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Surface-modified electrodes in the mimicry of oxidative drug metabolism

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ABSTRACT

This review discusses different approaches that have been taken to mimic oxidative drug metabolism as executed by members of the Cytochrome P450 (CYP450) family of enzymes in humans. Non-modified electrodes can be used to produce some of the oxidative drug metabolites observed *in vivo* but their scope is rather limited. Modifying electrodes with simple cofactors in analogy to those observed in CYP450 but without the protein scaffold extends these possibilities and notably allows driving reactions following a CYP450-like mechanism. The review ends with approaches to immobilize CYP450s or analogs thereof on electrodes to mimic the *in vivo* drug metabolism fully. We discuss future perspectives with respect to the advantages and the disadvantages of each level of complexity and possible ways forward.

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1. Introduction

It is a long journey before a new chemical entity enters the market as a pharmaceutical product. An important part of preclinical and clinical studies relates to the study of drug metabolism. A new drug may be converted into pharmacologically active, inactive or even toxic metabolites *in vivo*. The regulatory authorities therefore require studies summarized under the term ADME (absorption, distribution, metabolism and excretion) to assure the safety and the efficacy of newly developed pharmaceuticals [1,2]. Preclinical ADME studies are performed in experimental animals ranging from mice to monkeys prior to use in humans, due to safety considerations. In order to investigate drug metabolism at an early stage of new drug development, it is thus important to predict potential metabolites, to characterize them analytically and to synthesize them to evaluate their toxicity.

In vitro methods for drug metabolism research usually use extracts of rat or human liver microsomes that contain enzymes of the CYP450 family. The discovery of CYP450 enzymes traces back to the early 1960s when a pigmented protein that binds carbon monoxide and exhibits an absorption maximum at 450 nm was discovered in liver microsomes of pigs and rats [3,4]. It was subsequently shown that CYP450s contain an iron protoporphyrin IX (heme) as cofactor. This heme-containing protein was later identified as a *b*-type cytochrome and therefore called Cytochrome P450 [5].

The CYP450 family comprises many isoforms, such as CYP1A2, 2A6, 2B6, 2D6 and 2E1, with different substrate specificities [6], despite the fact that sequence similarity is as high as 80% [7]. CYP450 catalysis requires a constant supply of NADPH as the electron source

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Table 1
Overview of different modified electrodes

Species	Electrodes	Immobilization strategy	Techniques	Substrate catalysis	Ref.
PMMT	Platinum/carbon	Adsorption	CV	Cyclo-octene and stilbene	[40]
Co(*p*-OH)TPP	Vitreous carbon	Electropolymerize	CV	2-mercaptoethanol	[41]
cobalt corrin-polyion	Carbon	Electropolymerize	CV	No	[42]
$FePcCl_{16}$	Graphite	Adsorption	CV, CA	2-mercaptoethanol	[43]
CYP2C9	Gold	Thiophenol SAM	CV, HPLC	Tolbutamide	[49]
CYP2C9	Gold	Organic acid SAM	CV, HPLC	Warfarin	[50]
CYP2E1	Gold	Nanoparticles	EIS, TEM, CV	Rifampicin	[51]
CYP3A4	Gold	Organic acid SAM	QCM, LC-MS	Verapamil, quinidine	[52]
CYP2E1	Gold	Cysteamine SAM	CV	*P*-nitrophenol	[53]
CYP3A4	Gold	Thiolate SAM	CV, HPLC	Testosterone	[54]
CYP2C9/CYP2D6	Gold	SAM	CV, HPLC	Bufuralol and warfarin	[56]
CYP3A4	Glassy carbon	Nanoparticles	CV, HPLC	Nifedipine	[57]
CYP2B4	Screen-printed carbon	Amine SAM	CV, CA	Cocaine	[59]
Fungal monooxygenase	Glassy carbon	Mwcnt-nf-pei	AFM, CV	*N*-hexadecane	[60]
CYP6A1	EPG	Ddab film	CV, MS	Aldrin or heptachlor	[61]
CYP1A2	Carbon cloth	Sulfonate film	CV, GC	Styrene	[62]
CYP3A4	ITO	Electrostatic	CV	Testosterone	[63]
CYP2B4	Glassy carbon	adsorption	CV	Aminopyrine, benzphetamine	[65]

and CYP450 reductase to deliver electrons to the active site, both of which are costly and require continual addition upon extended incubations [8]. Additionally, it can be very difficult to detect drug metabolites or to isolate and to study reactive intermediates from incubations with microsomes, due to the complexity of the biological sample. Reactive intermediates may also react further, for example, with proteins, and escape detection. Thus, new approaches capable of generating oxidative drug metabolites and allowing their analysis are needed to gain better understanding of oxidative drug metabolism (Phase-I metabolism) and of synthesizing drug metabolites at a scale that allows toxicological studies in experimental animals (see review on "Electrosynthesis methods and approaches for the preparative production of metabolites from parent drugs" in this Special Issue).

