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Electrochemistry in the mimicry of oxidative drug metabolism

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Chapter 1
General Introduction*

Prediction of oxidative drug metabolism at the early stages of drug discovery and development requires fast and accurate analytical techniques to mimic the in vivo oxidation reactions by Cytochrome P450s (CYP). Direct electrochemical oxidation combined with mass spectrometry, although limited to the oxidation reactions initiated by charge transfer, has shown promise in the mimicry of certain CYP-mediated metabolic reactions. The electrochemical approach may further be utilized in an automated manner in microfluidics devices facilitating fast screening of oxidative drug metabolism. A wide range of in vivo oxidation reactions, particularly those initiated by hydrogen atom transfer, can be imitated through the electrochemically-assisted Fenton reaction. This reaction is based on homolytic activation of hydrogen peroxide and autoxidation by hydroxyl radicals, wherein electrochemistry is used for the reduction of molecular oxygen to hydrogen peroxide, as well as the reduction of Fe^{3+} to Fe^{2+}. Metalloporphyrins, as surrogates for the prosthetic group in CYP, utilizing metallo-oxo reactive species, can also be used in combination with electrochemistry. Electrochemical reduction of metalloporphyrins in solution or immobilized on the electrode surface activates molecular oxygen in an analogous manner as the catalytical cycle of CYP and different metalloporphyrins can mimic selective oxidation reactions. Chemoselective, stereoselective, and regioselective oxidation reactions may be mimicked using electrodes that have been modified with immobilized enzymes, especially CYP itself. This review summarizes the recent attempts in utilizing electrochemistry as a versatile analytical and preparative technique in the mimicry of oxidative drug metabolism by CYP.

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1.1 Introduction

Drug compounds may be converted in the body to therapeutically active or toxic metabolites. Study of oxidative drug metabolism is conventionally performed in animal models (*in vivo*) or perfused organs (*in vitro*). However, the increasing number of drug candidates and the critical role of metabolism in drug evaluation have raised the interest in the study of drug metabolism in the earlier stages of new drug developments. Therefore, there is a tangible need for the development of new analytical techniques capable of fast assessment and mimicry of oxidative drug metabolism [1].

1.1.1 Cytochrome P450s (CYP)

The firmly held view that oxygen in drug metabolism is derived from water, was discarded when the early isotope studies by Hayaishi in the 1950s pointed out that the oxygen source in *in vivo* oxidation is molecular oxygen [2, 3]. Since the triplet electronic structure of molecular oxygen prevents its direct reaction with mainly singlet organic compounds, the *in vivo* oxidation requires catalytic activation of molecular oxygen [4]. The main enzyme responsible for this activation reaction was first isolated from liver microsomes, and has a characteristic UV-visible spectroscopic signature at 450 nm when its reduced form is exposed to carbon monoxide. Therefore, it was named Cytochrome P450 (abbreviated as CYP) [5]. The most widely studied CYP, i.e. CYP101 or P450cam (a bacterial CYP), named for its camphor monooxygenation reaction, was the first CYP isolated in sufficient quantities for characterization by high resolution X-ray crystallography [6]. The CYP101 structure shown in **Figure 1** has a prosthetic group – iron protoporphyrin (heme) – that is anchored through coordination with the sulfur atom of the cysteine-357 residue. The prosthetic heme group is bracketed between two protein helices and lies close to the enzyme surface, although no part of it is directly exposed to bulk solvent. The substrate (e.g. a drug molecule) interacts with the hydrophobic active-site pocket, and the release of an active-site water molecule provides the thermodynamic driving force for substrate binding. The substrate-protein interactions aid in controlling stereospecificity of the oxidation reaction. Detailed reviews on the functional structure of CYPs can be found in the literature [2, 6, 7].

Our current knowledge about the catalytic activation of molecular oxygen by CYPs and about the consequent drug metabolism is mainly derived from direct observation of the intermediates through various spectroscopic techniques [8-10], the use of diagnostic substrates with mechanistically revealing rearrangements including radical clocks [11], and synthetic metalloporphyrins [12]. Advanced computational methods based on hybrid QM/MM (quantum mechanical/molecular

mechanical) models, that simulate heme by QM and protein by MM models, have enabled the study of active species in their native protein environmnent [13].

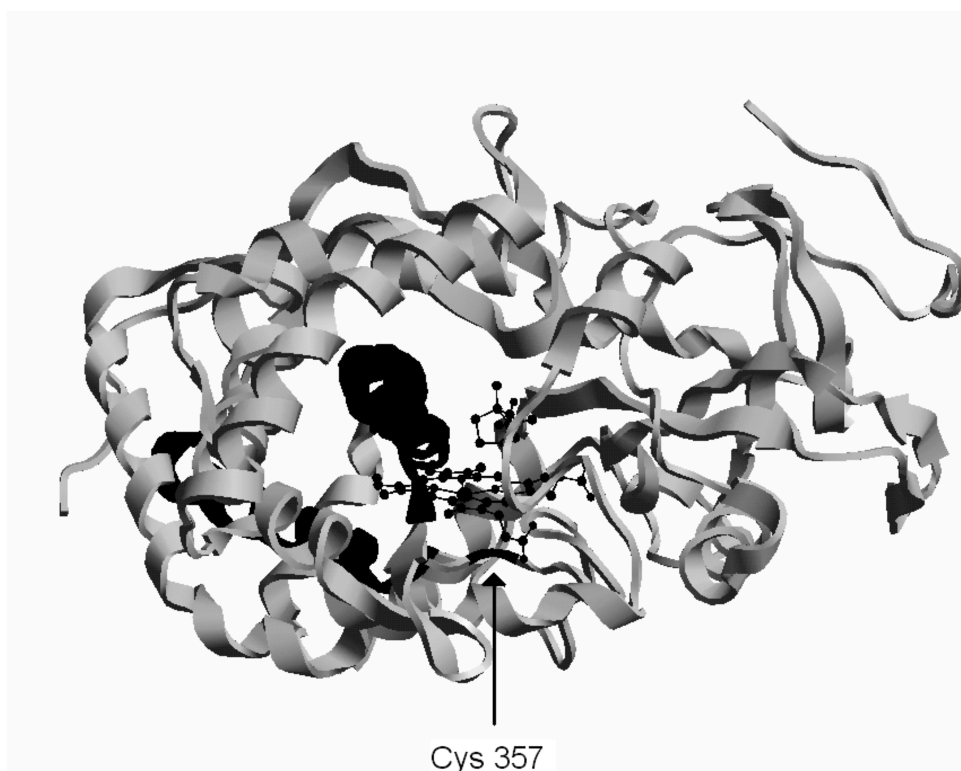


Figure 1. A schematic representation of CYP101 (P450 Camphor, PDB entry 2CPP). The prosthetic group iron protoporphyrin (heme) is bracketed between the helices I and L (dark). The heme is anchored through coordination with sulfur in the cysteine-357 residue (arrow). The subtrate, camphor, is shown in black above the heme [6].

CYPs activate molecular oxygen and transfer a single oxygen atom to a substrate while the other oxygen atom ends up in water [14]. These oxygen transfer reactions include: aromatic/aliphatic hydroxylations, olefin epoxidation, and heteroatom oxidations (nitrogen and sulfur) [15]. In addition, CYPs can promote other forms of oxidative transformations, for instance heteroatom-dealkylation, dehalogenation, and dehydrogenation. The widely accepted catalytic activation mechanism of molecular oxygen by CYPs is shown in **Figure 2** [13]. The hexa-coordinated Fe^{III}-porphyrin complex (**1**) in the resting state has the distal ligand

position occupied by water. Binding of a substrate molecule to the hydrophobic active-site pocket initiates the reaction by displacement of the water ligand leaving a penta-coordinated Fe^{III}-porphyrin (**2**). This complex is a slightly better electron acceptor than the resting state form and is reduced by the CYP reductase protein (CPR) to Fe^{II}-porphyrin (**3**). Coordination with molecular oxygen then generates oxy-Fe^{II}-porphyrin (**4**) which is a good electron acceptor. A subsequent reduction by CPR results in the Fe^{II}-peroxo species (**5**), which is a good base and is protonated easily to form an Fe^{II}-hydroperoxo species (**6**), also known as Compound 0. The second protonation on the proximal oxygen atom activates the O-O bond, and the splitting-off of a water molecule generates the high-valent Fe^{IV}-oxo radical cation species (**7**), known as Compound I, that can transfer an oxygen atom to the substrate. After oxidation, the substrate usually becomes more hydrophilic and leaves the hydrophobic pocket, water molecules re-enter and the resting state is restored. The details of the selective oxygen transfer reaction have, however, remained elusive. The large k_H/k_D kinetic isotope effect indicates a hydrogen abstraction reaction, and the large rearrangement of the fast radical clocks suggests the formation of a radical-in-cage at the active site [16]. The reactive species that are involved in CYP-mediated oxidations are presumably not limited to Compound I, and the nucleophilic and/or electrophilic reactions by Fe^{II}-(hydro)peroxo species (**5** and **6** in Figure 2) should also be considered [17, 18]. For detailed information about CYP-mediated oxidations in drug metabolism the reader is referred to the review articles by Guengerich [19, 20].

