Electrochemistry of Canis familiaris cytochrome P450 2D15 with gold nanoparticles: An alternative to animal testing in drug discovery

Electrochemistry of *Canis familiaris* cytochrome P450 2D15 with gold nanoparticles: an alternative to animal testing in drug discovery

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ABSTRACT

This work reports for the first time the direct electron transfer of the *Canis familiaris* cytochrome P450 2D15 on glassy carbon electrodes to provide an analytical tool as an alternative to P450 animal testing in the drug discovery process. Cytochrome P450 2D15, that corresponds to the human homologue P450 2D6, was recombinantly expressed in *Escherichia coli* and entrapped on glassy carbon electrodes (GC) either with the cationic polymer polydiallyldimethylammonium chloride (PDDA) or in presence of gold nanoparticles (AuNPs). Reversible electrochemical signals of P450 2D15 were observed with calculated midpoint potentials ($E_{1/2}$) of -191±5 and -233±4 mV vs Ag/AgCl for GC/PDDA/2D15 and GC/AuNPs/2D15, respectively.

These experiments were then followed by the electro-catalytic activity of the immobilized enzyme in presence of metoprolol. The latter drug is a beta-blocker used for the treatment of hypertention and is a specific marker of the human P450 2D6 activity. Electrocatalysis data showed that only in the presence of AuNps the expected α-hydroxy-metoprolol product was present as shown by HPLC. The successful immobilization of the electroactive *Canis familiaris* cytochrome P450 2D15 on electrode surfaces addresses the ever increasing demand of developing alternative *in vitro* methods for a more detailed study of animal P450 enzymes' metabolism, reducing the number of animals sacrificed in preclinical tests.

KEYWORDS

CYP, animal testing, carbon electrode, gold nanoparticle, immobilization, metoprolol.

1. Introduction

In view of the huge numbers of animals used during preclinical tests in drug discovery [1], both the European Union (European Commission and European Parliament, Directive 2010/63/EU) [2-3] and the US Food and Drug Administration (FDA) [4] are strongly supporting the development of alternative *in vitro* methods to implement the three Rs, that is "reduce, refine and replace" animal models [5].

Hepatic cytochromes P450 are central to toxicological studies due to their primary role in phase I metabolism of more than 80% of marketed drugs [6]. However to date, very little data is published on comparative *in vivo* interspecies studies with even less data available on the different animal P450 expression and/or their metabolic profile [7-10]. To this end, a fast and reliable *in vitro* method would offer the possibility of a more detailed study of the interaction of new drugs with the animal P450 enzymes therefore increasing the predictive value of these preclinical trials.

Canis familiaris is one of the most widely studied animal models used in safety determination of new pharmaceuticals [11-12] and, although the major isoforms of the human cytochromes P450 2D6, 3A4, 2E1, 2C19 and 1A2 have been identified in *C. familiaris* [13-16], there is still a lack of knowledge on their pharmacogenomic/metabolic diversity [9].

Electrochemical techniques already developed in our lab for human hepatic monooxygenases including cytochromes P450 [17-22] represent the ideal approach for a sensitive, accurate and rapid evaluation of animal P450-drug interactions obviating both the requirement for a redox partner and the addition of NADPH cofactor as already reported for some animal P450 enzymes recombinantly expressed in a soluble form [23-25].

In this work, *Canis familiaris* P450 2D15 was chosen as a model for the investigation of canine cytochromes P450 by adopting electrochemical approaches to provide a method for the screening of the safety of new chemical entities/drugs. This enzyme shares 75% identity with the human cytochrome P450 2D6 which alone is responsible for the metabolism of 20–25% of commonly used therapeutic drugs including antiarhythmics, adrenoceptor antagonists, and tricyclic antidepressants [6, 26]. The high P450 2D6 polymorphism profile is strictly related to either adverse drug reactions or no drug response [26-27] and to date six different variants have been already identified in the corresponding homologous *C. familiaris* P450 2D15 [28-30].

Generally, the study of the catalytic properties of canine P450 2D enzymes is hampered by the difficulty in their preparation from liver microsomes [14] or by their recombinant expression and purification in a stable form when not associated to the membranes [14-15, 28, 30]. To this end, in this work we report for the first time the recombinant expression and purification of the *Canis familiaris* P450 2D15 in a N-terminally modified form maintaining its structural integrity and function. Furthermore, the electrocatalytic functionality of the purified enzyme is tested whilst immobilized on glassy carbon electrodes (GC).

Two different immobilization strategies were adopted: a) entrapment within the cationic polymer polydiallyldimethylammonium chloride (PDDA) (GC/PDDA/2D15); b) immobilization in presence of gold nanoparticles (AuNPs) stabilized with didodecyldimethylammonium bromide (DDAB) (GC/AuNPs/2D15). Gold nanoparticles are widely used in electrochemical applications [31] especially because they act as excellent electron transfer relays by enhancing the electron transfer from the electrode to the protein [32].

