-0.00113	0.625	6.48		
-850 _{cor}	I	20.43	21.11	0.69	395	-0.00154	0.608	6.30	0.69	0.116
	II	21.80			593	-0.00113	0.670	6.94		
-950	I	7.11	8.86	1.74	316	-0.00179	0.566	5.86	0.31	0.101
	II	10.60			395	-0.00135	0.533	5.53		
-950 _{cor}	I	12.14	13.73	1.59	395	-0.00180	0.711	7.37	0.38	0.102
	II	15.32			475	-0.00129	0.613	6.35		
F87A										
-550	I	2.50	2.67	0.18	1662	-0.00037	0.610	6.32	0.09	0.006
	II	2.85			1662	-0.00031	0.507	5.26		
-650	I	12.11	12.40	0.29	1266	-0.00061	0.776	8.04	0.33	0.027
	II	12.69			1187	-0.00059	0.696	7.21		
-750	I	16.39	16.15	0.24	435	-0.00166	0.722	7.48	0.64	0.040
	II	15.90			475	-0.00099	0.469	4.86		
-750 _{cor}	I	20.58	20.65	0.08	475	-0.00116	0.551	5.71	0.69	0.061
	II	20.73			594	-0.00100	0.593	6.14		
-850	I	14.94	16.90	1.96	355	-0.00135	0.479	4.97	0.69	0.095
	II	18.86			316	-0.00128	0.404	4.19		
-850 _{cor}	I	20.78	23.02	2.25	395	-0.00135	0.533	5.53	0.94	0.112
	II	25.27			355	0.00128	-0.454	4.71		
-950	I	11.20	11.86	0.66	277	-0.00159	0.440	4.56	0.65	0.154
	II	12.52			237	-0.00157	0.372	3.86		
-950 _{cor}	I	15.96	17.34	1.38	356	-0.00150	0.534	5.53	0.70	0.152
	II	18.71			316	-0.00147	0.465	4.81		
M3										
-550	I	-2.73	2.36	n.d.	1652	-0.00046	0.753	7.81	0.06	0.003
	II	2.36			1652	-0.00045	0.750	7.77		
-650	I	3.78	4.06	0.28	1652	-0.00065	1.073	11.12	0.07	0.008
	II	4.34			1652	-0.00059	0.979	10.15		
-750	I	6.12	6.08	0.05	1076	-0.00114	1.227	12.71	0.10	0.007
	II	6.03			961	-0.00107	1.028	10.66		
-750 _{cor}	I	7.15	6.94	0.21	1268	-0.00113	1.433	14.85	0.11	0.013
	II	6.73			961	-0.00107	1.028	10.66		
-850	I	5.91	5.33	0.58	577	-0.00149	0.860	8.91	0.11	0.008
	II	4.75			615	-0.00139	0.855	8.86		
-850 _{cor}	I	8.02	7.02	1.01	730	-0.00149	1.088	11.27	0.13	0.015
	II	6.01			730	-0.00139	1.015	10.52		
-950	I	2.89	2.70	0.19	385	-0.00160	0.616	6.38	0.08	0.015
	II	2.51			423	-0.00162	0.685	7.10		
-950 _{cor}	I	3.58	3.50	0.08	423	-0.00161	0.681	7.06	0.09	0.003
	II	3.42			423	-0.00162	0.685	7.10		

