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Master Of Science Thesis

«Spectroscopic and photophysical studies of covalently and electrostatically linked Porphyrin – Laccase entities»

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ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ

ΣΧΟΛΗ ΘΕΤΙΚΩΝ ΚΑΙ ΤΕΧΝΟΛΟΓΙΚΩΝ ΕΠΙΣΤΗΜΩΝ

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Καφεντζή Π. Μαρία – Χρυσάνθη

Ιανουάριος 2013

To my parents and brothers

Στους γονείς μου και τα αδέρφια μου

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ABSTRACT

The improvements in existing energy networks will not be sufficient to meet our requirements in a sustainable manner. Finding new sources of renewable energy which will provide sufficient supplies of energy for the future is one of the largest and the most important challenges of our society.

The development of new materials that can be suitably designed to perform the conversion and the storage of solar energy is important and necessary. By mimicking the natural photosynthesis, solar energy can be used to drive catalysis and convert light to stored chemical energy.

Our target is to develop robust systems in which light absorption triggers electron transfer events that lead to the activation of a catalytic center. Herein, we report the activation of a catalytic centre of a metalloprotein, in particular, a laccase. So, we studied a laccase – porphyrin (ZnP) system, which is catalytically active for dioxygen reduction.

Porphyrins were selected as photosensitizers due to their high stability, the easy preparation in the lab, the high absorption at visible spectrum (400 – 600 nm) and they display long life time of the excited states. On the other hand, laccases are robust oxidoreductases which catalyze the oxidation of various organic and inorganic substrates with the concominant reduction of dioxygen to water as by-product (abundant, not dangerous and inexpensive) and that are easily available.

Three different approaches of new hybrid systems for the reduction of dioxygen to water were developed. Therefore, studies were initiated in systems which were created: (a) by interaction of the two components in solution, (b) after the covalent binding of ZnP on the surface of laccase and (c) after the adsorption of both components on TiO_2 nanoparticles. In all cases, the appropriate porphyrin ring was synthesized in order to interact either with the enzyme or with TiO_2 nanoparticles.

In the first approach, photophysical methods demonstrated the 4-electron transfer from the porphyrin to the laccase, enhancing the rate of dioxygen reduction. Thus, the mechanism of interaction of the two components was ascertained.

In the second approach, the porphyrin was linked covalently to the surface of the enzyme which has been appropriately modified. Therefore, a suitable method of enzyme reaction with the porphyrin was settled, obtaining 90% yield and maintaining the 80% of the initial activity of the enzyme.

Finally, in the third approach, the porphyrin and the laccase were adsorbed to the surface of titanium oxide nanoparticles (TiO_2). The enzyme in this case remains 97% active and the oxygen consumption rate in this system is 2-3 times greater than the corresponding speed of the bimolecular system porphyrin/laccase performed at the same concentrations.

Keywords: porphyrin, laccase, metalloprotein, dioxygen reduction, TiO_2 nanoparticles, photoreduction.

ΠΕΡΙΛΗΨΗ

Οι βελτιώσεις στα υπάρχοντα δίκτυα μετατροπής ενέργειας δεν είναι επαρκείς για την κάλυψη των αναγκών μας σε ένα βιώσιμο επίπεδο. Η εύρεση νέων ανανεώσιμων πηγών ενέργειας, η οποία θα παρέχει επαρκή αποθέματα ενέργειας για το μέλλον είναι μία από τις μεγαλύτερες και τις πιο σημαντικές προκλήσεις της κοινωνίας μας.

Συνεπώς, η ανάπτυξη νέων κατάλληλα σχεδιασμένων υλικών με σκοπό τη μετατροπή και την αποθήκευση της ηλιακής ενέργειας είναι σημαντική και απαραίτητη. Μιμούμενοι τη λειτουργία της φωτοσύνθεσης, η χρήση της ηλιακής ενέργειας μπορεί να εφαρμοστεί στην κατάλυση και σε φωτοβολταϊκές κυψελίδες.

Στόχος μας είναι να αναπτύξουμε σταθερά συστήματα στα οποία η απορρόφηση του φωτός να επάγει διαδικασίες μεταφοράς ηλεκτρονίων που οδηγούν στην ενεργοποίηση ενός καταλυτικού κέντρου. Στην παρούσα διατριβή, αναφέρουμε την ενεργοποίηση ενός καταλυτικού κέντρου μιας μεταλλοπρωτεΐνης, συγκεκριμένα, μια λακάσης. Επίσης, μελετήσαμε ένα σύστημα λακάσης - πορφυρίνης (ZnP), το οποίο είναι καταλυτικά ενεργό για την αναγωγή του οξυγόνου.

Οι πορφυρίνες επιλέχθηκαν ως φωτοευαισθητοποιητές λόγω της υψηλής σταθερότητάς τους, την εύκολη παρασκευή τους στο εργαστήριο, την υψηλή απορρόφησή τους στο ορατό φάσμα (400 - 600 nm) και εμφανίζουν μεγάλο χρόνο ζωής των διεγερμένων καταστάσεων. Από την άλλη πλευρά, οι λακάσες είναι οξειδαναγωγάσες που καταλύουν την οξείδωση των διαφόρων οργανικών και ανόργανων υποστρωμάτων με την ταυτόχρονη αναγωγή του οξυγόνου σε νερό ως παραπροϊόν (άφθονα, όχι επικίνδυνα, ανέξοδο) και ότι είναι εύκολα διαθέσιμες.

Τρεις διαφορετικές προσεγγίσεις ακολουθήθηκαν για την ανάπτυξη νέων υβριδικών συστημάτων με σκοπό την αναγωγή του οξυγόνου σε νερό. Συνεπώς, μελετήθηκαν ξεχωριστά συστήματα που δημιουργούνται: (α) με αλληλεπίδραση των δύο συστατικών σε διάλυμα, (β) μετά την ομοιοπολική δέσμευση της ZnP πάνω στην επιφάνεια της λακάσης και (γ) μετά την προσρόφηση και των δύο συστατικών σε TiO_2 νανοσωματίδια. Σε όλες τις περιπτώσεις, αρχικά συντίθεται ο κατάλληλος πορφυρινικός δακτύλιος που στοχεύει στην αλληλεπίδραση είτε με το ένζυμο είτε με τα νανοσωματίδια οξειδίου του τιτανίου.

Στην πρώτη προσέγγιση, φωτοφυσικές μέθοδοι απέδειξαν την 4- ηλεκτρονίων μεταφορά από την πορφυρίνη στη λακάση, με αποτέλεσμα την ενίσχυση της

ταχύτητας αναγωγής του οξυγόνου. Έτσι, διαπιστώνεται και ο μηχανισμός αλληλεπίδρασης των δυο συστατικών.

Στη δεύτερη προσέγγιση, η πορφυρίνη συνδέεται ομοιοπολικά στην επιφάνεια του ενζύμου που έχει κατάλληλα τροποποιηθεί. Εφαρμόστηκε, λοιπόν, μια κατάλληλη μέθοδος αντίδρασης του ενζύμου με την πορφυρίνη, αποκτώντας 90% απόδοση, διατηρώντας το ένζυμο στο 80% της αρχικής του ενεργότητας.

Τέλος, στην τρίτη προσέγγιση, η πορφυρίνη και η λακάση είναι προσδεδεμένες στην επιφάνεια νανοσωματιδίων οξειδίου του τιτανίου (TiO_2). Το ένζυμο και σε αυτή την περίπτωση παραμένει 97% ενεργό και η ταχύτητα κατανάλωσης οξυγόνου στο σύστημα αυτό είναι 2-3 φορές μεγαλύτερη από την αντίστοιχη ταχύτητα στο διμοριακό σύστημα πορφυρίνης/λακάσης που πραγματοποιείται στις ίδιες συγκεντρώσεις.

Λέξεις κλειδιά: πορφυρίνη, λακάση, μεταλλοπρωτεΐνη, αναγωγή οξυγόνου, TiO_2 νανοσωματίδια, φωτοαναγωγή.

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ABBREVIATIONS

B&R Britton & Robinson buffer (see Appendix)

CT charge transfer

Cu copper

d doublet

ε extinction co efficient

E^o redox potential

eq equivalents

EDTA ethylenediaminotetraacetic acid

EPR electron paramagnetic resonance

^{1}H NMR proton nuclear magnetic resonance

ICP-MS inductively coupled plasma mass spectrometry

J coupling constant

kDa kiloDalton

m multiplet

MALDI matrix assisted laser spray desorption ionization

Mr molecular weight

MS mass spectrometry

Lac3 isoenzyme of laccase

LMCT ligand to metal charge transfer

NaN_3 sodium azide

NHE normal hydrogen electrode

pI isoelectric point

PAGE polyacrylamide electrophoresis

s singlet

SDS sodium dodecyl sulfate

SGZ syringaldazine

t triplet

T1 Type 1 (copper site)

T2/T3 Type 2/Type 3 (copper site)

TOF time-of-flight

TiO_2 Titanium oxide

TEOA triethanolamine

TNC trinuclear cluster

Tris tris(hydroxymethyl)aminomethane

UniK mutated laccase with a unique amine group

UV-Vis ultraviolet/visible

ZnP zinc porphyrin

^{3}ZnP* triplet excited state of zinc porphyrin

$ZnP^{\bullet+}$ zinc porphyrin radical cation

I. INTRODUCTION: Porphyrin background

This chapter is an introduction on porphyrins: their structure, their general features and some of their applications. Porphyrins are compounds of great importance because in addition to their use in nature which is well known, the technological applications range from medicine to materials science. The synthesis of porphyrins and the investigation of the physical - and electro - chemical properties have attracted considerable interest in order to mimic the natural photosynthesis.

1.1 Overview

The porphyrins are very extensive aromatic compounds, with bright red (purple) color, to which they owe their name. Due to the intense color and the fact that they are present in every kind of living cells on our planet, they are named "The Color of Life."

The porphyrin molecule is a heterocyclic macrocycle composed of four pyrrole subunits connected via methine bridges. The structure of the unsubstituted macrocycle is called porphine, according to Fisher's nomenclature which was developed in the '20s and '30s.[1,2]

Later, in 1943, Corwin has adopted the name "porphyrin" for the system of porphine which prevails today. Below is the numbering of the atoms of the porphyrin and the distinction between the pyrrole rings, according to Corwin.[3] (see Figure 1.1.1)

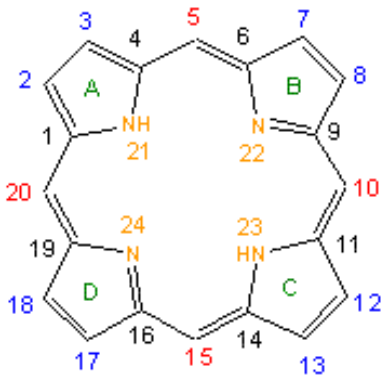


Figure 1.1.1: The skeleton and the numbering of carbons of the porphyrin according to Corwin.

Porphyrins are typically considered as derivatives of porphine by substituting some or all of the peripheral positions with various side groups. Numerous porphyrin derivatives can exist by replacing the exo-hydrogens at positions 2,3,7,8,12,13,17,18, or methine hydrogens at positions 5,10,15,20. The first are known as β-substituted and the second as meso-substituted porphyrins.

Porphyrins are aromatic compounds that obey the Hückel rule (4n +2). They have 18 π electrons which are delocalized over the entire circumference of the porphyrin ring. Consequently, because of their high conjugation, porphyrins and their derivatives absorb in the visible region which gives their color.

The extensive π conjugated system of porphyrins is suitable for application as electron donors in photoinduced electron transfer processes with direct applications in photonic systems and catalysis.

Porphyrins are unsaturated tetra dented macrocyclic ligands which can bind divalent metal ions that behave as Lewis acids (see Figure 1.1.2). The incorporation of the metal is an easy process including the departure of the two protons connected on the inner nitrogens.

Figure 1.1.2: Reaction of porphyrin's metallation.

1.2 Porphyrins in nature

The porphyrins are a group of compounds which are found in many biological and chemical processes in nature. In natural metal-porphyrins, all eight pyrrole carbon atoms are substituted.

The main function of the porphyrins in nature is their coordination with metals, which act as active centers for many biochemical processes. These molecules are key elements of the active sites of haemoglobin protein, P450 and chlorophyll.

For example, protoporphyrin IX, in haeme centers of haemoglobin and myoglobin, binds iron which can reversibly bind dioxygen in order to transfer it to the remaining body or storage in muscle tissues.

Chlorophyll is a porphyrin derivative which ensures photosynthesis in plants by triggering photochemical electron transfer events. The photosynthesis takes place in the chloroplast, where the principal receptor is chlorophyll A. This is a substituted tetrapyrrollic ring bearing a magnesium (II) ion.

1.3 General characteristics of porphyrins

The notable features of porphyrin molecules are:

1). The stability of the ring that can adopt different geometric conformations (planar or twisted structure).[4] Both carbon atoms and nitrogens participating in the porphyrin skeleton have sp^2 hybridization, so all the bond lengths range from 134 - 145pm and angles from 107^o-126^o.

2). The macrocyclic ligand is usually quite selective with regard to the size of the bound metal ion. Structural studies and computational models showed that ions with spherical radius of 60-70 pm are located in the central cavity of the tetrapyrrole ring. When the size of the ion is bigger than 70pm, e.g. for lanthanides (85-106pm), ions are located out of the plane defined by the four nitrogens of the porphyrin ring[5] (see Figure 1.1.3).

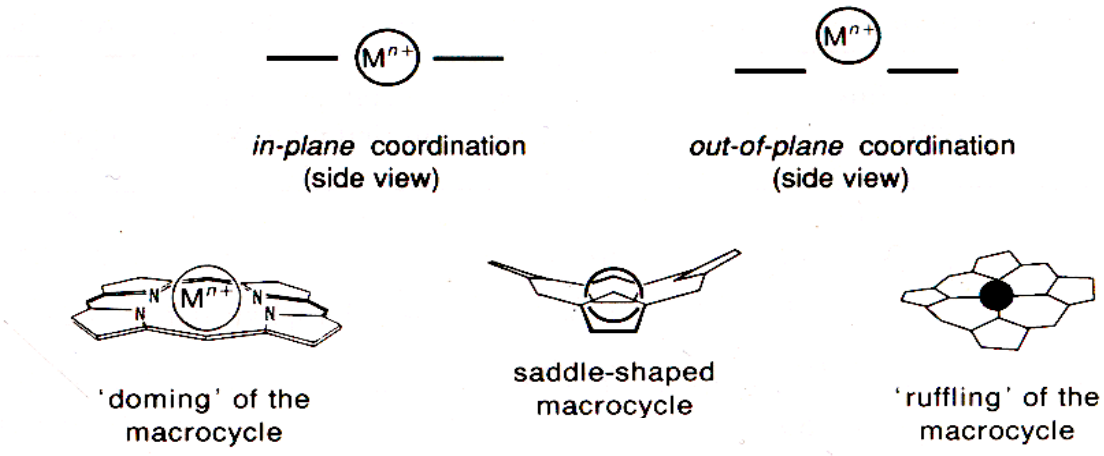


Figure 1.1.3: Typical geometric configurations of tetrapyrrolic complexes.

3). Provided that the coordination number is 6 in octahedral geometry, porphyrin binds the metal ions leaving two empty coordination sites X, Y in axial positions allowing trans substitution to occur in various reactions (see Figure 1.1.4).