Electrochemical properties of *C. familiaris* P450 2D15 immobilized on glassy carbon electrodes were characterized by cyclic voltammetry and the activity of this enzyme in presence of metoprolol, a selective β1 receptor blocker used in treatment of cardiovascular disease and a marker for human P450 2D6 activity, assayed by chronoamperometry. The separation and identification of the product

formed was carried out by HPLC leading to the calculation of the K_M value of metoprolol for the first time through the electrochemical system GC/AuNPs/2D15.

2 Material and methods

2.1 Reagents

Kits for plasmid and gene purification were purchased from Sigma Aldrich (Italy). Restriction enzymes, T4 DNA ligase, Vent Polymerase and dNTPS were from New England Biolabs (UK). Chromatographic resins were purchased from GE healthcare (Italy). Quinidine ((S)-[(2R,4S,5R)-5-ethenyl-1-azabicyclo[2.2.2]octan-2-yl](6-methoxyquinolin-4-yl)methanol) and (±)-Metoprolol ({2-hydroxy-3-[4-(2-methoxyethyl)phenoxy]propyl}(propan-2-yl)amine) (+)-tartrate salt, racemic mixture, were purchased from Sigma Aldrich (Italy). Analytical grade chemicals tetrachloroauric (III) acid (gold(III) chloride), DDAB (didodecyl-dimethylammonium bromide) and PDDA (poly(dimethyldiallylammonium chloride)) were all purchased from Sigma-Aldrich and their solutions prepared prior to their use in appropriate solvent for electrochemical experiments.

2.2 Cloning of recombinant P450 2D15

The gene coding for cytochrome P450 2D15 was amplified from the liver cDNA of *C. familiaris* (Biochain, UK) using the primers Fw: 5'-AGACAGCTATGGGGCTGCTG-3' and Rv: 5'-TGGTTTATTGTACCTCGGGCC-3'. The entire gene was blunt end ligated into pBS SK II(+) cloning vector using the *EcoR*V restriction enzyme.
Subsequently, the full-length cDNA coding for the canine P450 was cloned into a pCW expression vector [33] fused in frame with the sequence coding for the leader peptide of the bacterial OmpA protein (MKKTAIAIAVALAGFATVAQA). This system has been previously used to enhance the expression of native mammalian cytochromes P450 in *E. coli* [14, 34]. This leader peptide is cleaved in the bacterial membrane to yield the native P450 enzymes. A code of four histidine residues was added at the C-terminus before the STOP codon to facilitate the protein purification by affinity chromatography.

2.3 Expression and purification of C. familiaris P450 2D15

Large-scale expression (4 liters) of P450 2D15 in *E. coli* DH5α cells transformed with pCW- 2D15 was carried out as described previously for other P450 cytochromes [35] decreasing the post-induction expression time and temperature to 24 hours and 24 °C, respectively.
Purification of P450 2D15 was carried out starting from the isolated membrane fraction using an anion exchange DEAE sepharose column (GE-healthcare, Italy) and followed by a nickel ion affinity chromatography step (GE-healthcare, Italy) where the protein remained bound through the engineered His-tag and subsequently eluted using a 0–40 mM linear gradient of histidine. In order to preserve the P450 2D15 stability the protein was purified in presence of the human P450 2D6 inhibitor quinidine [36]. The UV-visible spectra of the oxidized, reduced and reduced-carbon monoxide bound forms of the protein were recorded on a Hewlett-Packard 8453 diode array spectrophotometer. The P450 concentration was calculated by the method described by Omura and Sato [37].

2.4 Synthesis of AuNPs

DDAB stabilized AuNPs were synthesized following the procedure described by Castrignanò et al. in 2012 [32]. In particular, 0.5 mL of 10 mM tetrachloroauric (III) acid aqueous solution were

mixed under vigorous stirring to 1 mL of a chloroform 0.1 M DDAB solution for 20 minutes to allow the complete transfer of Au(III) ions from the aqueous to the chloroform. After the two phases were separated, 0.2 mL of freshly prepared aqueous solution of 0.4 M sodium borohydride were slowly added. After additional two hours of vigorous stirring, the red organic phase containing the DDAB stabilized AuNPs was collected and stored at 4 °C before use. TEM morphological characterization showed that the synthesized DDAB stabilized AuNPs mainly consist of spherical particles of about 6.5-8.5 nm diameter, ranging from 2.5 to 13 nm in size.

2.5 Immobilization of P450 2D15 on glassy carbon electrode and electrochemical measurements

All electrochemical experiments were carried out at room temperature (25 °C) and in 50 mM phosphate buffer pH 7.4, containing 100 mM KCl as supporting electrolyte, using an Autolab PGSTAT12 potentiostat (Ecochemie, The Netherlands) controlled by GPES3 software. A conventional three-electrode glass cell of 0.5 mL volume, equipped with a platinum wire counter electrode, an Ag/AgCl (3 M NaCl) reference electrode and 3 mm diameter glassy carbon working electrode (BASi., UK), was also used. Before enzyme immobilization, GC electrodes were mechanically polished with alumina and subsequently rinsed and sonicated in ultra pure deionized water.