1.1.2 Instrumentation

In direct electrochemical oxidations, the electrode behaves as an oxidant which can be tuned by the applied potential in order to perform charge transfer reactions. An overview of the electrochemical reactions within the realm of organic chemistry can be found in the book Organic Electrochemistry [21]. In addition, there is a recent special issue of Chemical Reviews reviewing different aspects of electrochemistry in molecular and biomolecular reactions [22].

The first record of electrochemistry (EC) coupled with mass spectrometry (MS) was a study by Bruckenstein and Gadde on the detection of volatile intermediates generated during reactions on a porous platinum electrode of which one side contacted the solution while the other side contacted the vacuum inlet in the mass spectrometer [23]. Later, the development of thermospray ionization (TSP) allowed the direct introduction of oxidized samples whereby the solvent was forced by pressure from the working electrode into the heated capillary tube of the thermospray source [24, 25]. However, after the development of electrospray ionization (ESI), this became the ionization method of choice for analyzing nonvolatile, polar, and thermally labile compounds [26]. ESI-MS was used for the study of on-line linear sweep voltammetry [27]. A more practically suitable thin-layer, flow-through three-electrode cell was developed by Van Berkel and colleagues, with an Ag/AgCl reference electrode, and the working and counter electrodes separated by a spacing gasket [28, 29]. The same group demonstrated

the study of oxidation reactions by controlling the intrinsic electrochemistry of the electrospray ion source [30]. Electrochemistry can be coupled to MS through other ionization techniques, including atmospheric pressure chemical ionization (APCI), fast-atom bombardment (FAB), and particle-beam ionization (PB) [31].

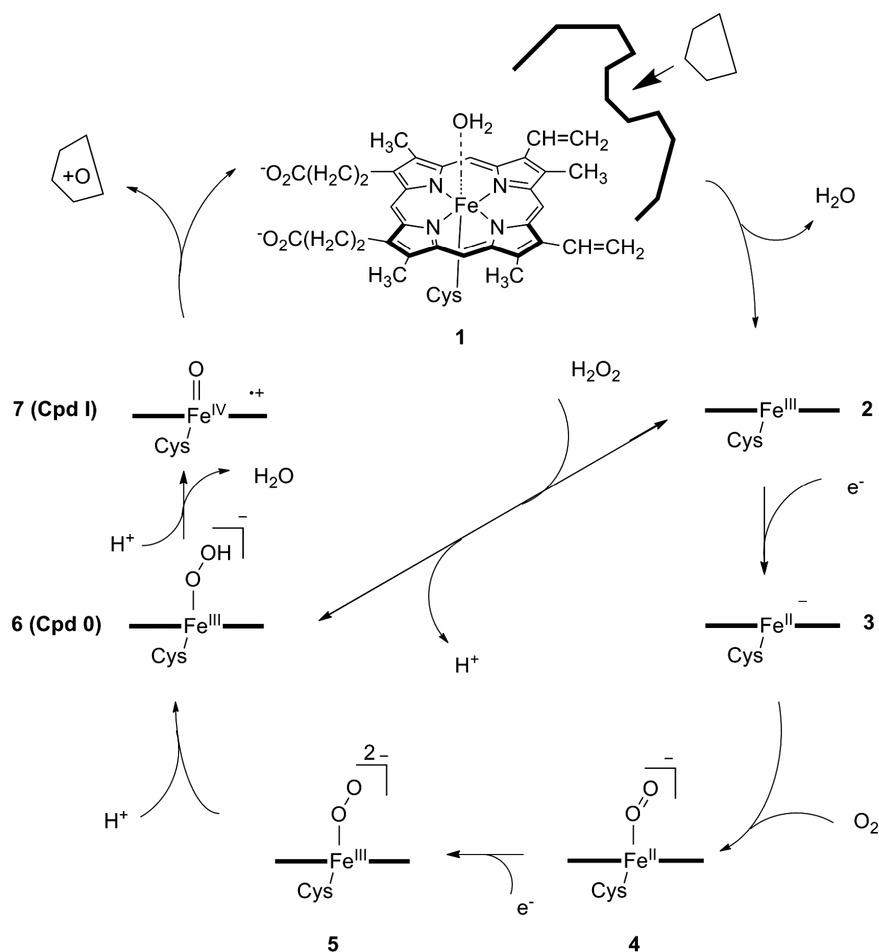


Figure 2. Schematic representation of the catalytic cycle of Cytochrome P450. The heme is depicted by two bold horizontal lines, and the cysteinate proximal ligand is abbreviated as Cys. The oxidation state of the iron center and the overall number of charges are shown by roman and arabic numbering, respectively [13]. The substrate shown here is a general cartoon presentation of a substrate.

The technical aspects of drug metabolism simulation in off-line EC batch reactors and on-line EC/(LC)/MS systems with different ionization techniques have been recently reviewed by Lohmann and Karst [32]. The various electrochemical cells used in online EC/(LC)/MS, including coulometric, amperometric, and in-source electrochemical cells, have been reviewed separately by Baumann and Karst [33]. Electrochemical cells that can be used for direct coupling to MS are commercially available from several companies, Thin-layer electrochemical cells with exchangeable working electrodes of different materials, including platinum, gold, silver, glassy carbon, and boron-doped diamond (BDD), are available from ESA-Dionex (Chelmsford, MA USA), Antec Leyden (Zoeterwoude, The Netherlands), and BASi (West Lafayette, Indiana, USA). In general, direct electrochemical oxidation applications (see section 2) are performed with these electrodes, but BDD can also be used to generate hydroxyl radicals which may produce additional oxidative drug metabolites (see section 3). Coulometric porous flow-through cells of glassy carbon and platinum (available from ESA-Dionex) provide a much large surface area, hence higher conversion rates, than thin-layer cells. The disadvantage is that the porous cells cannot be disassembled and cleaned thoroughly by surface polishing. The reference electrode in the described electrochemical cells is usually a palladium pseudoreference electrode, but in some types an Ag/AgCl reference electrode is used which offers more reliable potential measurements.

The important role of CYPs in phase-I drug metabolism has raised much interest in the development of fast and accurate analytical techniques to study their action during the early stages of drug discovery and development. Previously, we have reviewed the use of direct electrochemical oxidation in the mimicry of oxidative drug metabolism as well as proteomics [34]. The present review provides an overview of recent developments in the utilization of various electrochemical approaches (some combined with mass spectrometry) in the generation and analysis of oxidative drug metabolites, including direct electrochemistry, electrochemical generation of reactive oxygen species, Gif-chemistry, and metalloporphyrins and enzymes immobilized on electrodes.