M3 R471C										
-550	I	1.78	1.52	0.27	1652	-0.00037	0.611	6.34	0.05	0.009
	II	1.25			1652	-0.00038	0.629	6.52		
-650	I	5.29	4.77	0.52	1652	-0.00061	1.003	10.40	0.10	0.004
	II	4.25			1652	-0.00053	0.875	9.07		
-750	I	6.85	6.66	0.19	922	-0.00113	1.042	10.80	0.12	0.013
	II	6.47			1307	-0.00095	1.240	12.85		
-750 _{cor}	I	9.09	8.39	0.70	1230	-0.00111	1.365	14.15	0.12	0.008
	II	7.69			1422	-0.00094	1.340	13.89		
-850	I	4.49	4.69	0.20	500	-0.00147	0.735	7.62	0.11	0.004
	II	4.89			730	-0.00128	0.934	9.68		
-850 _{cor}	I	6.15	6.57	0.42	692	-0.00145	1.003	10.40	0.11	0.003
	II	6.99			961	-0.00127	1.220	12.65		
-950	I	2.35	2.66	0.31	385	-0.00162	0.624	6.46	0.08	0.005
	II	2.97			461	-0.00144	0.664	6.88		
-950 _{cor}	I	3.10	3.46	0.36	538	-0.00157	0.845	8.75	0.08	0.005
	II	3.82			615	-0.00143	0.879	9.11		
M3 N543S										
-550	I	1.10	0.95	0.14	1652	-0.00033	0.550	5.70	0.03	0.006
	II	0.81			1652	-0.00036	0.586	6.07		
-650	I	2.82	2.69	0.13	1652	-0.00052	0.858	8.90	0.06	0.001
	II	2.56			1460	-0.00054	0.792	8.21		
-750	I	5.16	5.49	0.33	1076	-0.00094	1.009	10.46	0.10	0.001
	II	5.82			1191	-0.00100	1.191	12.34		
-750 _{cor}	I	6.24	6.61	0.37	1384	-0.00094	1.300	13.47	0.10	0.003
	II	6.98			1384	-0.00100	1.384	14.34		
-850	I	4.62	4.77	0.15	692	-0.00127	0.879	9.11	0.10	0.002
	II	4.92			692	-0.00131	0.907	9.40		
-850 _{cor}	I	6.56	6.77	0.22	1115	-0.00126	1.405	14.56	0.09	0.002
	II	6.99			1153	-0.00130	1.499	15.53		
-950	I	2.48	2.87	0.39	500	-0.00141	0.705	7.31	0.07	0.008
	II	3.26			500	-0.00149	0.745	7.72		
-950 _{cor}	I	3.27	4.07	0.80	577	-0.00141	0.814	8.43	0.09	0.009
	II	4.87			692	-0.00149	1.031	10.69		
M3 DM I401D										
-550	I	0.00	1.08	n.d.	1662	-0.00025	0.423	4.38	0.04	n.d.
	II	1.08			1662	-0.00029	0.488	5.06		
-650	I	2.36	2.34	0.02	910	-0.00062	0.568	5.88	0.08	0.001
	II	2.32			950	-0.00063	0.598	6.20		
-750	I	3.73	3.65	0.08	910	-0.00112	1.019	10.56	0.07	0.001
	II	3.56			791	-0.00113	0.894	9.26		
-750 _{cor}	I	4.59	4.13	0.46	1108	-0.00111	1.230	12.75	0.07	0.001
	II	3.67			791	-0.00113	0.894	9.26		
-850	I	3.67	3.86	0.19	632	-0.00129	0.815	8.45	0.09	0.006
	II	4.05			514	-0.00143	0.735	7.62		
-850 _{cor}	I	4.79	5.15	0.36	830	-0.00128	1.062	11.01	0.09	0.002
	II	5.51			790	-0.00142	1.122	11.63		
-950	I	2.24	2.46	0.22	435	-0.00147	0.639	6.63	0.09	0.012
	II	2.68			316	-0.00183	0.578	5.99		
-950 _{cor}	I	3.29	3.39	0.11	593	-0.00144	0.854	8.85	0.11	0.027
	II	3.50			316	-0.00183	0.578	5.99		
M3 DM nd										
-550	I	0.52	0.77	0.25	1662	-0.00021	0.341	3.53	0.04	0.012
	II	1.02			1662	-0.00023	0.382	3.96		
-650	I	1.48	2.09	0.62	514	-0.00050	0.257	2.67	0.11	0.006
	II	2.71			989	-0.00051	0.508	5.26		
-750	I	3.12	3.55	0.43	396	-0.00099	0.393	4.08	0.16	0.006
	II	3.98			396	-0.00120	0.475	4.93		
-750 _{cor}	I	3.87	4.27	0.40	475	-0.00100	0.473	4.90	0.17	0.009
	II	4.68			475	-0.00120	0.570	5.91		
-850	I	3.34	4.60	1.26	434	-0.00128	0.556	5.76	0.15	0.008
	II	5.86			672	-0.00109	0.732	7.59		
-850 _{cor}	I	5.18	6.49	1.32	790	-0.00124	0.980	10.15	0.12	0.008
	II	7.81			948	-0.00124	1.176	12.18		
-950	I	1.66	2.42	0.76	356	-0.00150	0.534	5.53	0.10	0.026
	II	3.18			356	-0.00116	0.413	4.28		
-950 _{cor}	I	2.37	3.47	1.11	395	-0.00147	0.581	6.02	0.11	0.030
	II	4.58			435	-0.00148	0.644	6.67		

