

P450-Based Nano-Bio-Sensors for Personalized Medicine

Camilla Baj-Rossi, Giovanni De Micheli and Sandro Carrara
*EPFL - École Polytechnique Fédérale de Lausanne
Switzerland*

1. Introduction

Cytochromes P450 (P450s or CYPs) belong to a multigene family of more than 3,000 heme proteins which catalyse the NADPH-dependent monooxygenation and other about 60 distinct classes of biotransformation reactions. Cytochromes P450 are known to be involved in the metabolism of over 1,000,000 different xenobiotic and endobiotic lipophilic substrates (Shumyantseva, Bulko, Archakov, 2005). Cytochrome P450s carry out a wide array of metabolic activities that are essential to homeostasis, apart from their roles in steroid biosynthesis and biotransformation and drug or toxin clearance. In figure 1, the tridimensional structure of cytochrome P450 3A4 is reported.

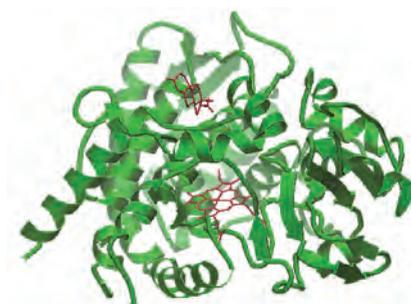


Fig. 1. Structure of cytochrome P450 3A4 (obtained by PDBe Protein Databank Europe <http://www.ebi.ac.uk/pdbe/>).

The liver is the main organ responsible for the biotransformation of drugs and chemicals, even if the gut metabolizes many drugs, and the CYPs and other metabolizing enzymes reside in the hepatocytes (Fig. 2). Basically, the primary function of CYPs and other biotransforming enzymes is to make very oil-soluble molecules highly water-soluble, so that they can be easily cleared by the kidneys into urine and they will be finally eliminated. When the drugs or toxins reach the hepatocytes in the liver, they basically flow inside the walls of the tubular structure of the smooth endoplasmic reticulum (SER), entering into the path of the CYP monooxygenase system. This is a highly lipophilic environment that keeps the lipophilic molecules away from the aqueous areas of the cell and allows the CYPs to metabolize them into more water-soluble agents (Coleman, 2010).

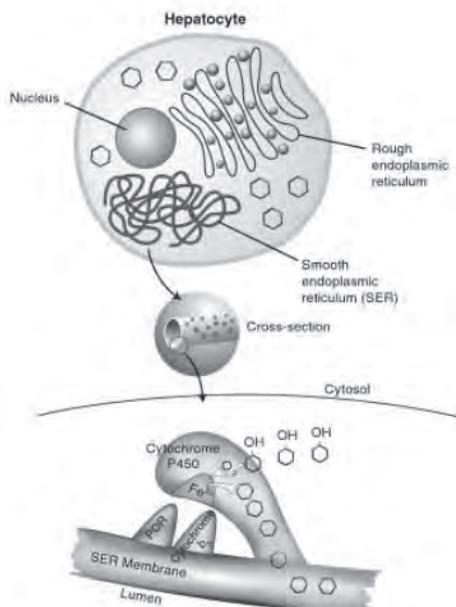


Fig. 2. Location in the hepatocyte of CYP enzymes and their redox partners, cytochrome b_5 and P450 oxidoreductase (POR), (Coleman, 2010). Reprinted with permission from Coleman, 2010. Copyright 2010 Wiley-Blackwell (John Wiley & Sons, Ltd).

Cytochromes P450 are enzymes involved in the metabolism of ~75% of all drugs (Figure 3A). Of the 57 human P450s, five are involved in ~95% of biotransformation reactions (Figure 3B), and each one is specific for a certain fraction of reactions which involved different substrates (Guengerich, 2008). In all living things, over 7,700 individual CYPs have been described and identified, although only 57 have been identified in human hepatocytes; of these, only 15 metabolize drugs and other chemicals.

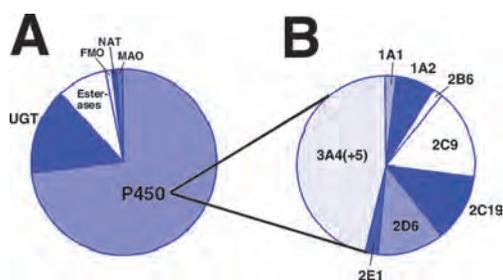


Fig. 3. Contributions of enzymes to the metabolism of marketed drugs. (A) Fraction of reactions on drugs catalyzed by various human enzymes. FMO, flavin-containing monooxygenase; NAT, Nacetyltransferase; and MAO, monoamine oxidase. (B) Fractions of P450 oxidations on drugs catalyzed by individual P450 enzymes (Guengerich, 2008). Reprinted with permission from Guengerich, 2008. Copyright 2008 American Chemical Society.

2. Cytochrome P450: classification and polymorphism

Cytochromes P450 are classified according to their amino acid sequence homology, that is, if two CYPs have 40 per cent of the full length of their amino acid structure in common they are thought to belong to the same 'family'. More than 780 CYP families have been found in nature in total, but only 18 have been identified in humans. Subfamilies are identified as having 55 per cent sequence homology and there are often several subfamilies in each family (Coleman, 2010). The nomenclature for P450s is based on naming cytochromes P450 with CYP followed by a number indicating the gene family (such as CYP1, CYP2, CYP3, etc.), a letter indicating the subfamily (i.e. CYP1A, CYP2A, CYP2B, CYP2C, etc.) and a number for the gene that identify the so named 'isoform'. In order to have the same gene number the genes must have the same function and exhibit high conservation (Ingelman-Sundberg, 2004). That is, two isoforms (e.g. CYP1A1 and CYP1A2) have 97 per cent of their general sequence in common. The completion of the sequence of the human genome revealed the presence of about 107 human P450 genes: 59 active and about 48 pseudogenes (Ingelman-Sundberg, 2004). The majority of genes exhibit a certain polymorphism (which is generally defined as 1% frequency of an allelic variant in a population) which leads to classify the CYPs even according to these allelic differences. A polymorphic form of a CYP is usually written with a * and a number for each allelic variant, or translated version of the gene. Regarding the polymorphic forms, they might contain one or more single nucleotide polymorphism (SNP, i.e. a change in one nucleotide of the genetic code) in the same allele (Coleman, 2010). For example, CYP2B6 has eight other significant allelic variants besides its major form, and among this variants, CYP2B6*4 has just one SNP, whilst CYP2B6*6 possesses two SNPs. The clinically most important polymorphism is seen with CYP2C9, CYP2C19 and CYP2D6. The functional importance of the polymorphisms of the xenobiotics metabolizing CYPs is summarized in Table 1.

The mutations in the CYP genes can cause the enzyme activity to be abolished, reduced, altered or increased, with substantial consequences in drug metabolism (Ingelman-Sundberg, 2004). Based on the composition of the alleles, the affected individuals might be divided into four major phenotypes: poor metabolizers (PMs), having two nonfunctional genes, intermediate metabolizers (IMs) being deficient on one allele, extensive metabolizers (EMs) having two copies of normal genes and ultrarapid metabolizers (UMs) having three or more functional active gene copies (Ingelman-Sundberg, 2004; Rodriguez-Antona, 2006). Phenotyping usually involves administering a single probe drug for a particular enzyme and measuring clearance and comparing it with data from other patients. The clinical influence of differences in CYP activity can be schematized as reported in figure 4. In this model example, only EMs and PMs are reported as the general population of interest. Referring to the EMs metabolizers (upper panel in figure 4), it is visible that after drug administration the plasma concentration rises to a peak ($C_{p,max}$) following the first dose and then decrease to a lower level prior to the next dose. With subsequent doses, the plasma concentration remains within this region and yields the desired pharmacological effect. Without prior knowledge about a problem with this drug, the PM (lower panel of Figure 4) and EM would be administrated the same dose. For PMs, a limited metabolism would occur between doses, and the plasma concentration of the drug will rise to an unexpectedly high level. The simplest effect would be an exaggerated and undesirable pharmacological response (Ortiz de Montellano, 2005).

CYP enzyme	Substrates	Polymorphism		
		Frequency	Functional effects	Most important polymorphic variants
CYP1A1	Carcinogens	Relatively high	-	-
CYP1A2	Drugs, carcinogens	High	Some	CYP1A2*1F, CYP1A2*1K
CYP2A6	Nicotine, drugs, carcinogens	High in orientals, less frequent in Caucasians	Important for nicotine metabolism	CYP2A6*1B, CYP2A6*4, CYP2A6*9, CYP2A6*12
CYP2B6	Drugs	High	Reduced drug metabolism	CYP2B6*5, CYP2B6*6, CYP2B6*16
CYP2C8	Some drugs	High	Reduced drug metabolism	CYP2C8*3
CYP2C9	Drugs	Relatively low	Very significant	CYP2C9*2, CYP2C9*3
CYP2C19	Drugs	High	Very significant	CYP2C19*2, CYP2C19*3, CYP2C19*17
CYP2D6	Drugs	High	Very significant	CYP2D6*2, CYP2D6*4, CYP2D6*5, CYP2D6*10, CYP2D6*17
CYP2E1	Carcinogens, solvents, few drugs	Low	No	-
CYP3A4	Drugs, carcinogens	Low	No or small	CYP3A4*1B

Table 1. Polymorphic cytochromes P450 of importance for drugs and carcinogens metabolism (Guengerich, 2001; Ortiz de Montellano, 2005).

At present state-of-the-art the only available monitoring system for personalized therapy is a check of the genetic predisposition of patients. In order to know which patient is at risk of having sub-therapeutic or toxic drug concentrations, a genetic test is done on alleles, which correspond to patient genetic predisposition for expressing the CYP proteins (Kirchheiner & Seeringer, 2007). A genetic test based on microarray has been introduced into the market by Roche: the Amplichip CYP450 (Amplichip, Figure 5).

It is the first FDA-cleared test for analysis of CYP2D6 and CYP2C19, two genes in the cytochrome P450 system that can greatly influence drugs metabolism. This test identifies the patient's genotype group and predicts his phenotype in order to classify patients as an either poor, intermediate, extensive, or ultra-rapid metabolizer. It was proven that this classification affects the actual amount of mean plasma concentration after a single drug dose (Lin, 2007).

However, the Amplichip can only "predict" the patient's phenotype and can only allow the adjustment of drug dose according to patient's genotype (as schematized in Figure 6). In Fig. 6, the theoretical dosages for different genotypes including ultrarapid, extensive, intermediate or slow metabolic activity are reported. They have been calculated from the differences in pharmacokinetic parameters and are depicted as schematic genotype-specific dosages, in order to have the same plasma-concentration course for all genetic group (Kirchheiner & Seeringer, 2007). Thus, in order to individually optimize an ongoing drug therapy, it is required to know how the patient metabolize drugs at the moment of the pharmacological cure, i.e. it is necessary to measure the plasma concentrations of drugs or their metabolites after the administration. This is a strong need since still most effective drug therapies for major diseases provide benefit only to a fraction of patients, typically in the 20 to 50% range (Lazarou et al., 1998).