The P450 2D15 enzyme was immobilized on glassy carbon (GC) with two different approaches: a) P450 2D15 entrapment in PDDA film by mixing equal volumes of 30 μM protein and surfactant solutions before drop-coating on the electrode surfaces (GC/PDDA/2D15); b) P450 2D15 immobilization on GC electrodes using didodecyldimethylammonium bromide (DDAB) stabilized gold nanoparticles (AuNPs) (GC/AuNPs/2D15). 5 μl of 5 mM colloidal gold in 0.1 M DDAB/chloroform was placed on the electrode surface. After evaporation of the chloroform (10 min), 10 μl of 30 μM P450 2D15 solution was added onto the electrode surface. The electrodes were kept overnight at 4 °C in a humid chamber to prevent their total drying.

Cyclic voltammetry experiments were carried out under anaerobic conditions in a glove box with < 5 ppm oxygen (Belle Technologies, UK). Cyclic voltammograms were collected between +100 and −500 mV (vs Ag/AgCl) at a scan rate range of 20–120 mVs^{-1} in the supporting electrolyte solution. Electrocatalysis experiments were carried out using chronoamperometry applying a potential bias of −650 mV (vs Ag/AgCl) or in cyclic voltammetry at 25°C in presence of metoprolol. The product formed was separated and analyzed by HPLC as previously reported [38]. All electrocatalytic experiments were carried out in triplicates and performed at 200 rpm rotation speed using a RDE-2 rotator system (BASi, USA).

2.6 HPLC analysis of electrocatalysis products

After chronoamperometry in the presence of metoprolol substrate, electrocatalysis solution was collected and an aliquot of 100 μL was injected in HPLC for product quantification. Electrocatalysis products were identified by comparison of retention times to those obtained with authentic standards. For this purpose, standard solutions of metoprolol, α-OH-metoprolol and O-desmethyl-metoprolol were separated isocratically by HPLC (Agilent Technologies-1200 series, Italy) coupled with diode array detector using a 4.6 × 150 mm 5 μm Eclipse XDB-C18 column (Agilent Technologies, USA). Retention times were 3.8 min, 4.7 min and 10.5 min respectively for α-OH-metoprolol, O-desmethyl-metoprolol and metoprolol.

3. Results and discussion

3.1 Recombinant P450 2D15

The gene coding for the full length cytochrome P450 2D15 was amplified from *Canis familiaris* liver cDNA and three mutations were identified by full DNA sequencing. A silent substitution of an adenine in guanine was found 325 bases after the 2D15 start with two other mutations leading to the amino acid substitutions of Ile109Val and Phe115Leu in the full-length protein (g345c and g738a). Cytochromes P450 variants in *C. familiaris* 2D15 are not unusual and have been previously reported [28-30] as they are a consequence of the genetic variability existing between dog colonies [39].

"Here Figure 1"

The gene encoding for the P450 2D15 variant with the three above-mentioned mutations was cloned in the expression vector pCW and the OmpA leader peptide sequence was introduced at its N-terminus to enhance the protein expression levels as described in the materials and methods. The protein was heterologously expressed in *E. coli* and purified from the membrane fraction with a yield of 4.1 mg of P450 per liter of culture. Cytochrome P450 2D15 (57 kDa) (Fig.1A) was purified in presence of quinidine, a known inhibitor of human P450 2D6. The purified protein was also stored in the presence of this inhibitor which has been shown to preserve the stability of the protein [36]. The inhibitor was removed prior to each experimental assay through buffer exchange. Contrary to all previous literature data, in this work the purified *C. familiaris* P450 2D15 protein and not the microsomal or membrane-associated protein was studied.
Binding of carbon monoxide to the dithionite reduced protein confirmed the presence of the correctly incorporated haem cofactor inside the protein scaffold. The protein in its oxidized form presents a Soret peak at 417 nm, the α and β bands at 570 nm and 535 nm, respectively (Fig.1B). As expected, the Soret peak shows the characteristic shift to 450 nm upon reduction and bubbling of carbon monoxide due to the reduced and carbon monoxide-bound adduct and the α and β bands are replaced by a single broad peak centered at 550 nm as shown in Fig.1B. Similarly to the microsomal P450 2D15 reported by Roussel and colleagues [29], a partial amount of inactive protein was also detected at 420 nm (Fig.1B).