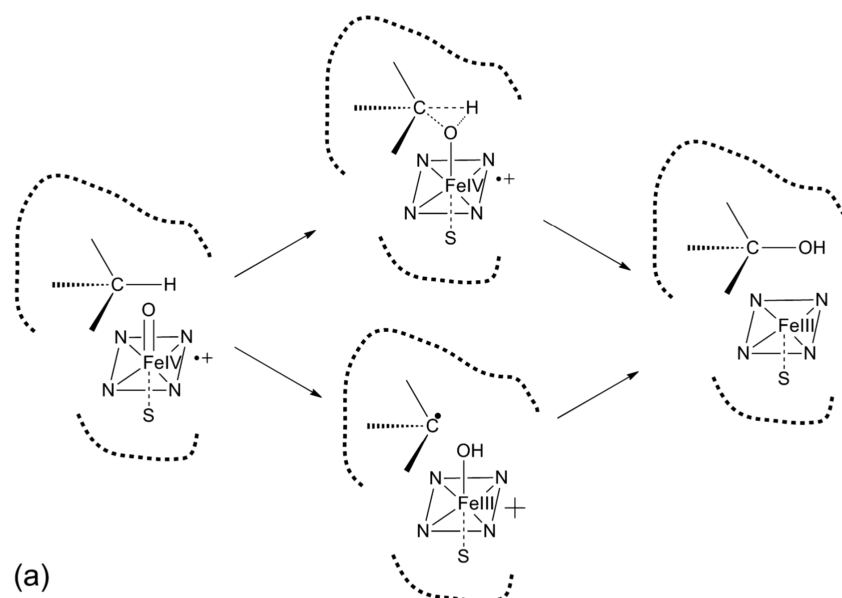
1.2 Direct electrochemical oxidation

Direct electrochemical oxidation is the most straightforward method to imitate drug metabolism. In 1981 Shone *et al*. were the first to utilize direct electrochemical oxidation successfully in the imitation of biotransformation and preparation of drug metabolites, for the N-dealkylated metabolites of lisuride, diazepam, methysergide, and imipramine [35]. In a systematic study by Bruins and colleagues, reactions such as the N-dealkylation of lidocaine were readily performed electrochemically, whereas some reactions failed, for example the O-dealkylation of 7-ethoxycoumarin [36]. They eventually concluded that direct electrochemical oxidation provides oxidative drug metabolites only when initiated by one or more one-electron oxidations, and only if they do not require potentials higher than the

oxidation potential of the solvent (usually water) [37]. In addition to N-dealkylation, biotransformations that could be imitated by direct electrochemical oxidation included S-oxidation, P-oxidation, alcohol oxidation and dehydrogenation [37]. In contrast, oxidative metabolites resulting from hydrogen atom abstraction, such as O-dealkylation and hydroxylation of the nonactivated aromatic rings, cannot be imitated by direct electrochemistry [37]. Although direct electrochemical oxidation is limited to the oxidation reactions initiated by single electron transfer, it has received considerable attention due to its simplicity and the potential for preparative synthesis of drug metabolites for further structural analysis, for instance by NMR [38]. Furthermore, interest in EC-MS has increased due to the possibility of using direct electrochemical oxidation in miniaturized devices such as microfluidics chips [39, 40], and its ability to detect unstable, reactive, and volatile intermediates of the oxidation products by direct coupling with mass spectrometry [41]. In the next sections we specifically discuss the mechanisms of different classes of CYP oxidation reactions in relation to analogous reactions performed with direct electrochemistry.

1.2.1 Aromatic and aliphatic hydroxylation

One of the outstanding oxidative capabilities of CYP is the hydroxylation of saturated aliphatic hydrocarbons. This kind of oxidation in synthetic chemistry can only be done under vigorous oxidative conditions. As illustrated in **Figure 3-a**, there are two mechanisms that have been proposed to explain aliphatic hydroxylation by Compound I, namely a radical-in-cage mechanism (oxygen rebound) and a concerted mechanism (oxygen insertion). According to the first mechanism the reaction occurs through an initial hydrogen atom abstraction followed by oxygen rebound that proceeds through a radical-in-cage intermediate, while the oxygen insertion mechanism does not postulate a radical intermediate [42]. Early studies found retention of stereochemistry and therefore favoured the oxygen insertion mechanism. In contrast, the loss of stereochemistry observed during the oxidation of fast radical clocks suggested the generation of radical intermediates. The large D/H kinetic isotope effect also suggested a reaction involving hydrogen atom abstraction. Compound I might not be the only electrophilic oxidant in aliphatic hydroxylation reactions, since some studies have found evidence for involvement of iron-hydroperoxo species [17, 43]. Since the C-H bond in aromatic compounds is stronger than in aliphatic ones, *in vivo* aromatic hydroxylation does not necessarily obey the same mechanism as aliphatic hydroxylation. The widely accepted mechanism for aromatic hydroxylation is an oxygen insertion mechanism, the so-called NIH (National Institutes of Health) mechanism, that proceeds through an arene oxide intermediate **(Figure 3-b)**. The small D/H kinetic isotope effect supports the NIH mechanism for aromatic hydroxylation. The shifted-deuterium effect observed for aromatic hydroxylation could also be explained by a nonconcerted addition of Compound I, that does not involve an arene intermediate, as shown in **Figure 3-b** [15].



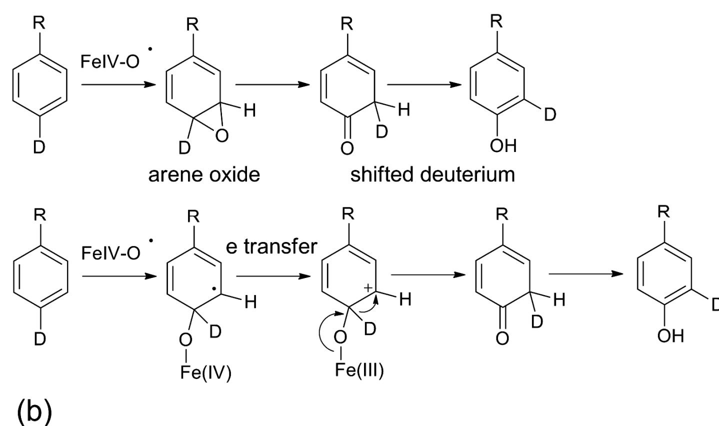


Figure 3. (a) Aliphatic hydroxylation by CYP through oxygen rebound and oxygen insertion mechanisms; (b) Aromatic hydroxylation through the NIH mechanism (top), and through the nonconcerted oxygen addition mechanism (bottom), adapted from Meunier *et al* [15].

Direct electrochemical oxidation has been used widely to perform hydroxylation of organic compounds. At positive electrochemical potentials it is possible, in principle, to abstract electrons from any chemical bond, but electron transfer from

saturated aliphatic hydrocarbons requires such a high positive potential that any solvent would usually be oxidized. For instance, Jurva *et al.* showed that the CYP4A11-catalyzed 12-hydroxylation of lauric acid could not be imitated electrochemically at positive potentials of up to 1.5 V versus Pd/H_2 in aqueous acetonitrile with acetic acid [37]. However, n-alkane oxidation in water-free acetonitrile with Et_4NBF_4 as electrolyte can be achieved at a peak potential of 3.5 V versus Ag/Ag^+ [21].

Electrochemical oxidation of aromatic rings is initiated by electron abstraction. This electron transfer requires a lower positive potential than for aliphatic hydrocarbons due to resonance stabilization of the aromatic radical cations. The electron transfer potential from an aromatic ring that is activated by electron-donating substituents is even less positive. For instance, the oxidation potential for benzene compounds with one to six methyl substituents decreased from 1.93 to 1.20 V versus Ag/Ag^+ [44]. The presence of electron-donating groups on the aromatic ring stabilizes a positive charge or a radical electron localized in the ortho and para positions. In a comparative study by Johansson *et al.* [45] aromatic hydroxylation of the dopamine agonist N-0437 was observed with direct electrochemical oxidation as it has a strong electron-donating group in the aromatic ring. In contrast, aromatic hydroxylation of mephenytoin was only achieved by an EC-Fenton reaction (see section 3.2) and metalloporphyrin in solution (see part 4.1). The absence of a strong electron-donating group in mephenytoin prevents aromatic hyrdoxylation using direct electrochemical oxidation. Hydroxylation requires the presence of water or hydroxyl anions to allow the anodic substitution reaction [46]. Since the oxidation products may be oxidized further at lower potentials than the starting compound, overoxidation is unavoidable. For the dopamine agonist N-0923, oxidation started at potentials as low as 200 mV versus Pd/H_2. The phenol moiety is oxidized readily to a catechol or p-hydroquinone, but these are oxidized further to quinone, as shown in **Figure 4** [36]. Therefore, despite some mechanistic resemblance of electrochemical oxidation to CYP-mediated reactions, the radical intermediate generated after initial oxidation will react further in various ways as determined by the ring substituents [37]. In another example, aromatic hydroxylation of clozapine at 400 mV versus Pd/H_2 was observed to be followed by substitution reactions [47]. A complicated mechanism of oxidation of tetrazepam has been shown to proceed through consecutive hydroxylation and dehydration steps that result in a wide range of allylic and aromatic hydroxylations products [48]. Solvent conditions, such as proton donor availability, also affect hydroxylation reactions. Benzylic hydoxylation imitated by direct electrochemistry was shown for metoprolol at a higher yield in acidic than basic conditions [45].