A.3. Protein purification

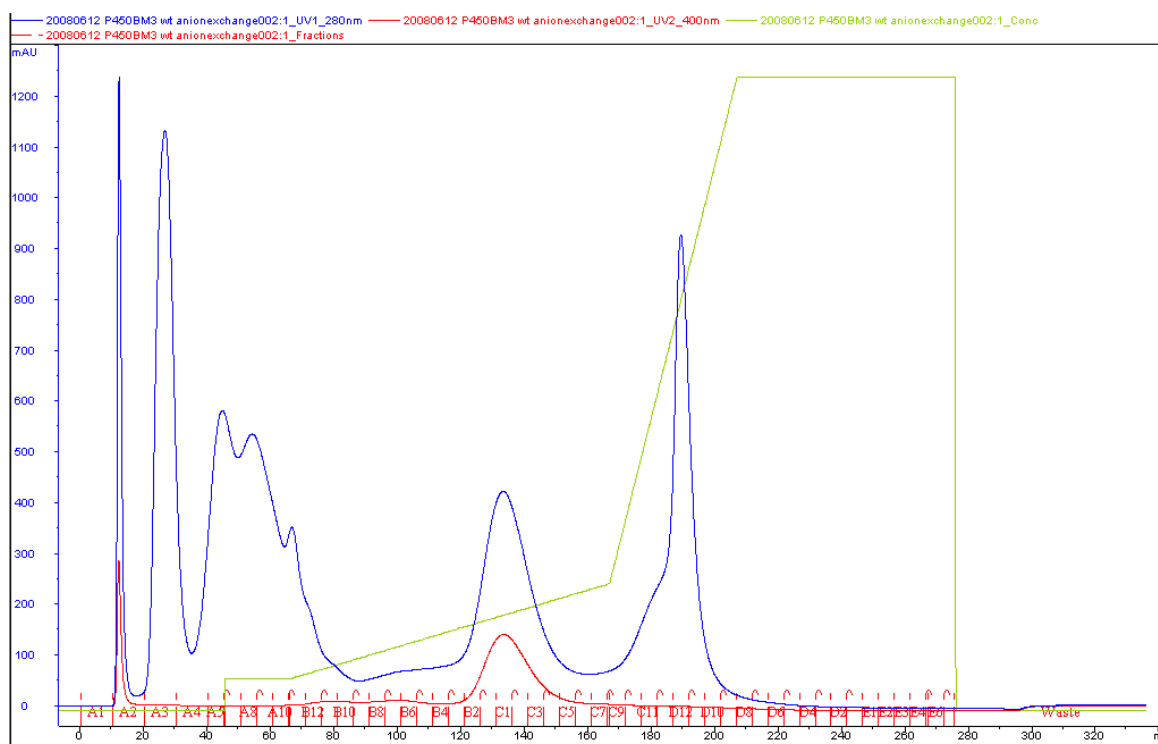
Affinity purification of His-tagged P450 BM3

Figure A1: Documentation of the affinity purification of His-tagged P450 BM3 F87A via a 5 mL HisTrap HP column. The amount of total protein (blue) was determined by the BCA assay and the P450 content (red) was estimated via CO difference spectroscopy. w = wash fractions, e = fractions collected during elution, retentate = after buffer exchange (for imidazole removal).



Ion exchange chromatography

Figure A2: Chromatogram of the purification of P450 BM3 WT via ion exchange chromatography with an Äkta Explorer System (as described in chapter 3.1.2.2.). Fractions B1, C1, C2 and C3 were recombined and used for further work up.



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