3.2 Direct electrochemistry of P450 2D15 on glassy carbon electrodes

Recombinant P450 2D15 was immobilized on glassy carbon electrodes in a non-oriented fashion either by entrapment within the cationic polymer polydiallyldimethylammonium chloride (PDDA) (GC/PDDA/2D15) or in the presence of gold nanoparticles (AuNPs) (GC/AuNPs/2D15) which are one of the most stable metal nanoparticles that provide an effective electron transfer to-and-from the glassy carbon electrode [32, 40].
Once the protein was successfully immobilized on glassy carbon electrodes, cyclic voltammetry experiments were carried out at 25°C under anaerobic conditions (< 5 ppm O_2) to prevent the formation of the Fe^{2+}-dioxygen complex related to the second electron transfer step within the catalytic cycle of cytochromes P450. Cyclic voltammograms of P450 2D15 entrapped with PDDA (GC/PDDA/2D15) or in presence of AuNPs (GC/AuNPs/2D15) are shown in Fig.2A and B, respectively. In both cases the ratio between the anodic and cathodic peak currents were found to be one but the magnitude of the current observed was nearly 4x higher in the presence of AuNPs. As stated by Laviron's theory [41], the linear dependence of the anodic and cathodic peak currents on the scan rate (within the range 20-120 mV) for both GC/PDDA/2D15 and GC/AuNPs/2D15, indicated that the electron transfer from/to the modified GC electrode is quasi-reversible and it is a surface-controlled process as expected for an immobilized cytochrome P450 on electrode surfaces.

"Here Figure 2"

The resulting anodic and cathodic peak currents for GC/PDDA/2D15 were detected at −256±6 mV (Ea) and −127±5 mV (Ec), respectively, with a calculated midpoint potential (Em) of -191±5 mV (vs Ag/AgCl). In the GC/AuNPs/2D15 immobilization strategy, the anodic and cathodic peak currents were measured at -267±5 mV (Ea) and −200±4 mV (Ec) (Fig.2D) and the resulting midpoint potential was calculated to be -233±4 mV. These values are in the range of other electrochemically determined midpoint potentials reported in the literature for different human P450 enzymes [21].

Peak-to-peak separation value was calculated to be -129±6 for GC/PDDA/2D15 and -67±5 mV for GC/AuNPs/2D15 indicating that the presence of the gold nanoparticles facilitates a much faster electron transfer of P450 2D15 when immobilized on glassy carbon electrodes.

Apparent surface coverage of the electroactive protein was calculated from the slope of I_p versus ν plot in accordance with the Brown Anson model using the equation:

$$i_p = n^2 F^2 A \Gamma \nu / 4RT$$

where n represents the number of electrons involved in the reaction, A is the electrode surface area (0.071 cm^2), Γ is the surface coverage, ν is the scan rate, F is the Faraday constant, T is the temperature and R is the gas constant. The surface coverage value on GC/PDDA was calculated to be $1.1X10^{12}$ molecules/cm^2 in the same range previously reported for the *Macaca fascicularis* P450 2C20 immobilized in the same manner [25]. As expected the same calculation of the surface coverage in the presence of AuNps resulted in a value of $4.6x10^{12}$ molecules/cm^2, around 4 times higher than using PDDA alone.

The electron transfer efficiency of the GC/AuNPs/2D15 was further investigated recording a series of cyclic voltammograms varying the pH within the range 6.5< pH <8.5 (Fig. 3). A negative shift in peak potential upon lowering pH was observed which is indicative of proton-coupled electron transfer [42]. The measured shift in the formal potential was around 60 mV per pH unit, close to the theoretical value of 59 mV/pH expected at 25°C for one proton, one electron transfer. The protonation site of the reduced P450 remains unknown but it is reasonable to assume that it is either an amino acid in close proximity of the haem iron or the water bound in the sixth coordination, as has been suggested in the case of other cytochromes P450 [42-43]. Nocera's group [44] have also suggested that proton-coupled electron transfer in P450 enzymes is from a structured water, which is the sixth coordinated H_20 mentioned above.

"Here Figure 3"

3.3 Electrocatalysis of 2D15 immobilized on glassy carbon electrodes

Electrocatalysis by the immobilized P450 2D15 (GC/AuNPs/2D15) was followed by both chronoamperometry and cyclic voltammetry in the presence of metoprolol. The latter drug (Scheme 1) is a cardioselective β1-adrenergic blocking agent metabolized principally by the human P450 2D6 and converted into the α-OH-metoprolol and O-desmethyl-metoprolol products [38]. The amount of O-desmethyl-metoprolol (scheme1-left) after electrocatalysis was found to be below the

detection limits. For this reason, only α-OH-metoprolol (scheme 1-right) was considered as a product formed.

"Here Scheme 1"

As can be seen in Fig. 4A, a catalytic current was observed in air saturating conditions in the presence of metoprolol in cyclic voltammetry experiments. Furthermore, using chronoamperometry at regular internals additions of metoprolol were carried out in the range of 12.5-250 μM under aerobic conditions (Fig. 4B) with an applied bias of -650 mV. The change in the chronoamperometric currents registered due to the different concentrations of the drug showed a Michaelis–Menten kinetic mechanism with a plateau or saturation at higher than 70μM concentrations of metoprolol. The K_{Mapp} calculated from these currents was 39.5±3.5 μM (Fig. 5A).