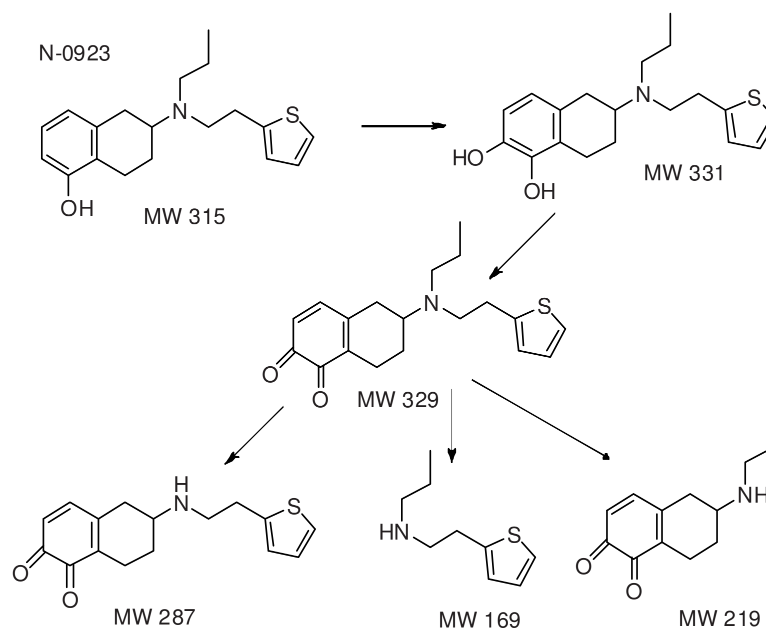


Figure 4. Proposed electrochemical oxidation pathway of the dopamine agonist N-0923 [36].

A great advantage of direct electrochemical oxidation compared with oxidation by enzymes, namely the ability to separate and isolate reactions in different on-line compartments, has been highlighted in work of the Karst group. Coupling of a second electrochemical cell under reductive conditions has been used in the imitation and generation of metabolites from the oxidative metabolism, particularly for hydroxylation and dehydrogenation reactions (sections 2.3). Post EC cell addition of reagents such as glutathione produce the same covalent adducts as formed *in vivo* by phase II drug metabolism (conjugation reactions), in an integrated on-line system [49-52].

Polycyclic aromatic compounds such as naphthalene and anthracene can be oxidized electrochemically, due to the low oxidation potential of polycyclic compounds, they are converted to the corresponding radical cations, which may react further with solvent components to generate hydroxylation products. These hydroxylation products can be detected by using negative-ion mode APCI, as they can be deprotonated efficiently [53].

1.2.2 Heteroatom dealkylation and oxidation

For *in vivo* N-dealkylation by CYP two competing mechanisms are proposed, one initiated by the hydroxylation of the alpha-carbon (hydrogen atom transfer, HAT), and the other by a one-electron oxidation of the heteroatom itself (single electron transfer, SET), as depicted in **Figure 5-a**. The HAT mechanism is initiated by transfer of the alpha-carbon hydrogen to Compound I, and proceeds by oxygen rebound to a carbinol intermediate that after intramolecular rearrangement results in N-dealkylation. The SET mechanism is initiated by electron transfer from the heteroatom to Compound I which generates a radical cation intermediate that, after a second electron transfer and deprotonation reactions leads to an iminium intermediate (**Figure 5-a**). Reaction with hydroperoxo-iron gives the carbinol intermediate, which then follows the same reaction as for the HAT mechanism [15]. The SET mechanism is more widely accepted, since it is supported by the observation of a small D/H kinetic isotope effect which suggests the absence of a hydrogen transfer reaction in the kinetically rate determining step [15]. In contrast, O-dealkylation is considered to proceed via the HAT mechanism and hydroxylation on the alpha-carbon [15]. *In vivo* N-oxidation may either occur via the concerted oxygen atom transfer from Compound I to the amine, or it could be initiated by electron transfer and N-O bond formation, as shown in **Figure 5-b** [15]. S-oxidation is also considered to be a result of concerted oxygen atom transfer from Compound I [54].

Electrochemistry has been used extensively in the imitation of the N-dealkylation metabolism of aliphatic amines, tertiary amides and cyclic tertiary allylamines by CYP [55-57]. The electrochemical reaction of the tertiary amine group of lidocaine [36], shown in **Figure 6**, proceeds through one-electron transfer to generate an imine intermediate that, after hydrolysis and intramolecular rearrangement, gives the N-dealkylation product [58]. The electrochemical mechanism resembles to a large extent the SET mechanism by CYP. Electrochemical N-demethylation of N,N-dimethylamides has shown small D/H kinetic isotope effects indicating the same direct electron transfer mechanism for aliphatic amines and amides [55]. N-dealkylation of other drug compounds with tertiary amine groups such as zotepine [59], and clozapine [47] as well as secondary amines such as metoprolol [45] has been performed successfully.

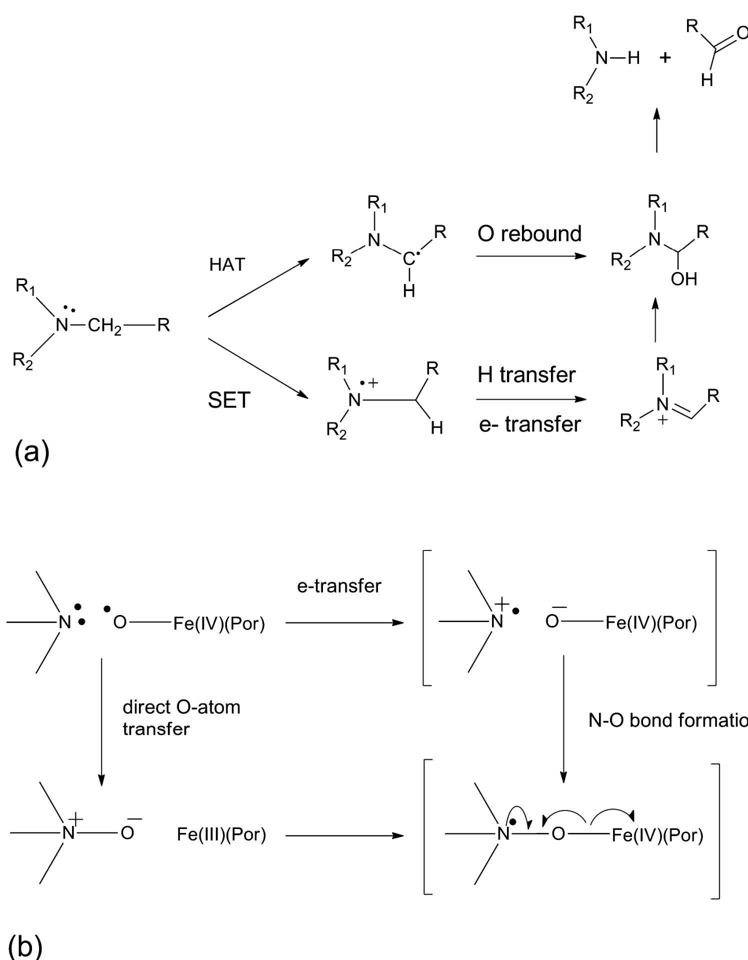


Figure 5. (a) Proposed *in vivo* N-dealkylation mechanism by CYP proceeding via two pathways intiated by hydrogen atom transfer (HAT) and single electron transfer (SET), respectively; (b) Proposed *in vivo* N-oxidation mechanism by CYP resulting from direct oxygen transfer, or via initial electron transfer, followed by N-O bond formation [15]. Por indicates heme porphyrin.

Competition between different oxidation reactions are often an issue, for instance for clozapine and N-0923, where the application of higher positive potentials diverts the major oxidation reaction pathway from ring hydroxylation to N-dealkylation [36, 53]. In a recent study by Jurva and colleagues, the addition of KCN during electrochemical oxidation completely blocked the N-dealkylation of haloperidol, whereas in liver microsomes experiment the reaction was not

impaired. This suggests that an exocyclic iminium intermediate (which is reactive toward KCN) may not be formed in the CYP-catalyzed reactions, again highlighting a different mechanism for electrochemistry and CYP reactions, in this case for N-dealkylation [60].