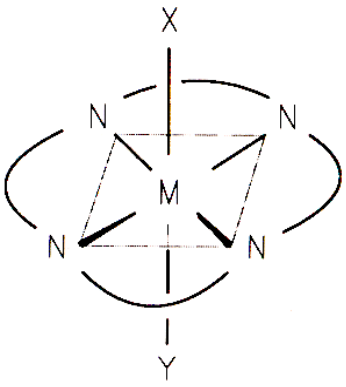


Figure 1.1.4: Axial positions X, Y at six coordinated complexes.

4). These molecules are able to "take" and "give" electrons and the first oxidation and also the first reduction are performed with great ease. The anions or cations, respectively formed, are quite stable.

Therefore, the absorption of light and the easy redox processes make these tetrapyrrole molecules the most important energy convertors in biological systems.

1.4 Synthetic meso - water-soluble porphyrins

In this study we worked with meso - substituted porphyrins. The tetra - pyridyl - porphyrins (TPyP), like the tetra - phenyl - porphyrins (TPP) (see Figure 1.1.5) belong to the class of meso - substituted synthetic porphyrins which are widely used in many chemical and photochemical studies. A number of water soluble porphyrins can be prepared by adding carboxylate or sulfonate groups ($-COO^-$, SO_3^-) on the phenyl substituents of TPP. In the case of TPyP, nitrogen atoms can be found in ortho, meta or para position of pyridyl groups and can become water soluble by a simple methylation of nitrogen atoms.[6] (see Figure 1.1.6).

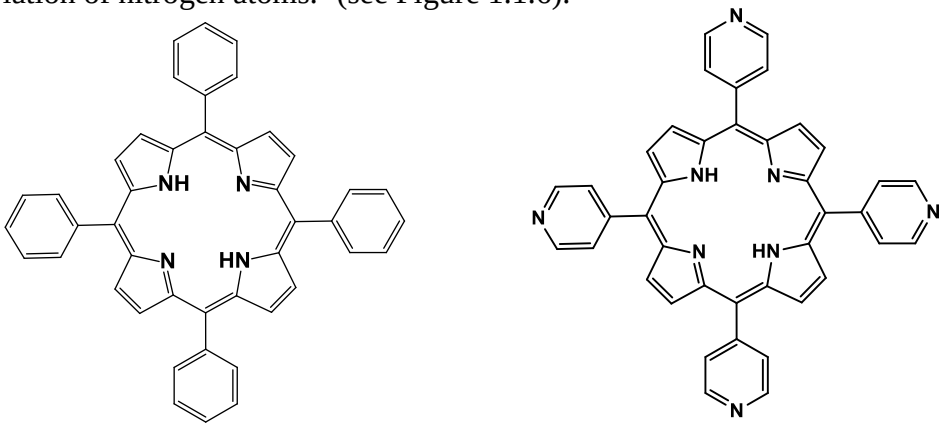


Figure 1.1.5: Left: Tetra – phenyl - substituted porphyrin (TPP), right: tetra - pyridyl porphyrin (TpyP).

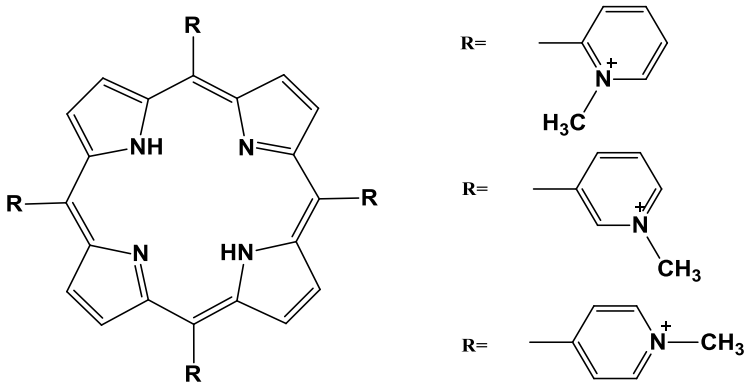


Figure 1.1.6: Water soluble pyridyl porphyrins in the ortho, meta and para positions.

The properties of meso – substituted water soluble porphyrins depend on the type of charge on peripheral ligands. The porphyrins that have substituents with negative charges have a greater tendency to form aggregates than those having positively charged substituents. The most commonly studied porphyrins are those charged at the para position.

All porphyrins have characteristic absorption spectrum with high absorption at 400 - 450nm (Soret band) and four Q bands for free bases or two for the metallated porphyrins (mainly Zn^{2+}) which have much lower intensity at 500 - 650 nm.[7] In addition, they display long life-time of single or triple excited states.[6]

The above characteristic properties, namely broadband absorption in the visible region, high stability under irradiation, make the porphyrins and their derivatives suitable for direct application as sensitizers for photodynamic therapy from cancer[8], as synthetic enzyme models[9], in catalysis[10] and in solar cells.[11]

I. INTRODUCTION: Laccases

Introduction

Laccases are oxidoreductases which catalyze the oxidation of substrates at the expense of molecular oxygen. They have received much attention during the past decades because of their potential use in biotechnology and bioremendation.[12] They are characterized as environmental friendly biocatalysts, as they produce only water as by-product, which make them available to exploitation in many industrial uses. In this chapter are described the structural and spectroscopic features of laccases, the way they work, their optimum working conditions, as well as some of their applications.

2.1 Overview

Laccases are among the few enzymes that have been the subject of study since the 19th century. Yoshida first described laccases in 1883 from the exudates of the Japanese lacquer tree *Rhus vernicifera*.[13] In 1896, for the first time, both Bertrand and Laborde demonstrated that these enzymes are produced in fungi as well.[14] Also, laccases have been identified in insects and bacteria.[15,16] Laccases are members of a large family of blue copper oxidases; other members of this group are mammalian plasma ceruloplasmin and ascorbate oxidase in higher plants.[14]

Laccases are copper containing oxidoreductases which catalyze the oxidation of various substituted aromatic substrates, such as phenols, anilines and metal ions with the concominant reduction of dioxygen to water (see Figure 1.2.1).

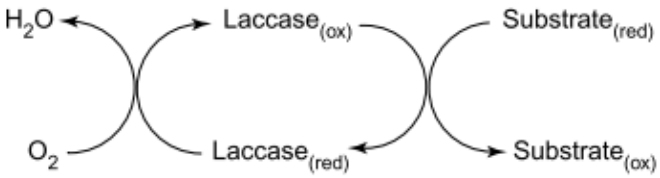


Figure 1.2.1: Schematic representation of laccase – catalyzed oxidation redox cycles for substrates oxidation.[12]

Especially, the mono electronic oxidation of a substrate generates the corresponding reactive radical. Consequently, the oxidation of four substrates leads to the formation of four free radicals. These intermediates are generally unstable and then they can undergo further oxidation, dimerization or oligomerization.

The biotechnological exploitation of these enzymes is possible thanks to their resistance and tolerance in organic solvents used in industrial applications. Laccases provide many advantages of great interest for technological applications. They are important resistant biocatalysts which can be used to remove xenobiotics and pollutants found in industrial waste and water. Also, they can be used in paper pulping, in textile and food industries, for personal and medical care applications and finally as biosensors.[12,17]

2.2 Occurrence in biological systems

Laccases are found in plants (trees, cabbages, apples, asparagus, potatoes, pears, and various vegetables), in insects (Diploptera, Drosophila, etc.) and in fungi (ascomycetes, deuteromycetes and basidiomycetes). Among the latter ones, white rot fungi are the most important laccase producers. Well known producers are the wood rot fungi, such as *Trametes versicolor*, *Trametes villosa*, etc.[17]

Most laccases are extracellular, but intracellular ones have been detected in fungi and insects.[18]

The physiological function of laccases is different in various organisms but they all catalyze polymerization or depolymerization processes. In plants, laccases are involved in cell wall formation and together with peroxidases, in lignification. Also, it has been proposed that laccases are involved in cuticle sclerotization in insects and in the assembly of UV resistant spores in bacteria. There is no doubt that laccases from white rot fungi achieve depolymerization on lignin together with other enzymes.

2.3 Structural and Spectroscopic properties of laccases.

2.3.1 Structure

Laccase contains four copper ions per enzyme and its scaffold consists of triplicated domain homologous to the blue copper proteins cupredoxins. It is considered that evolving from a (mono) cupredoxin domain into three – domains laccase (domains D1, D2, D3), pro – laccase lost the copper ions in D1 and D2 and acquired a trinuclear copper cluster (TNC) in D2 and D3. Substrates bind in neoformed clefts near the D3 domain (see Figure 1.2.2).[10]

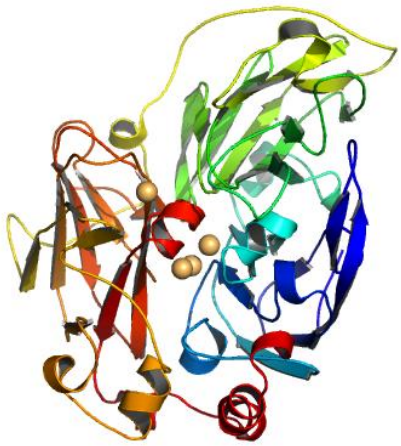


Figure 1.2.2: Three dimensional representation of fungal laccase. Cupredoxin domains are coloured from blue (D1) to green (D2) to red (D3). Light orange spheres depict copper atoms, an isolated close to the surface of the enzyme in D3 and a tri-nuclear cluster embedded in the enzyme at the D1-D3. [10]

In most cases, the molecular weight of laccases extracted from fungi is between 60 and 100kDa with isoelectric points (pI) from 3 to 7.

Laccases are glycosylated proteins generally having fewer saccharide compounds (10-25%) in fungi and bacteria than in plants (20-25%). The N-linked glycans to the polypeptide chain include glucose, galactose, fructose, arabinose, xylose and mannose as the most important carbohydrates. Finally, it has been suggested that glycosylation is responsible for copper retention, thermal stability as well as resistance to proteolysis.[18]

2.3.2 Active sites

Four copper atoms can be distinguished into three categories based on their spectroscopic features: a type 1 copper (T1 Cu^{II}) or blue copper, a type 2 copper (T2 Cu^{II}) or normal copper and a pair of type 3 coppers or binuclear copper site (T3 Cu^{II} - Cu^{II}) (see Figure 1.2.3).

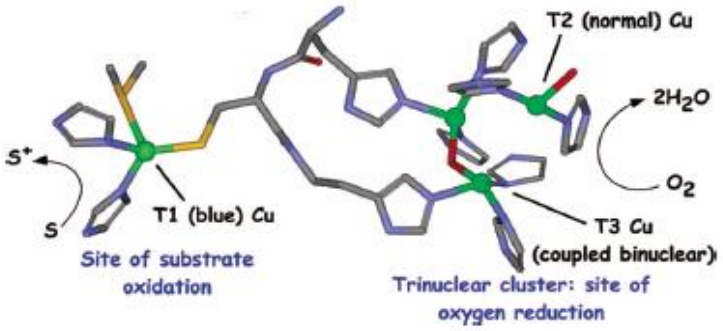


Figure 1.2.3: Active sites of multicopper oxidases. In this figure, is appeared the crystal structure of ascorbate oxidase. Cu sites are shown in green spheres.[19]

These types of copper are distinguished in the oxidized state using UV-visible spectroscopy (UV-Vis) and electron paramagnetic resonance (EPR) spectroscopy. Type 1 copper is responsible for the blue color of the protein displaying an absorbance at approximately 610 nm ($\varepsilon \sim 5600\ M^{-1}\ cm^{-1}$) and is EPR detectable, Type 2 copper does not confer color but is EPR detectable and Type 3 coppers display an absorbance in the near UV region (330 nm, $\varepsilon \sim 5000\ M^{-1}\ cm^{-1}$) but have no detectable EPR signal (see Figure 1.2.4 and Figure 1.2.5). Typical UV-Vis spectra of laccase also display a peak around 280 nm ($\varepsilon \sim 90000\ M^{-1}\ cm^{-1}$) due to the absorbance of aromatic amino acids. [19]

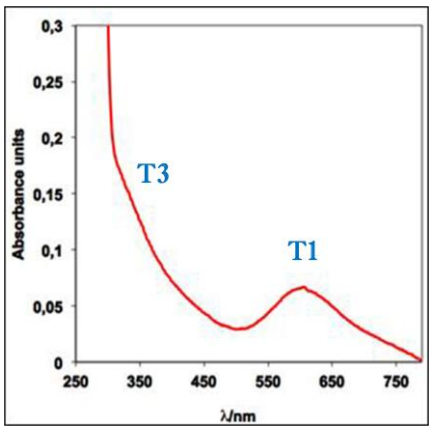


Figure 1.2.4: UV-Vis absorption band arising from T1 Cu and T3 Cu pair.

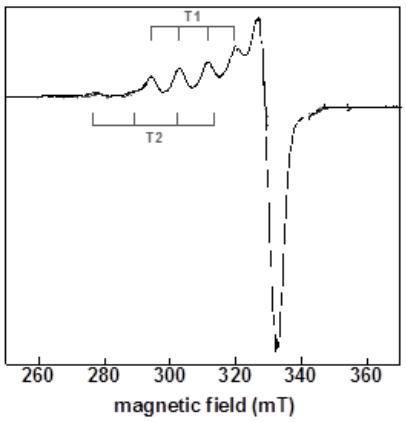


Figure 1.2.5: EPR spectrum shows the signals arising from T1 and T2 Cu(II) ions.[10]

T1 Cu Site

The Type 1 Cu site is present at +2 oxidized state in the resting enzyme. It is usually coordinated to two nitrogens from two histidines and one sulphur from a cysteine. Type 1 Cu is coordinated to the sulphur atom through strong covalent π bond that is responsible for the characteristic blue color of typical laccase enzymes. This is due to a charge transfer transition $(S)_{Cys} \rightarrow Cu(II)$ (LMCT) at *ca.*600 nm in the electronic absorption spectrum.

Moreover, T1 Cu exhibits a weak parallel hyperfine coupling constant $A_{//}$ in EPR spectrum, because the unpaired electron in the orbital $3d_{x^2-y^2}$ of the copper (II) is delocalized on the cysteine, thereby reducing the interaction with the nuclear spin (I=3/2) of the copper.[20]

In some cases of multi-copper proteins, a methionine ligand occupies the fourth coordination site, whereas in others there is an uncoordinated ligand, leucine or phenylalanine. This methionine residue is absent in the laccase studied in our laboratory.

The redox potentials of the T1 Cu site vary depending on the source of laccases. For example, the E^o of laccases extracted from fungi is much higher than that of laccases extracted from plants and bacteria. So, the electrochemical potential of laccases extracted from plant laccase *Rhus vernicifera* is around 0.4 V, while the redox potential of T1 Cu site from fungi is measured between 0.4-0.8 V *versus* hydrogen electrode (NHE).[21]

T2/T3 Cu Sites

The T2 Cu in the resting state is triply coordinated to two nitrogens from two histidines and one water – ligand. So, it is EPR detectable and it does not display characteristic band in UV-Vis absorption spectrum.