"Here Figure 4"

Further electrocatalysis experiments were carried out by chronoamperometry for both the GC/AuNPs/2D15 and the GC/PDDA/2D15 systems applying a potential bias of −650 mV (vs Ag/AgCl) for 30 min at 25 °C in presence of different concentrations of metoprolol (Fig. 5B). For each concentration of the drug tested, the contents of the electrochemical cell were analyzed after the 30 min reaction by HPLC in order to separate and quantify the product(s) formed by the immobilized enzyme. No products were detected from the HPLC analysis for the *C. familiaris* P450 2D15 enzyme entrapped with the polycationic agent PDDA. On the other hand, the enzyme immobilized in the presence of AuNps (GC/AuNPs/2D15) was capable of hydroxylating the metoprolol producing 30.7±2.3 pmol of product at the higher metoprolol concentrations used. No O-desmethylated product was observed in line with previously published data on microsomal P450 2D15 [28].

"Here Figure 5"

Control experiments were also carried out in the absence of the protein and as expected no product was detected (data not shown). Quantification of the reaction products in the presence of increasing amounts of metoprolol (Fig. 5B) resulted in a calculated apparent K_M value of 36.2±1.4 μM for GC/AuNPs/2D15 (Table 1) very similar to the K_{Mapp} obtained by titrating different concentrations of metoprolol (see Fig. 5A). The electrochemically determined K_M values are also in the same range as those reported by Ellis and colleagues for human P450 2D6 [45] therefore it can be concluded that the *C. familiaris* P450 2D15 possesses similar enzymatic activity to human P450 2D6, at least for metoprolol metabolism.

"Here Table 1"

4. Conclusions

The development of an electrochemical platform for the screening of animal cytochromes P450 represents an interesting and feasible solution to the ever increasing demand to reduce animal testing, maintaining a high level of predictivity of the animal models used in preclinical tests and providing the opportunity to study the P450 metabolism of new drugs and chemical entities.

To this end, the cloning of a new *Canis familiaris* P450 2D15 variant and its expression in a pure form for its electrochemical application reported in this work, brings us a step closer to the

realization of an *in vitro* electrochemical toxicity screening, an alternative to *in vivo* animal testing and sacrifice.

The P450 2D15 enzyme was successfully immobilized on glassy carbon electrodes in a stable form achieving an efficient electron transfer between the electrode and the enzyme. The presence of gold nanoparticles enhanced the sensitivity of the electrode-protein system and allowed for the estimation of kinetic parameters for metoprolol, a typical drug marker of the human P450 2D6.

These results represent the first step towards the development of electrochemical platform for the rapid exploration of the metabolic diversity existing between animals and humans cytochromes P450 contributing to a more reliable interpretation of the data generated in canine species during the pharmacological preclinical studies.

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Figure legends:

Fig. 1: Purification and spectral characterization of P450 2D15. **(A)** SDS-PAGE gel, lane 1: molecular weight markers, lane 2: purified protein (57 kDa). The absorbance spectra of the purified P450 2D15 **(B)** in the oxidized (solid black line), dithionite reduced (dotted line), and the reduced carbon monoxide-bound form (dashed line).

Fig. 2: Electrochemical characterization of P450 2D15 immobilized on glassy carbon electrodes **(A)** entrapped with the cathionic surfactant PDDA (GC/PDDA/2D15), and **(B)** in presence of gold nanoparticles (GC/AuNPs/2D15). Cyclic voltammogram of P450 2D15 measured at a scan rate of 120 mV/s in 50 mM potassium phosphate buffer pH 7.4 with100 mM KCl at 25 °C. Shown are the original and baseline corrected (upper traces) cyclic voltammograms. Bottom: plot of cathodic (filled symbols) and anodic (empty symbols) peak currents versus scan rate. $R^2 > 0.99$.

Fig. 3: Cyclic voltammograms of GC/AuNPs/2D15 at a scan rate of 120 mVs^{-1} under anaerobic conditions in different pH value buffer. The cyclic voltammogram at pH 7.4 is shown in black.

Fig. 4: Electrochemical responses of GC/AuNPs/2D15 to metoprolol. (A) Cyclic voltammograms of the GC/AuNPs/2D15 in the air-saturated buffer in presence (black) and absence (grey) of metoprolol shown together with control trace obtained in the absence of the protein (dashed); (B) Chronoamperometric response of GC/AuNPs/2D15 at −650 mV followed for 600 sec in 50 mM potassium phosphate buffer pH 7.4. The arrows indicate the addition of different concentrations of metoprolol (12.5-250 μM).

Fig. 5: Michaelis–Menten plot of the turnover of P450 2D15 immobilized on glassy carbon electrode in presence of AuNps (GC/AuNPs/2D15) and metoprolol obtained from: (A) the current registered in the titration experiments (Δ: current increase upon metoprolol addition) and, (B) product quantified by HPLC after 30 min electrocatalysis.