Electrochemistry has been used as a versatile technique for the mimicry of oxidative drug metabolism [9–24], since it allows generating drug metabolites and reactive intermediates under controlled conditions, avoiding the complexity of the *in vitro* or *in vivo* systems [25]. Direct electrochemistry on metal or carbon electrodes proved to be a powerful tool for the synthesis of a wide range of metabolites due to N-dealkylation, N-oxidation, S-oxidation or P-oxidation reactions [26]. However, a number of reactions that are catalyzed by CYP450s could not be reproduced on non-modified electrodes, including aliphatic hydroxylation. Moreover, electrochemistry on metal-based or carbon-based electrodes produces these metabolites through mechanisms that do not resemble CYP450-mediated reaction pathways, making them unsuitable to mimic and to study such reactions in greater mechanistic detail.

In order to mimic the natural enzyme system more closely, approaches to immobilize CYP450s on different electrodes have been promoted [27–29], including different bare electrodes, clay-modified electrodes, phospholipid-modified electrodes and electrodes modified with multilayer films, in which the electrode serves as the supplier of electrons to drive the CYP450 catalytic cycle and thus to expand the range of reactions leading to oxidative drug metabolites. Immobilizing redox-active metalloporphyrins or CYP450 enzymes on electrodes has been shown to facilitate their use as catalysts in electrochemical cells, due to a fast, direct, reversible electron transfer between the redox center and the electrode surface [30]. Different strategies to immobilize CYP450 were reported [31], for example, adsorption to bare electrodes or thin films, layer-by-layer adsorption, encapsulation in polymers of gels, and covalent attachment to a self-assembled monolayer (SAM). However, the active redox site in CYP450s is deeply embedded in the interior of the enzyme, which insulates it and impedes electron transfer from the electrode, resulting in a diminished biocatalytic activity of directly immobilized enzymes on bare electrodes [32]. Regioselective immobilization of CYP450s on SAM-modified electrodes was shown to be beneficial in this respect (Table 1).

In this review, we summarize the applications of metalloporphyrin and CYP450 modified electrodes in the field of drug metabolism research, and introduce the reader to different strategies for the preparation of such modified electrodes.

2. Metalloporphyrin-modified electrodes

Due to the importance and the versatility of the porphyrin macrocycle and its metalated complexes in nature, considerable efforts have been devoted to understanding, mimicking and expanding the role of metalloporphyrins [33]. The past two decades witnessed considerable progress in biomimetic applications of synthetic metalloporphyrins in, among others, olefin epoxidation [34,35], alkane hydroxylation [36] and the electrocatalytic reduction of O_2 [37,38]. As iron is located in the active center of CYP450s, iron complexes are the most widely studied metalloporphyrins, although other metal ions, such as manganese, ruthenium and cobalt, have also been investigated. One of the shortcomings of immobilized metalloporphyrins in view of mimicking drug metabolism is their poor regioselectivity, for example, with respect to aromatic hydroxylation reactions.

Mimicking the catalytic reaction mechanism of CYP450s with immobilized metalloporphyrins is a challenge. Reduction of the central metal ion in the presence of O_2 to produce an oxo-iron intermediate or H_2O_2 has been reported [39]. For example, a manganese porphyrin (Fig. 1a) polymer film deposited on a platinum or carbon electrode surface by electropolymerization of a pyrrole-monosubstituted manganese tetraphenylporphyrin (PMMT) was designed [40], where the pyrrole was covalently linked to the phenyl groups. However, steric hindrance limited the efficiency of the electrochemical polymerization process of the metalloporphyrin monomer. Cauquis et al. reported that connecting the polymerizable group to the macrocycle through a flexible chain improved the electropolymerization efficiency. Platinum or carbon electrodes coated with electropolymerized pyrrole-substituted manganese tetraphenylporphyrin films were used for the catalytic epoxidation of cyclo-octene and stilbene with molecular oxygen.

An electropolymerized cobalt porphyrin-modified vitreous carbon electrode was reported by Bedioui et al. to catalyze the oxidation of 2-mercaptoethanol [41]. Cobalt (*p*-tetrakishydroxyphenyl) porphyrin (Co(*p*-OH)TPP, Fig. 1b) was immobilized by repeated potential scans between −1.4 and 1.4 V [*versus* saturated calomel electrode