The T3 Cu ions are each one fourfold coordinated with three histidine ligands and one hydroxyl bridging group. Because of a strong anti ferromagnetic coupling, it is EPR silent. On the contrast, it is characterized by a bridging hydroxide to Cu(II) CT transition at 330 nm ($\varepsilon \sim 5000\ M^{-1}cm^{-1}$).[22]

Magnetic circular dichroism and X-rays absorption spectroscopy of laccases have shown that the type 2 and 3 centers combine to function as a trinuclear copper cluster with respect to exogenous ligand interaction including reaction with dioxygen.

No exogenous bridge is evidenced between the T2 Cu center and T3 Cu center, but the T2 Cu is located at a 4Å distance from the binuclear center. This trinuclear cluster is responsible for the reduction of dioxygen to water.[23]

Data about redox potentials of copper sites in TNC are rare but both T2 Cu and T3 Cu display redox potentials comparable to that of T1 Cu site.

2.4 Mode of action of laccases

Laccases have low substrate specificity, and as confirmed by structural data, there is no real binding pocket for substrates but rather a shallow depression at the bottom of which there is a histidine coordinating the T1 copper ion. The oxidation of the substrate is strictly outer – sphere and this reaction is very fast (10^5 – 10^6 s^{-1} M^{-1}).

Substrate oxidation and dioxygen reduction sites are located approximately 13 Å away from each other and are connected through a histidine – cystein – histidine tripeptide (H – C – H) involved in the coordination of the metal ions.

The main electron transfer steps involved in the reaction mechanism are[19]:

i) Reduction of T1 Cu by the substrate

ii) Internal electron transfer from T1 Cu to the trinuclear cluster T2/T3 Cu

iii) O_2 reduction by the trinuclear T2/T3 cluster (see Figure 1.2.6)

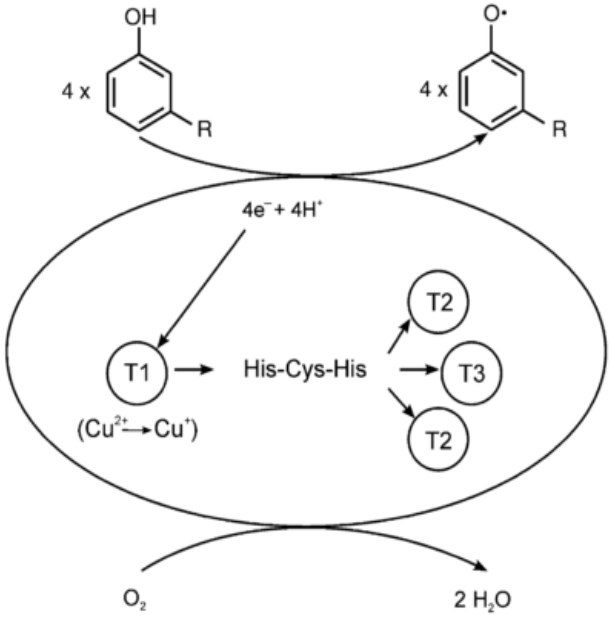


Figure 1.2.6: Schematic representation of a laccase catalytic cycle producing two molecules of water from the reduction of one molecule of dioxygen and the concomitant oxidation of substrate molecules to the corresponding radicals.

The electrons from substrates are transferred to the TNC where the reduction of oxygen takes place. Dioxygen reacts with the fully reduced TNC to generate Cu (II) – (O_2) – Cu(II) intermediate preceding the cleavage of O – O bond which triggers movements of protonated oxygen atoms away from the cluster.[24]

2.5 Infuence of pH, temperature and inhibitors on laccase activity

- *pH*

Fungal laccase's stability is higher in acidic pH, because OH^- ions bind to T2/T3 coppers, resulting in the inhibition of the laccase activity.[25]

- *Temperature*

The optimal temperature of laccase can differ greatly from one strain to another. Our laccase LAC3 was found stable at 30 – 40 ^oC for at least 24 hours, but rapidly lose activity at temperatures above 50 ^oC.[26]

- *Inhibitors*

Studies have shown that azides, floride, thioglycolic acid, diethyldithiocarbamic acid inhibit laccase's activity, whereas EDTA did not affect laccase activity. Small molecules such as N_3^-, CN^- , F^- and OH^- bind to T2/T3 coppers, leading to the interruption of the internal electron transfer and activity inhibition.[17]

2.6 Laccase used in this study

The laccase used for this study is a fungal enzyme, LAC3 from *Trametes* sp. strain C30[27]. It is produced in the filamentous fungus *Aspergillus niger* in large – scale production (300mg of enzyme/L of culture medium).[26]

I. INTRODUCTION: Aim of the study

In this work we intended to couple a photosensitizer to a metalloprotein. There are many ways to produce photosensitizer/metalloprotein hybrid systems and to improve their performance. The use of different photosensitizers and the mode of interaction of the components (i.e. the photosensitizers can be covalently bound, adsorbed on an electrode etc.) can be modulated to improve the efficiency of electron transfer events.

In this field Gray et al. pioneered the coupling of photoactive units to metalloenzymes to access the buried active sites leading to great advances on the understanding of electron transfer processes in biological systems[28] Moreover, it was recently reported that TiO_2 nanoparticles modified with Ru – bipyridyl complexes and a hydrogenase or CO_2 reducing enzyme, provide intresting catalysts for H_2O and CO_2 photoreduction, respectively[29].

Previously in the laboratory, Simaan et al., described the first example of dioxygen photoreduction by a multicopper oxidase (laccase) using a Ru – based photosensitizer[30]. Taking advantages of the interesting physical properties of zinc-containing porphyrins, we wanted to explore the possibility of substituting the expensive Ru-based complex and to develop improved hybrid systems.

In this study three approaches were developed to create ZnP/laccase hybrid systems: (a) interaction of two components in solution, (see Figure 1.3.a) (b) covalent attachment of ZnP on the surface of laccase, (see Figure 1.3.b) (c) the co – adsorption of the two components on TiO_2 nanoparticles (see Figure 1.3.c)

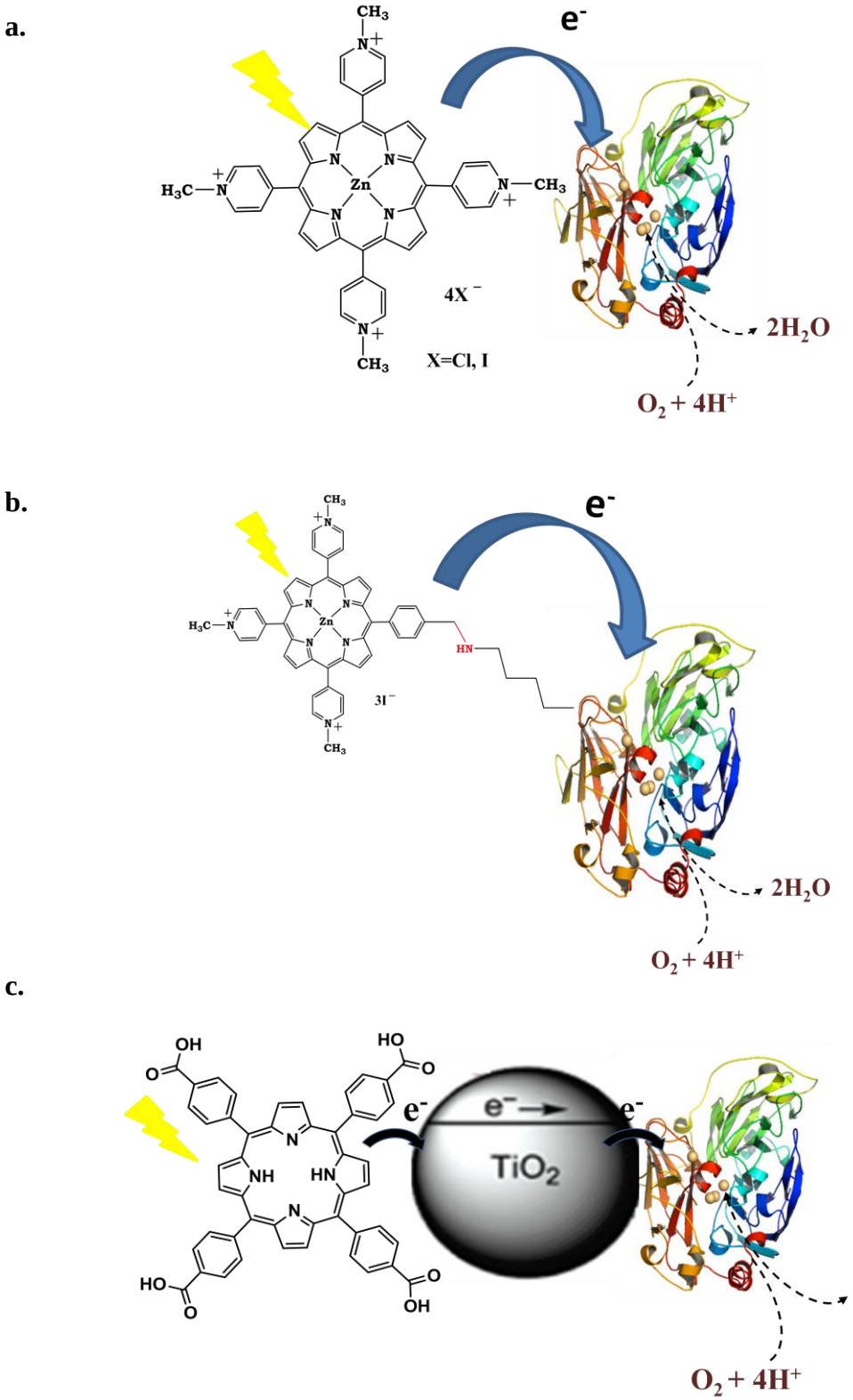


Figure 1.3.1: Dioxygen reduction representation. (a) porphyrin and laccase in solution, (b) porphyrin and laccase covalently linked, (c) porphyrin and laccase adsorbed on TiO_2 nanoparticles.

II. Bimolecular porphyrin/laccase system

Introduction

In a first approach, the photocatalytic system was prepared by mixing the porphyrin and the laccase in solution.

Within the context of collaboration between Bioinorganic Chemistry laboratory (Crete) and Biosciences (Marseille), studies on the electronic interaction between a water soluble porphyrin and the laccase were initiated. Photoreduction of laccase was monitored under anaerobic conditions, in the presence of EDTA as sacrificial electron donor and with or without the porphyrin, shown in Figure 2.1. The absorption band of T1 Cu(II) at 605 nm was bleached within 15 minutes of irradiation, whereas in the absence of the porphyrin, the T1 Cu (II) was barely photoreduced, suggesting a promoting role of the porphyrin in photoinduced electron transfer to the enzyme.

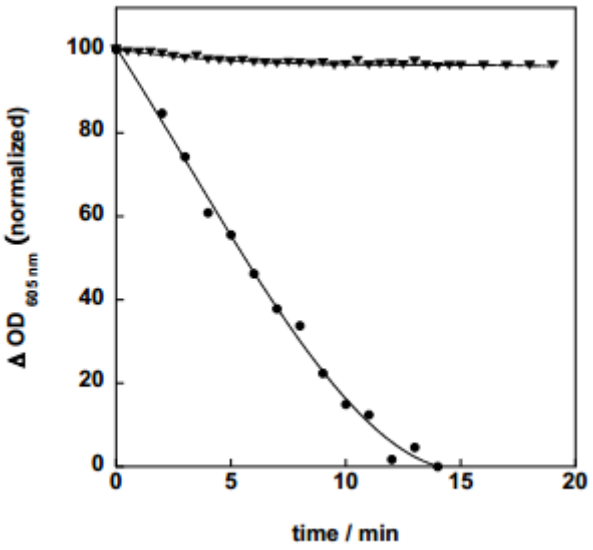


Figure 2.1: Kinetics of bleaching of T1 Cu(II) absorption band.

More experiments on porphyrin/laccase complex were needed in order to better understand these results. In particular, UV-Vis experiments show that the T1 Cu (II) can be reduced. We intended to know if the TNC could be reduced and if the reduced laccase could react with dioxygen.

Strategy

The porphyrin which was selected is the positively charged and water soluble photosensitizer: the zinc tetra – methyl pyridinium one ($ZnTMePyP^{4+}$), shown in Figure 2.2.

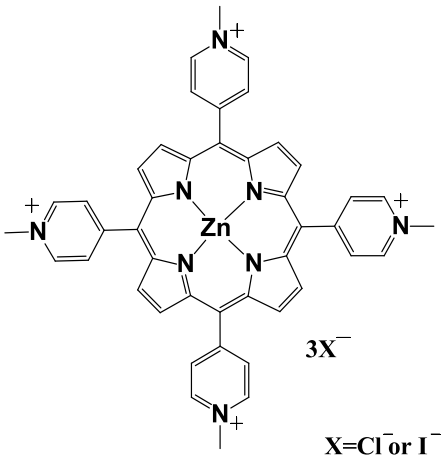


Figure 2.2: Positively charged zinc porphyrin.

Experimental

Laccase production: The laccase (LAC3 from fungus *Trametes* sp. strain C30)[27] is produced by heterologous expression of the laccase in the filamentous fungus *Aspergillus niger* in large – scale production (300mg/L) as described elsewhere.[26]

Synthesis of the porphyrin

5,10,15,20-tetrakis(4-N-methylpyridinium)porphyrinato Zinc chloride was synthesized using a modified literature process.[31] The porphyrin was a ready prepared and purified at the beginning of these experiments.

EPR measurements

X – band EPR spectra were obtained using a BRUKER Elexsys E500-9.5/2.7 spectrometer equipped with a BVT 3000 digital temperature controller (100-400K). The experiments were performed using 1:1 (Laccase: $ZnTMPyP^{4+}$) ratio and 100 eq. of EDTA as sacrificial electron donor. The sample (450 μM of laccase, 1 eq. of $ZnTMPyP^{4+}$, 100 eq. of EDTA in B&R buffer set at pH 4) was placed in J. Young valved EPR tube in absence of dioxygen. Irradiations were performed at room temperature using a Dolan – Jenner MI-I50 illuminator (EDMUND). The samples were analyzed in the spectrometer at 120 K as frozen solutions.

Dioxygen consumption

Dioxygen consumption was measured by polarography using a model 781 oxygen meter (Strathkelvin Instruments) with a micro Clark electrode fitted to a temperature controlled glass chamber (1.5 ml). Irradiation of the sample was performed through the glass chamber using a Dolan-Jenner MI-150 illuminator (Edmund) equipped with a 150W EKE Quartz Halogen lamp with optic fibers (∅ 0.8 cm) adapted to the respiratory chamber. A power density of about 230 mW/cm^2 was measured with a power meter Vector H410 connected to a Scientech head after a 2 cm thick water lens corresponding to the water cooled glass respiratory chamber.

Results and Discussion

EPR measurements

Previous experiments were performed but the full reduction of the T2 Cu was never observed using sub-stoichiometric amounts of porphyrin. Therefore, we reproduced the experiments using stoichiometric amount of the photosensitizer and of laccase. The samples (LAC3 + ZnTMPyP^{4+} + EDTA in B&R buffer at pH 4) were placed in EPR quartz tubes and irradiated in the absence of dioxygen. Before irradiation, the 120K spectra displayed the characteristic signals of the Cu (II) ions of the enzyme: the T1 Cu(II) ion ($g_{//}$=2.19; $A_{//}$=87 G; $g_{\perp}$=2.04) and the T2 Cu(II) ($g_{//}$=2.25; $A_{//}$=162 G). After a few seconds of irradiation, the blue color disappeared as well as the EPR signal arising from the T1 Cu(II) indicating that at least one electron was transferred to the enzyme. The complete reduction of the T2 copper was achieved within minutes (after *ca.* 4 minutes) indicating the transfer of at least two electrons.