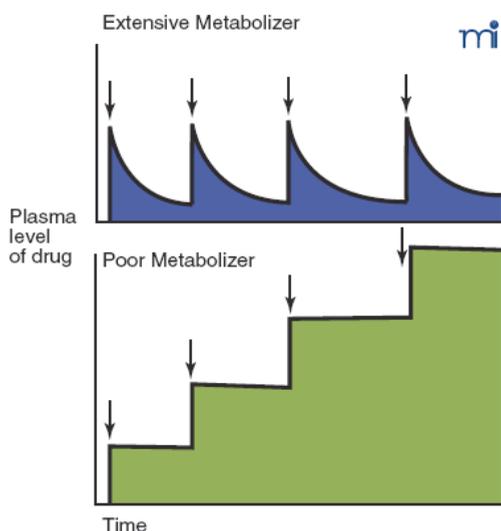


Fig. 4. Examples of unexpectedly low metabolism of a drug by P450s. The typical pattern seen with the majority of the population (extensive metabolizers) is shown in the upper panel, where the plasma level of the drug is maintained in a certain range after the administration of several consecutive doses (arrows indicate multiple doses). Unusually slow metabolism (lower panel) occurs when a poor metabolizer receives the same dose, resulting in unexpectedly high plasma level of the drug (Guengerich, 2003). Reprinted with permission from Guengerich, 2003. Copyright 2003 Molecular Interventions Online by the American Society for Pharmacology and Experimental Therapeutics.



Fig. 5. AmpliChip CYP450 (Roche). ROCHE and AMPLICHIP are trademarks of Roche. AFFYMETRIX and POWERED BY AFFYMETRIX are trademarks of Affymetrix, Inc.

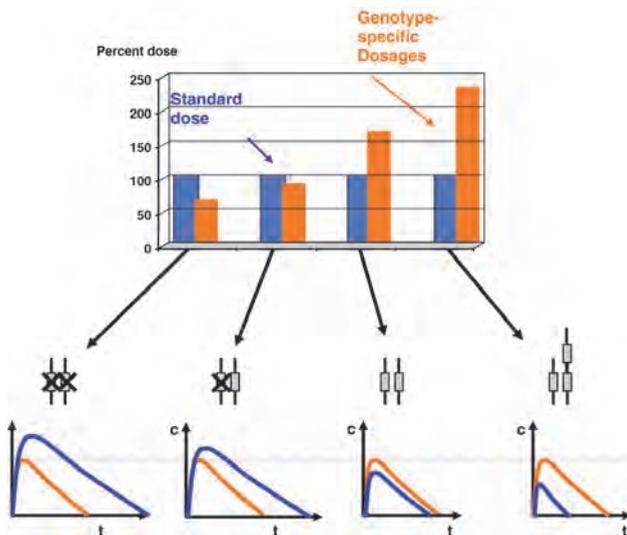


Fig. 6. Principle of calculation of genotype based dose adjustments based upon differences in pharmacokinetic parameters such as clearance and AUC. (Kirchheiner & Seeringer, 2007). Reprinted from *Biochimica et Biophysica Acta*, Vol. 1770, Julia Kirchheiner, Angela Seeringer, "Clinical implications of pharmacogenetics of cytochrome P450 drug metabolizing enzymes", Pages No. 489–494, Copyright (2007), with permission from Elsevier.

In general, there are only three CYP families which are mainly involved in drug and toxin biotransformation for humans, including:

- CYP1 family (CYP1A1, CYP1A2 and CYP1B1);
- CYP2 family (CYP2A6, CYP2A13, CYP2B6, CYP2C8, CYP2C9, CYP218, CYP219, CYP2D6, CYP2E1);
- CYP3 family (CYP3A4, CYP3A5, CYP3A7).

It is known that the 90% of marketed drugs are metabolized by only five of these isoforms (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4/5), (Ingelman-Sundberg, 2004).

3. Active-site structure of P450 enzymes

Although CYPs in general are capable of metabolizing almost any chemical structure and catalyse around 60 different classes of biotransforming reactions, they have a number of features in common, including (Coleman, 2010):

- a. Most mammalian CYPs exist in the lipid smooth endoplasmic reticulum (SER) microenvironment, being partially embedded in the liphophilic membrane of the SER and their access channels are actually positioned inside the membrane ready to receive substrates, which are normally liphophilic (see Figure 2).
- b. CYPs possess in their inner structure a heme group in their active site containing iron, which is a highly conserved part of their structures, and it is crucial for their catalytic activity. This active-site area is quite rigid, but it is surrounded by much more flexible complex binding areas, which can be regulated with different conformational changes in order to allow the entrance of different-size molecules.

- c. CYPs have one or more binding area inside their active site, which determine for most part their variations and their ability to metabolize specific groups of chemicals.
- d. Thanks to the heme group, CYPs exploit the ability of a metal to gain or lose electrons, thus catalyzing substrate oxidations and reductions, according to the NADPH-monoxygenation pathway.
- e. All CYPs have closely associated redox partners, the cytochrome b_5 and P450 oxidoreductase (POR), able to supply them with electrons for their catalytic activities (Figure 2).
- f. All CYPs bind and activate oxygen in their catalytic cycle as part of the metabolism process, but they are also able to carry out reduction reactions that do not require the presence of oxygen.

CYP enzymes share a common overall fold and topology (Figure 7) despite the differences in the genetic sequences and the genetic polymorphism. The conserved CYP structural core is formed by a four- α helix bundle composed of three parallel helices labelled D, L, and I and one antiparallel helix E (Denisov et al., 2005). The whole enzyme structure is usually anchored in the membrane of the smooth endoplasmic reticulum by an N-terminal α helix. The α helices hold in place the active site of the enzyme, the heme-iron group. In most CYPs the heme group is a relative rigid part of the protein's structure. The heme moiety (Figure 8), also known as ferriprotoporphyrin-9, has a highly specialized lattice structure that supports a iron molecule, which is the core of the enzyme and is the responsible of the substrate oxidation (Coleman, 2010).



Fig. 7. Ribbon representation (distal face) of cytochrome P450s fold. Substrate recognition sites (SRS) are shown in black and labelled. α -Helices are labelled with capital letters (Denisov et al., 2005). Reprinted with permission from Denisov et al., 2005. Copyright 2005 American Chemical Society.

The iron atom is normally bound to five other molecules which keep it secured; four of them are pyrrole nitrogens and hold it in the horizontal plane while the fifth group, a sulphur atom from a cysteine amino acid residue links the iron in a vertical plane. This structure, known as the pentacoordinate state, represent the resting state, whilst the hexacoordinate state occurs when the iron binds another ligand. In this latter configuration, the iron atom appears to move 'upwards' and draws level with the nitrogens to bind a water molecule which is hydrogen bonded to an amino acid residue and is involved in the movement of protons during the catalytic activity. The ferriprotoporphyrin-9 is held in place by hydrogen bonding and a number of amino acid residues. The iron is crucial to the catalytic function of CYP enzymes.

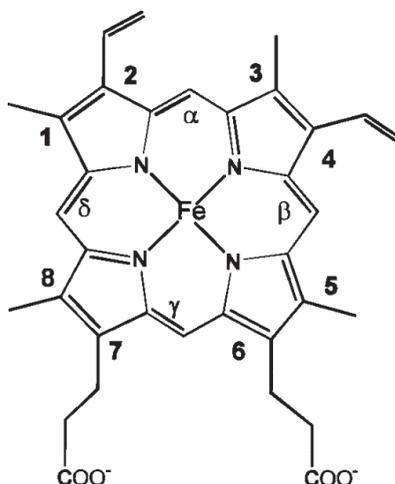


Fig. 8. Heme group chemical structure (Sono et al., 1996). Reprinted with permission from Sono et al., 1996. Copyright 1996 American Chemical Society.

The other helices that cover the active site are the flexible regions of the structure. They are normally partially clenched, but can open out to accommodate large substrates. The lipophilic substrate molecules diffuse through the membrane and enter the isoform through a sort of access path, which is defined as the widest, shortest and usually the most lipophilic pathway to reach the heme iron active site. The active site is supplied with electrons by the dual redox partners, the cytochrome b₅ and P450 oxidoreductase (POR), through another access channel from the other side of the CYP (see Figure 9). These redox partners are also embedded in the smooth endoplasmic reticulum membrane right next to the CYP (see Figure 2). After the catalytic reaction, the transformed products naturally exit the isoform through another channel (Coleman, 2010). Figure 9 shows the structure of cytochrome P450 14 α -sterol demethylases (CYP51) with the open substrate access channel between the β -sheet and helical domains (channel 2) oriented approximately 90° relative to CYP51 channel 1. Those channels, delimited by the F, G, and H helices and loops in between, are known to undergo synchronized motions in CYPs when substrate binds. This synchronization allows the channel 1 to open, whereas channel 2 remains closed, thus providing a means for substrate to enter one channel and product to depart from the other (Podust et al., 2001).

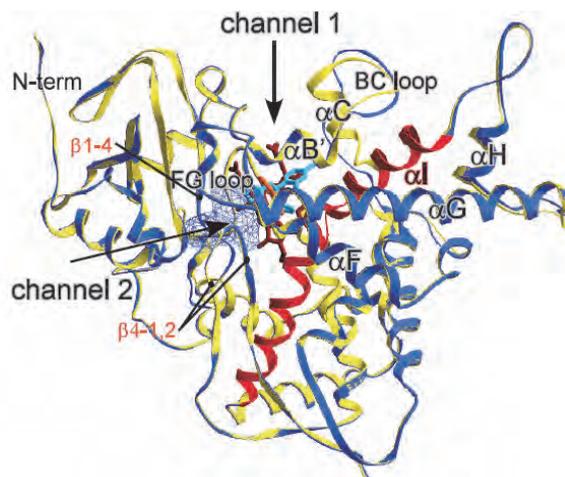


Fig. 9. Ribbon representation of the CYP51 structures with the azole inhibitors bound (Podust et al., 2001), which shows the two access channels (channel 1 and 2). Reprinted with permission from Podust et al., 2001. Copyright (2001) National Academy of Sciences, U.S.A.

3.1 Substrate binding in CYPs

The active site of an enzyme usually refers to a binding area which holds the substrate in a proper orientation capable to present the molecule to the structure of the enzyme that catalyze the reactions. In many enzymes, the dimensions and properties of the active and binding sites are quite well defined and mapped in detail, but crystallographic studies (Coleman, 2010), have shown that in the case of CYPs is difficult to define what constitutes the active binding site and to correctly identify their structure. As an example, it is known that CYP3A4, CYP2C8 and CYP2C9 have very large active sites, whilst that of CYP2A6 is quite small. Cytochrome P450 undergo big changes in movement and binding area to accommodate substrates of differing sizes, thanks to the small-intermediate hydrophobic pockets placed into the CYP active site, with the capability as the α -helices to extend its area to bind larger substrates. The hydrophobic pocket (Figure 10) includes many amino acid residues that can bind a molecule with hydrogen bonding, weak van der Waals forces, or other interactions between electron orbitals of phenyl groups, such as π - π bond stacking. It is known that the isoform CYP3A4 can increase its active site area by 80 per cent to accommodate erythromycin (Coleman, 2010).