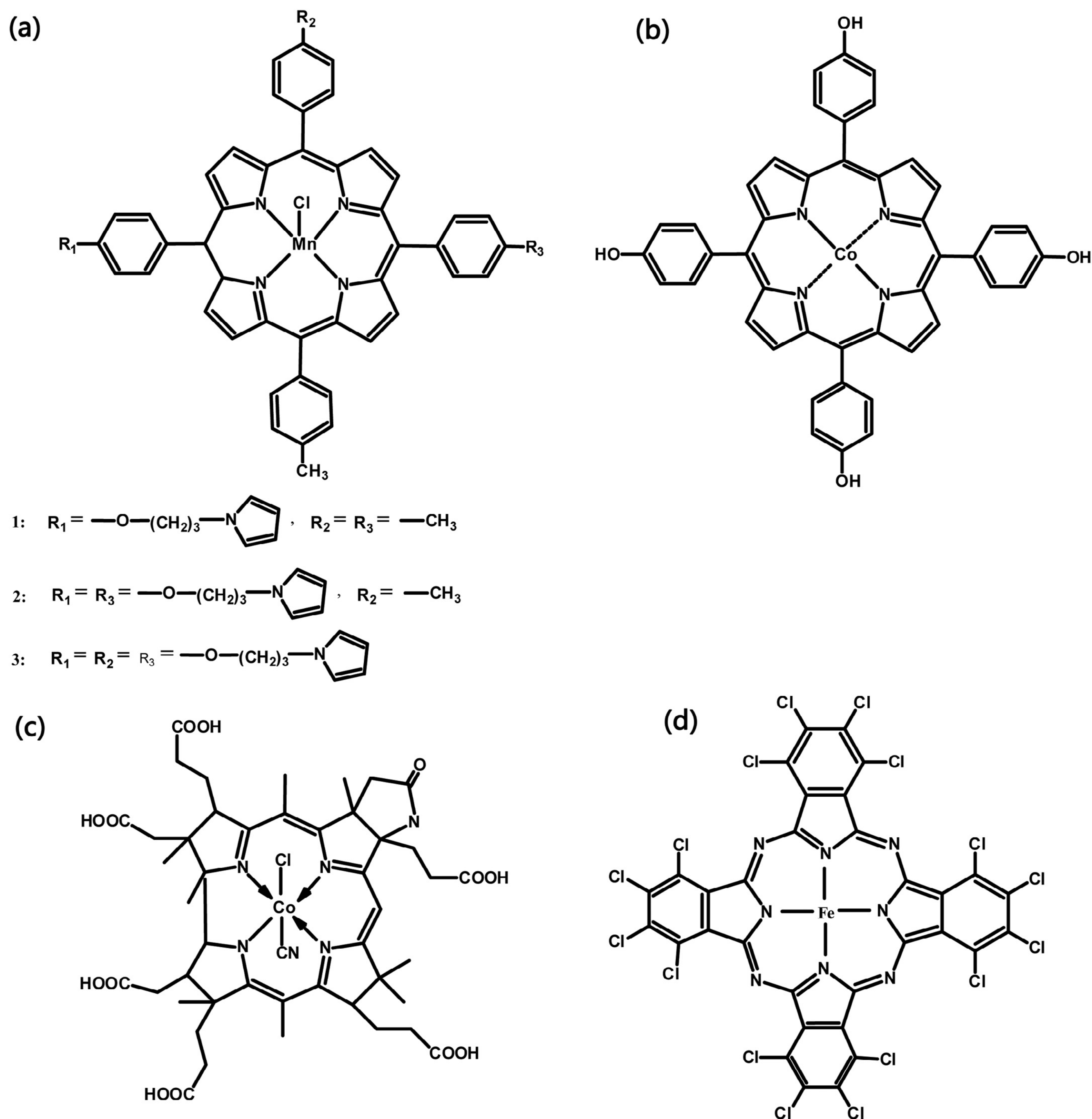

Fig. 1. Chemical structures of different metalloporphyrins discussed in this review: (a) pyrrole-monosubstituted manganese tetraphenylporphyrin [40]; (b) cobalt (*p*-tetrakishydroxyphenyl) porphyrin (Co(*p*-OH)TPP) [41]; (c) cobalt corrin vitamin B12 hexacarboxylic acid [42]; and, (d) iron perchlorinated phthalocyanine ($FePcCl_{16}$) [43].

(SCE)] in an acetonitrile solution containing 1 mM Co(*p*-OH)TPP. Surface coverage of the porphyrin on the electrode was calculated to be 4×10^{-10} mol/cm^2, and the formal potential was −1.01 V (*versus* SCE), corresponding to the redox couple of $Co^{2+/1+}$. An enhanced oxidation current was observed in the presence of 2 mM 2-mercaptoethanol, while no oxidation of 2-mercaptoethanol was observed on the non-modified electrode, indicating electrocatalytic oxidation of 2-mercaptoethanol. While the products of the oxidized 2-mercaptoethanol were not identified, this is the first report of porphyrin-based modified electrodes applied to the electrocatalytic activation of thiols.

The thickness of the metalloporphyrin films on the electrode was found to play a key role in the catalytic activity of the modified electrode. Rusling's group reported a cobalt corrin vitamin B12 hexacarboxylic acid (Fig. 1c)-modified carbon electrode [42], which was immobilized by covalent amide bonding to a poly-L-lysine (PLL) layer on a carboxyl-group-functionalized carbon electrode. An enhanced current was observed at the forward Co^{2+} reduction

potential of –0.75 V (*versus* SCE) in the presence of trans-1,2-dibromocyclohexane, while only a minimal current was observed for the oxidation of Co^{+} to Co^{2+}, indicating a characteristic electrochemical catalytic reduction. Optimal conversion was obtained at a vitamin B12 hexacarboxylic acid coverage of 2 nmol/cm^2. Electron and reactant mass transport within the films became limiting factors above the optimal coverage, while kinetic control became predominant below the optimal coverage.