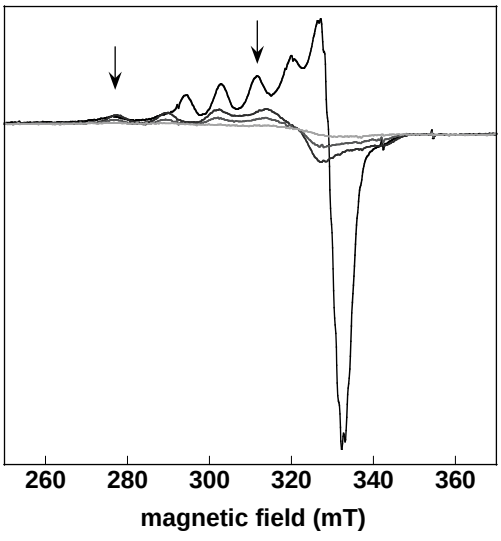


Figure 2.3: Evolution of the EPR spectrum of LAC3 in the presence of 1 eq. of ZnTMPyP^{4+} and 100 eq. of EDTA under white light irradiation at pH = 4.0 under inert atmosphere. X-band EPR spectra were recorded in frozen solutions using 490 μM of laccase; before irradiation (black line) and after 30 s, 120 s and 300 s of irradiation (grey gradient for the corresponding lines). Conditions used : temperature 115K, microwave power 20mW, modulation 3G, gain 10^5.

Dioxygen consumption rates

In order to know if the enzyme is fully reduced, we studied the photodriven dioxygen consumption. As previously reported, we used EDTA as sacrificial electron donor. The photodriven dioxygen consumption rates were monitored as a function of laccase concentration or of the [$ZnTMPyP^{4+}$]. The experiments were conducted in the presence or absence of sodium azide, a strong inhibitor of laccases. Upon irradiation of solutions containing $ZnTMPyP^{4+}$ + EDTA with or without laccase (0-60 µM), the dioxygen concentration decreased proportionally to the concentration of enzyme. Light induced dioxygen consumption rates obtained with variable concentrations of $ZnTMPyP^{4+}$ ranged from 3 to 100 x 10^{-6} $mol.L^{-1}.min^{-1}$ upon addition of laccase. The enzyme dependent dioxygen consumption was inhibited in the presence of 10 mM of NaN_3. At this concentration of azide the enzyme is totally inhibited. The residual consumption – *i. e.* the enzyme independent consumption – represents less than 25% of the maximum rate (2 to 20 x 10^{-6} $mol.L^{-1}.min^{-1}$).

The presence of enzyme-dependent dioxygen consumption indicates that the laccase accomplishes full catalytic cycles and therefore that 4 electrons are transferred per cycle.

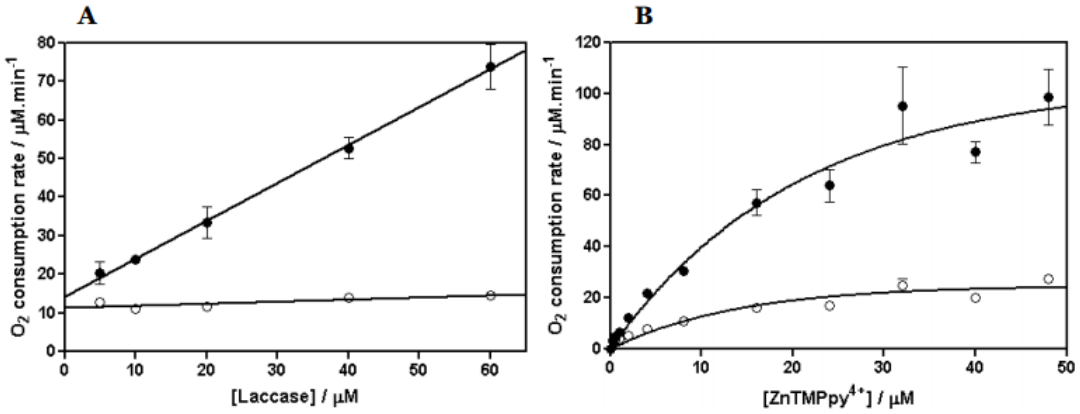


Figure 2.4: Dioxygen consumption dependence on either [laccase] or [$ZnTMPyP^{4+}$] in the presence or absence of sodium azide. A: 10µM of $ZnTMPyP^{4+}$; 4 mM of EDTA, 0-60 µM laccase; B: 0.24 to 48 µM $ZnTMPyP^{4+}$, 4 mM of EDTA, 40 µM laccase. (●) in the absence of sodium azide; (○), in the presence of 10 mM sodium azide. Data points represent the mean of at least 3 independent measurements; error bars reflect standard deviations.

Conclusion

In conclusion, assembling a zinc photosensitizer and a multicopper oxidase in a photocatalytic system links the oxidation of an electron donor (here EDTA) to the four electrons of dioxygen. All the experiments allow us to propose the schematic scheme shown in Figure 2.5.

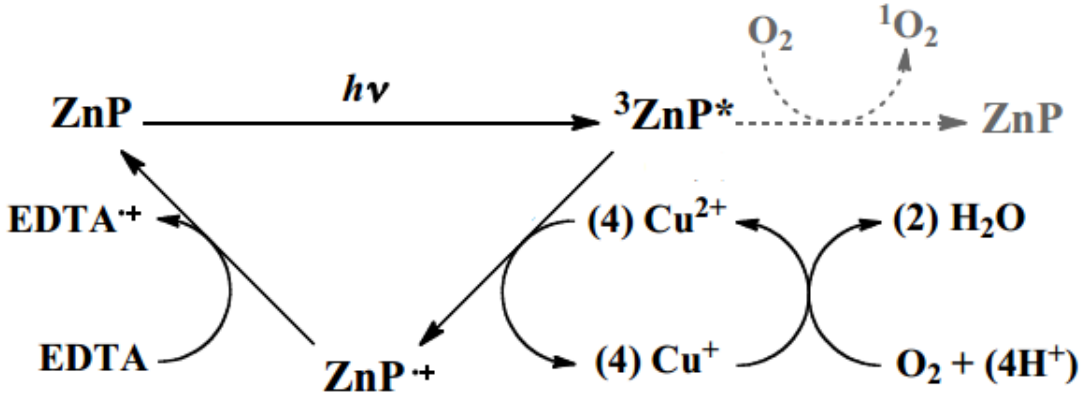


Figure 2.5: Schematic representation of charge transfer upon irradiation of our system after addition of EDTA under presence of dioxygen.

Upon irradiation, the excited triplet state of $ZnTMPyP^{4+}$ is produced ($^3ZnP^*$). $^3ZnP^*$ can be quenched by laccase that can be reduced step-wise by four electrons. Addition of EDTA results in the re-generation of the initial state ZnP by reduction of the $ZnP^{\bullet+}$ produced during the charge transfer reaction. In the presence of dioxygen, $^3ZnP^*$ can be quenched not only by laccase but also by O_2 to form singlet oxygen O_2 ($^1\Delta g$) in an energy transfer process and O_2^- in an oxidative process, although the formation of the latter is probably largely compensated by a fast back electron transfer. Laccase dependent dioxygen consumption (*i. e.* the rate of O_2 consumption sensitive to the inhibitor azide) is reaching a value that is at least 4 times larger than the enzyme-independent one highlighting the influence of the enzyme in the whole process.

These results are accepted for publication to the Journal of the American Chemical Society (JACS, 2013, in press).

III. Grafting the porphyrin on the laccase

Introduction

Site – selective chemical modification of a protein requires two key features: an efficient reaction and the preparation of the components (porphyrin and laccase). Performing chemical reaction on a biological macromolecule and maintaining its activity is not an easy task. The most widely used strategies rely on electrophilic reagents that can react with nucleophilic functional groups of amino acids.

Strategy

Many methods have been developed enabling chemical modifications of proteins. Most of them use nucleophilic residues such as lysines, cysteines and tyrosines.[32] In this case, we chose to perform the modification on laccase's surface through lysine residues. Indeed, the laccase available in our lab (LAC3 from *Trametes* sp.) only contains three free amine groups included the N – terminus and two lysine residues which are located away from T1 Cu site. Construction of a site directed mutant with a unique lysine near the T1 Cu site (K161), removing the two others (K40 and K71) was achieved at Biosciences and is named LAC3 –UniK. So, with this mutant we can perform orientated functionalization on laccase's surface.

In order to develop successful strategies for the derivatization reaction, key requirements have to be followed in order to maintain the activity of the enzyme. Firstly, the reaction should be performed in water. Enzymes can tolerate a small quantity of alcohol or DMSO but the use of these solvents can induce structural and functional changes. The pH needs to be rather neutral (4 to 8) and the temperature has to be near ambient. Also, the low concentration of reactants is an important factor because these molecules can be deleterious to the enzyme at high concentrations.[32]

Therefore, we had to select the effective and selective reaction which follows all the above conditions. Since our laccase is glycosylated, a lot of functionalized groups which can react with amine groups, can react with hydroxyl groups on sugars as well. So, our chosen "grafting" approach relies on the reductive amination of an aldehyde. In particular, we have chosen the reaction between an aldehyde coming from a functionalized porphyrin and an amine coming from a lysine pointing on the surface of the laccase. So, we had to prepare an "aldehyde – containing" porphyrin. The synthetic pathway, which was followed, was the synthesis of the carbonyl functionalized water soluble porphyrin which then was reduced to hydroxyl functionalized porphyrin and finally reoxidized to the formyl one (see Figure 3.1). The last step was the metallation and methylation of the porphyrin.

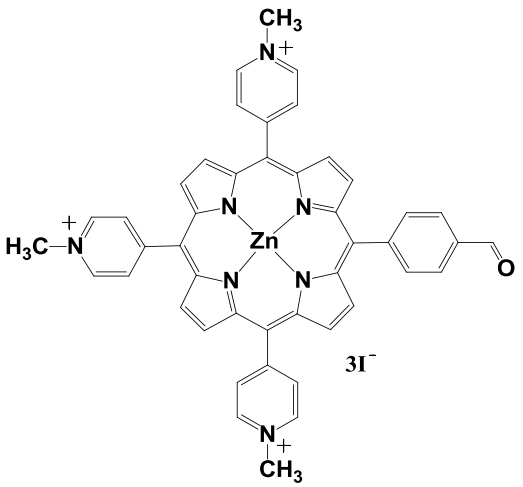


Figure 3.1: Structure of the desired porphyrin

Typically, this reaction is carried out by the addition of reductive agents such as $NaBH_4$ which can reduce the imines formed by condensation of lysine amino groups or the N – terminus with aliphatic or aromatic aldehydes.[32,33] High concentrations typically used can be deleterious for the enzymes. Numerous transition metal catalysed reactions performed in aqueous media and minimal quantities of non toxic reagents have been published.[33] Ogo, Fukuzumi et al[34] have published the ability of an easily prepared water soluble Ir-complex to form hydrid species in the presence of formate ions (see Figure 3.2).

By this study, McFarland and Francis[35] have shown that Ir(III) –complexes can be used as an alternative reagent to reduce imines in the reductive amination of lysines and we selected this catalyst for our derivatization reactions.

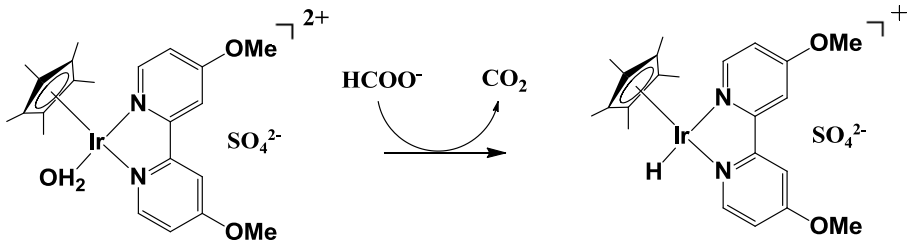


Figure 3.2: Schematic representation of Ir – catalyst reaction in the presence of formate ion.

Our studies began with the development of reaction conditions which would afford the best yield in the minimum derivatization time maintaining the activity of the enzyme. Firstly, we had to find the optimum ratios between enzyme and porphyrin. Secondly, we should find the best pH value from 5 to 8 in which HCOONa (pKa 3.6) acts as $HCOO^-$ to bind the Ir-center[36]. Also, we had to check the influence of the temperature on the yield and rate of reaction (below to 30 ^oC to avoid denaturation).

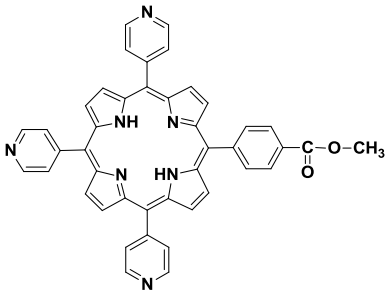
Experimental

Laccase – UniK.

The mutant enzyme was prepared at Biosciences. It was provided ready and pure at the initiation of this project.

Synthesis of the 5-(4-formyl-phenyl)-10,15,20- tris-methyl-(4-pyridyl) porphyrin zinc iodine (5).

Synthesis of 5-(4-methoxy-carbonyl-phenyl)-10,15,20-tris(4-pyridyl) porphyrin (1). 5-



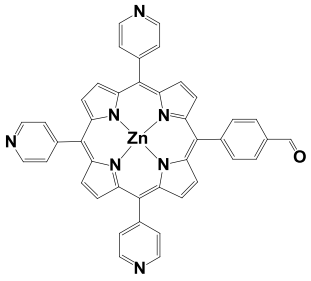
(4-methoxy-carbonyl-phenyl)-10,15,20-tris(4-pyridyl) porphyrin was obtained according to the standard literature procedure.[37] 2,1 g of methyl formyl ester (0,04 mol) and 2,5 ml of 4-pyridyl aldehyde (0,03 mol) were added in a 250 ml round bottom flask, containing 100 ml of propionic acid at 140^oC. 2,6 ml pyrrole (0,013 mol) were then added portion – wise and the reaction was refluxed for 1,5 hours. The propionic acid was removed by distillation and the mixture was dried under reduced pressure. The 6 different products were separated using silica gel chromatography (gradient of DCM to 4% MeOH/DCM), affording 300

mg,(yield:4,5%) of the desired purple product. ^{1}H NMR (300 MHz, $CDCl_3$): -2,89 (2H, s), 4,12 (3H, s), 8,16 (6H, m), 8,28 (2H, d, J=8,1Hz), 8,46 (2H, d, J=8,1 Hz), 8,84 (8H, m) 9,05 (6H, m); UV-Vis (CH_2Cl_2), λ/nm, (ε/M^{-1} cm^{-1}) : 417 (200000), 512 (14270), 546 (4860), 587 (4500), 643 (1950); MS (MALDI-ToF): m/z=676,77 [M+H]+ for $C_{43}H_{29}N_7O_2$

Synthesis of 5-(4-hydroxy-phenyl)-10,15,20-tris(4-pyridyl) porphyrin (2). 5-(4-hydroxy-phenyl)-10,15,20-tris(4-pyridyl) porphyrin was obtained according to the standard literature procedure.[38] 300 mg of **(1)** (0,46 mmol) and 140 mg $LiAlH_4$ (3,6 mmol) were mixed in 45 ml anhydrous THF at room temperature. After stirring for 30 min, H_2O was very carefully poured in the mixture in order to neutralize $LiAlH_4$. Solvents were then evaporated under reduced pressure and the product was purified by basic Aluminum oxide, (Al_2O_3, pH=9.5) chromatography with 3% MeOH/DCM elution. 150 mg of **(2)** were obtained (52% yield). ^{1}H NMR (300 MHz, $CDCl_3$/MeOH): -2,98 (2H, s), 4,95 (2H, s), 7,69 (2H, d, J=8,1 Hz), 8,01 (8H, m), 8,72 (14H, m); UV-Vis ($CHCl_3$), λ/nm, (ε/M^{-1} cm^{-1}): 417 (384720), 512 (17530), 546 (5800), 588 (5430), 644 (2570); MS (MALDI-ToF): m/z=648,53 [M+H]+ for $C_{42}H_{29}N_7O$.