The presence of several amino acid residues allows the active site to provide a grip on the substrate in a number of places in the molecule preventing excessive movements while, when not binding substrates, CYP active site area is full of water molecules displaced upon the binding site. Moreover the substrate binding may occur in the interior and exterior structure of the isoform, and also the binding may involve internal rearrangement of the isoform or involve simultaneous binding of other substrates. At the simplest structural level it is possible to identify six different active sites or "substrate recognition sites" (SRS, see Figure 7), which predetermine CYP substrate specificity (Denisov et al., 2005). It has been reported that several CYP isoforms, including 3A4, 1A2, 2E1, 2D6, and 2C9, exhibit allotropic kinetics *in vitro* (Atkins, 2005). This atypical kinetic behaviour is typical of the enzymes that shows multiple substrate recognition sites in their active site, as CYPs, and is basically due to the conformational or chemical changes that occur in the active site of the enzyme after binding a first substrate (the effector). These changes in the conformation of

enzyme tertiary and quaternary structure result in the enzyme catalytic activity alteration which can affect the metabolism of a second substrate (Houston & Galetin, 2005).

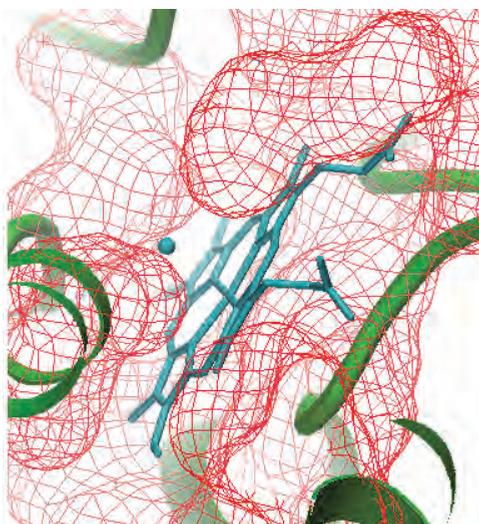
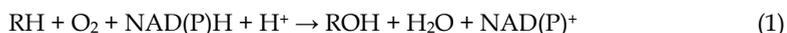


Fig. 10. The substrate binding cavity (meshed surface) of human cytochrome P450 3A4 (obtained by PDBe Protein Databank Europe, <http://www.ebi.ac.uk/pdbe/>). Molecular oxygen is reduced by the heme prosthetic group (the cyan stick figure) to form a reactive intermediate that oxygenates the substrate.

3.2 Redox partners and electron transport system

In catalyzing monooxygenation reactions of a substrate RH, CYP is able to utilize either NADH or NADPH (nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate respectively) as the electron donor (equation 1).



However, the two electrons derived from NAD(P)H must be transferred to CYP via electron transport proteins called redox partners. The main CYP redox partners are the P450 oxidoreductase and the cytochrome b_5 (Sevrioukova et al., 1999):

- a. *P450 oxidoreductase (POR)* is a flavoprotein complex, that consists of a large butterfly-shaped protein which locates and binds two flavins that work as electron carriers, FAD (flavin adenine dinucleotide) and FMN (flavin mononucleotide) in two separate lobes of the P450 oxidoreductase structure. The oxidative system, which consumes the glucose in the liver by the pentose phosphate pathway, produces NADPH to power all reductive reactions related to CYPs, and other reactions. P450 oxidoreductase complex operates as follows (Figure 11): electrons from reduced *NADPH* (released as NADP^+), are taken up by the FAD moiety, which is reduced to FADH_2 . Then FADH_2 reduces FMN to FMNH_2 , which in turn passes its two electrons to the CYP heme group, which is associated with the P450 oxidoreductase by electrostatic interactions (Huang et al., 2008). This electron transfer becomes a current of electron that P450 oxidoreductase must supply in the presence of high substrate concentrations.

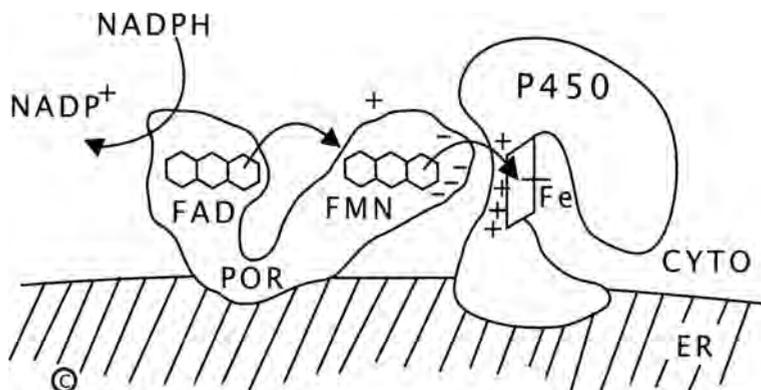


Fig. 11. Relationship of P450 oxidoreductase to a microsomal cytochrome P450 enzyme (Huang et al., 2008). ER is the endoplasmic reticulum and CYTO is the cytoplasm. Adapted from (Miller, W.L., 2005). Reprinted with permission from Huang et al., 2008. Copyright (2008) National Academy of Sciences, U.S.A.

- b. *Cytochromes b₅* (Figure 12) are electron transport heme proteins which, similarly to cytochrome P450, are built around a central heme group. In nature these proteins convert in the cells NADH to NAD⁺, building up proton gradients which stimulate the flow of electrons. There are numerous forms and structures of cytochrome b₅, but the microsomal cytochrome b₅ is of greatest interest in drug metabolism. Microsomal cytochrome b₅ is anchored by a helix into the smooth endoplasmic reticulum membrane (see Figure 2), but its heme structure, which is not-covalently linked with its CYP isoform, is closely associated with P450 oxidoreductase. Cytochrome b₅ has a complex but also essential role in CYP function. The standard CYP catalytic cycle (see paragraph 4) requires two electrons to reach a complete turn. The second electron is the rate limiting step in the enzymatic catalytic activity, and the cytochrome b₅ can supply this electron with high speed, even quicker than the system composed by P450 oxidoreductase and NADPH (Coleman, 2010).

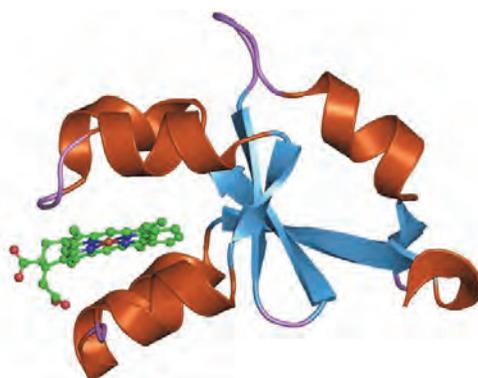
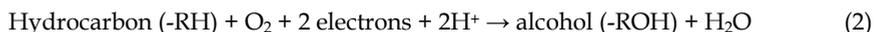


Fig. 12. Cytochrome b₅ structure (obtained by PDBe Protein Data Bank Europe, <http://www.ebi.ac.uk/pdbe/>).

4. Cytochrome P450 catalytic cycle

After having understand the complex structure of these enzymes, it is necessary to know that all of them essentially function in the same way, despite the genetic differences. CYPs can carry out substrate (RH) reductions through a catalytic cycle after substrate and oxygen binding. However, their main function is to insert an oxygen molecule into a usually stable and hydrophobic compound, and the global reaction can be schematized with the following equation (Coleman, 2010):



The CYP reduction activity is carried out through the so-called catalytic cycle (figure 13), which will be briefly reported in a simple way, considering the many details involved in this complex process (Sligar, 1976).

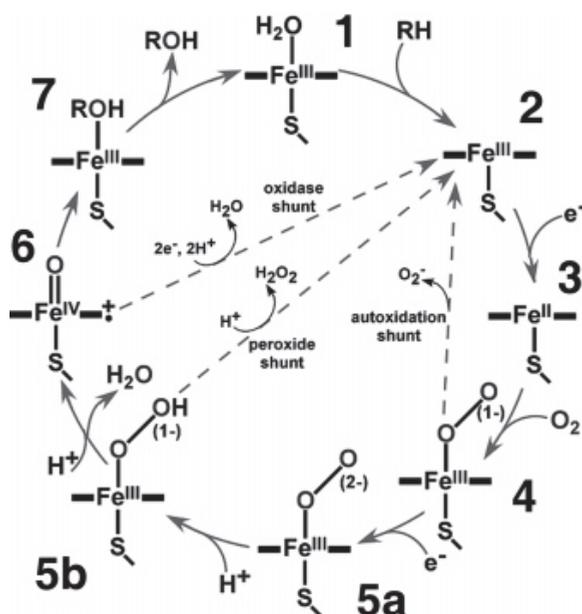
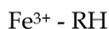


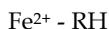
Fig. 13. CYP catalytic cycle (Denisov et al., 2005). Reprinted with permission from Denisov et al., 2005. Copyright 2005 American Chemical Society.

- a. *Substrate binding (1-3).* The first step is the binding and orientation of the substrate molecules. The iron is usually in the ferric form when the substrate is bound:

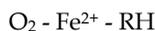


The substrate binds to the active site of the enzyme, in close proximity to the heme group. The bound substrate induces a change in the conformation of the active site, displacing a water molecule from the distal axial coordination position of the heme iron, and changing the state of the heme iron from a low-spin (LS) to an high-spin (HS) substrate-bound complex (2). The HS ferric enzyme ($\text{Fe}^{\text{3+}}$) has a more positive reduction potential and thus in CYP is much easier reduced. Once the substrate has been bound,

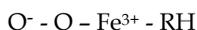
the next stage is to receive the first of two electrons from the redox partners, so reducing the iron to its ferrous state Fe^{2+} (3):



- b. *Oxygen binding (3-4)*. The next stage involves the binding between ferrous substrate-bound complex and molecular oxygen. This process is usually faster than the previous one, since in the cell there is much more oxygen than substrate:

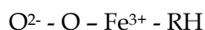


Oxygen binding leads to a slow rearrangement of the Fe^{2+}O_2 complex to form an oxy-P450 complex (4), which is the last relatively stable intermediate in this cycle:



However, this sometimes allows the bond to dissociate, through the so-called "autoxidation shunt", releasing a reactive superoxide radical, with the return of the enzyme to its resting state, thus interrupting the catalytic cycle (Denisov et al., 2005).

- c. *Oxygen scission (splitting), (4-5_a)*. This is the crucial stage that decides if the substrate will be oxidized or not and it is the rate-limiting step of the cycle. A second electron supplied by the redox partners feeds into the complex and forms a *peroxy-ferric intermediate (5_a)*:



As explained earlier, cytochrome b_5 may supply this second electron faster than NADPH reductase.