Iron perchlorinated phthalocyanine ($FePcCl_{16}$, Fig. 1d) adsorbed on a graphite electrode displayed good electrocatalytic activity for the reduction of O_2 [43]. Its suitability for the electrooxidation of 2-mercaptoethanol was further evaluated by adsorption of a drop of 0.1 mM $FePcCl_{16}$ in dimethylformamide on a pyrolitic graphite disk for 30 min [44]. The electrode showed two pairs of characteristic redox peaks centered at ~–0.1 V and ~–0.3 V (*versus* SCE), respectively, with a calculated surface coverage of 0.31 nmol/cm^2. The current peaks increased linearly with the square root of the potential scan rate indicating a mass-transport controlled process. In the presence of 3 mM 2-mercaptoethanol, a peak at –0.8 V (*versus* SCE) was observed, which was attributed to the reduction of the disulfide bond formed during the positive scan. Such an electrode is also applicable to the detection of other thiols, such as aminoethanethiol, cysteine and glutathione, which is of great importance in deodorizing reactions, such as the treatment of wastewater.

3. CYP450-modified electrodes

As one of the most versatile enzyme families in nature, CYP450s utilize molecular oxygen and two reducing equivalents of NADPH to catalyze a great variety of stereospecific and regioselective oxygen-insertion reactions [45]. Such processes are of great significance in biosystems, where the enzyme participates in detoxification and biodegradation. In humans, CYP450s are the most important Phase-I drug-metabolizing enzymes, contributing about 75% to the metabolism of all drugs [46]. The active species of the enzyme is the iron protoporphyrin IX heme cofactor, which has two axial ligands. The proximal ligand is a thiolate from a cysteine residue of the protein, while the distal ligand is variable and can be a substrate molecule.

Electrochemistry has attracted much attention in recent years for driving redox-enzyme-mediated catalytic processes. However, some obstacles still exist for efficient electron transfer between heme-containing enzymes and the electrode surface. First, it is difficult to transfer electrons between the surface of electrodes and the active site because of the long distance between the electroactive center of the heme-containing enzyme and the electrode surface [47]. Second, loss of electrochemical activity and bioactivity often occurs when heme-containing enzymes are immobilized on an electrode due to adsorptive denaturation and an inappropriate orientation [48]. One strategy to immobilize heme-containing enzymes is to incorporate them into modified films on the electrode surface. In this section, we discuss different strategies based on the material of the working electrode.

3.1. Gold electrodes

A nanostructured gold surface prepared by sputtering was modified with 4-aminothiophenol to make a self-assembly monolayer (SAM) and CYP2C9 was covalently immobilized through reaction of a carboxylic-acid residue in the enzyme and amino groups on the SAM [49]. A pair of voltammetric peaks was observed with a formal potential of –0.399 V (*versus* Ag/AgCl) corresponding to direct electron transfer between CYP2C9 and the electrode. This CYP-modified electrode was applied to the electrochemical analysis of drug-metabolism reactions using tolbutamide as the substrate. At the formal potential of the enzyme, an increase in current that was observed correlated with an increase in drug concentration, demonstrating the electrochemically-driven drug-metabolism reaction by CYP2C9. For comparison, the electrochemical responses of CYP2C9 were also evaluated using two other linkers, p-hydroxythiophenol and p-carboxythiophenol, but no electron transfer was observed, demonstrating that these two linkers are unsuitable for direct electron transfer to the active site of CYP2C9.

In another approach, CYP2C9 was immobilized on an 11-mercaptoundecanoic acid-modified SAM by coupling the N-terminus of the enzyme to the carboxylic-acid group on a gold electrode [50]. Direct electrochemistry of the covalently-coupled CYP2C9 to the SAM-modified electrode was observed by cyclic voltammetry (CV) with a formal potential of –0.45 V (*versus* Ag/AgCl). CV measurements of the modified electrodes displayed an increase in the reduction current at –0.49 V in the presence of the known CYP2C9 substrate warfarin (Fig. 2), which depended on the warfarin concentration. To assess the electrochemical catalytic activity of the immobilized CYP2C9 further, warfarin was incubated with the modified electrode at a constant potential of –0.38 V under aerobic conditions. The typical metabolite 7-hydroxywarfarin was observed by high-performance liquid chromatography (HPLC) after 2 h of electrolysis, which is comparable to a control experiment where CYP2C9 was incubated with NADPH and CYP reductase.

Nanoparticles have been used to fabricate modified electrodes. An ethylene glycol bis(succinic acid N-hydroxysuccinimide ester) (EG) CYP2E1-modified gold electrode was designed as a biosensor for the determination of rifampicin [51]. The gold electrode was first modified with a polyvinylpyrrolidone-modified silver nanoparticle/poly(8-anilino-1-naphthalene sulfonic acid) nanocomposite followed by electrodeposition of EG-CYP2E1 at a potential of 700 mV (*versus* Ag/AgCl) for 10 min. The assembly procedure was followed by electrochemical impedance spectroscopy (EIS) comparing the charge-transfer resistance after each assembly step. The modified electrode was used for the analysis of rifampicin in 50 mL phosphate buffer (0.1 M, pH 7.4) with a detection limit of 50 nM.