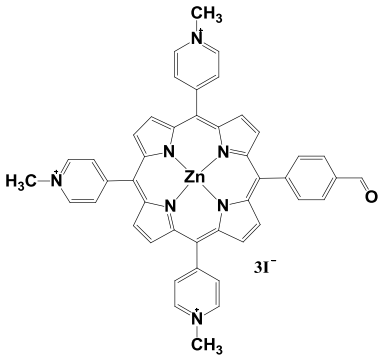
Synthesis of 5-(4-formyl-phenyl)-10,15,20-tris(4-pyridyl) porphyrin (3). 40 eq. of activated manganese oxide (1,85 mmol) was added to a solution of 30 mg of porphyrin **(2)** (0,44 mmol) in 6 ml chloroform under stirring. The heterogeneous mixture was stirred vigorously at room temperature. The reaction was monitored by thin layer chromatography (TLC) and when all the alcohol was oxidized into the aldehyde, manganese dioxide was removed by filtration through Celite, washed three times with chloroform and the combined organic phases were concentrated and dried under vacuum to afford 28 mg (yield:93%) of the porphyrin **(3)**. ^{1}H NMR (400 MHz, $CDCl_3$): -2,86 (2H, s), 8,17 (6H, m), 8,30 (2H, d, J=7,6 Hz), 8,40 (2H, d, J=7,6 Hz), 8,86 (8H, m), 9,06 (6H, m), 10,4 (1H, s); UV-Vis ($CHCl_3$), λ/nm, (ε/M^{-1} cm^{-1}): 417 (446860), 512 (18530), 547 (6060), 587 (5660), 643 (2210)

Synthesis of Zn-5-(4-formyl-phenyl)-10,15,20-tris(4-pyridyl) porphyrin (4). In a 25 ml



round bottom flask 20 mg of porphyrin **(3)** (0,028 mmols) were dissolved in 10 ml of chloroform and and 68 mg of zinc acetate (0,310 mmols) in 2 ml methanol were added. The mixture was left overnight at room temperature. The end of reaction was identified by thin layer chromatography (TLC) and was confirmed by UV – Vis spectroscopy. The mixture was washed 3 times with water and the organic phase was concentrated under reduced pressure to afford 16 mg of product (yield:93%).

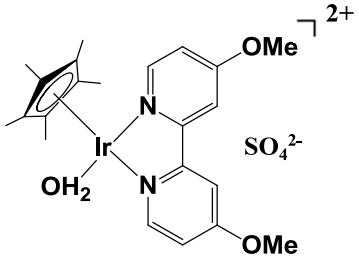
Synthesis of 5-(4-formyl-phenyl)-10,15,20- tris-methyl-(4-pyridyl) porphyrin zinc iodine (5). In a 25 ml round bottom flask 16 mgr of **(4)** were dissolved in 6 ml DMF and 6 ml CH_3I was added. The mixture was left stirring at room temperature for 5 days. The resulting solution was poured into 50 ml of diethyl ether and the precipitated solid was collected by filtration, washed 3 times with diethyl ether and dried under vacuum to give the porphyrin with iodine as counter ion (25 mgr, yield:80%). It is easy to convert the counter ion from iodine salt to the chloride one using Dowex 2 anion exchange but in our cases there was no observed influence of the anion on the results. ^{1}H NMR (400 MHz, DMSO): 4,72 (9H, s), 8,32 (4H, m) 8,79 (8H, m), 8,99 (6H, m), 9,49 (6H, m); 10,3 (1H, s) UV-Vis (H_2O), λ/nm, (ε/M^{-1}*cm^{-1}): 437 (120000), 560 (9955), 602 (3285)



Synthesis of Ir – catalyst

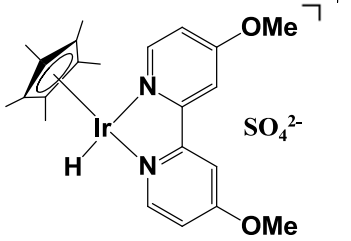
An adapted procedure was used according to the literature.[3]

*Cp*Ir(4,4'-dimethoxy-2,2'-bipyridine)Cl_2.(1)* 32 mg of dichloro(pentamethylcyxlo-



pentadienyl)iridium (III) dimer (40,2 μmol), 17,4 mg of 4,4'-dimethoxy-2,2'-bipyridine (80,4 μmol) and 4 ml of methanol were combined in a scintillation vial with a magnetic stirring bar. The heterogeneous mixture was stirred at room temperature until it became homogeneous (<10 min). The solution was concentrated under reduced pressure and the residue was redissolved in a minimum amount of chloroform. The product was then precipitated by the dropwise addition of hexane until no more precipitate appeared. The precipitate was collected by filtration, washed with hexane (3 x 1ml) and dried in vacuo to yield the product as light yellow solid (48 mg, yield: 97%)

*Cp*Ir(4,4'-dimethoxy-2,2'-bipyridine)SO_4.* 48 mg of **(1)** (78,4 μmol), 16,8 mg of



silver (I) sulfate (53,8 μmol) and 4 ml of water were combined in a scintillation vial charged with a magnetic stirring bar. The heterogeneous mixture was stirred at room temperature. The mixture was filtered to remove the precipitate and the collected material was washed with water (3 x 1ml). The filtrate and washings were combined and the solvent was removed under reduced pressure. The product was obtained as yellow solid (32,6 mg, yield: 95%)

Grafting of porphyrin (5) on the surface of UniK.

In 1 ml aqueous phosphate buffer 50mM set at pH=7.4, 50μM of UniK, 500μM of porphyrin **(5)**, 250μM of Ir-catalyst solution and 25mM HCOONa were mixed. The reaction mixture placed in an eppendorf tube is left for 2-5 days at 30^{o}C in an Eppendorf Thermomixer confort device.

At desired times, 150μl from the reaction mixture were collected, the buffer was exchanged from 50 mM Na_2HPO_4 pH 7.4 to 0,1M B&R pH 4 containing 200mM NaCl using Sartorius Stedin Biotech Vivaspin 500, with a cut – off 10.000 MWCO

and by centrifugation. The sample was then passed through PD Spintrap G-25 size exclusion column and the ratio of concentrations (enzyme/porphyrin) was estimated by UV – Visible Perkin – Elmer Lambda 650 spectrophotometer and 1.0 cm path–length quartz micro cuvettes (100µl).

Activity test of grafted laccase

Laccase activity was assayed at room temperature using siringaldazine (SGZ) as substrate. Oxidation of SGZ was detected by following the absorbance at 525 nm (ε=65.000 $cm^{-1}M^{-1}$) during 1 min using VARIAN, Cary 50 spectrophotometer using disposable plastic cuvettes of 1.00 cm path-length. The reaction was performed in 1 ml of 25mM B&R buffer pH 6.0 containing 0,15µM of enzyme and 66µM siringaldazine (0,8 mg/ml in methanol). The reaction started after the addition of SGZ solution and immediate mixing by inversion.

The enzymatic unit 1U is defined as the µmol of substrate which are consumed by the enzyme per minute.

Electrophoresis conditions

SDS – PAGE experiments were performed on 8% acrylamide-containing gels. The samples were denaturated by boiling them for 5 min in Laemmli buffer containing dithiothreitol (DTT). The samples and the molecular mass markers were loaded and the electrophoresis was performed at 200V using BioRAD Mini – Protean tetra cell device. The running buffer contained 25 mM Tris, 192 mM glycine, 0.1% SDS. Gels were revealed by fluorescence and by Coomassie Blue staining.

Dioxygen consumption

Dioxygen consumption was measured using the same conditions as in the chapter II (Bimolecular porphyrin/laccase system).

Results and Discussion

Grafting of porphyrin (5) on the surface of UniK.

According to McFarland and Francis publication and to other derivatization reactions performed in the laboratory, the concentration of the porphyrin was set 10 times higher than that of the enzyme. Indeed, it appeared to be the optimum ratio between porphyrin and laccase in which the least unreacted enzyme was obtained. The best concentrations of reactants were obtained using 50 μM of laccase, 10 equivalents of porphyrin, 5 equivalents of the Ir – catalyst and 25mM of hydride source (formate). Different temperatures (25 and 30°C) and pH values were tested. The best reaction yield was obtained in phosphate buffer at pH 7.4 and performing the reaction at 30 °C. (see Figure 3.3).

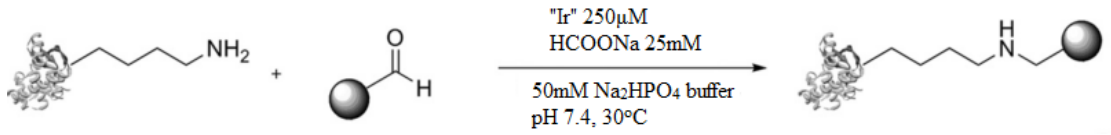


Figure 3.3: Grafting conditions of porphyrin on the surface of laccase – UniK.

The progress of the derivatization was monitored every 6, 24 and 48 hours. In order to measure only the concentration of the porphyrin grafted on the laccase, the removal of the free (unreacted) porphyrin is necessary. The separation of the enzyme (grafted or not) from the unreacted porphyrin was performed using size exclusion chromatography column. Firstly, we found that a good separation required the exchange of buffer from 50mM phosphate buffer pH 7.4 to 0.1M B&R buffer pH 4 and the addition of salt (NaCl). This is probably due to the electrostatic interactions between the laccase (bearing negatively charges at pH>pI≈4) and unreacted porphyrin (positively charged). The ratio of both concentrations (porphyrin and enzyme) was measured by UV-Vis spectroscopy. The concentration of the enzyme was calculated from the absorbance at 280 nm (ε=90.000 $cm^{-1}M^{-1}$) and the concentration of the porphyrin at 437 nm (Soret) (ε~120.000 $cm^{-1}M^{-1}$).

In Figure 3.4 is shown the yield of derivatization versus reaction time. At t=0, when the reagents are mixed together no derivatization is obtained with 0% yield.

The yield of the hybrid system increases with the time and after 48 hours the reaction reaches the 90% yield. No further evolution is observed up to 72 hours.

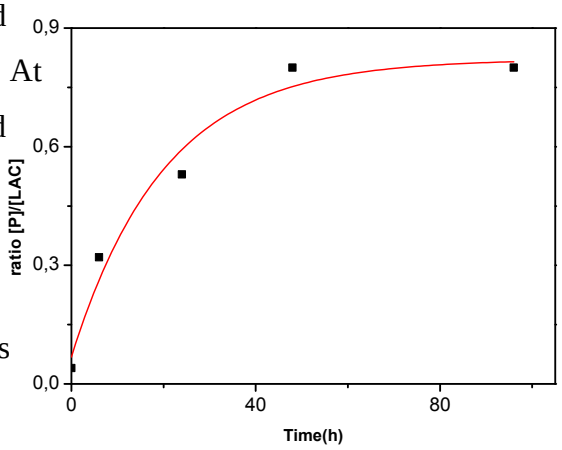


Figure 3.4: Schematic representation of reaction progress.

Atomic absorption and ICP – MS measurements

Atomic absorption and ICP – MS measurements were performed in order to confirm the content in copper and zinc in the hybrid system and therefore the ratio between laccase and porphyrin (Zn ion comes from the porphyrin and Cu ions from the enzyme). The analysis showed that the ratio between the metal ions [Zn]/[Cu] ranges from 0,18 to 0,25, which confirms that the hybrid system contains approximately 1 Zn ion and 4 copper ions per molecule - system, as expected. This is also, an evidence of the modification of laccase.

Activity of laccase

The activity of the laccase was estimated along the reaction. When all the reagents are mixed (t=0) as well as after 6 hours, the laccase is fully active (100% activity). The laccase keeps approximately 80% of the initial activity after 24 hours of reactions and no further activity loss is observed until the end of the reaction (see Table 3.1).

Time	Specific activity (µmol min^{-1}/mg)	% Enzyme activity
0h	20,5 U/mg	100%
6h	20,5 U/mg	100%
24h	17 U/mg	82%
48h	17 U/mg	82%

Table 3.1: Laccase - UniK activity at different derivatization reaction times.

SDS – PAGE staining

The SDS – PAGE analysis shown in Figure 3.5 presents the same gel revealed either by fluorescence (which arises from the porphyrin) or by the protein staining. The mixture was analysed at different reaction times (0,6,24,48, hours). At t=0, UniK enzyme does not display any fluorescence. The fluorescence of the porphyrin appears after 6 hours of reaction. Colour protein staining confirms the presence of the enzyme at the same time. Consequently, as concluded, the modification of laccase has been achieved without any apparent degradation of the enzyme.

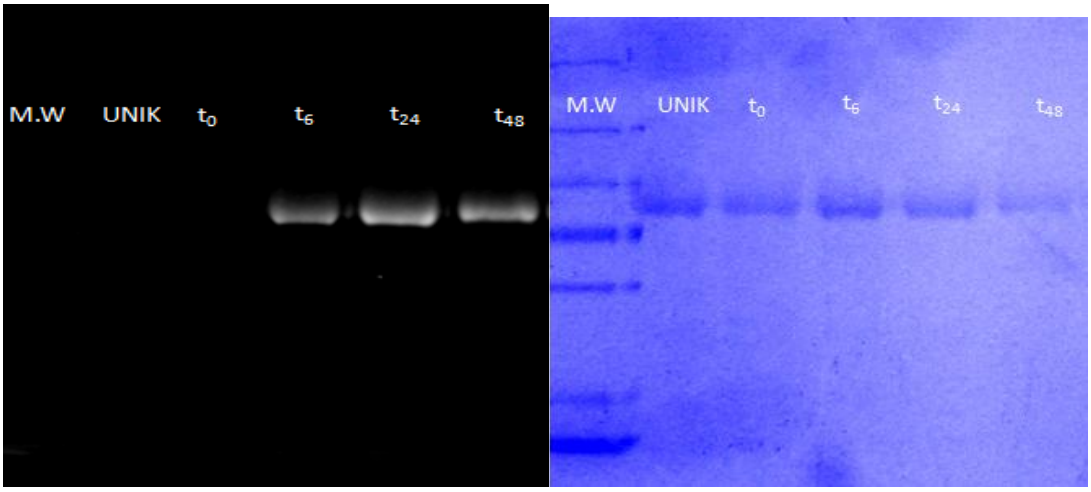


Figure 3.5: SDS – PAGE (8%) of the hybrid system during the derivatization time. Left: Fluorescence of the porphyrin. Right: Coomasie Blue staining of the enzyme. SDS (8%) PAGE; line 1: molecular mass standards; line 2: 7,5 µg of native UniK; line 3: 7,5 µg of grafted enzyme at t_o; line 4: 7,5 µg of grafted enzyme at t_6; line 5: 7,5 µg of grafted enzyme at t_{24}; line 6: 7,5 µg of grafted enzyme at t_{48}.