- d. *Protonation of peroxy-ferric intermediate (5_a-6)*. The peroxy group formed in the previous step is rapidly protonated by surrounding amino-acid side chains forming an hydroperoxy-ferric intermediate (5_b) and then is secondly protonated at the distal oxygen atom. Then, a subsequent heterolysis of O-O bond occurs with the release of a mole of water and the formation of a highly reactive iron (IV)-oxo species (6).
- e. *Insertion of the oxygen into substrate (6-7)*. Depending on the substrate and enzyme involved, P450 enzymes can catalyse a wide variety of reactions (a complete list is reported in Figure 14). A hypothetical hydroxylation is shown in the illustration (Sono et al., 1996). The substrate can be activated by either removing hydrogen (hydrogen abstraction) or an electron (e.g. from nitrogen atoms), from the substrate molecule. The hydrogen abstraction leaves the carbon with a spare electron making it a reactive radical (6) and then much more likely to react with the hydroxyl group. The final stage is the reaction between the newly created hydroxyl group and the carbon radical, leading the alcohol formation (7).
- f. *Release of product (7-new cycle)*. Now the substrate, converted to a metabolite, has changed both structurally and chemically and cannot be longer bound to the active site of the CYP. The product is thus released and the CYP isoform returns to its original resting state (1), with a water molecule returning to occupy the distal coordination position of the iron nucleus, now ready for binding another substrate molecule.

In addition the P450 reaction cycle contains at least three branch points, where multiple side reactions are possible and often occur under physiological conditions. The three major abortive reactions are (i) *autoxidation (autoxidation shunt)* of the oxy-ferrous enzyme (4) with

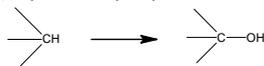
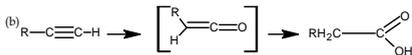
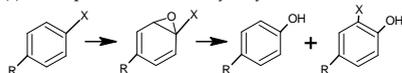
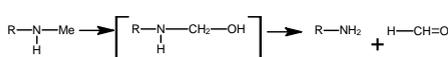
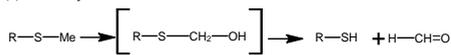
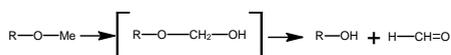
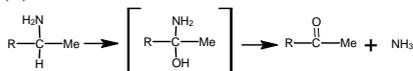
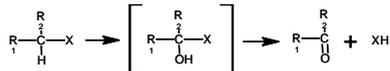
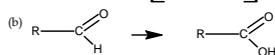
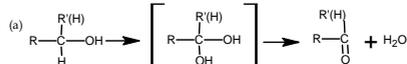
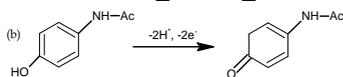
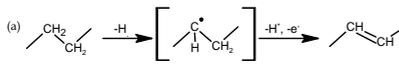
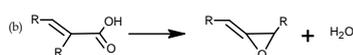
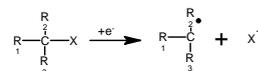
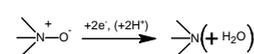
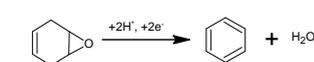
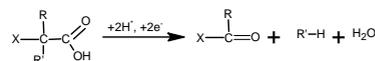
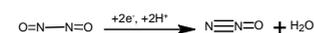
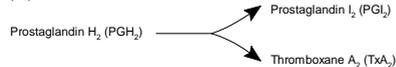
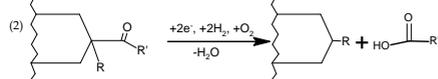
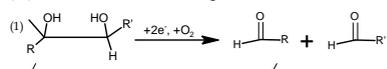
(1) Hydrocarbon hydroxylation**(2) Alkene epoxidation / Alkyne oxygenation****(3) Arene epoxidation, aromatic hydroxylation, NIH shift****(4) N-Dealkylation****(5) S-Dealkylation****(6) O-Dealkylation****(7) N-Hydroxylation****(8) N-Oxidation****(9) S-Oxidation****(10) Oxidative deamination****(11) Oxidative dehalogenation****(12) Alcohol and Aldehyde oxidations****(13) Dehydrogenation****(14) Dehydrations****(15) Reductive dehalogenation****(16) N-Oxide reduction****(17) Epoxide reduction****(18) Reductive β -scission of alkyl peroxides****(19) NO reduction****(20) Isomerizations****(21) Oxidative C-C bond cleavage**

Fig. 14. Schematic summary of the diverse P450-catalyzed reactions (Sono et al., 1996).

the production of a superoxide anion and return of the enzyme to its resting state (2), (ii) a *peroxide shunt*, where the hydroperoxide anion (5_b) dissociates from the iron forming hydrogen peroxide, thus completing the unproductive reduction of oxygen, without substrate turnover, and (iii) *an oxidase uncoupling* wherein the ferryl-oxo intermediate (6) is oxidized to water, which results effectively in the reduction of dioxygen molecule with the formation of two molecules of water. These processes are often categorized together and referred to as uncoupling reactions (Denisov et al., 2005). Cytochrome P450 catalyzes different types of reactions, illustrated in the Figure 14, which are all based on the same catalytic cycle.

5. Cytochrome P450 for drug-biosensor

Electrochemical investigations are usually performed on enzymes to determine fundamental parameters, such as the redox potential, and the electron transfer between the enzymes and the electrodes (Armstrong & Wilson, 2000). Electrochemical studies of cytochromes P450 are of great interest due to the possibility of developing applications such as biosensors for analyte detection and electrochemical catalysis for product synthesis (Honeychurch, 2006). CYPs ability to metabolize a broad spectrum of endogenous substances, e.g., fatty acids, steroid hormones, prostaglandins and foreign compounds (e.g. drugs and environmental toxins), has made this enzyme family interesting as recognition element for biosensing (Bistolos et al., 2005). At the present state-of-the-art, methods such as gas chromatography-mass spectrometry and high-performance liquid chromatography are used for in vitro quantifying the levels of drugs and their metabolites in blood and plasma. These commercially-available methods are time-consuming and expensive. Although with these methods is possible to obtain rapid quantitative in vitro diagnosis with high accuracy, they require time-consuming sample preparation. Thus, these techniques are not so widely spread for practical industrial and medical applications (Iwuoha et al., 2007; Shumyantseva et al., 2004). A cytochrome P450 biosensor may be a promising alternative that would provide quick measurements for drugs and metabolites with a cheap, simple to use, rapid and in some instances disposable equipment, which also supply good selectivity, accuracy and sensitivity.

After the first studies in the 1970s (Bistolos et al., 2005), when CYPs contained in intact hepatocytes and microsomes were used in extracorporeal detoxification reactions, choosing as target a wide range of substrates which are subjected to hydroxylation by CYPs, many attempts were carried out in order to built up a CYP-based biosensor for the detections of molecules of interest and drugs. In order to provide stability to the enzyme-system and in order to avoid the need of the regeneration of cofactor NADH and NADPH, which naturally provide electrons to the cytochrome active site, amperometric biosensors of second and third generations (Figure 15) were thought to be the most suitable for such applications and were designed. Some attempts have been recently made to improve the electron transfer between the CYP-enzymes and the electrodes, through the employment of an electrochemically active mediator and using cathodic current for the substrate reduction, thus obtaining highly efficient electrochemical second-generation biosensor (Shumyantseva et al., 2000). As found in literature, cobalt(III) sepulchrate trichloride was used as mediator for the electrocatalytical reduction of proteins containing different cytochrome P450s and NADPH-P450 reductase for catalyze the hydroxylation of steroids and the N-demethylation of drugs (Estabrook et al., 1996). Otherwise mediators such as FMN, FAD or riboflavins

(Shumyantseva et al., 2000, 2001) were covalently bound to cytochrome P450 2B4 and 1A2 cross-linked onto a screen-printed rhodium graphite electrode for direct amperometric measurement of cholesterol or aminopyrine. Unfortunately, this kind of redox mediators used in conjunction with redox enzymes facilitates not only the electron transfer between electrode and enzyme but also other various interfering reactions, resulting in a low-specificity detection.

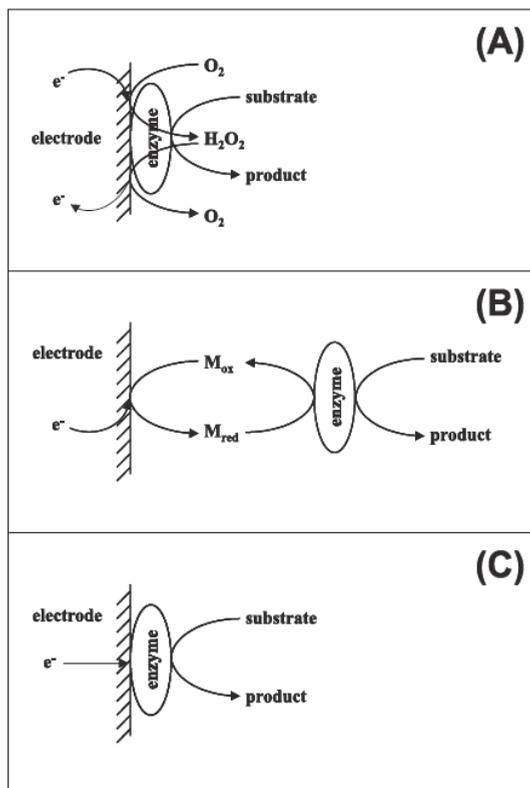


Fig. 15. Representation of three different amperometric biosensors generations (Freire et al., 2003). The first-generation biosensors (Fig. 15 A) are based on direct electrochemical detection of substrate or product of the enzyme reaction, while in the second and third-generation biosensor the detection is based on the quantification of the electron transfer. In the second-generation biosensors (Fig. 15 B), molecular oxygen is replaced with other reversible oxidizing agents, called mediators M_{ox} , which, are small redox active molecules (e.g., ferrocene derivates, ferricyanide, conducting organic salts and quinones) that could react both with the active site of the enzyme and with the electrode surface, realizing the electron transfer between the enzyme and the electrode. In the third generation-biosensors (Fig. 15 C) a direct electron transfer occurs, without using any kind of mediators. In third generation-biosensors several enzymes able to catalyse direct (mediatorless) electron transfer are used. Reprinted with permission from Freire et al., 2003. Copyright 2003 Sociedade Brasileira de Química.

The most suitable approach for the design of a CYP-based biosensor is the direct mediatorless electron supply from an electrode to the redox active group of the CYP, thus leading a direct flow of electrons between the enzyme and the electrode. This kind of biosensor (third-generation biosensors) usually offers better selectivity, because they are able to operate in a potential range closer to the redox potential of the enzyme itself, becoming less exposed to reactions with interferents. The electrode is used as electron source for the CYP cathodic reduction which is coupled with the substrate transformation. The generation and the measurement of a catalytic current is the direct indicator of CYP-dependent electrocatalysis. In the figure 16 is reported a scheme for the electrocatalytic oxygenation reaction of a substrate (RH) bound to CYP immobilized onto the electrode surface. In this scheme is possible to observe the active role of electrode as electron source in the P450 catalytic cycle.

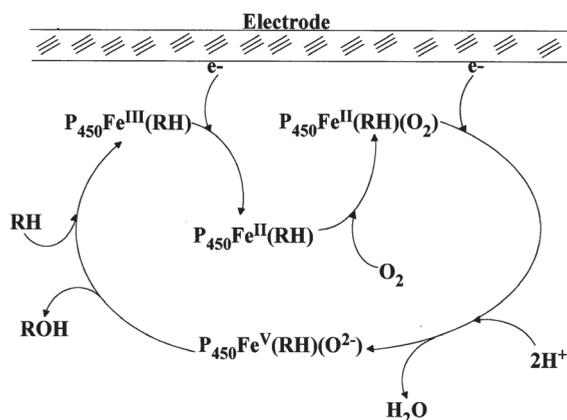


Fig. 16. Suggested scheme for the electrocatalytic oxygenation reaction of substrate-bound cytochrome P450 (Iwuoha et al., 1998). Reprinted from Journal of Pharmaceutical and Biomedical Analysis, Vol. 17, Emmanuel I. Iwuoha, Shiba Joseph, Z. Zhang, Malcolm R. Smyth, Uwe Fuhr, Paul R. Ortiz de Montellano, "Drug metabolism biosensors: electrochemical reactivities of cytochrome P450cam immobilised in synthetic vesicular systems", Pages No. 1101-1110, Copyright (1998), with permission from Elsevier.