Joseph et al. reported a CYP3A4-modified gold electrode by first coating the gold surface with a monolayer of 3-mercapto-1-propenesulfonic acid, and then alternate adsorption from poly(diallyldimethylammonium chloride) and CYP3A4 solution for multiple times [52]. A formal potential of 98 mV [*versus* normal hydrogen electrode (NHE)] was observed on the modified electrode, which was attributed to the heme $Fe^{3+/2+}$ couple in CYP3A4. A quartz-crystal microbalance (QCM) was used for monitoring the formation of the enzyme films on the electrode by measuring changes in the

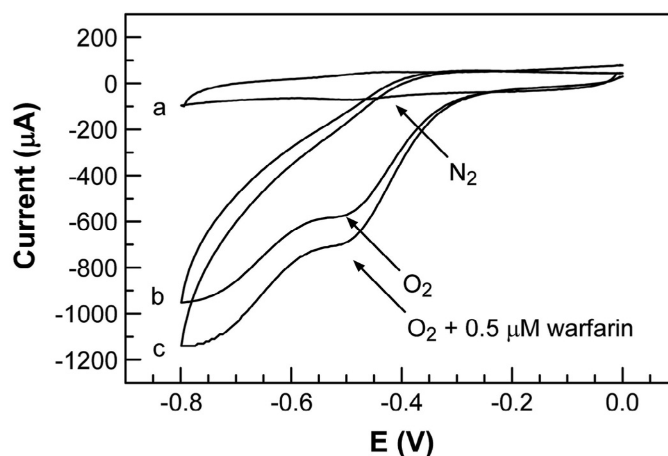

Fig. 2. Cyclic voltammograms of a CYP2C9-modified gold electrode in 40 mM phosphate buffered saline containing 154 mM NaCl. (a) In the absence of warfarin under anaerobic condition. (b) In the absence of warfarin under aerobic condition. (c) In the presence of 0.5 μM warfarin under aerobic condition [50].

resonance frequency of the electrode after each modification step. A linear change of the resonance frequency with cycles of adsorption on the electrode was observed, indicating a linear increase of film mass with increasing number of cycles. This modified electrode was used for the catalytic conversion of three classic substrates of CYP3A4, namely verapamil, quinidine and progesterone. A 2-h electrolysis experiment was carried out in the presence of substrate under aerobic conditions. Subsequent product analysis by LC-MS displayed the same metabolites as for CYP3A4 *in vivo*, confirming the catalytic activity of the modified electrode.

Direct electrochemistry of CYP enzymes adsorbed on bare electrodes was generally thought to be difficult due to the instability of the enzymes and the deeply buried heme cofactor. Non-specific adsorption of enzymes results in a randomly oriented layer on the electrode, which is difficult to control. However, when the enzyme binds to the electrode covalently through a thiol linker, an oriented, compact monolayer covalently bound to the gold surface is obtained. Gilardi's group reported the electrochemical investigation of different CYP2E1-modified electrodes by adsorption and covalent linking [53]. Electron transfer on a CYP2E1-modified gold electrode (formal potential –177 mV *versus* NHE), where the enzyme was covalently bound to a cysteamine monolayer, was twice as fast after adsorption on a glassy-carbon electrode (GCE), probably due to the well-controlled orientation. Conversion of *p*-nitrophenol into *p*-nitrocatechol was achieved at a potential of –300 mV, indicating electrocatalytic activity of the modified electrode.

Hydrophobicity of the SAM is one of the critical parameters in the fabrication of electrocatalytic CYP-modified gold electrodes. Mie's group thoroughly investigated the influence of hydrophobicity by preparing different hydrophobic and hydrophilic thin layers [54]. A well-defined redox peak was observed on a naphthalenethiolate hydrophobic SAM with a formal potential of –0.4 V (*versus* Ag/AgCl), while no voltammetric peaks were observed on an aminoethanethiolate hydrophilic SAM. Interestingly, another hydrophobic SAM based on ethanethiolate displayed redox peaks that were only 10% of that on the naphthalenethiolate SAM, although both have similar surface hydrophobicities. This demonstrates that aromatic hydrophobic layers are more effective for direct electron transfer, because unsaturated oligophenylene thiolates show better electronic conductance than saturated alkanethiolates [55]. Testosterone was used as a substrate of CYP3A4 to study the electrocatalytic activity of the modified electrode, and the main metabolite 6β-hydroxytestosterone was detected by HPLC.

A CYP450-based amperometric platform was proposed by Gilardi et al. for measuring the individually variable, polymorphic response in Phase-I drug metabolism depending on drug therapy [56]. Since no catalytically active protein was observed after direct immobilization of CYP450 enzymes on the electrode surface, chemical spacers were used to form a SAM by covalently binding to the gold electrode on one side, while the other side bound the protein (Fig. 3). Two isoforms of CYP450 with relevance for polymorphism in drug metabolism, CYP2C9 and CYP2D6, were covalently linked to a sputtered