Dioxygen consumption

Dioxygen comsumption measurements were performed as described in the previous chapter I (Bimolecular system) and preliminary results were obtained. In the dark or in the absence of EDTA, no significant dioxygen consumption was detected. Upon irradiation of a solution containing 4mM of EDTA and 5µM of grafted laccase in 1.0 ml of 0,1M B&R buffer pH 4, the dioxygen concentration decreases (see Figure 3.6). In the presence of NaN_3 the enzyme should be totally inhibited but the consumption rate remains unchanged. This unexpected behavior, that was unfortunately only studied once, is not understood yet. It seems to indicate that there is no direct electron transfer between the porphyrin and the laccase and that the observed consumption of dioxygen is only due to the reaction of O_2 with the photosensitizer. This could be the case if the porphyrin is attached on the N- terminal amine or if the position of the lysine (residue 157) is not suitable for electronic communication between the redox partners. Another UNIK mutant is available in the laboratory and could then be tested.

However, it is also possible that the absence of enzyme – dependent dioxygen consumption is due to the instability of the hybrid system. Indeed, we observed a high sensitivity of the grafted –UNIK that required to be handled with care (storage at low temperature, sensitivity to light). From that observation, samples were kept in dark during derivatization reactions or treatment in order to avoid degradation of the system. The experiments will soon be reproduced with fresh samples.

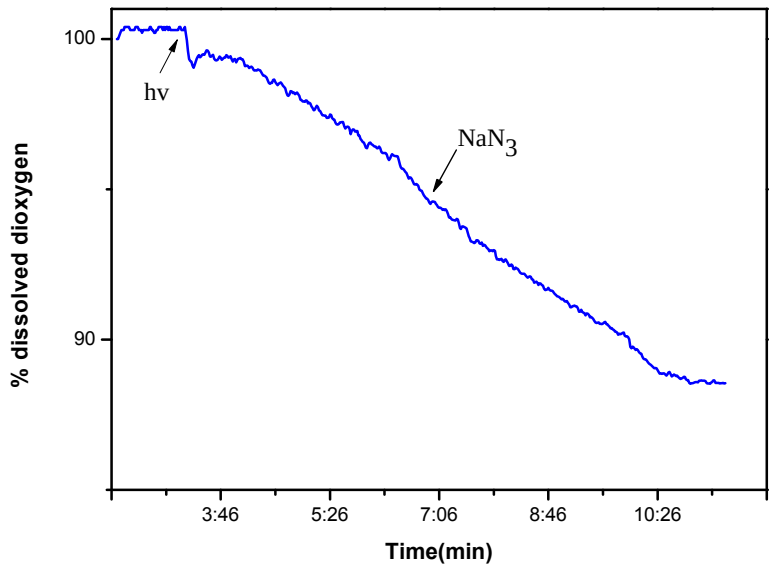


Figure 3.6: Dioxygen consumption rate in the presence of NaN_3.

Conclusion

Overall, we have successfully settled a strategy for the covalent attachment of a porphyrin on the surface of a single – lysine containing mutated laccase (UniK). A single photosensitizer was attached in an almost quantitative yield (90%). Also, within the hybrid system, the laccase is active and keeps 80% of the initial activity of the unmodified enzyme. The attachment site (N-terminus or lysine) will be verified by mass spectrometry and light – driven electron transfer and dioxygen consumption measurements will be performed to characterize the hydrid system.

IV. Grafting of porphyrin and laccase on TiO_2 nanoparticles

Introduction

TiO_2 is among the most popular photocatalyst although its inactivity in the visible light region limits its practical applications. TiO_2 is close to being an ideal photocatalyst in several respects: Its chemical stability, non-toxicity, low cost, and other advantageous properties. Herein, we report the photocatalytic activity of a new hybrid system constituted of TiO_2 nanoparticles modified with free base water soluble porphyrins and our laccase.

Strategy

The principle of this prototype system is that visible light excites the photosensitizer, which injects electrons into the TiO_2 conduction band (the oxidized dye is recovered by the sacrificial electron donor) and the electrons are then transferred into the enzyme.

The electronic coupling between dyes and TiO_2 is significantly influenced by various physicochemical properties. It includes the type of the ligand, the anchoring group of the dye and the surface coverage of the dye, as well as the properties of TiO_2 nanoparticles (i.e anatase vs rutile).[39]

The photosensitizer attached on TiO_2 should fulfil some requirements included i) an absorption in the visible spectrum, ii) stable attachment to TiO_2, iii) efficient charge separation and iv) long – term stability upon irradiation.

The kind and the number of anchoring groups govern its stability on TiO_2. Typical anchoring groups for such semiconductors include anionic groups like phosphonate and carboxylate. One of the most popular anchoring groups is the carboxylate and it is known to bind to TiO_2 mainly through a bidentate bridging mode.

On the other hand, stable adsorption of laccase on TiO_2 surface is required for the photoreduction of dioxygen. Strong interactions between TiO_2 and aspartate or glutamate carboxylates have been reported, which allow the enzyme to be efficiently adsorbed.[40]

The photosensitizers which were used are a porphyrin with four carboxylate groups (**C4**) or one with one carboxylate group (**C1**), as shown in Figure 4.1.

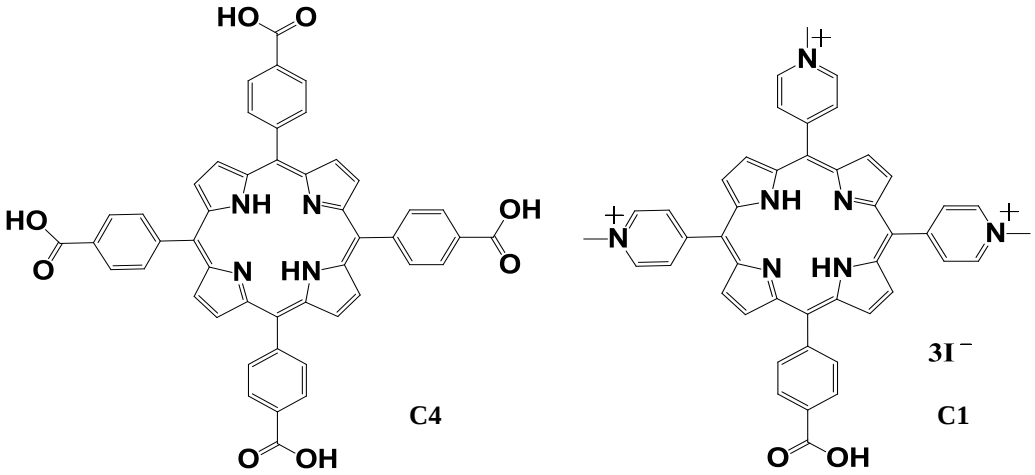


Figure 4.1: Structures of porphyrinic complexes used in this study.

In this chapter, we report the photocatalytic reduction of dioxygen by adsorbing both the photosensitizer and the laccase on TiO_2 nanoparticles (see Figure 4.2).

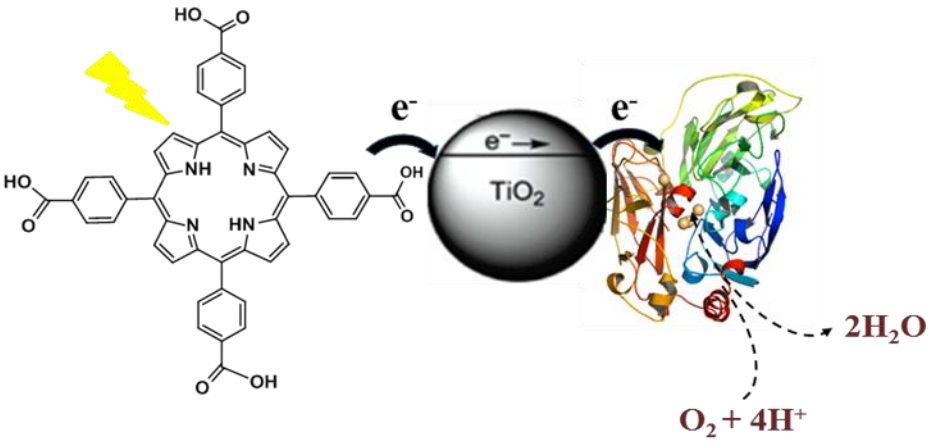


Figure 4.2: Oxygen reduction representation, using laccase conjugated with porphyrin via TiO_2 nanoparticles.

Experimental

Chemicals.

The nanoparticles TiO_2 (AEROXIDE, P25 particles from Evonik Industries) were an anatase/rutile (8:2) mixture with an average size of 21 nm. Triethanolamine buffer (TEOA, 25mM) was used as a medium for adsorbing the components on TiO_2 nanoparticles and also, as sacrificial electron donor. The triethanolamine solution was titrated with dilute HCl to the pH 6 at room temperature.

Laccase production.

Laccases were produced as previously described in "Bimolecular porphyrin/laccase system". Samples were kept in freezer in aqueous phosphate buffer pH 6.0 + 20% glycerol.

Synthesis of porphyrins.

Synthesis of 5,10,15,20 – (4 – carboxy – phenyl) porphyrin (C4).

The 5,10,15,20 (4 – methoxy – carbonyl phenyl) porphyrin was synthesized following the literature procedure.[41]

Synthesis of 5,10,15 – (4-pyridyl) - 20 – (4 – carboxy – phenyl) porphyrin (C1).

The *5,10,15 – (4-pyridyl) - 20 – (4 – carboxy – phenyl)* porphyrin was synthesized following the literature procedure.[42]

Adsorption of photosensitizers to TiO_2 nanoparticles.

The dye (0.6 μmol) was dissolved in 0.4 ml of 25 mM triethanolamin (TEOA) buffer pH 6.0. 30 mg of TiO_2 was sonicated in 4.6 ml of the same solvent and the dye solution was then added to the stirred dispersion. The mixture was left stirring for 20 min for **C4** and overnight for **C1** under protection from light. The mixture was then centrifuged and the clear supernatant was analyzed by UV-Vis spectrophotometry. The amount of the adsorption of each sensitizer on TiO_2 was estimated by comparison of the absorbances at Soret band before and after the adsorption procedure. Desorption of the photosensitizers was studied by re dispersing the centrifuged dye – sensitized TiO_2 nanoparticles in fresh TEOA buffer (5 ml, pH 6.0, 25 mM) and stirring the resulting dispersion for 20 more min for **C4** and overnight for **C1**. The

amount of desorbed complex was quantified by spectrophotometry on the centrifuged supernatant.

Adsorption of laccase to TiO_2 nanoparticles.

Laccase was added in 5ml final volume of TEOA buffer pH 6.0 (3 μM). Attachment of the laccase to the TiO_2 nanoparticles was quantified by analyzing the absorbance difference at 280 nm of enzyme solution before and after stirring for 15 min with variable amounts of TiO_2 nanoparticles (5, 10, 30 mg). The extinction coefficient for the laccase is 90.000 M^{-1} cm^{-1} at 280 nm.

Adsorption of photosensitizer and laccase on TiO_2.

First photosensitizer, then laccase

A mixture of dispersed TiO_2 nanoparticles (30 mg) and photosensitizer (120μM) was stirred in 5 ml final volume of 25 mM TEOA buffer pH 6 for 20 min. The particles were separated from the supernatant solution by centrifugation and dried. All the amount of particles are resuspend with fresh 5 ml of 25 mM TEOA buffer pH 6 and laccase (3μM) was added and left stirring for 15 min.

First laccase, then photosensitizer

A mixture of dispersed TiO_2 nanoparticles (30 mg) and laccase (3μM) was stirred in 5 ml of 25 mM TEOA buffer pH 6 for 15 min. The particles were separated from the supernatant solution by centrifugation and dried. All the amount of particles are resuspend with fresh 5 ml of 25 mM TEOA buffer pH 6 and the porphyrin (200μM) was added stirring for 20 min for **C4** and overnight for **C1**.

Activity tests

Laccase activity was assayed at room temperature using siringaldazine (SGZ) as substrate, as already referred in chapter "Grafting of porphyrin on laccase". The reaction was performed in 1 ml of 25mM B&R buffer pH 6 containing 3nM of enzyme and 66μM siringaldazine (0,8 mg/ml in methanol).

Dioxygen consumption rates

Dioxygen consumption was measured using the same conditions as in the chapter II (Bimolecular porphyrin/laccase system). 10µl of suspension was added in 1 ml final volume of TEOA buffer pH 6. EDTA was not added because TEOA acts also as sacrificial electron donor.

Results

Adsorption of porphyrin to TiO_2 nanoparticles.

UV – Vis spectra showed that >90% of the **C4** porphyrin is attached on TiO_2 nanoparticles after only 20 minutes. On the contrary only 58% of **C1** porphyrin is adsorbed after the same time, as shown in Figure 4.3.

UV-Vis results after washing the porphyrin adsorbed on TiO_2 nanoparticles indicate that the **C4** binds strongly on TiO_2 since no desorbed porphyrin is observed compared to **C1** which is unstable on the particles and can be desorbed very easily (see Figure 4.3).

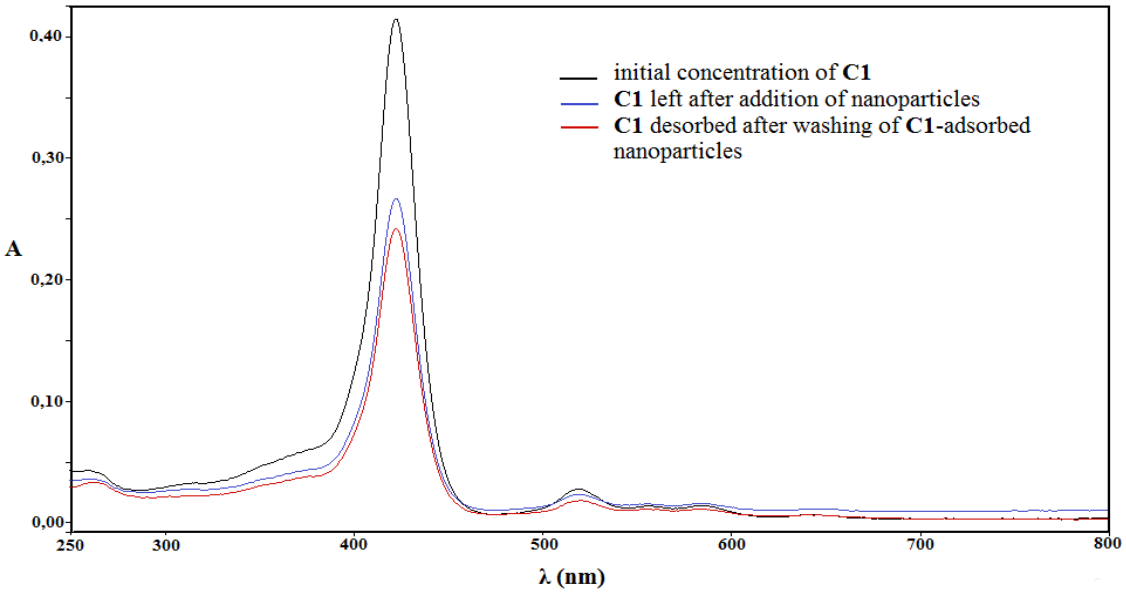


Figure 4.3: Electronic absorption spectra of aqueous solution of 200µM **C1** before (red) and after (green) addition of TiO_2 (30 mg) stirring for 20 min. The supernatant solution was separated from the TiO_2 nanoparticles by centrifugation. Blue line shows the **C1** that desorbed after washing the grafted particles for 20 min. The samples were diluted 50 times in order to observe the Soret band.