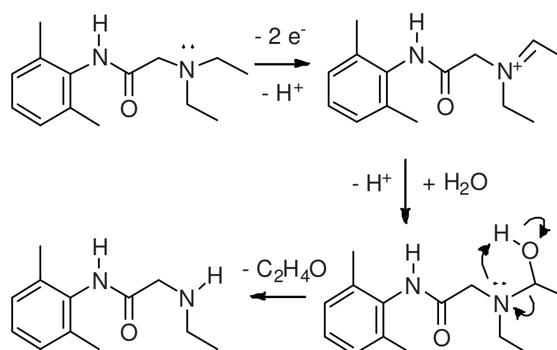


Figure 6. Proposed electrochemical N-dealkylation mechanism for lidocaine [58].

Direct electrochemistry is not generally capable of imitation of O-dealkylation reactions, as mentioned earlier, and it also does not generate N-oxides, but S-oxidation of sulfides to sulfoxides has been reported to occur readily [37, 59]. O-dealkylation of metoprolol under acidic conditions has been shown with a very low yield. S-oxidation of S-methylthiopurine was shown under neutral conditions [45]. Nozaki et al. showed both N-dealkylation and S-oxidation, but not O-dealkylation, of zotepine by direct electrochemistry [59].

1.2.3 Dehydrogenation and epoxidation

The dehydrogenation reaction is a special feature of CYP in which it actually behaves as an oxidase-dehydrogenase, and converts molecular oxygen to water. A HAT mechanism for the dehydrogenation of valproic acid (VPA) has been suggested, since the D/H kinetic isotope effect on the C-4 position was the same for 4-ene-VPA and 4-hydroxy-VPA [61]. Epoxidation of olefins occurs with the retention of stereochemistry, which suggests a concerted oxygen insertion mechanism by Compound I [15].

Electrochemical dehydrogenation has been observed for few drug compounds. Paracetamol is oxidized by several CYP isoforms through dehydrogenation to its toxic metabolite N-acetyl-p-benzoquinoneimine (NAPQI). The same dehydrogenation product can be obtained electrochemically, but this reaction is proposed to proceed through two successive electron/proton transfer reactions [49,

62]. Since the *in vivo* dehydrogenation mechanism is HAT-initiated, its mimicry would presumably be difficult by electrochemistry. Simulation of the *in vivo* phase II detoxification mechanism of the NAPQI metabolite by adduct formation with glutathione was shown in an on-line EC/LC/MS system [49]. In a similar study by Madsen *et al.,* the stability of NAPQI was studied, and its reaction kinetics with glutathione and other nucleophiles was determined using electrochemical measurements [63]. Electrochemical oxidation is useful for electrophilic metabolites of which the chemical reactivity is so high, that it prevents their detection *in vivo*. Electrochemical oxidation in the absence of nucleophiles could help isolate and detect the reactive intermediates, for example the quinoneimine intermediate metabolite of amodiaquine [63, 64].

Electrochemical studies can aid in the better understanding of charge/proton transfers in dehydrogenation and other oxidative pathways. Oliveira-Brett *et al.* have studied the oxidation of drugs including metolazone, apomorphine, and thalidomide at glassy carbon electrodes using cyclic, differential pulse, and square-wave voltammetry [65-67]. Two oxidation processes were observed, a reversible diffusion-controlled oxidation with two-electron and two-proton transfer reactions, and an irreversible diffusion-controlled oxidation at more positive potentials with one-electron and one-proton transfer reactions. The first reaction caused the dehydrogenation of the sulfonamide moiety of metolazone, and the latter an aromatic hydroxylation reaction [65-67].

Jurva *et al*. showed that the 4,5-epoxidation of benzo[*a*]pyrene could not be imitated electrochemically as it resulted instead in benzo[*a*]pyrene quinones [37]. Jeftic and Adams have proposed that this oxidation is initiated by a one-electron transfer process to the radical cation, which can either undergo a polymerization reaction, or, after several hydrolysis and oxidation steps, ends as benzo[*a*]pyrene quinones [68]. Xu *et al*. later studied the same reactions using an on-line EC/ESI-MS method [69]. Ultimately, electrochemical oxidation of benzo[*a*]pyrene resulted in several quinones, instead of the desired epoxidation metabolite.

1.3 Indirect electrochemical oxidation by reactive oxygen species (ROS)

Direct electrochemical oxidation is limited to the generation of oxidative drug metabolites initiated by electron transfer reactions which do not exceed the oxidation potential of the solvent. On the other hand, electrochemistry can be used to generate reactive oxygen species (ROS), which may mediate the production of other oxidative drug metabolites, including HAT-initiated reaction products.

Electrochemical reduction of molecular oxygen initiates activation of molecular oxygen toward reaction with organic compounds. This activation through successive electrochemical reduction and protonation steps will be discussed first, and then extended to the catalytic activation using iron cations (Fenton reaction

and Gif chemistry), which are more reminiscent of the *in vivo* catalytic cycle of CYP.

1.3.1 Electrochemically generated reactive oxygen species

Electrochemical reduction of molecular oxygen in aprotic solvents, such as acetonitrile, results in superoxide anion production (**reaction 1**). The superoxide anion is sufficiently stable in the absence of proton donors, and can be further reduced at higher negative potentials to peroxide anions (**reaction 2**) [70]**.** Electrochemically generated superoxide anions can promote different reactions including proton transfer, hydrogen atom transfer (e.g. from reduced flavins), and one-electron reduction [71]. Superoxide anions can undergo dismutation and form perhydroxyl anions in the presence of residual water or weak acids (**reaction 3**) [72, 73]. However, in the presence of stronger acids, molecular oxygen is reduced directly to perhydroxyl radicals, which can be chemisorbed to the electrode surface and disproportionate to hydrogen peroxide and molecular oxygen [74]. Perhydroxyl radicals are readily reduced to peroxide anions, directly on the electrode or by superoxide anions, but since the recombination of radicals has a very small activation energy barrier, two perhydroxyl radicals may combine to generate hydrogen peroxide and molecular oxygen (**reaction 4**) [70].

$O_2 + e^- \rightarrow O_2^{-\bullet}$ $\quad E^0 = -0.75$ V vs SCE (1)

$O_2^{-\bullet} + e^- \rightarrow O_2^{2-}$ $\quad E^0 = -2.05$ V vs SCE (2)

$O_2^{-\bullet} + H_2O \rightarrow HO_2^{\bullet} + OH^-$ (3)

$HO_2^{\bullet} + HO_2^{\bullet} \rightarrow H_2O_2 + O_2$ (4)

Perhydroxyl radicals are relatively strong oxidizing species in HAT reactions, and are capable of abstracting hydrogen from allylic positions, but they are unlikely to play a major role in oxidation reactions of organic substrates, since they are highly susceptible to recombination and the generation of hydrogen peroxide. A study by Lorenzola *et al*. showed that the final product of oxygen reduction on Pt and Au electrodes in acetonitrile in the presence of residual water is hydrogen peroxide [75], even though surface studies showed the reaction of the intermediate electrogenerated reactive oxygen species with the metallic surface [76]. Reduction of hydrogen peroxide by superoxide anions, known as the Haber-Weiss reaction (**reaction 5**), generates hydroxyl radicals capable of autoxidation and the promotion of HAT reactions [77, 78].

$H_2O_2 + O_2^{-\bullet} \rightarrow OH^{\bullet} + OH^- + O_2$ (5)

A recent study showed that the reduction of molecular oxygen in acetonitrile containing 1% water resulted in hydrogen peroxide, which reacted with lidocaine leading to N-oxidation, but not hydroxylation, suggesting that the amount of hydroxyl radicals was very small [58]. The N-oxide, presumably generated through adduct formation of the tertiary amine and hydrogen peroxide ($R_3N{\cdot}O_2H_2$) and subsequent decomposition [79], which could not be produced by direct electrochemical oxidation (section 2). On the other hand, direct electrochemical oxidation occurs on the counter electrode while reduction is performed at the working electrode, resulting in N-dealkylation in case of lidocaine. A combination of direct and indirect electrochemical oxidation by ROS thus provides access to a wider range of drug metabolites in a single electrochemical experiment [58].