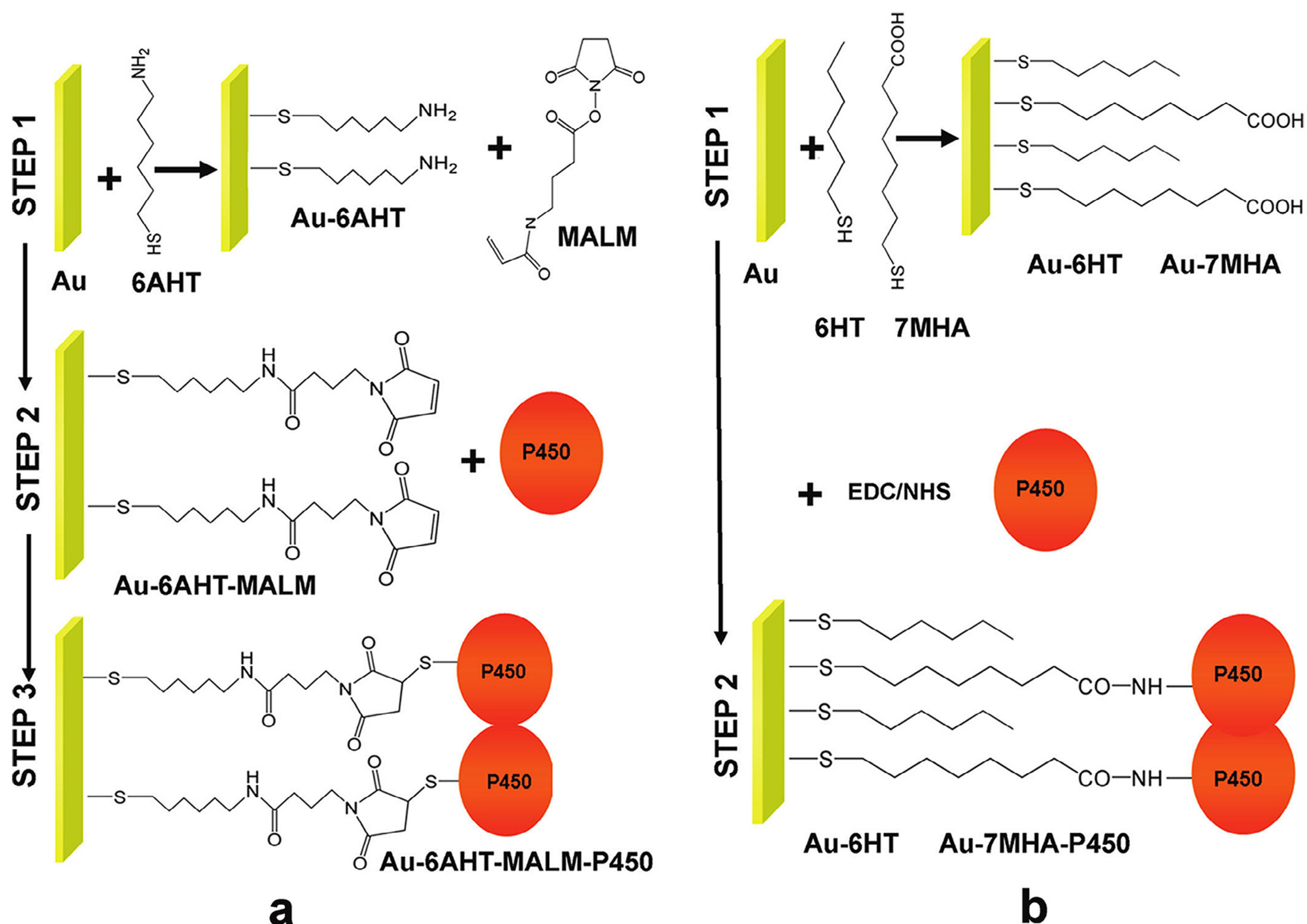

Fig. 3. Two strategies for the immobilization of CYP2D6 on a gold electrode via SH groups (left side) and via NH_2 groups (right side) [56].

polycrystalline gold working electrode surface, as depicted in Fig. 3. The electrochemical response of the enzyme electrodes was verified by CV. Redox couples corresponding to the $Fe^{3+/2+}$ transition of the heme cofactor were observed on all enzyme-modified electrodes under anaerobic conditions. Electrocatalysis was observed at a constant potential of –0.21 V over 30 min in the presence of the substrates bufuralol and warfarin, resulting in the generation of 1-hydroxy bufuralol and 7-hydroxy warfarin, as determined by HPLC analysis, indicating the catalytic activity of the enzyme electrodes.

3.2. Carbon-based electrodes

A new platform for rapid *in vitro* mimicking of CYP450 metabolic pathways on a nanoparticle-modified GCE was reported by Liu et al. [57]. The electrode was first covered with poly(diallyldimethylammonium chloride)-functionalized graphene containing gold nanoparticles to increase the surface area and the conductivity of the electrode. CYP3A4, which is positively charged, can thus be electrostatically bound to the negatively charged surface of the modified electrode. A couple of reversible redox peaks was observed with a formal potential of –0.482 V (*versus* SCE). Nifedipine was used as a CYP3A4 substrate, and predominant metabolite dehydronifedipine was observed by HPLC-MS, when a constant potential of –0.5 V (*versus* SCE) was applied for 1 h. The Michaelis-Menten constant K_M was measured to be 1.3 μM, which is 75 times lower than the values reported before [58], indicating a high electrocatalytic activity.