In the development of this mediator-less approach, the immobilization of CYP onto the electrode surface has to be deeply controlled in order to obtain a high probability for the protein to be attached to the electrode in a proper orientation that could optimize the electron transfer to the heme group since it is deeply immersed in the cytochrome structure. In addition, the immobilization techniques of CYPs onto the electrode should avoid the formation of an insulating protein layer which prevents electron transfer, due to the adsorptive denaturation of proteins onto metal electrodes (Eggs, 2003).

5.1 CYPs immobilization techniques

The electron transfer between the enzyme and the electrode depends mostly on the technique used for the protein immobilization, on the electrode material or on the method used for modify the electrode surface, because all these factors change the orientation and the distance of the protein from the electrode. A variety of metal electrodes such as Au, Pt,

Tin oxide, oxide electrodes (such as In_2O_3), as well as non-metal electrodes such as glassy carbon, graphite (pyrolytic graphite and edge-plane graphite), and carbon cloth has been used for the fabrication of CYP-based amperometric biosensors (Bistolos et al., 2005; Eggins, 2003). Electrodes fabricated by screen-printed technology have been widely used for the design of amperometric-enzyme biosensors, thanks to their high selectivity and sensitivity, low cost, portable field-based size and their great versatility in the wide range of ways in which the electrodes can be modified (Renedo et al., 2007). Enzymes tend to denature and to passivate the electrode on not-modified metal electrodes, forming an insulating protein layer which prevents electron transfer. Electrochemical measurements has been carried out with CYPs immobilized onto bare electrode (Bistolos et al., 2005), showing that CYPs were absorbed onto the electrode surfaces but the electron transfer estimated with these systems was low and poorly efficient (Fantuzzi et al., 2004). It is necessary to keep under control both the concentration at the electrode surface and the orientation of the enzyme, in order to improve the sensor sensitivity. The inclusion of CYPs in an appropriate medium like a conductive polymer or the non-specific absorption of CYPs onto metal electrode modified with nanomaterials, can lead to the formation of a non-controllable and randomly orientated layer of enzymes. On the other hand, an orientated packed enzyme-monolayer covalently bonded to the electrode surface could be realized through the specific functionalization of both the CYPs ends and the metal surface. More in general, there are different techniques for the CYPs immobilization and the electrode surface modification which depend on the applications, but all of them avoid protein denaturation and preserve an appropriate orientation thus increasing electron transfer. Here, the main techniques used for modify the electrode surfaces are reported:

- a. *Clay nanoparticles-modified electrodes.* Clay minerals are usually ion-exchangeable aluminosilicates, very widespread in geologic deposits. The mineral group of clays includes sodium montmorillonite, smectite, laponite, kaolinite, talc, goethite and orche. Clay particles are very small and their size is comparable with protein sizes so that they are really effective for mediate the electron transfer between electrode and biocomponent. In aqueous solutions clay produces colloid solutions with 1-100 nm size. Thus, clay materials can form films by dropping, casting and drying of a colloidal clay suspension, while with ionic surfactants they can generate biomembrane-like structures (Eggins, 2003; Shumyantseva et al., 2004) or can create protein-films by layer-by-layer technique. For instance, direct electrochemistry of cytochrome P450 at layer-by-layer clay modified carbon electrode was achieved (Lei et al., 2000). Sodium montmorillonite, kaolinite, talc, goethite and orche have been used to modify electrodes and investigate the electrochemistry of heme proteins (Bistolos et al., 2005).
- b. *Phospholipid and vesicle-systems modified electrodes.* In order to mimic the physiological environment of CYP enzymes, i.e. the hydrophobic environment in the endoplasmic reticulum of cell, synthetic phospholipids has been used for the construction of biosensors. These films which mimic the cellular membrane structure are surfactant or bilayer lipid films made by didodecyldimethylammonium bromide (DDAB), dimeristoyl-1-phosphatidylcholine (DMPC), dilauroylphosphatidylethanolamine (DLPE) and distearoyl-phosphatidyl-ethanolamine (DSPE). The phospholipid layer-structure may facilitates electron transfer between the enzymes redox centre and the electrode. Stable films can be cast onto surfaces from solution of surfactants in

organic solvents or from aqueous vesicle dispersion, since the surfactants are insoluble in water. Then evaporation of solvent leads the surfactant to auto-assembly into phospholipid biomembrane-like films (Wu & Hu, 2007). In some studies (Iwuoha et al., 1998), CYPs were incorporated in vesicle dispersion of the synthetic material DDAB and then immobilized by cross-link onto glassy carbon electrode. Other applications involving CYPs for biosensor production used glutaraldehyde and synthetic phospholipids for the immobilization of semisynthetic flavocytochromes based on CYP2B4, CYP1A2 and mitochondrial P450_{scc} onto rhodium-graphite electrodes (Shumyantseva et al., 2001), or films of surfactants with CYP176A (Aguey-Zinsou et al., 2003).

- c. *Electrodes modified with multilayer films.* In order to improve the direct electron transfer between electrodes and heme proteins like CYP, multilayer-deposition techniques, such as layer-by-layer (LBL) polyionic films of CYPs, have been studied (Bistolos et al., 2005). The repetition of cycles of enzyme and polyion adsorption alternately with intermediate washing provides multilayer stable films with reproducible amounts of enzyme in each layer and nanometer-scale control of thickness. More than 20 enzymes and proteins have been incorporated into films by this electrostatic layer-by-layer self-assembly technique. Synthetic polyions, metal oxide nanoparticles, and biological polyions such as DNA have been used to alternate with the enzyme layers. Some examples are (i) Au and pyrolytic graphite electrodes modified by LBL film of poly-(styrenesulfonate) (PSS) and/or branched poly(ethyleneimine) (PEI) and CYP101 thus creating CYP101-multilayer films (Munge et al., 2003), (ii) PSS-CYP1A2 multilayers grown on carbon cloth electrodes (Estavillo et al., 2003), (iii) LBL enzyme films on Au electrodes by alternate adsorption of a layer of CYP3A4 on top of a layer of PDDA (poly(dimethyl diallyl) ammonium chloride), (Joseph et al., 2003), and (iv) Polyaniline (Pan)-doped glassy carbon electrode (GCE) and CYP2D6 (Iwuoha, Wilson, Howel, Mathebe, Montane-Jaime, Narinesingh, Guiseppi-Elie, 2000).
- d. *Nanomaterials.* Materials in the nanometric size regime display size-dependent optical, electronic, and chemical properties. Nanoparticles have several advantages, such as large surface area, high-powered catalysis, excellent affinity, and also their nanometric-size, since is comparable with protein size, influence the rate of electron transfer thus enhancing the electrochemical catalytic activity of enzymes. Several material nanoparticles have been used to immobilize proteins, including gold, colloidal gold (Wu & Hu, 2007), SiO₂ (He et al., 2004; Liu et al., 2004), MnO₂, ZrO₂ (Liu et al., 2008; Peng et al., 2008) and TiO₂ nanoparticles and carbon nanotubes (see paragraph 5.2.1). The immobilization of protein on gold nanoparticles can help the protein to keep a favoured orientation or to create conducting channels between the prosthetic groups and the electrode surface, thus reducing the effective electron transfer distance (Wu & Hu, 2007). It has been demonstrated (Shumyantseva, Carrara, Bavastrello, Riley, Bulko, Skryabin, Archakov, Nicolini, 2005) that gold nanoparticles improve the sensitivity of cholesterol biosensor with cytochrome P450_{scc}. Other studies (Liu et al., 2008; Peng et al., 2008) showed the feasibility in enhance redox current of CYP2B6 to be incorporated into films made by zirconium dioxide nanoparticles and platinum components (Peng et al., 2008) and into a chitosan modified colloidal gold nanoparticles (Liu et al., 2008).

- e. *Self-Assembled monolayers (Figure 17) and Langmuir-Blodgett protein films.* Redox proteins can be adsorbed via electrostatic interaction or covalently immobilized on Self-assembled monolayers (SAM), which can lead to a better-controlled electron transfer between the protein and the electrode (Yang et al., 2009). With SAMs is possible to regulate the distance between the heme group of CYP and the electrode surface, changing the chain length (the so called 'tail') of SAMs which allow the film to be tightly packed and oriented on the surface thanks to the weak interactions between the tails. Usually alkane-thiol or other thiol-terminated chains covalently bind the self-assembled molecule to the metal surface of the electrode. At the other end of the chains a group is able to specifically interact with a group on the protein surface, thus adding selectivity to the modified electrode for particular proteins. Figure 17 shows the model of CYP2C9 bonded to the Gold-SAM electrode surface. The Langmuir-Blodgett technique allows one to form highly ordered amphiphilic lipid monolayers at the air-water interface which can be used to study the interaction of proteins and phospholipids and to immobilize enzymes such as CYPs onto the electrode surface. LB monolayers offer high thermal stability thanks to the close packing of molecules and it has been demonstrated that its behaviour on electrodes is dependent on the number of monolayers (Nicolini et al., 2001). Recombinant and wild types of CYP11A1 (cytochrome P450sc) have been immobilized onto electrode surfaces with LB technique for cholesterol biosensing (Nicolini et al., 2001). Other cytochrome P450 isoforms were immobilized by the LB technique, and used as biological recognition element for biosensor fabrication. The substrates chosen for the detection utilized were clozapine for CYP1A2, styrene for CYP2B4, and cholesterol for CYP11A1 (Paternolli et al., 2004).

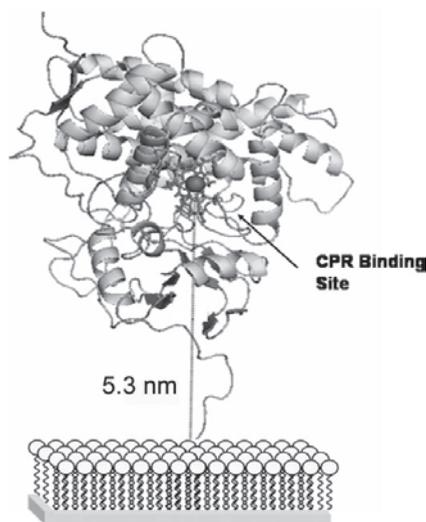


Fig. 17. Model of CYP2C9 bonded to the Gold-SAM electrode. The gold surface/SAM to iron atom separation is shown (Yang et al., 2009). Reprinted with permission from Yang et al., 2009. Copyright 2003 Drug Metabolism and Disposition by American Society for Pharmacology and Experimental Therapeutics.