Oxidation of water on the various electrode materials may generate reactive oxygen intermediates, mainly hydroxyl radicals. On the electrode surfaces with a low molecular oxygen evolution overpotential, such as platinum and carbon, the main product of water oxidation is molecular oxygen. A very small fraction of reactive species generated on the surface may be capable of promoting specific oxidation reactions at low yields. In general, these electrode materials have higher oxidation states available so that chemisorbed hydroxyl radicals may interact with the electrode to generate higher oxide species [80]. Boron-doped diamond (BDD), which has a high oxygen evolution overpotential and is a poor catalyst for oxygen evolution, can produce hydroxyl radicals upon water oxidation, as verified by using spin trap, and NMR studies [80]. The electrochemistry of water oxidation on BDD electrodes has been studied by differential-pulse voltammetry, showing that water oxidation to hydroxyl radicals at pH values lower than 9 proceeds through a concerted transfer of one electron, and one proton [81]. Interestingly, anodic and cathodic pretreatments result in different surface terminations of BDD, significantly influencing the surface resistance and capacitance, as shown by electrochemical impedance spectroscopy, and cyclic voltammetry [82]. The electrochemical oxidation of hydroquinone, resorcinol and catechol, which has been shown using a BDD electrode, probably proceeds through reaction with hydroxyl radicals, rather than through direct electrochemical oxidation of the substrate [83].

1.3.2 Electrochemically-assisted Fenton reaction

Electrochemical reduction of molecular oxygen in acetonitrile containing small amounts of water only results in generation of hydrogen peroxide as the final ROS product [58, 75]. The kinetics of hydroxyl radical generation through the Haber-Weiss reaction (0.13 $M^{-1}s^{-1}$, measured by radiolysis) is very slow compared with catalytic reactions (63 $M^{-1}s^{-1}$ for the Fenton reaction, described below) [84, 85]. As a consequence, in order to generate hydroxyl radicals in sufficient amounts to initiate oxidation through hydrogen atom transfer, it is necessary to catalyze formation in the presence of metallic cations.

The homolytic activation of hydrogen peroxide using Fe^{2+} cations is known as the Fenton reaction. It is considered to be a free-diffusing reaction and its selectivity depends on the choice of metallic cations, their relative concentration, the dissolved gas and pH [86, 87]. The general theme is that lower-valence metallic cations, such as Fe^{2+} cations, reduce hydrogen peroxide to a hydroxyl radical and a hydroxyl anion (**reaction 6**) [85]. A catalytic amount of Fe^{2+} cations is sufficient to initiate the reaction since they can be regenerated through **reaction 7**. Sawyer and coworkers have proposed a different oxidation pathway for this reaction, where there is a large excess of hydrogen peroxide to Fe^{2+} and whereby the generation of free radicals is excluded [88, 89]. Although this hypothesis was criticized later [90], the exact mechanism of the Fenton reaction remains elusive.

$$H_2O_2 + Fe^{2+} \rightarrow OH^{\bullet} + OH^{-} + Fe^{3+} \quad (6)$$

$$H_2O_2 + Fe^{3+} \rightarrow HO_2^{\bullet} + H^{+} + Fe^{2+} \quad (7)$$

Aliphatic hydroxylation by hydroxyl radicals proceeds through hydrogen atom transfer and radical recombination. Aromatic hydroxylation of benzoic acid to mono- and polyhydroxylated products by hydroxyl radicals generated by concurrent electrochemical reduction of molecular oxygen and Fe^{3+} cations allowed the measurement of the kinetics of each hydroxylation step [84]. The rate of hydroxylation shows a clear tendency to increase with the number of hydroxyl groups already present in the aromatic ring. The reaction is initiated with a very fast nucleophilic addition of hydroxyl radicals to the aromatic ring, and the resulting radical then undergoes different reactions depending on the medium, including dimerization, dismutation, oxidation, water elimination, or reaction with dissolved molecular oxygen to generate hydroxylation products through a peroxy intermediate (Dorfman mechanism) [84].

Electrochemistry can be used both in the generation of hydrogen peroxide through the reduction of molecular oxygen (section 3.1), and the reduction of Fe^{3+} cations to trigger the Fenton reaction. **Figure 7** illustrates the different stages of the electrochemically-assisted Fenton reaction and the reactions leading to aliphatic and aromatic hydroxylations, due to hydrogen atom abstraction and nucleophilic addition by hydroxyl radicals, respectively. Jurva *et al*. used the radical scavenger 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) to trap the generated radicals [91]. Although non-hydroxyl radicals resulting from autoxidation reactions were also detected, the Fenton reaction proved useful for oxygen atom insertion into organic molecules in case direct electrochemistry methods fail [91]. Successful imitation of the enzymatic oxidation of metoprolol to benzylic and aromatic hydroxylation as well as O- and N-dealkylation products was shown. In addition, a systematic study using different drug compounds proved that aliphatic, benzylic and aromatic hydroxylation as well as N- and O-dealkylation, N- and S-oxidation, and

dehydrogenation reactions are possible [45]. The electrochemically-assisted Fenton reaction has also been used successfully for the various oxidation reactions (mainly hydroxylations) of the antimicrobials triclosan and triclocarbon [92], the β-blocker atenolol [93], and for clofibic acid [94].

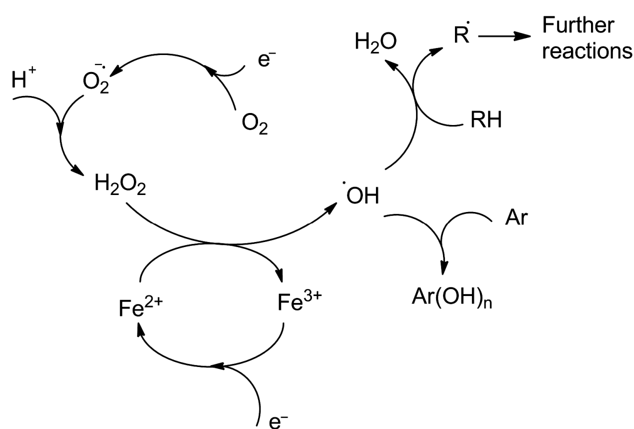


Figure 7. Schematic illustration of generation of hydroxyl radicals through the electrochemically-assisted Fenton reaction, and their subsequent reactions with aliphatic and aromatic compounds [84].

The electro-Fenton reaction is not the only method for the electrochemical generation of hydroxyl radicals. As shown earlier, water oxidation on BDD electrodes also produces hydroxyl radicals. It was found that by operating electrochemical cells at a high current, within the water oxidation potential, reactive hydroxyl radicals adsorbed on the BDD electrode were formed that promoted aromatic and aliphatic hydroxylations [85]. The kinetics of oxidation of paracetamol on the BDD electrode was high compared with that on platinum or glassy carbon electrodes [95], suggesting that BDD is an attractive electrode material for drug oxidation studies.

1.3.3 Electrochemically assisted Gif chemistry

Whereas the Fenton reaction proceeds through homolytic activation of the O-O bond and the generation of freely diffusing radicals, the activation of hydrogen peroxide by CYP (hydrogen peroxide shunt pathway, **Figure 2**) proceeds through heterolytic activation of the O-O bond and generation of Compound I. A model system (named $GoAgg^{II}$) resembling Compound I was first developed by Barton, who used Fe^{2+} cations in the presence of hydrogen peroxide in a pyridine/acetic

acid solution to promote a non-radical oxidation pathway [96]. Later, he showed that the addition of catalytic amounts of picolinic acid decreases the reaction half-time from 4-6 h to 5-10 min (model system named $GoAgg^{III}$) [97]. The main difference between CYP and the GoAgg models (collectively called Gif chemistry) is the absence of a porphyrin ligand that binds the iron to transfer electrons and to reduce Fe^{V} to Fe^{IV}. Barton suggested that the carbon-centered radicals do not play a role in Gif chemistry because they would be quenched by hydrogen atom transfer from the S-H bond in hydrogen sulfide that used to be added in the early Gif reactions. In addition, a radical reaction was excluded because the kinetics of oxidation of primary to tertiary hydrocarbons with Gif chemistry did not follow the pattern of their bond energies. A comparative study showed a different product distribution for alkanes obtained with Gif chemistry compared with halogen radical reaction [98]. Newcomb and co-workers suggested the presence of free alkyl radicals in Gif chemistry by using hypersensitive cyclopropane-based radical clocks (with very fast rearrangement kinetics) [99]. Clearly, mechanistic studies of Gif reactions should be interpreted carefully, as most current results are consistent with a free oxygen-centered radical mechanism rather than one involving high-valency iron-oxo species [100]. A review by Gozzo discusses different possible mechanisms involved in Gif chemistry [101].