Arcos-Martinez et al. reported a screen-printed carbon electrode and used it for cocaine analysis [59]. The electrode was first incubated in a 4-nitrobenzenediazonium tetrafluoroborate solution to form an amine-functionalized monolayer after sweeping the potential between –0.4 V and 0.8 V (*versus* Ag/AgCl), and then incubated with CYP2B4, *N*-hydroxysuccinimide (NHS) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC). EDC was used to activate the carboxylic acid groups of CYP2B4 and the resulting NHS esters reacted with amine groups on the electrode surface by covalent formation of amide bonds. Cocaine was selected as one of the preferred substrates of CYP2B4 to study its electrocatalytic activity. Street samples of cocaine were analyzed by chronoamperometry (CA) on the modified electrodes and the results were in agreement with those obtained by HPLC analysis.

Fungal (*Aspergillus terreus MTCC 6324*) CYP450 monooxygenase, isolated from an *A. terreus* culture grown in the presence of *n*-hexadecane, has been immobilized on a multi-walled carbon nanotube-Nafion-polyethyleneimine (MWCNT-NF-PEI) modified GCE [60]. To prepare the modified electrode, a suspension of MWCNTs and NF was layered on the surface of a GCE, followed by incubation with the CYP450 solution overnight and the addition of PEI. Atomic force microscopy (AFM) was employed for characterization of the surface morphology. A formal potential of –0.53 V (*versus* Ag/AgCl) was observed, which was attributed to the $Fe^{3+/2+}$ couple in CYP450. In the presence of CYP450 substrate *n*-hexadecane, a positive shift in the redox potential to –0.475 V was observed, and the current response increased linearly with the concentration of *n*-hexadecane, demonstrating electrocatalytic activity.

A CYP6A1-modified electrode was prepared by immobilizing the enzyme on a dioctadecyl dimethylammonium bromide (DDAB) film on an edge-plane pyrolytic graphite (EPG) electrode for aldrin metabolism research [61]. Voltammetry showed that the CYP6A1-modified electrode displayed a pair of redox peaks with a formal potential of –0.36 V (*versus* Ag/AgCl). An oxygen-reduction peak current was observed on the modified electrode in the presence of CYP6A1 substrates aldrin or heptachlor. Electrolysis was performed at –0.45V for 1 h, and samples were collected and analyzed by MS. Epoxidized products were detected, indicating that the immobilized CYP6A1 on the electrode displayed comparable catalytic activity to the enzyme *in vitro*.

A carbon-cloth electrode was used to construct a CYP1A2-modified electrode after prior adsorption of polystyrene sulfonate by repeated, alternating incubation with sodium poly-(styrene-sulfonate) and the protein solution [62]. A reduction-oxidation peak pair with a formal potential of –0.31 V (*versus* SCE) was observed under anaerobic conditions, corresponding to the reversible $Fe^{3+/2+}$ redox pair. After sparging oxygen through the buffer, the reduction peak increased, while the oxidation peak disappeared, indicating the expected electrochemical catalysis behavior of CYP1A2. To measure the catalytic activity of the modified electrode, a constant potential of –0.6 V (*versus* SCE) was applied in the presence of styrene, and product styrene oxide was detected by gas chromatography (GC), demonstrating electrocatalytic activity. The turnover rates of CYP1A2 in solution and on the modified electrode were comparable.

3.3. Other types of electrode

Direct electron transfer (DET) of adsorbed CYP450 with most non-modified electrode materials proved to be difficult. However, DET on indium-tin oxide (ITO) electrodes was reported to be possible. Niwa's group observed direct electron transfer on a CYP450-modified polycrystalline ITO electrode by electrostatic interaction without any surface modification [63]. At neutral conditions, the surface of the ITO is negatively charged while CYP3A4 is positively charged. Two different surface nanostructures of ITO films were used in this study, both polycrystalline and amorphous. The electron-transfer rate of oxygen reduction on polycrystalline ITO was 15 times greater than that of the amorphous film due to the larger surface area. A clear reduction peak at –0.52 V was detected (Fig. 4), which was due to the reduction of oxygen. In the presence of CYP inhibitor ketoconazole, the oxygen-reduction current disappeared, while, in the presence of CYP3A4 substrate testosterone, this reduction current increased 3.7-fold, demonstrating catalytic activity of CYP3A4 on the ITO. Comparable results were obtained on an ITO electrode to which liver CYP3A4 microsomes were adsorbed.

As an alternative, Niwa's group evaluated carbon nanomaterials, including nanofibers, nanotubes and carbon black, as electrode materials for CYP3A4 inhibitor screening [64]. The unique properties of high conductivity, large surface area and sufficient edge planes make carbon nanofibers the preferred material. Due to their simple preparation, such modified electrodes have potential for the evaluation of CYP activity in drug-metabolism research.

Clay material, such as montmorillonite, is widely used to modify electrodes, since it easily forms films on conductive material after dropping of a colloidal clay suspension on the electrode surface. Wollenberger reported a CYP2B4-modified GCE by incubating the electrode with a mixture of clay colloid and a CYP2B4 solution [65]. In the presence of 0.33% of non-ionic detergent Tween 80, CYP2B4 is in its monomeric state, and can thus be reduced faster on the modified electrode. A reversible redox peak was observed with a formal potential of –0.298 V (*versus* Ag/AgCl). The electrocatalytic performance was investigated for 1 h by constant potential electrolysis at –0.5 V in the presence of typical substrates, such as aminopyrine and benzphetamine. When incubating with methyrapone, an inhibitor of CYP2B4, the reaction was totally suppressed, indicating that substrate conversion was due to electrocatalytic turnover.