- f. *Sol-Gel matrix systems.* This kind of bioelectrode is suitable for applications in organic phases. In the sol-gel immobilization onto electrode, the silicate matrix is formed through the acid or base hydrolysis of an alkoxide precursor, such as tetramethyl orthosilicate (TMOS), or more in general of the silane monomer followed by condensation reaction, which generate a high-density silica gel. Therefore, if a protein is added after the partial hydrolysis of the TMOS precursor, a porous matrix is formed around the protein molecule, entrapping it in an aqueous microenvironment similar to that of an aqueous solution of protein (Eggins, 2003). The resulting sol-gel has been reported to be chemically, thermally and structurally stable, with a various pore-size structure which is large enough to allow substrate/product diffusion into and out of the sol-gel network but still being small enough to prevent leaching of the enzyme. In a study (Iwuoha, Kane, Ania, Smyth, Ortiz de Montellano, Fuhr, 2000), a composite amperometric biosensor has been prepared by encapsulating a cytochrome P450cam /didodecyl-dimethylammonium bromide (DDAB) liquid crystal system in a methyltriethoxysilane (MTEOS) sol-gel.

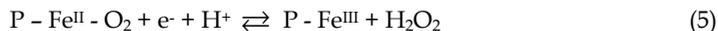
5.2 Electrochemical characterization of cytochrome P450 for drug sensing

Using an amperometric biosensor, it is possible to follow the catalytic cycle of CYP by the quantification of the electron transfer between the electrode and the enzyme. Thus it is possible to indirectly measure the concentration of the drug or metabolites present in the sample by measuring the current in the electrochemical cell. There are several electroanalytical techniques for the measure of substrate concentration, such as cyclic voltammetry, square wave voltammetry and amperometry (Thévenot et al., 2001). Cyclic voltammetry (CV) is one of the mostly considered technique in electrochemistry for biosensing applications. Compared to other electroanalytical techniques, CV is the most appropriate in the case of CYP, because of the uncertainty of CYP peaks potential and because with CV is possible to identify more substrate molecules at a time with the same biological recognition element (Eggins, 2003). The biosensing is carried out by the measurement of the current-peaks height found in the voltammograms resulting from the application of a potential scan, since the height of the peaks is proportional to the drug/metabolite concentration, while its position in potential gives information on the substrate chemistry. Several attempts to measure the presence of drugs in sample have been carried out with cytochrome P450-based biosensors (Bistolos et al., 2005). Table 2 reports peaks position of different CYPs obtained trough cyclic voltammetry. It is important to know that the redox potential mostly depends on the method used for the CYP immobilization onto the electrode surface.

Cyclic voltammograms registered with a CYP-based biosensor have different shape depending on some factors, such as the pH and the ionic strength of the buffer solution, the temperature, the immobilization technique and the scan rate, but mostly on the presence/absence of oxygen (O₂), and obviously of the substrate (Johnson et al., 2005).

In the absence of the CYP substrate, the mechanism of the reaction between the CYP immobilized onto electrode surface and O₂ is outlined as follows (Guengerich, 2001):





In the absence of oxygen in the buffer solution, one electron is first transferred from the electrode to the immobilized CYP (P is for the protein), leading to the reduction of the ferric heme iron (Fe^{III}) to the ferrous form (Fe^{II}), according to the equation (3). This is a reversible electrode reaction, where one proton and the iron heme ion participate in the electron transfer process (Liu et al., 2008). In the presence of dissolved oxygen, the shape of the cyclic voltammogram visibly changed with an increase of the reduction peak current and the decrease of the oxidation peak current (Fig. 18). The mechanism of the reaction between the CYP and O_2 is reported in the equations 4 and 5. Following CYP reduction, the ferrous heme iron of CYP (Fe^{II}) quickly binds to O_2 to form the ferrous-dioxygen complex ($\text{Fe}^{\text{II}}-\text{O}_2$, equation 4). This unstable highly-reactive ferrous-dioxygen complex can easily accept a second electron to be oxidized back to its ferric form, while the oxygen is catalytically reduced to H_2O_2 (Estavillo et al., 2003). The CYP ferric form could be reduced again, resulting in pronounced reduction currents observed that are typical for electrocatalytic oxygen reduction (Liu et al., 2008; Peng et al., 2008). In figure 18 a comparison between cyclic voltammograms obtained with argon-saturated buffer, air-saturated buffer and air-saturated buffer in presence of the CYP2B6 substrate, is reported (Shumyantseva et al., 2004).

CYP species	Electrode modification	E° potential	Reference
CYP1A2	Multilayer with PSS-Carbon cloth	-310mV(vs SCE)	(Estavillo et al., 2003)
	PEI(/PSS/CYP) multilayer on pyrolytic graphite	-330mV(vs Ag/AgCl)	(Krishnan et al., 2009)
CYP2B4	DDAB/Au/CYP	-323mV(vsAg/AgCl)	(Shumyantseva et al., 2009)
CYP2B6	ZrO ₂ /Pt/PLL-film	-449mV(vsAg/AgCl)	(Peng et al., 2008)
	CYP/Au-chitosan/GCE	-454mV(vsAg/AgCl)	(Liu et al., 2008)
CYP2C9	DDAB/CYP film on PGE electrode	-41mV(vs NHE)	(Johnson et al., 2005)
CYP2C18	DDAB/CYP film on EPG electrode	-70mV(vs NHE)	(Shukla et al., 2005)
CYP2C19	DDAB/CYP film on EPG electrode	-45mV(vs NHE)	(Shukla et al., 2005)
CYP2D6	Polyaniline doped GCE	-120mV(vs SCE)	(Bistolas et al., 2005)
CYP2E1	PEI(/PSS/CYP) multilayer on pyrolytic graphite	-347mV(vs Ag/AgCl)	(Krishnan et al., 2009)
CYP3A4	Au-MPS-PDDA multilayers	-470mV(vs Ag/AgCl)	(Joseph et al., 2003)
	Pt-CYP-DDAB-BSA-Glu multilayers	-689mV(vs Ag/AgCl)	(Ignaszak et al., 2009)
CYP11A1	Langmuir Blodgett films ITO glass plate	-295 to -318mV (vs Ag/AgCl)	(Bistolas et al., 2005)

Table 2. List of CYP used for biosensor and their electrochemical parameters obtained with cyclic voltammetry technique.

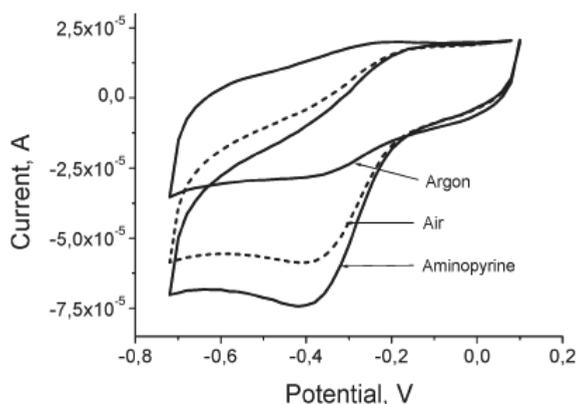


Fig. 18. Effect of oxygen and aminopyrine on the CYP2B4 electrode with mixed film of CYP2B4, Tween 80, and clay (Shumyantseva et al., 2004). Reprinted with permission from Shumyantseva et al., 2004. Copyright 2004 American Chemical Society.

When the drugs (substrates) are added to a buffer solution in presence of oxygen, a further increase of the CYP reduction current is observed (Fig. 18, spectrum of Aminopyrine), (Shumyantseva et al., 2004). It is known that the binding of substrates to the ferric CYP (Fe^{III}) induces a change in spin state, from low spin to high spin and this change in spin state makes the redox potential of the enzyme shift towards more positive values, thus thermodynamically facilitating the transfer of the first electron from the electrode (Liu et al., 2008). So, the binding of the drug substrate to the enzyme promote the CYP reduction to its ferrous form, resulting in a faster oxygen binding and making more favorable the transfer of the second electron to the ferrous-dioxygen complex (Liu et al., 2008; Peng et al., 2008).

It is possible to conclude that the coupling of the bioelectrocatalytic oxygen reduction current and the bioelectrocatalytic substrate conversion occurs when the substrate is added to a buffer solution in presence of oxygen. Indeed, this phenomenon has been verified for a number of different P450 enzymes in solution, including CYP101, CYP102 and CYP11A1 (Johnson et al., 2005), and for a number of different CYPs immobilized onto electrodes, such as CYP2C9 (Johnson et al., 2005), CYP2B4 (Shumyantseva et al., 2004,2007), CYP3A4 (Joseph et al., 2003), CYP2B6 (Liu et al., 2008; Peng et al., 2008), CYP2D6 (Iwuoha et al., 2007), and CYP1A2 (Antonini et al., 2003; Estavillo et al., 2003). The simplified reaction scheme reported in figure 19, explains both the bioelectrocatalytic oxygen reduction current and the bioelectrocatalytic substrate conversion (Shumyantseva et al., 2004).

The first three reactions reported in Figure 19 are respectively the substrate binding (reaction 1), the first electron transfer (reaction 2) and the oxygen binding (reaction 3), with the formation of the dioxygen-ferrous complex. The latter accepts a second electron (reaction 4), leading to the formation of an highly reactive peroxy intermediate. The addition of protons H^+ to this intermediate results in the insertion of an oxygen atom into the substrate (ROH), which is then released and the CYP is restored to its starting ferric state (reaction 5). The uncoupling can occur on the level of the dioxygen (reaction 6) or peroxy complex (reaction 7). The catalytic oxygen reduction (with the peroxide formation), regenerates the ferric enzyme, which may again be reduced in a further reaction cycle (reaction 2), thus enhancing the electrochemical reduction (Estabrook et al., 1996).

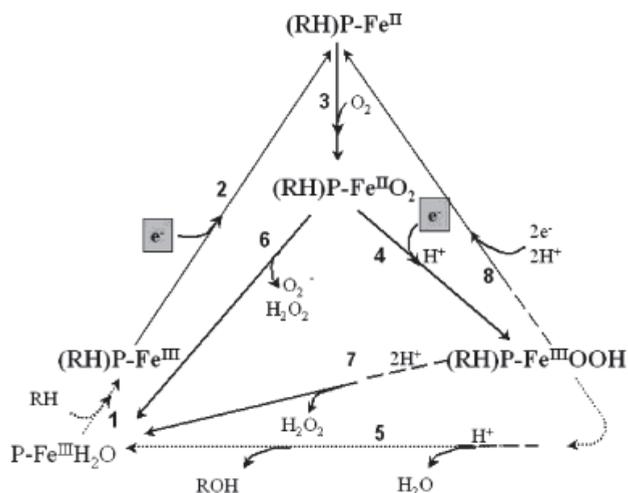


Fig. 19. General scheme of the electrocatalytic reaction of CYP in presence of oxygen and its substrate (RH), (Shumyantseva et al., 2004). Reprinted with permission from Shumyantseva et al., 2004. Copyright 2004 American Chemical Society.

Biosensors based on individual P450 proteins have already been proposed to develop novel tools for drug detection in human serum (Bistolos et al., 2005; Cavallini et al., 2010; Ghindilis, 2000). The biosensing is mainly based on the biochemical reaction schematized in figure 20 where a cytochrome P450 transform the redox form of a drug (the Cyclophosphamide in the example) in its oxidized form by using one oxygen molecules and two electrons.