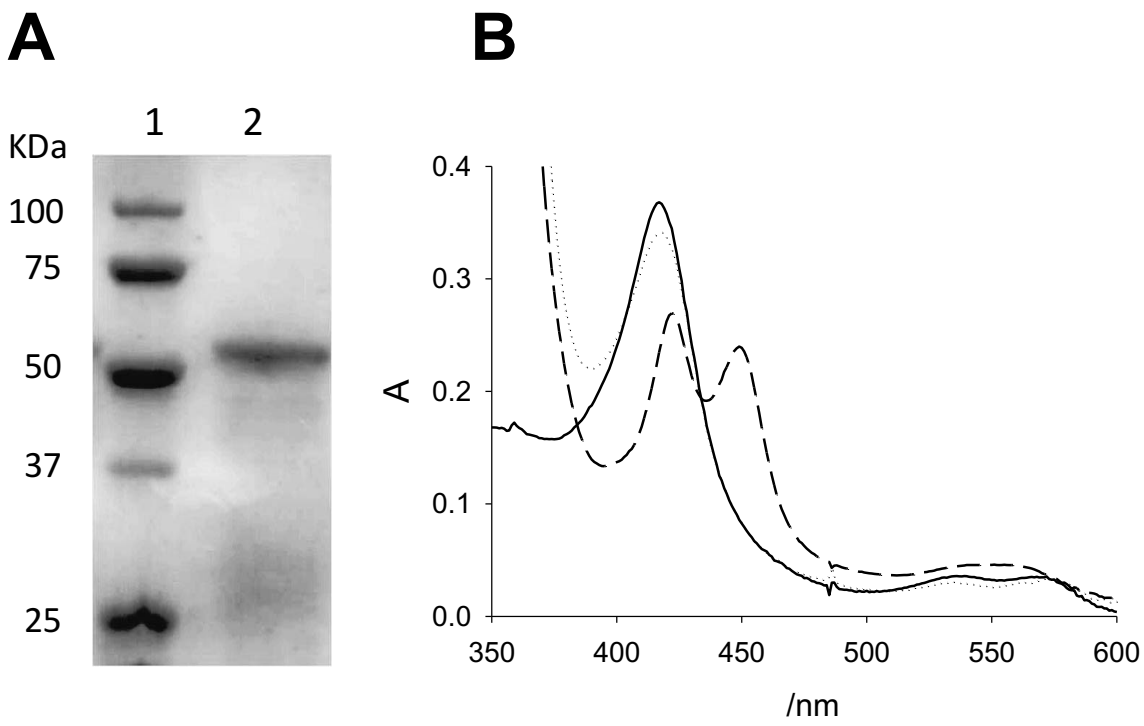

Figure 1

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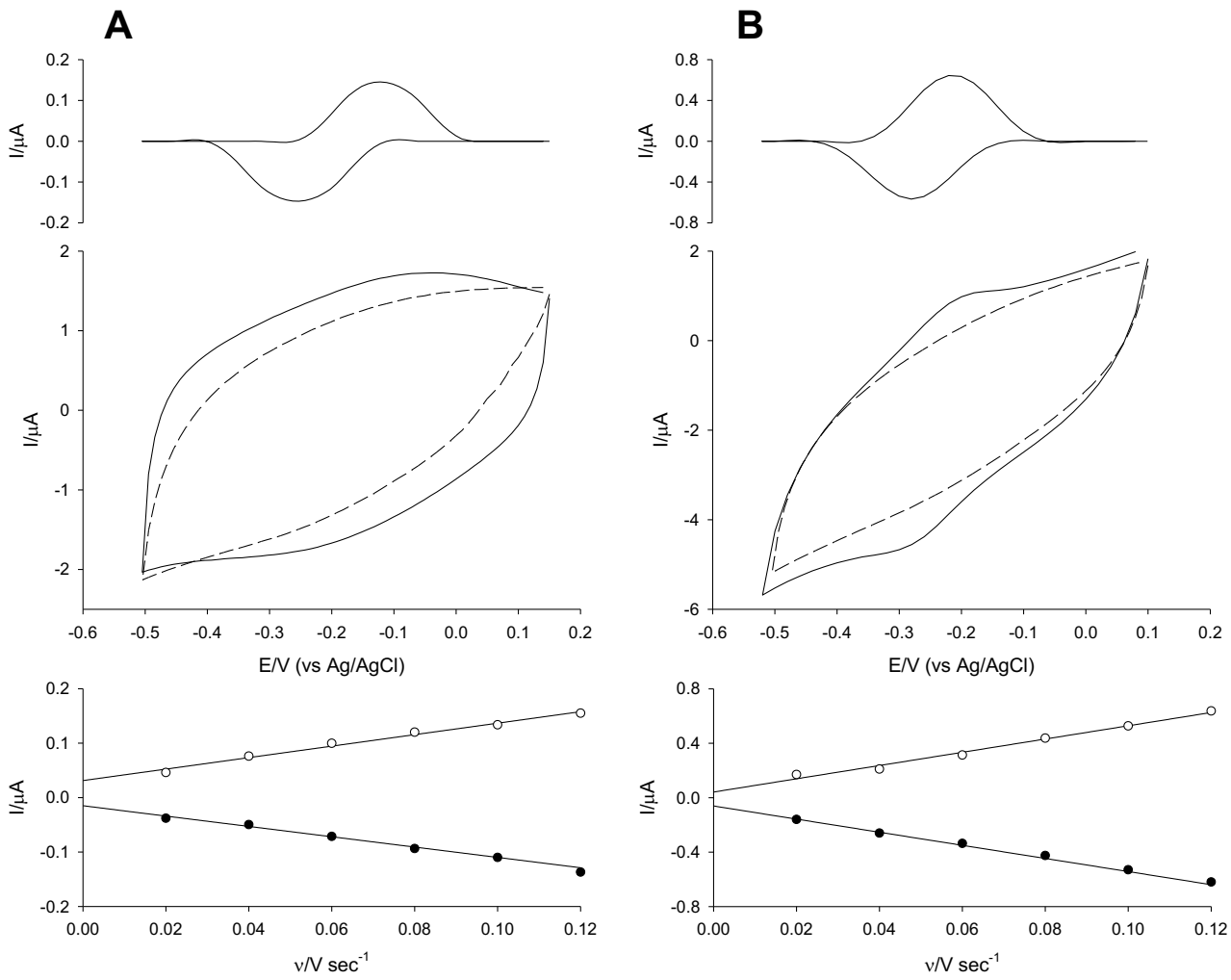