Gif chemistry can be induced electrochemically by reducing molecular oxygen to an active form, which is probably the superoxide anion. The electrochemically-assisted Gif chemistry (so-called GO) promotes aliphatic hydroxylation as well as ketone formation reactions of test compounds [102]. A constant potential of -0.6 to -0.7 V vs. SCE with tetraethylammonium salt as electrolyte permits the reduction of molecular oxygen in pyridine/acetic acid solution without direct reduction of the pyridinium ion. Acetic acid can be replaced by α-picolinic or trifluoroacetic acid which protonate pyridine sufficiently to avoid the need for additional electrolytes.

1.4 Electrochemically assisted oxidation by metalloporphyrins as biomimetic models of CYP

Free hydroxyl radicals generated during the Fenton reaction promote a variety of oxidation reactions, which, however, lack selectivity. Metalloporphyrins can be used to generate metallo-oxo species, similar to Compound I, and to act as biomimetic models in the oxidative drug metabolism toward oxygen transfer reactions [103]. Unsubstituted metalloporphyrins were used as early biomimetic models, but since they are easily degraded, they were replaced by sterically hindered metalloporphyrins such as meso-tetraphenyl porphyrins (TPP). Metalloporphyrins with iron and manganese as metallic centers are the most widely used porphyrins in oxygen transfer reactions. Halide-substitution of the phenyl groups of TPP activates its metallic center and makes it a stronger oxidant [1]. The oxygen source in most of these biomimetic models is not molecular oxygen, but a single oxygen atom donor, such as iodosyl benzene (PhIO), and meta-chloro

perbenzoic acid (*m*-CPBA). These reactions are mainly performed in organic solvents, in which a basic nitrogen atom is required as proximal ligand to increase reactivity. They have benefits over reactions with freely diffusing radicals since a wide range of chemoselective [1], enantioselective [104], and regioselective [105] oxidation reactions can be achieved by proper selection of porphyrins with different metallic centers and substituents, solvent, and proximal ligand. In a review by Balogh and Keseru, differently substituted metalloporphyrins were used in the mimicry of oxidative drug metabolism. Oxidation products were collected and were found to be comparable to the *in vivo* metabolites, demonstrating the usefulness of metalloporphyrin-based chemical models in metabolic studies [106]. In a systematic study by Johansson *et al*. aliphatic and aromatic hydroxylation (even for non-activated aromatic rings), heteroatom dealkylation and oxidation, and dehydrogenation were shown to be mimicked successfully using different substituted iron and manganese meso-tetraphenyl porphyrins (FeTPP and MnTPP) activating hydrogen peroxide [45]. The chemical biomimetic models and their extension to drug metabolism have been reviewed in detail by Karst *et al.* [32]. In a recent review by Nam, different non-heme systems are presented in comparison with heme-based reactive species in the promotion of various oxidation reactions [107].

1.4.1 Metalloporphyrins in solution

Electrochemistry can assist metalloporphyrin models in a catalytic cycle identical to CYP in the activation of molecular oxygen. The oxygen source is molecular oxygen, and the reaction requires electrochemical reduction for the activation of the molecular oxygen bond.

Metalloporphyrins in solution have been used for the electrocatalytic activation of molecular oxygen. An early study suggested that the catalytic cycle could be initiated by the reduction of the metal center followed by coordination with molecular oxygen, and further reduction and protonation which would ultimately lead to the generation of metallo-oxo species [108]. There is also a need for an axial coordinator, such as 1-methyl imidazole. To sustain the catalytic cycle, and in case protic solvents are used, protons are required for the O-O bond activation, whereas in aprotic solvents this can be achieved with acid anhydrides [109]. Epoxidation of olefins has been shown to occur upon electrocatalytic activation of molecular oxygen by metalloporphyrins in solution [108]. Simándi has reviewed reactions of various metal complexes, including metalloporphyrins, in the catalytic activation of molecular oxygen in the oxidation of saturated and unsaturated hydrocarbons, and of aromatic compounds [110].

1.4.2 Metalloporphyrins immobilized on the electrode surface

Metalloporphyrins immobilized on the electrode surface suffer less from degradation. In addition, electron transfer from the surface facilitates the electrocatalytic activation of molecular oxygen. Different methods for immobilization of metalloporphyrins on a surface have been reviewed by Meunier and Brule *et al.*, which include coordinative binding, encapsulation (within zeolites, silica or clay), electrostatic adsorption, covalent anchorage and polymerization [111, 112]. The catalytic behavior of metalloporphyrin complexes in molecular sieves, in the epoxidation of cyclohexene was reported by Rani *et al.* [113]. Parton and colleagues proved that the zeolite-encaged iron complexes in a polymer membrane activated by peroxides resembles CYP in the oxidation of tertiary hydrocarbons [114]. Electropolymerized manganese porphyrin films have also been shown to be a versatile model system for the electrocatalytic activation of molecular oxygen and the oxidation of various small organic compounds and drug molecules [115]. Direct reduction of electropolymerized metalloporphyrins followed by coordination of molecular oxygen (the identical catalytic cycle as for CYP) has been proposed for the generation of metallo-oxo species [116]. On the other hand, Collman and coworkers suggested that in thick electropolymerized metalloporphyrin films, the direct reduction of molecular oxygen on the electrode surface to hydrogen peroxide, and its subsequent activation by metalloporphyrins through the peroxide shunt would generate high-valent metallo-oxo species capable of epoxidation of olefins [117].

Immobilized metalloporphyrins on the electrode surface may allow selective oxidation and generation of a wider range of drug metabolites compared with direct electrochemical oxidation which is merely capable of initiating charge transfer oxidations. Functionalized electrodes with metalloporphyrins could be used in combination with mass spectrometry for fast assessment of oxidative drug metabolism. For this purpose, metalloporphyrins were bound to an electrode via axial ligation to a self-assembled monolayers (SAM) [118]. Specifically, a 4-pyridinethiol SAM on a gold electrode was generated through thiol linkage and used to anchor metalloporphyrins by axial coordination [119]. The presence of metalloporphyrins anchored on the electrode surface was verified by the characteristic surface enhanced Raman spectroscopy (SERS) peaks.

Heme, the prosthetic group of CYP, has been shown to be useful as the electrocatalyst for the activation of molecular oxygen in alkene epoxidation reactions [120]. In an ongoing study in our group, a SAM of 4-pyridinethiol on gold and roughened silver electrodes was used to anchor heme, as shown in **Figure 8-a**. The generation of the SAM from a diluted solution of 4-pyridinethiol in ethanol on silver was studied by surface enhanced resonance Raman spectroscopy (SERRS). The presence of the SAM on the surface was verified by characteristic SERRS peaks. Following exposure of the modified surface to heme in dimethyl sulfoxide (DMSO) for ten hours, extra peaks were observed between 1200-1500 cm^{-1} in the SERRS spectrum (**Figure 8-b**) which can be attributed to the axial coordination of

heme via the SAM. An *in situ* SERRS study with electrochemistry was used to study the physical state of the iron center during the electrocatalytic activation of molecular oxygen in an acetonitrile solution. Similar spectra have been shown for the reduction of the iron center in Cytochrome c adsorbed on SAMs of 4-pyridinethiol on a silver electrode [121]. No spectral change was observed after the application of reductive potentials which may be explained by the generation of stable iron-oxygen species on the electrode surface in the absence of strong proton donors.