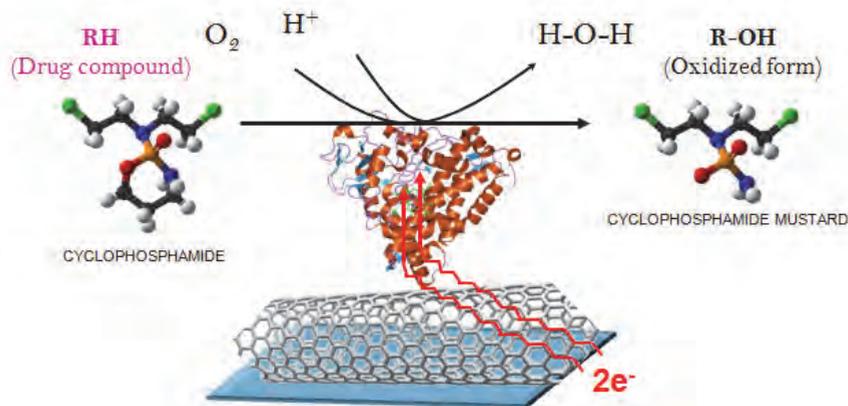


Fig. 20. The oxidation of a drug (Cyclophosphamide) catalyzed by the cytochrome P450.

The two electrons (which in nature are provided by the NADPH molecules) are supplied by the flowing current from the electrode. As explained previously, is possible to measure this catalytic current and, thus, to indirectly measure the drug concentration through the

CYP species	Drugs	Description	Reduction potential (vs Ag/AgCl)	Reference
CYP1A2	Clozapine	Antipsychotic for schizophrenia	-265mV	(Antonini et al., 2003)
	Ftorafur	Anticancer	-430mV	*
CYP2B4	Aminopyrine	Analgesic, anti-inflammatory and antipyretic	-400mV	(Shumyantseva et al., 2004)
	Benzphetamine	Anorectic	-250mV	(Shumyantseva et al., 2007)
CYP2B6	Bupropion	Antidepressant	-450mV	(Liu et al., 2008)
	Cyclophosphamide	Anticancer and immunosuppressive	-450mV	(Liu et al., 2008)
	Ifosfamide	Anticancer and immunosuppressive	-430mV	*
	Lidocaine	Anesthetic and antiarrhythmic	-450mV	(Peng et al., 2008)
CYP2C9	Diclofenac	Analgesic and anti-inflammatory	-41mV	(Johnson et al., 2005)
	S-Warfarin	Anticoagulant	-36mV	(Johnson et al., 2005)
	Sulfaphenazole	Antibacterial	-41mV	(Johnson et al., 2005)
	Tolbutamide	Stimulator for insulin secretion (treatment of type II diabetes)	-37mV	(Johnson et al., 2005)
	Torsemide	Diuretic	-19mV	(Johnson et al., 2005)
CYP2D6	Fluoxetine	Antidepressant	-327mV	(Iwuoha, Wilson, Howel, Mathebe, Montane-Jaime, Narinesingh, Guiseppi-Elie, 2000)
	Sertaline	Antidepressant	-275mV	(Iwuoha et al., 2007)
CYP2E1	P-Nitrophenol	Intermediate in the synthesis of paracetamol	-300mV	(Fantuzzi et al., 2004)
CYP3A4	Cyclophosphamide	Anticancer and immunosuppressive	-450mV	*
	Erythromycin	Antibiotic	-625mV	(Hendricks et al., 2009)

Ifosfamide	Anticancer and immunosuppressive	-435mV	*
Indinavir	Anti-HIV	-750mV	(Ignaszak et al., 2009)
Midazolam	Anxiolytic, anaesthetic, sedative, anticonvulsant, and muscle relaxant	-	(Joseph et al., 2003)
Quinidine	Beta blocker	-	(Joseph et al., 2003)
Progesterone	Steroid hormone	-	(Joseph et al., 2003)
Verapamil	For the treatment of hypertension, angina pectoris, cardiac arrhythmia	-100mV	(Joseph et al., 2003)

* Measurements obtained in studies performed by the authors, immobilizing CYPs isoforms onto carbon nanotubes.

Table 3. List of CYPs used for the detection of drugs for common diseases and their reduction potential obtained with cyclic voltammetry technique.

analysis of reduction peaks obtained in the cyclic-voltammograms. The electron transfer can be enhanced by electrodes nanostructuring, as using metallic or zirconium dioxide nanoparticles, carbon-nanotubes (Bistolas et al., 2005; Eggin, 2003), or other techniques for the enzyme immobilization onto the electrode surface, which have been already explained in the previous paragraph. Different studies demonstrated that carbon-nanotubes (schematized in figure 20) promote the electron transfer between the CYP active site and the electrode and enhance biosensor sensitivity (Lyons & Keeley, 2008; Wang, 2005). In table 3 a list of a target drugs which have been detected with several CYP isoforms used as biological recognition element of biosensors is reported.

So, the cytochromes P450 may be used to detect drug compounds commonly used in medical treatments by using nanoparticles or carbon nanotubes for improving the device sensitivity to reach the therapeutic ranges found in the patients' serum. Since for the treatments some of the most common diseases, as in anti-cancer therapies, more than one drug are administrated contemporaneously, an array-based biosensor able to measure multiple-drug concentrations at the same time, by using different CYP isoforms, would be very useful and it would find several practical applications. The development of such as biosensor has to overcome several difficulties, first of all the fact that each cytochrome P450 isoform detects many drugs and that different isoforms can detect the same drug (Carrara et al., 2009).

5.2.1 Carbon Nanotube (CNTs)

CNTs can be described as sp^2 carbon atoms arranged in graphitic sheets wrapped into cylinders and can have lengths ranging from tens of nanometers to several microns (Lyons & Keeley, 2008). CNTs can display metallic, semiconducting and superconducting

electron transport, possess a hollow core suitable for storing guest molecules and have the largest elastic modulus of any known material. CNTs can be made by chemical vapour deposition, carbon arc methods, or laser evaporation (Wang, 2005) and can be divided into single-walled carbon-nanotubes and multi-walled carbon-nanotubes (see figure 21). Single-walled carbon nanotubes (SWCNTs) provide good chemical stability, mechanical strength and a range of electrical conductivity. They are around ten times stronger and six times lighter than steel and they can behave as metals, semiconductors or insulators depending on their chirality and diameter (Lyons & Keeley, 2008). The chirality of the SWNT is related to the angle at which the graphene sheets are rolled up (Gooding, 2005). It has been also demonstrated (Gooding, 2005) that the conductivity properties of SWNTs can depend by the presence of catalytic particles, deriving from the fabrication process, the presence of defects in their chemical structure, ion-doping and side-wall functionalizations.

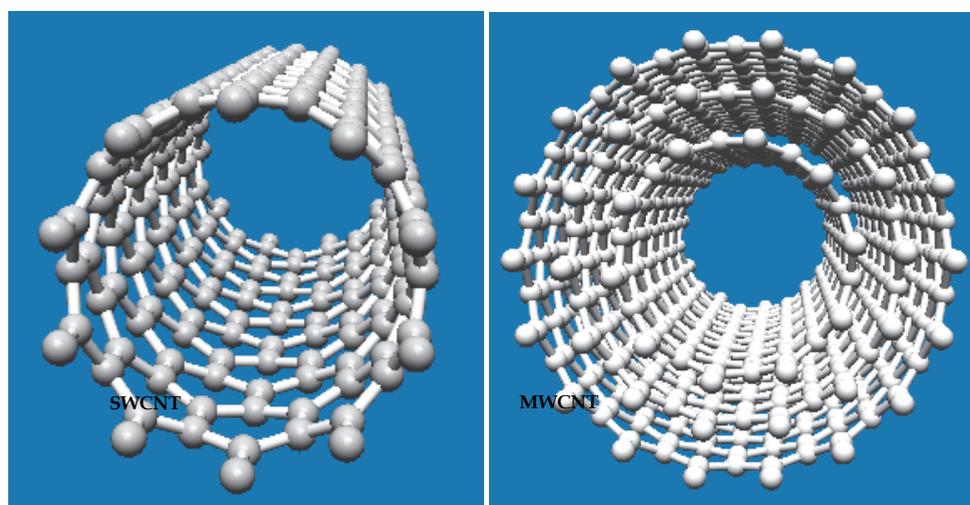


Fig. 21. MWCNT and SWCNT (obtained with Nanotube Modeler © JCrystalSoft, 2010).

Due to their high surface energies, SWCNTs are usually found in bundles or small aggregates composed of 10-100 tubes in parallel and in contact with each other. Multi-walled carbon nanotubes (MWCNTs) are composed of several layers of concentric graphitic cylinders. They are regarded entirely as metallic conductors, making them more suitable for electrochemical applications (Lyons & Keeley, 2008). Anyway, thanks to their electrochemical properties, both multi and single-walled carbon nanotubes could be excellent candidates for the nanostructuring of electrodes used in amperometric biosensor devices. Pre-treatments of CNTs before their deposition onto electrode surfaces, cause the formation of open-ended tubes with oxygenated functional groups, crucial for the electrochemical properties of CNTs. Because of the hydrophobicity due to the CNT walls, in aqueous solution or in polar solvents the tubes have a tendency to rapidly coagulate. Thus, dispersing tubes is usually performed in non-polar organic solvents such as in dimethylformamide (DMF) or chloroform, or with the aid of surfactants or polymers, such as Nafion. The difficulty in dispersing nanotubes in aqueous solution though has been used

as an advantage in preparing nanotube modified electrodes where nanotubes dispersed in an organic solvent are dropped onto an electrode surface and the solvent allowed evaporating. It has been demonstrated that this kind of CNT deposition allows the nanotubes to be strongly adsorbed onto the electrode surface (Gooding, 2005).

5.2.1.1 Electron transfer CNTs-protein

The best strategy for successful enzyme biosensor fabrication is to devise a configuration by which electrons can directly transfer between the redox center of the enzyme and the underlying electrode. This is achievable because the physical adsorption or covalent immobilization of enzymes onto the surface of immobilized carbon nanotubes allows a direct electrical communication between the electrode and the active site of redox-active enzymes. It has been reported (Wang, 2005) that a redox enzyme, such as the glucose oxidase or cytochrome P450, adsorbs preferentially to edge-plane sites on nanotubes. Such sites contain a significant amount of oxygenated functionalities such as hydroxyl groups or carboxylic moieties formed during the purification of CNT, which provide sites for covalent linking of CNT to biorecognition elements (or other materials) or for their integration onto polymer surface structures (Wang, 2005). Other oxygenated moieties, useful for the protein immobilization, can be also formed by the breaking of carbon-carbon bonds at the nanotube ends and at defect sites present on the side-walls. The nanotubes and enzyme molecules are of similar dimensions, which facilitate the adsorption of the enzyme without significant loss of its shape or catalytic function. It is thought that the nanotube directly reaches the prosthetic group such that the electron tunnelling distance is minimized. In this way, loss of biochemical activity and protein denaturation are prevented (Lyons & Keeley, 2008).