Figure 2

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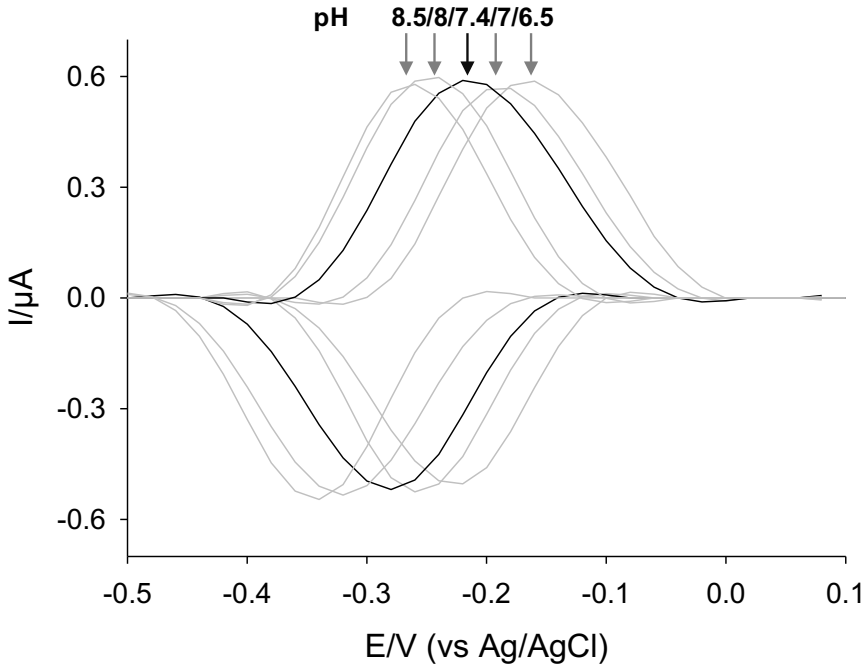

Figure 3

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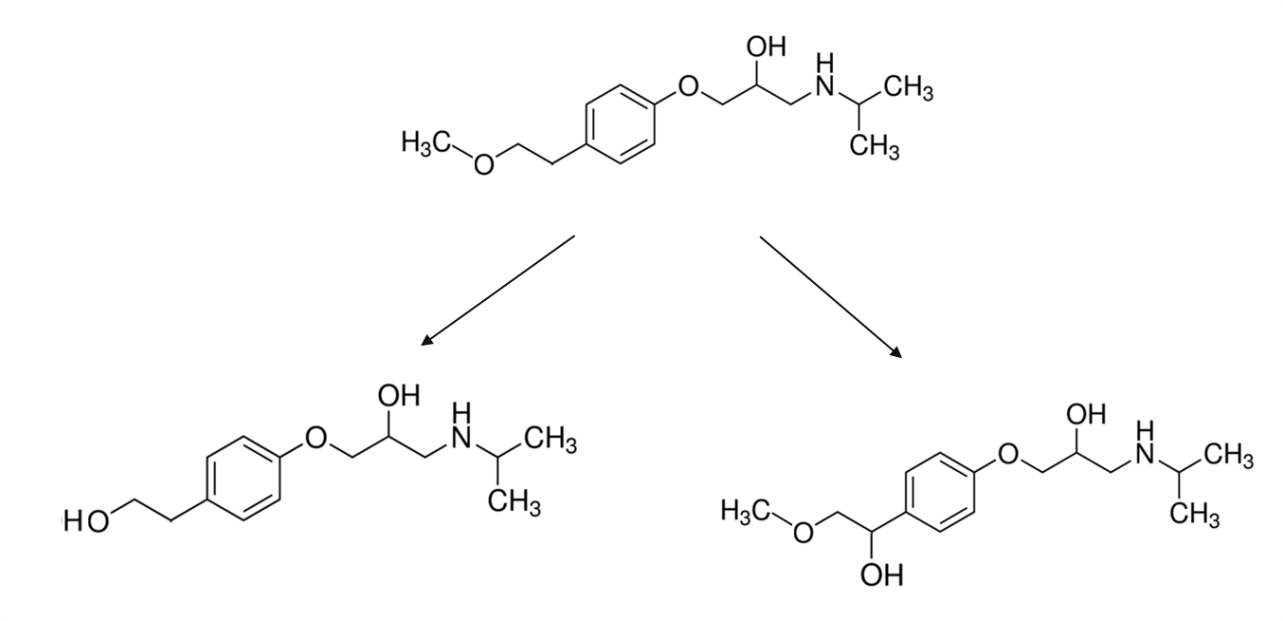