4. Conclusions and perspectives

Electrochemistry is a powerful tool for *in vitro* drug-metabolism research. A number of Phase-I metabolites of common, marketed drugs can be produced by direct electrochemistry. However, direct electrochemistry shows limitations when it comes to some specific reactions, such as regioselective aromatic and aliphatic

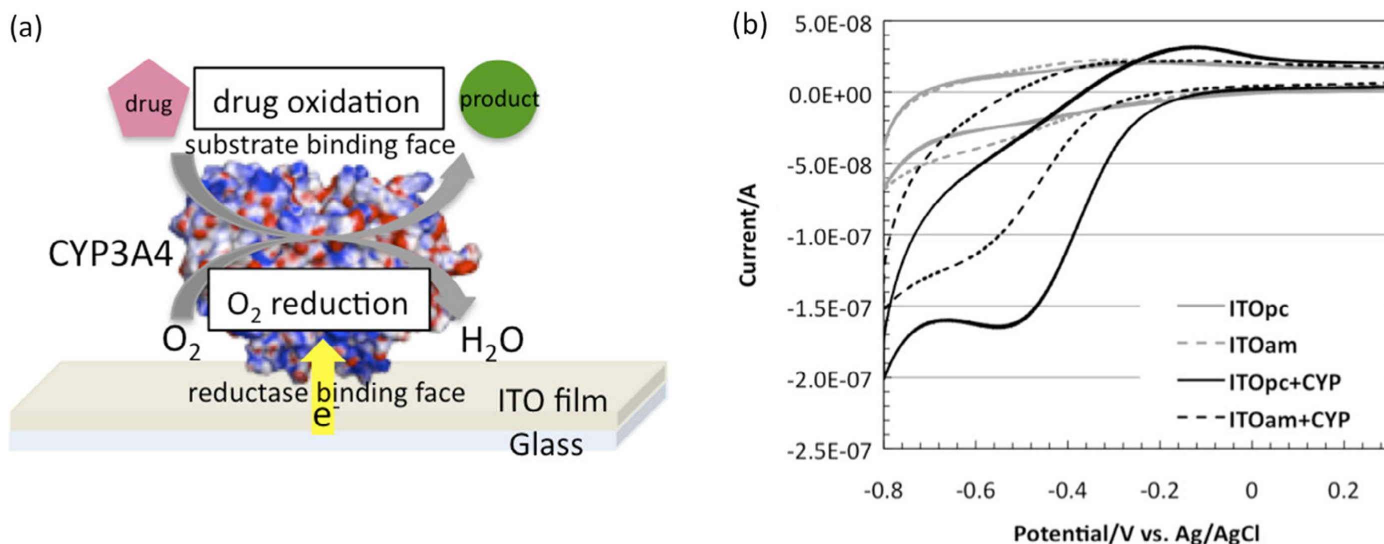

Fig. 4. (a) CYP450 redox reactions on an ITO electrode. (b) Cyclic voltammograms of CYP3A4 adsorbed on bare ITO electrodes (pc: polycrystalline; am: amorphous) in 50 mM Tris-HCl buffer (pH 7.4) at a scan rate of 20 mV/s [63].

hydroxylations. To address this issue, surface-modified electrodes have been proposed to extend the range of electrochemically-driven reactions and as a new tool for more extensive drug-metabolism studies. Notably, the mimicry of CYP450-mediated reactions has greatly benefitted from surface-modified electrodes.

Adsorbing CYP450 directly on metal-based or carbon-based electrodes proved to be unsuccessful in most cases, since catalytic activity was lost, probably because conformational changes of the enzyme on the electrode surface are sterically hindered. Anchoring CYP450 through a linker to the electrode surface provides more conformational flexibility, so that the substrate can access the active site of the enzyme. Modified electrodes have been characterized by a number of analytical techniques. Electrochemical methods, such as CV, QCM and EIS offer quantitative information about the surface layer, while AFM and SEM give insights into surface morphology. Other analytical methods have been used to follow drug conversion, such as HPLC and MS.

Modified electrodes cannot mimic the complexity of all CYP450-mediated reactions taking place *in vivo*. Hence, there is no doubt that modified electrodes will not replace existing *in vivo* and *in vitro* methods. But, with the modified electrodes as electron supplier, they may serve to generate metabolites in sufficient quantities for further studies, obviating the use of NADPH and CYP reductase in complex biological matrices. Notably, few studies report conversion yields or measures of robustness of the modified electrodes, so there is a need for further development to make this promising technique suitable for routine use and for the generation of sufficient quantities of drug metabolites for preclinical toxicology or activity studies. While most of the reported work focuses on specific CYP450 enzymes and their respective preferred substrates, there are other drug-metabolizing enzymes of interest, such as the FAD-dependent monooxygenases. Studying this class of enzymes provides room for future investigations.

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