5.2.1.2 Nanostructuring electrode surfaces with carbon nanotubes

There have been a number of approaches to randomly distributing the CNTs on electrodes by dispersing the nanotubes with a binder such as dihexadecyl-hydrogen phosphate or Nafion, forming the nanotube equivalent of a carbon paste which can be screen printed, forming a nanotube-*teflon* composite, drop coating onto an electrode without any binders, preparing a nanotubes paper as the electrode and abrasion onto the basal planes of pyrolytic graphite. The resultant electrode has randomly distributed tubes with no control over the alignment of the nanotubes. To better control the alignment of nanotubes a more versatile approach to producing aligned carbon nanotube arrays is by self-assembly, by using self-assembled monolayers (after the functionalization of the carboxylic-ends of CNTs with carbodiimide groups and thiols), or by directly growing of aligned nanotubes onto the surface. To do this plasma enhanced chemical vapor deposition using a nickel catalyst on a chromium coated silicon wafer can be used (Gooding, 2005). Advantages in using this method are the robustness of these electrodes and also the control over the density of the CNT film by controlling the distribution of the catalyst on the surface (Salimi et al., 2005). Figure 22 reports a comparison between SEM images of MWCNTs (on the bottom) and MWCNTs covered by 1 layer of CYP3A4 (on the top). The CNTs has been deposited by drop casting technique onto the electrode surface (30 μ L of a solution 1mg/ml of MWCNTs in chloroform). In the figure is visible the increase of apparent CNTs diameter due to the presence of a layer of CYP3A4 (on the top), that has been deposited by drop casting onto the CNT-surface.

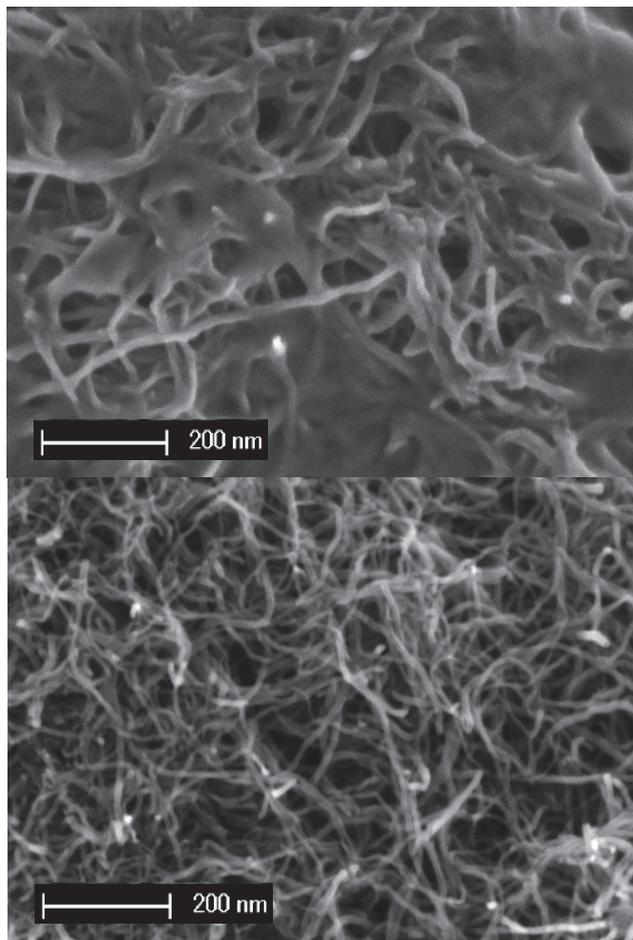


Fig. 22. Comparison between SEM images of MWCNTs (on the bottom) and MWCNTs covered by 1 layer of CYP3A4 (on the top), both at 80,000X of magnification.

5.2.1.3 Enhancement of catalytic current with CNTs

The chemical modification of electrode surfaces with carbon nanotubes has enhanced the activity of electrode surfaces with respect to the catalysis of biologically active species such as hydrogen peroxide, dopamine and NADH. Furthermore, multi-walled carbon nanotubes have exhibited good electronic communication with redox proteins where not only the redox center is close to the protein surface such as in Cytochrome c (Zhao et al., 2005) and horseradish peroxidase, but also when it is deeply embedded within the glycoprotein such as is found with glucose oxidase (Gooding, 2005). A recent study (Carrara et al., 2008) demonstrated the enhancement of the catalytic current in a P450-based enzyme sensor in the case of electrodes modified with MWCNT, with respect to the case of both the bare electrodes and the electrode modified with gold nanoparticles. In figure 23, a comparison between cyclic voltammograms of screen-printed bare electrode (1), electrode modified with

Au nanoparticles and CYP11A1 (2) and with MWCNTs and CYP11A1 (3) is reported. In these voltammograms, a huge increase of the current peak is observable in the case of the P450 working electrode modified with gold nanoparticles respect to the bare electrode, but a further enhancement of the peak current is clearly visible in the case of MWCNTs-modified electrode with P450 (Carrara et al., 2008).

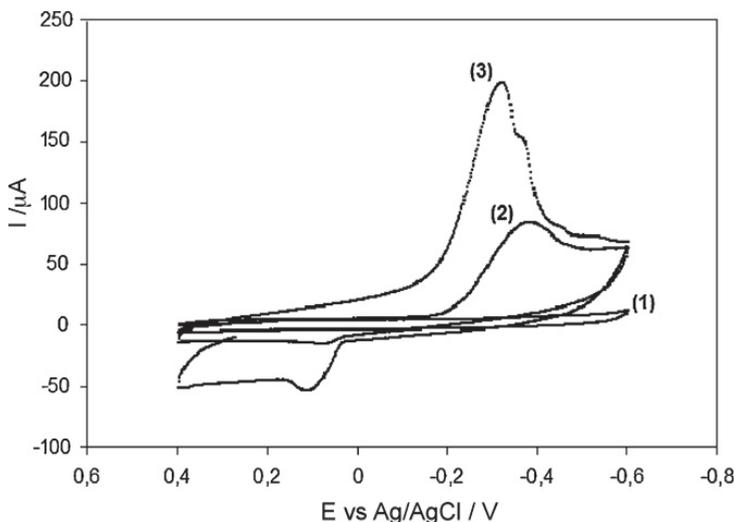


Fig. 23. Cyclic voltammograms of screen-printed bare electrode (1), electrode modified with Au nanoparticles and CYP11A1 (2) and with MWCNTs and CYP11A1 (3), (Carrara et al., 2008). Reprinted from *Biosensors and Bioelectronics*, Vol. 24, Sandro Carrara, Victoria V. Shumyantseva, Alexander I. Archakov, Bruno Samori, "Screen-printed electrodes based on carbon nanotubes and cytochrome P450scc for highly sensitive cholesterol biosensors", Pages No. 148–150, Copyright (2008), with permission from Elsevier.

This is the direct proof that the CNT improve the electron transfer between the electrodes and the heme groups of the cytochromes. Moreover, in the presence of MWCNT, the peak is shifted in the positive direction of the voltage axis, because P450 is easier reduced in the presence of CNT, i.e. it is easier to reduce the heme iron incorporated in the protein core.

6. Conclusions

In this chapter the feasibility of cytochrome P450 as probe molecule for the design of an electrochemical biosensor for drug detection in biological fluids has been investigated. Cytochromes P450 have been chosen since they are known to be involved in the metabolism of over 1,000,000 different xenobiotic and endobiotic lipophilic substrates, in particular in the metabolism of ~75% of all drugs. The majority of cytochromes involved in drug metabolism exhibits a certain genetic polymorphism, i.e. mutations in the CYP genes that can cause the enzyme activity to be abolished, reduced, altered or increased, with substantial consequences in drug metabolism, such as an exaggerated and undesirable pharmacological response. In order to individually optimize an ongoing drug therapy, it is required to measure the plasma concentrations of drugs or their metabolites after the

administration. This is needed for really understand how the patient metabolize drugs at the moment of the pharmacological cure. It is a strong need since most effective drug therapies for major diseases still provide benefit only to a fraction of patients, typically in the 20 to 50% range. At the present state-of-the-art the technology allows only to check the genetic predisposition of patients to metabolize a certain drug, without taking into account the many factors that can influence drug metabolism, such as lifestyle, drug-drug interactions and cytochrome P450 daily variation of the polymorphism. Although CYPs are capable in general of catalyse around 60 different classes of reactions, they have a number of features in common, such as the overall fold structure, the presence in their active site of the heme group, that allow the electron transfer to catalyze substrate oxidations and reductions, and the typical catalytic cycle which requires oxygen and electrons as part of the process of metabolism.

CYPs ability to metabolize a broad spectrum of endogenous substances, e.g., fatty acids, steroid hormones, prostaglandins and in particular foreign compounds such as drugs, has made this enzyme family interesting as recognition element for biosensing. P450-based biosensors are of great interest due to the possibility of developing applications such as the detection of analytes and drugs, since the currently-available methods used for in vitro quantifying the levels of drugs in biological fluids are time-consuming and expensive. A cytochrome P450 biosensor may be a promising alternative that would provide quick measurements for drugs and metabolites with a cheap, simple to use, rapid and, in some instances, disposable equipment, which also supplies good selectivity, accuracy and sensitivity. The most suitable approach for the design of a CYP-based biosensor is the direct mediatorless electron supply from an electrode to the redox active group of the CYP, thus leading a direct flow of electrons to the enzyme. In the development of this mediator-less approach, the immobilization of CYP onto the electrode surface has to be deeply controlled in order to obtain a high probability for the protein to be attached to the electrode in a proper orientation that could optimize the electron transfer to the heme group. In this chapter different techniques for the immobilization of CYPs onto the electrode surface have been described as reported in literature, focusing the attention also on the use of nanostructures (e.g. carbon nanotubes), to improve the biosensor sensitivity.

Finally, a list of drugs which have been detected with several CYP isoforms has been reported with data found in literature as well as data obtained by the authors. It is possible to conclude that cytochromes P450 may be used to detect drug compounds also reaching the therapeutic ranges found in the patients' blood, thanks to improved performances due to nanostructured-electrodes. Since for the treatments of some of the most common diseases (e.g. in anti-cancer therapies), more than one drug are administrated contemporaneously, an array-based biosensor able to measure multiple-drug concentrations at the same time, by using different CYP isoforms, would find several practical applications and it could be a first step toward the development of a real chip for personalized-medicine. Electrode miniaturization is the next mandatory step in order to test the real feasibility of this cytochrome-based biosensor as a fully-implantable device for the detection of drugs and metabolites, as much as the evaluation of the biocompatibility of all chip's components, with particular regard to nanostructures and cytochrome cytotoxicity. Finally, kinetics studies of drugs should be carried out in order to better understand drug-drug interaction phenomena and the reactions between drugs and cytochrome P450, with regard to enzyme heterotropic kinetics and its effects on drug metabolism.

A cytochrome P450-based biochip for drug detection should be a very powerful platform for personalization of drug therapy thanks to the key role of P450. However, as it has been shown in this chapter, different P450 isoforms may have the same drug compound as substrate and different drugs may be substrates of the same P450 protein. Proper strategies to develop the multiplexing P450-based biosensor arrays must be studied, considering problems due to multiple enzyme-substrate interactions and in the meanwhile maintaining high reliability and low cost of experimentation.

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