Although 20 minutes is enough time for **C4** to be totally attached on TiO_2 nanoparticles, it is not enough for **C1**. So, the mixture solution with **C1** porphyrin was left overnight to see if it is possible to attach efficiently (see Figure 4.4). It seems that

approximately 95% is attached but is not stable on the particles for more than a few hours (data not shown).

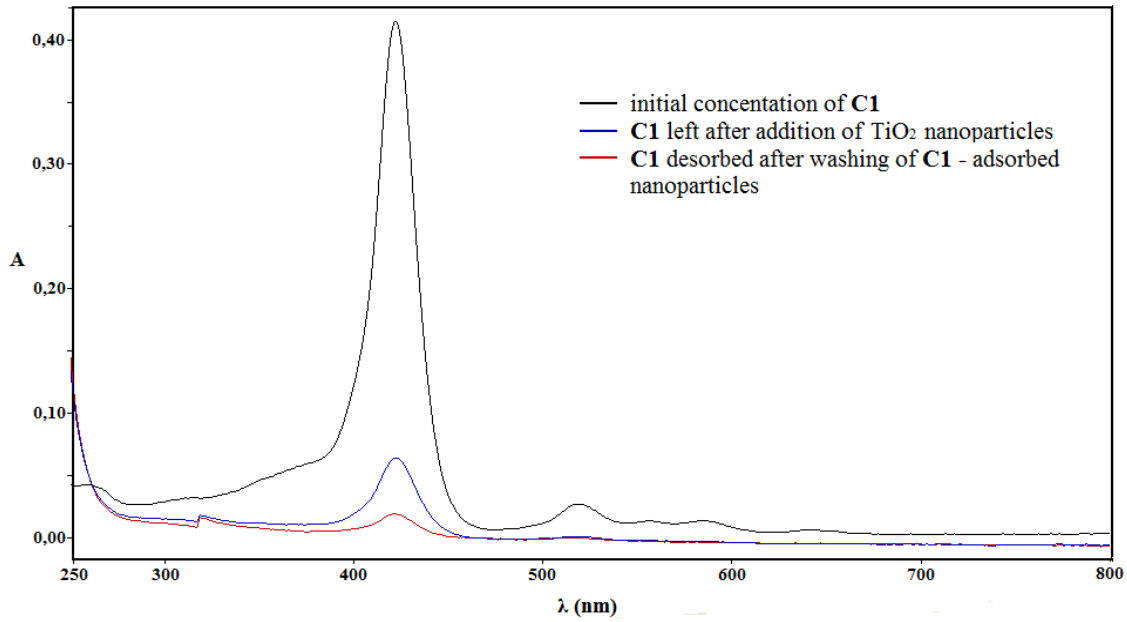


Figure 4.4: Electronic absorption spectra of aqueous solution of 200μM **C1** before (green) and after (blue) addition of TiO_2 (30 mg) stirring overnight. The supernatant solution was separated from the TiO_2 nanoparticles by centrifugation. Red line shows the **C1** that desorbed after washing the grafted particles for 20 min. The samples were diluted 50 times in order to observe the Soret band.

Adsorption of laccase to TiO_2 nanoparticles.

Laccase are kept in phosphate buffer pH 6. Firstly, adsorbing laccase in the presence of pH 6 phosphate/TEOA 25 mM buffer, resulted in unmodified particles with laccase (data not shown). It is well known that phosphate has a high affinity for TiO_2. Therefore, phosphate blocks the binding sites on TiO_2 for laccase. So, the phosphate buffer was exchanged by 25 mM TEOA buffer pH 6 to attach the laccase on the particles.

UV-Vis spectra showed that increasing the amount of TiO_2 nanoparticles in dispersion keeping the concentration of laccase (3μM) constant in 5ml of buffer, the attachment of the enzyme is getting higher. We verified that for 3μM in 5ml final volume of buffer per 30 mg TiO_2 nanoparticles is the best minimum ratio in which laccase is totally attached on the particles (see Figure 4.5).

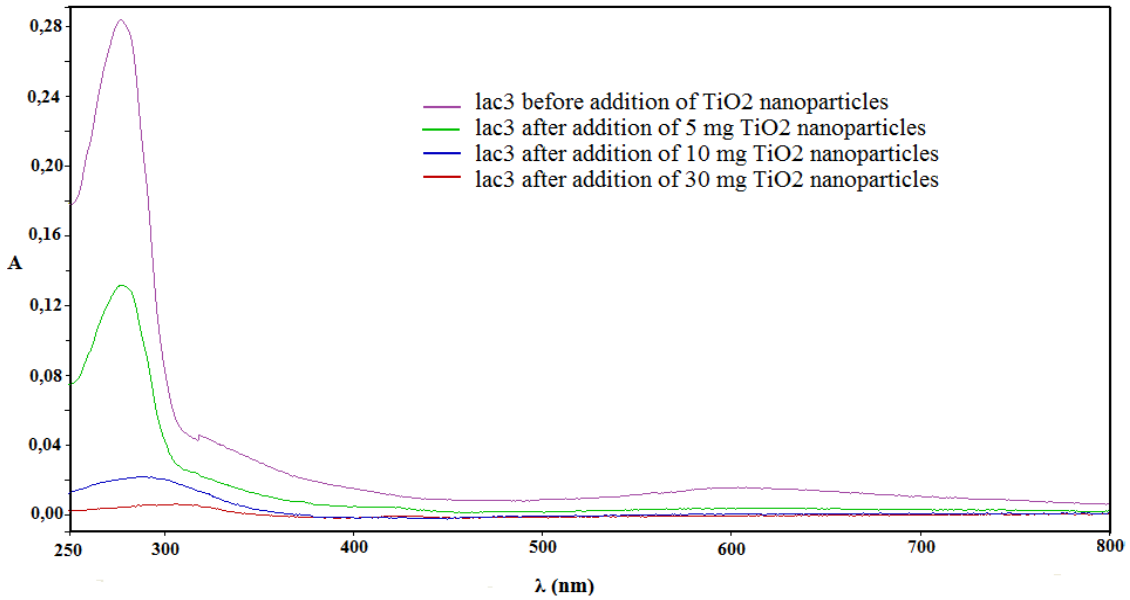


Figure 4.5: Electronic absorption spectra of aqueous solution of laccase (3μM) in 5 ml of 25 mM TEOA buffer pH 6 before addition of TiO_2 nanoparticles (purple), after addition of 5 mg TiO_2 nanoparticles (green), 10 mg TiO_2 nanoparticles (blue), 30 mg TiO_2 nanoparticles (red).

Adsorption of photosensitizer and laccase on TiO_2.

First photosensitizer, then laccase

Adsorbing first the porphyrin on the particles and subsequent addition of enzyme, laccase is not attached on the particles, as shown in Figure 4.6. A hypothesis for this behavior is that the porphyrin at high levels forms a dense coverage on the oxide, leaving no space for the much larger enzyme molecules to adsorb.

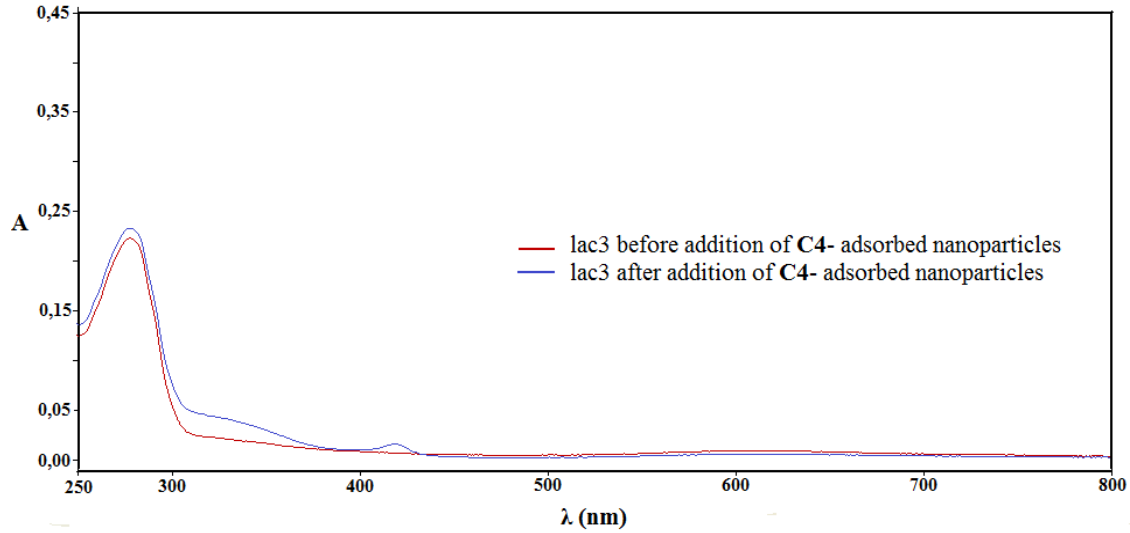


Figure 4.6: Electronic absorption spectra of aqueous solution of laccase in supernatant after mixing the dye – sensitized nanoparticles (30 mg) and free laccase (3μM) in 5ml of 25 mM TEOA buffer pH 6.

First laccase, then photosensitizer

On the other hand, adsorbing laccase on TiO_2 prior to addition of the porphyrin resulted in efficient enzyme - modified particles, shown in Figure 4.7. This means that even if the larger laccase was adsorbed, there should always remain sites at which the much smaller porphyrin molecules can bind.

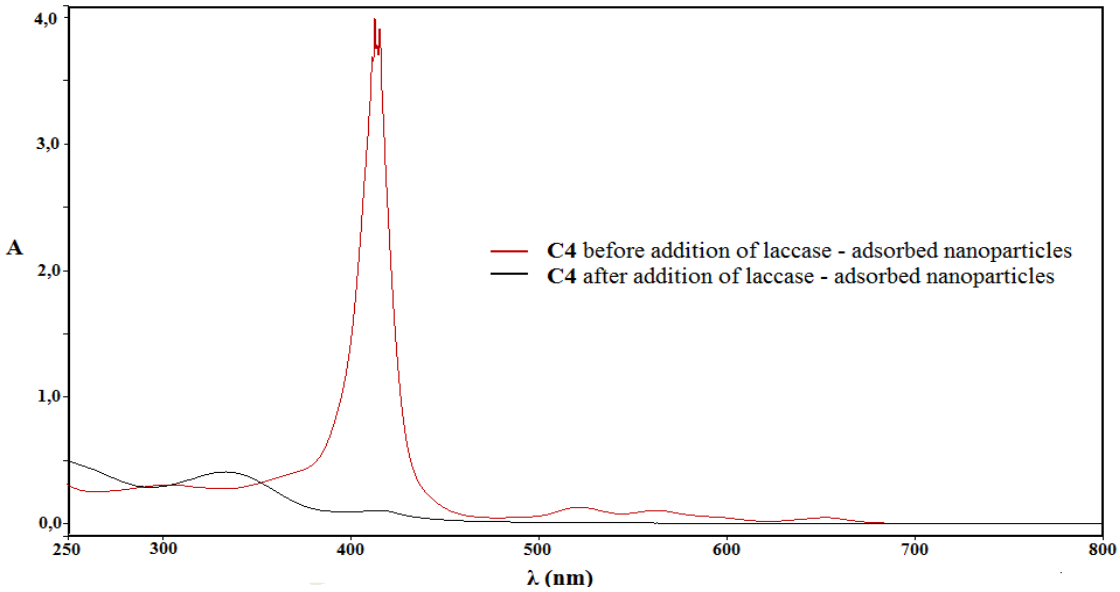


Figure 4.7: Electronic absorption spectra of aqueous solution of porphyrin in supernatant after mixing the laccase – sensitized nanoparticles (30 mg) and free **C1** porphyrin (200µM) in 5 ml of 25 mM TEOA buffer pH 6.

Activity of laccase

The activity of laccase was checked using syringaldazine as substrate. The activity remains the same as compared to laccase enzyme in solution of 25 mM TEOA buffer pH 6 and enzyme modified particles.

Laccase	Specific activity (µmol min^{-1}/mg)	% Enzyme activity
In solution TEOA buffer pH 6	19,3 U/mg	100%
Attached on TiO_2 nanoparticles	18,8 U/mg	97%

Table 4.1: Activity test of the enzyme attached on TiO_2 nanoparticles compared to that of free enzyme in TEOA buffer.

Dioxygen consumption rates

Light-driven dioxygen consumption was then analyzed. When TiO_2 nanoparticles are used alone without attachment of either porphyrin or laccase or using particles modified with laccase, no dioxygen consumption was observed. In the Figure 4., using porphyrin/TiO_2 nanoparticles, the dioxygen consumption rate ranges from 5 to $7x10^{-6}$ $mol.L^{-1}.min^{-1}$. When both porphyrin and laccase are attached on the particles, the decrease in dioxygen concentration is 2-3 times higher than that in absence of laccase. The enzyme – dependent dioxygen consumption was inhibited in the presence of 10 mM of NaN_3. As a control, we tested the same concentration of porphyrin and laccase in solution (bi-molecular system). Porphyrin alone (2μM) in (aerated) solution consumes dioxygen at a rate ranging from 8 to 12 $x10^{-6}$ $mol.L^{-1}.min^{-1}$. Adding laccase did not increase O_2 consumption (see Figure 4.8). However, it is noteworthy that the concentration of laccase in this experiment is very low (30nM) as compared with the bimolecular setting described previously (40μM, see chapter II) and an absence of effect upon laccase addition is then rather expectable. These experiments are going to be performed again with fresh samples to verify and confirm the results.