Schematic 1

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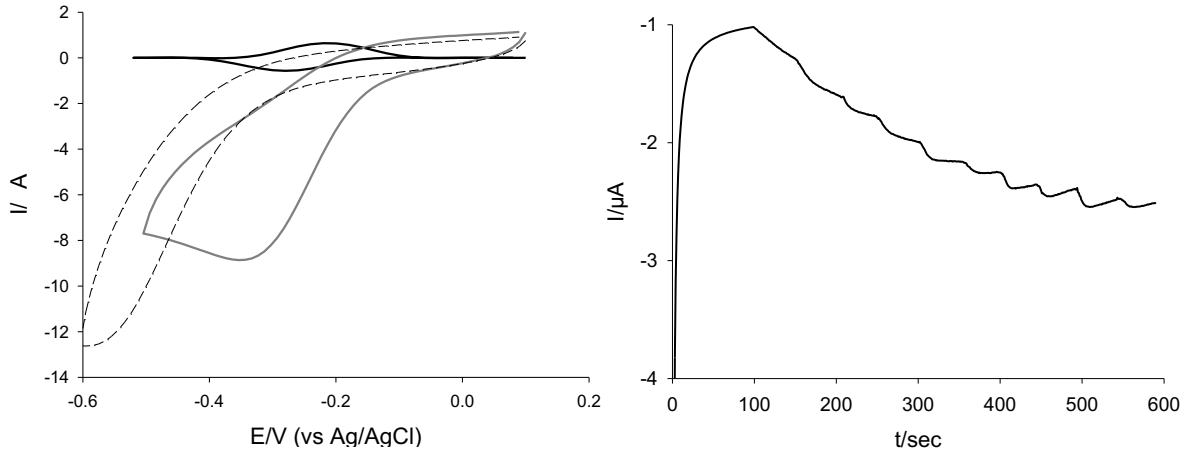

Figure 4

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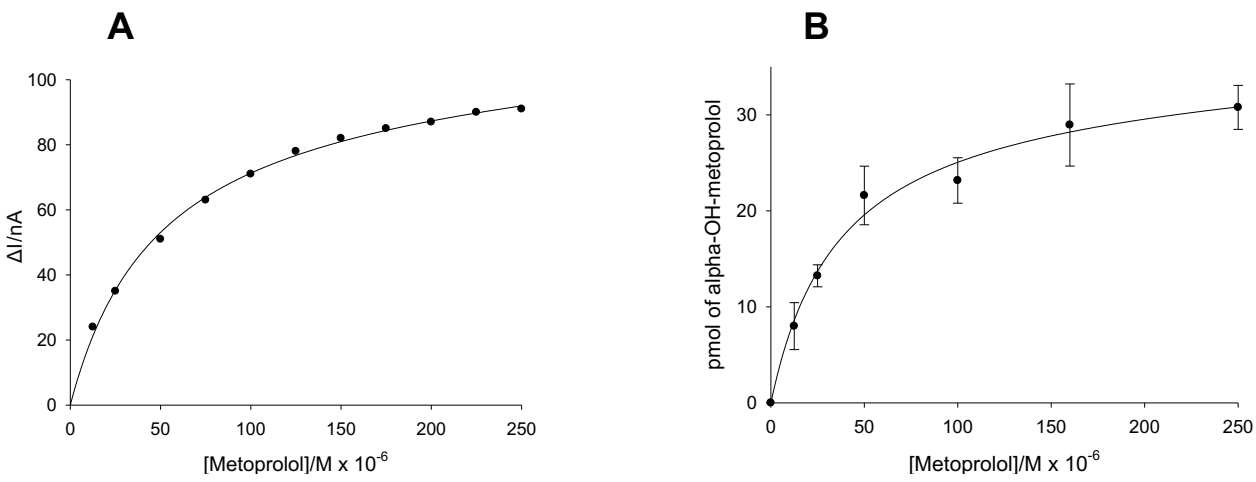
A
100
80
60
40
20
0
ΔI/nA
0
50
100
150
200
250
[Metoprolol]/M x 10-6
B
30
20
10
0
pmol of alpha-OH-metoprolol
0
50
100
150
200
250
[Metoprolol]/M x 10-6

Figure 5

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