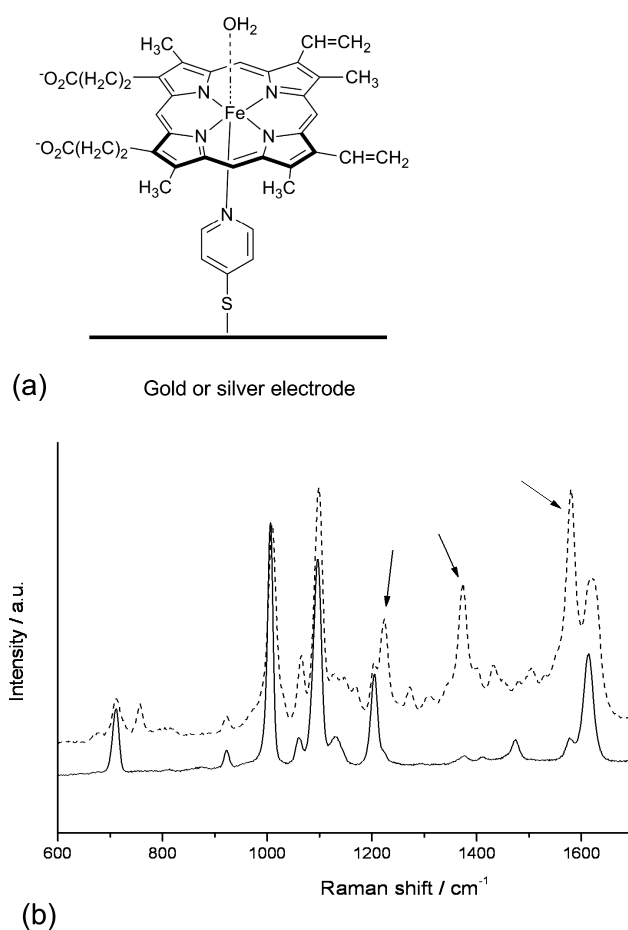


Figure 8. (a) Schematic representation of heme anchored through coordination via a self-assembled monolayer (SAM) of 4-pyridinethiol on a gold electrode, and (b) the experimental surface enhanced resonance Raman spectrum of the electrode surface before (lower trace) and after (upper trace) heme coordination [136].

1.5 Enzyme-modified electrodes

The final electrochemical mimicry method that we discuss employs enzymes anchored to the electrode surface. Peroxidases containing a heme prosthetic group anchored via histidine to the protein backbone have been used as biomimetic models. In peroxidases, unlike CYP, the catalytic cycle is triggered by the replacement of water from the distal ligand by hydrogen peroxide and the subsequent splitting off of one water molecule results in Compound I (similar to the CYP hydrogen peroxide shunt). Peroxidases are the catalysts of choice for sulfoxidation, hydroxylation and epoxidation on account of their high activity and enantioselectivity [122]. Immobilized peroxidases on poly-(γ-methyl-L-glutamate) catalyze the hydroxylation of benzene and exhibit an even higher activity than peroxidases in solution [123]. Peroxidases in solution or immobilized on a surface for the activation of hydrogen peroxide do not require a reduction step. A study by Sheldon and coworkers showed that peroxidases promote selective oxidation reactions with molecular oxygen in the presence of a chemical reductant [124].

Synthetic heme peptides, containing a heme group bound either covalently or by coordination to (helical) peptides, have been shown to reproduce the catalytic activity of heme oxidases and to provide an environment for heme which is similar to the native enzyme as shown in **Figure 1** [125]. Synthetic heme peptides were immobilized on a gold electrode via hydrophobic interactions on a surface coated with a SAM of decane-1-thiol. The electrochemical response of reduction of the metallic center, and its role in the catalytic activation of molecular oxygen, were verified using electrochemical techniques [126].

Although isolated CYPs suffer from low stability, and need an additional reductase cofactor to regenerate the enzyme, purified CYPs on electrode surfaces have been succesfully used at high turn-over rates [127]. A study by the group of Rusling showed that it is possible to oxidize styrene and cis-β-methylstyrene by using CYP101 and myoglobin embedded in thin films of polyion and surfactant. Myoglobin and CYP101 mediate the electrochemical reduction of molecular oxygen to hydrogen peroxide, which is used further through the peroxide shunt [128]. The same group later showed the possible direct charge transfer to CYP from the electrode by reduced redox partners on a polycation-coated electrode, and accordingly they observed electrocatalytic oxidation of styrene [129]. Mie *et al.* advanced this study by using different hydrophobic coating materials and showed that CYP and CYP-reductase on a simple hydrophobic electrode surface can promote drug metabolism reactions [130].

In order to eliminate the need for a reductase, Gilardi and colleagues utilized a protein engineering strategy to obtain sufficient interaction of CYP with the electrode surface by assembling a redox module and the enzyme domain to generate an enzyme-reductase fusion protein [131]. They used covalent bonding of the mutated CYP to the electrode surface via self-assembled monolayers [132].

Direct immobilization of human flavin-containing monooxygenase on a glassy carbon electrode derivatized with the cationic surfactant didodecylammonium bromide (DDAB) was shown to be efficient in the mimicry of N-oxide formation of tamoxifen, when a potential of -600 mV versus Ag/AgCl was applied for 30 min in the aerated cell [133]. Oriented immobilization of human CYP 2E1 was achieved by engineering two multisite mutants of P450 2E1, where all the exposed cysteines were modified into serines, except for one cysteine which was used to link the protein covalently to the gold electrode. As shown in **Figure 9**, the enzyme and its mutants were immobilized on a dithio-bismaleimidoethane SAM, and the transformation of p-nitrophenol to p-nitrocatechol was used as a test reaction. Activation at -500 mV for 30 min showed higher levels of products for the mutants that could be ascribed to the controlled immobilization on the gold electrode. Presumably, in the mutated CYPs the proximal side involved in electron transfer is linked to the electrode surface, while the distal side is exposed to the bulk solution [134]. Gilardi and co-workers recently showed that the CYP 3A4 fused with the electron transfer module flavodoxin (FLD), immobilized covalently on a SAM of 6-hexanethiol and 7-mercaptoheptanoic acid, could be integrated in a microfluidic platform [135]. In the microfluidic system, cyclic voltammometry was used to study the reduction and oxidation of the fused enzyme. The platform was also used to identify the specific kinetic parameters of oxidation of different drug compounds.

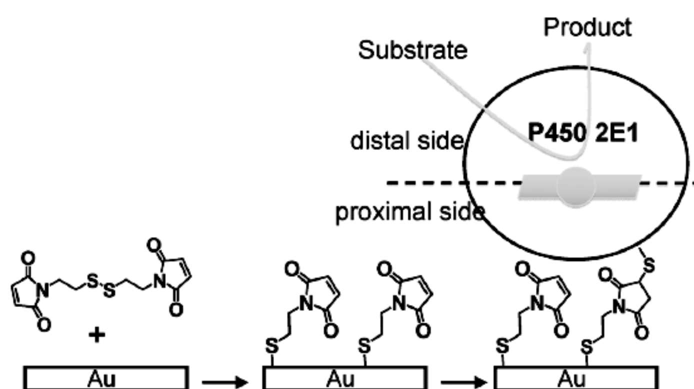


Figure 9. Surface immobilization of CYP 2E1, through covalent linkage to a dithio-bismaleimidoethane (DTME) SAM on a gold electrode surface [134].

1.6 Perspective

Direct electrochemical oxidation is a versatile analytical technique in the fast assessment of oxidative drug metabolism (particularly in combination with mass spectrometry), but it is limited to the oxidation reactions initiated by charge transfer. There is a continuing trend toward utilization of new electrochemical techniques to more effectively and selectively mimic oxidative drug metabolism. Indirect electrochemical oxidation mediated by reactive oxygen species generated through electrochemical oxygen reduction, and through the (electrochemically mediated) Fenton reaction and Gif chemistry helps in the generation of metabolites that cannot be obtained by direct electrochemical oxidation. Metalloporphyrins with different substituents and different metallic centers are useful as more specific biomimetic models in selective oxidation. The ongoing research to anchor metalloporphyrins on electrode surfaces to activate molecular oxygen could provide new possibilities for studying oxidative drug metabolism, and would allow the benefit of on-line coupling of functionalized electrodes with mass spectrometry or other analytical techniques. Mutated CYP enzymes that are anchored on the electrode and receive electrons directly from the surface are the closest mimicry model to *in vivo* CYP. Finally, the method of choice for electrochemical mimicry depends on the purpose of the study. For preparative synthesis of metabolites, direct electrochemical oxidation is a good option, though limited to the metabolites generated with electron transfer reactions (see section 2). For the metabolites not obtainable from this technique, the two other mimicry techniques should be tried, i.e. electrochemically generated reactive oxygen species (section 3), and metalloporphyrines (section 4). For the prediction of oxidative drug metabolism, the electrodes modified with enzymes (section 5), a field which is expanding fast, would be the method of choice.

1.7 References

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