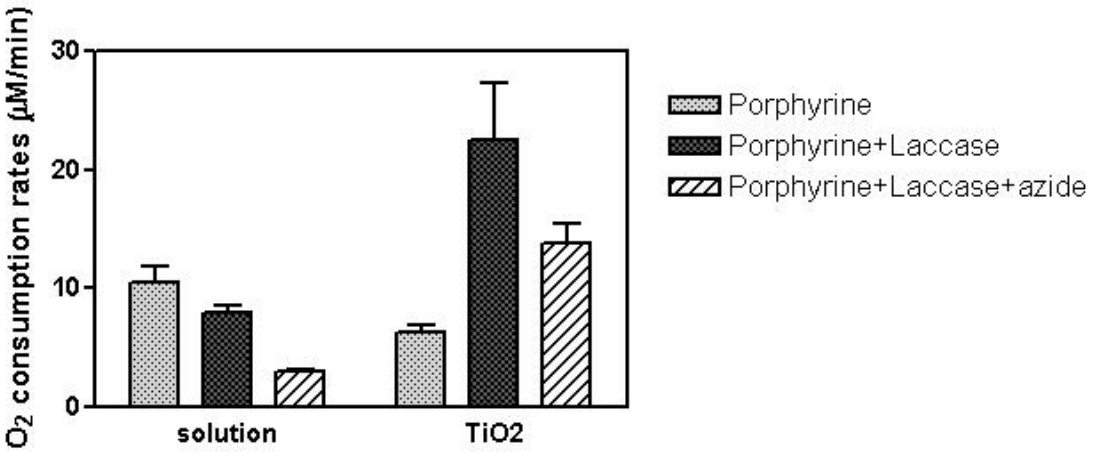


Figure 4.8: Schematic representation of dioxygen consumption rates of porphyrin and porphyrin/laccase system in the presence or absence of sodium azide either in solution or attached on TiO_2 nanoparticles.

In order to better understand the system, porphyrin and laccase were mixed in solution (2μM/30nM). Upon irradiation, the dioxygen consumption of bimolecular system is approximately 9 $x10^{-6}$ $mol.L^{-1}.min^{-1}$. Addition of TiO_2 nanoparticles induces an enhancement of dioxygen consumption (ranging from 15 to 17 $x10^{-6}$ $mol.L^{-1}.min^{-1}$). In the presence of sodium azide, the dioxygen consumption decreases and the rates reach the 10 $x10^{-6}$ $mol.L^{-1}.min^{-1}$ which is approximately the initial consumption of the system before the addition of the particles (see Figure 4.9). Apparently, the effect of TiO_2 particles used as mediator to link the porphyrin and laccase is very important. So, the electronic connection between porphyrin and laccase through TiO_2 nanoparticles is more effective than in solution.

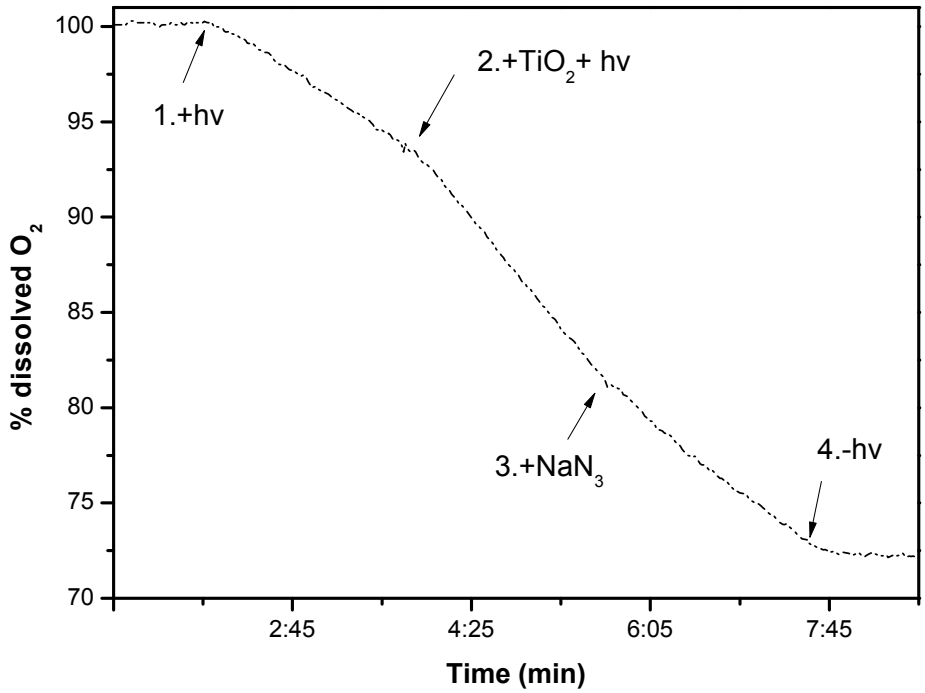


Figure 4.9: Schematic representation of dioxygen consumption rates:
1. After irradiation of porphyrin/laccase system (bi-molecular), 2. After addition of unmodified TiO_2 nanoparticles under irradiation, 3. After addition of NaN_3, 4. After stopping irradiation. Final concentration of porphyrin: 2μM and laccase: 30nM

Conclusion

We have investigated that the surface binding on TiO_2 is highly dependent on the number of anchoring groups (carboxylate) of the porphyrin. As a result, the **C4**-TiO_2 system was used for further experiments since this porphyrin is very stable on TiO_2. The laccase also binds strongly on TiO_2 and maintains its activity. So, adsorption of both porphyrin and laccase was achieved and preliminary results show that the dioxygen consumption rate is approximately 2-3 times higher in the presence of TiO_2 than in the bimolecular system.

Appendix

Preparation of aqueous buffer solutions

- Deionized water used was ultrapure and prepared by Millipore Milli-Q system.
- To adjust the pH of the buffer solutions was used Mettler Toledo meter, since it has been calibrated with buffers pH 4 and pH 7 derived from the company.
- The preparation of Britton - Robinson (B&R) buffer solutions were made as follows:

0.1 M aqueous solution of acetic acid

0.1 M aqueous solution of phosphoric acid

0.1 M aqueous solution of boric acid

Then they were mixed in a 1:1:1 ratio and the pH adjusted to the desired value by adding concentrated first and then dilute aqueous solution NaOH 1M and 0.1M respectively.

- The preparation of aqueous sodium phosphate solution (phosphate buffer) at desired pH value was done by mixing aqueous solution of 0.2 M Na_2HPO_4 and 0.2 M NaH_2PO_4 in a ratio according to the following table.

pH	% of total phosphate as monobasic form	% of total phosphate as dibasic form
5.7	93.5	6.5
5.8	92.0	8.0
5.9	90.0	10.0
6.0	87.7	12.3
6.1	85.0	15.0
6.2	81.5	18.5
6.3	77.5	22.5
6.4	73.5	26.5
6.5	68.5	31.5
6.6	62.5	37.5
6.7	56.5	43.5
6.8	51.0	49.0
6.9	45.0	55.0
7.0	39.0	61.0
7.1	33.0	67.0
7.2	28.0	72.0
7.3	23.0	77.0
7.4	19.0	81.0
7.5	16.0	84.0

7.6	13.0	87.0
7.7	10.5	90.5
7.8	8.5	91.5

- EDTA.2H_2O was weighed and stirred vigorously with a stirrer in water. However, the EDTA will not dissolve if the pH of the solution is not reached the value 8. Therefore, adjust the pH by adding aqueous NaOH solution and EDTA was successfully dissolved.
- The Gilson pipettes used for any dilutions were bought Pipetman ® Company 20, 200 and 1000 µl.

PUBLICATIONS

In part of the Master thesis, the following publications were resulted in (one of which in a well known journal and an international conference):

- Theodore Lazarides, Igor V. Sazanovich, A. Jalila Simaan, Maria - Chrisanthi Kafentzi, Yasmina Mekmouche, Bruno Faure, Marius Réglier, Julia Weinstein, Athanassios G. Coutsolelos, Thierry Tron
  "*Visible Light-Driven O_2 Reduction by a Porphyrin-Laccase System*"(**J.Am.Chem.Soc., in press**).
- Maria-Chrysanthi P. Kafentzi, Athanassios G. Coutsolelos , Yasmina Mekmouche, Pierre Rousselot–Pailley, Viviane Robert, A.Jalila Simaan, Thierry Tron
  "*Synthesis of new covalently linked laccase-porphyrin hybrid system*"
  Poster Presentation, Oxizymes - 2012 (10^{th} International Symposium on Peroxydases) Marseille, France, 16-19 September 2012).

Bibliography

[1] D. Dolphin, "*The Porphyrins*", Academic Press **1978**, Vol. 1 "*Porphyrins and Metalloporphyrins*", Elsevier Scientific Publishing Company, **1975**, 2-28.

[2] F. H. Seyler, *Phys. Chem.* **1987-1988**, *1*, 121.

[3] P. Schaeffer, R. Ocampo, H. J. Callot, P. Albrecht, *Nature (London)*, *364*, 133.

[4] G. A. Spyroulias, A. Despotopoulos, C. P. Raptopoulou, A. Terzis, A. G. Coutsolelos, *Chem. Commun.*, **1997**, 783.

[5] (a). W. K. Wong, L. Zhang, W. T. Wong, F. Xue, T. C. W. Mak, *J. Chem. Soc., Dalton Trans.*,**1999**, 615. (b). W. K. Wong, L. Zhang, F. Xue, T. C. W. Mak, *J. Chem. Soc., Dalton Trans.*,**1999**, 3053. (c). J. W. Buchler, M. Kihn-Botulinski, J. Löffler, B. Scharbert, *New J. Chem.*, **1992**, *16*, 54. (d). J. H. Fuhrhop, *Angew. Chem. Int. Ed. Engl.*, **1974**, *15*, 321.

[6] K. Kalyaanasundaram, *Inorg.Chem.*, **1984**, 23, 2453-2459.

[7] R.F.Pasternack, L.Francesconi, D.Raff, E.Spiro, *Inorg. Chem.*, **1973**, 12, 11, 2606 - 2611.

[8] J. MacDonald, T. J. Dougherty, *J. Porphyrins Phthalocyanines*, **2001**, *5*, 105.

[9] K. Ladomenou, G. Charalampidis, A.G. Coutsolelos, *Inorg. Chim. Acta.*, **2010**, 363, 2201 – 2208.

[10] T.Lazarides, I. Sazanovich, A.J.Simaan, M.C.Kafentzi, Y.Mekmouche, B.Faure, M.Réglier, J.Weinstein, A.G. Coutsolelos, T. Tron, *J.Am. Chem. Soc.*,**2013,** in press.

[11] M. K. Panda, K. LAdomenou, A. G. Coutsolelos, *Coord. Chem. Rev.*, **2012**, 256, 2601 – 2627.

[12] S. Riva, *TRENDS in Biotechnology*, **2006**, 24, 5, 219 – 226.

[13] H. Yoshida, *J.Chem.Soc.*, **1883**, 43, 472-486.

[14] C.F. Thurston, *Microbiology*, **1994**, 140, 19-26.

[15] K. J. Kramer, M. R. Kanost, T. L. Hopkins, H. Jiang, Y.C. Zhu, R. Xu, J. L. Kerwin, F. Turecek, *Tetrahedron*, **2001**, 57, 385 – 392.

[16] C. Raghukumar, *Mycol. Res.*, **2000**, 1222 – 1226.

[17] V. Madhavi and S. S. Lele, *BioResources*, **2009**, 4, 4, 1694 – 1717.

[18] P. Baldrian, *FEMS Microbiol. Rev.*, **2006**, 30, 215 – 242.

[19] L. Quintanar, C. Stoj, A.B. Taylor, P.J. Hart, D.J.Kosman and E.Solomon, *Acc.Chem.Res.*, **2007**, 40, 445 – 452.

[20] D.J. Kosman, *J.Biol.Inorg.Chem.*,**2010**, 15, 15 – 28.

[21] V.K. Gochev, A.I. Krastanov, *Bulgarian Journal of Agricultural Science*, **2007**, 13, 75 – 83.

[22] E.I. Solomon, U.M Sundaram, T.E Machonkin, *Chem. Rev.*, **1996**, 96, 2563 – 2605.

[23] O. Loera Corral, C. I. Pérez Pérez Ma., J. R. Barbosa Rodrigues, and F.Villasenor Ortega, "15. Laccases" in *Advances in Agricultural and Food Biotechnology*, ISBN: 81 – 7736 – 269 – 0, **2006**, India, 323 – 340.

[24] E.I. Solomon, A.J. Augustine, J. Yoon, Dalton Trans. 2008, 30, 3921 – 3932

[25] F. Xu, *J.Biol.Chem*, **1997**, 272, 2, 924 – 928.

[26] Yasmina Meckmouche, S. Zhou, A. M. Cusano, E. Record, A. Lomascolo, V. Robert, A.J. Simaan, P. Rousselot – Pailley, F. Chaspoul, T. Tron submitted to *Applied Microb. Biotech*.

[27] A. Klonowska, C.Gaudin, M. Asso, A. Fournel, M. Réglier, T.Tron, *Enz. Microb. Technol.*, **2005**, 36,34

[28] H. B. Gray, J.R. Winkler, *Biochim. Biophys. Acta Bioenergetics*, **2010**, 1797, 1563 – 1572.

[29] (a). E. Reisner, D.J. Rowell, C.Cavazza, J.C.Fontecilla-Camps, F.A.Armstrong, *J.Am.Chem.Soc.*, **2009**, 131, 18457 – 18466, (b) T. Woorlerton, S. Sheard, E. Reisner, E. Pierce, S.W.Ragsdale, F.A. Armstrong, *J.Am.Chem.Soc.*, **2010**, 132, 2132 – 2133.

[30] A. J. Simaan, Y. Mekmouche, C.Herrero, P. Moreno, A. Aukauloo, J. A. Delaire, M. Reglier, T.Tron, *Chem. Eur. J.*, **2011**, 17, 11743 – 11746.

[31] A. Harriman, G. Porter, P. Walters, *J.Chem.Soc.*, Faraday Trans.1, **1983**, 79, 1335

[32] E. Baslé, N.Joubert, M. Pucheault, *Chemistry and Biology Review*, **2010**, 213 – 227

[33] J.M.Antos, M.B.Francis, *Curr. Opin. Chem. Biol.*, **2006**, 10,253 – 262

[34] T. Abura, S.Ogo, Y.Watanabe, S. Fukuzumi, *J. Am. Chem. Soc.*, **2003**, 125, 4149

[35] J. M. McFarland and M.B.Francis, *J.Am. Chem. Soc.*, **2005**, 127, 13490 – 13491

[36] S.Ogo, K.Uehara, T.Abura, S.Fukuzumi, *J. Am. Chem. Soc.*, **2004**, *3020 – 3021*

[37] Y. Ishikawa, A. Yamashita, T. Uno, *Chem. Pharm. Bull.*, **2001**, 49, 287 – 293

[38] K. Okuda, C. Abeta, T. Hirota, M. Mochizuki, T. Mashino, *Chem. Pharm. Bull.*, **2002**, 50, 7, 985 – 987

[39] Hyunwoong Park, Eunyoung Bae, Jae – Joon Lee, Jaiwook Park and Wonyong Choi, *J. Phys. Chem. B*, **2006**, 110, 8740 – 8749.

[40] E. Reisner, D.J. Rowell, C.Cavazza, J.C.Fontecilla-Camps, F.A.Armstrong, *J.Am.Chem.Soc.*, **2009**, 131, 18457 – 18466.

[41] Joao P.C. Tomé, Maria G. P. M. S. Neves, Augusto C. Tomé, José A. S. Cavaleiro, Marina Soncin, Michela Magaraggia, Stefania Ferro and Giulio Jori, *J. Med. Chem.*, **2004**, 47, 6649 – 6652.

[42] Teresa Gianferrara, Alberta Bergamo, Ioannis Bratsos, Barbara Milani, Cinzia Spagnul, Gianni Sava and Enzo Alessio, *J. Med. Chem.*, **2010**, 53, 4678 – 4690.