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Michael Holzinger, Serge Cosnier, Paulo Henrique M. Buzzetti. The versatility of pyrene and its derivatives on sp² carbon nanomaterials for bioelectrochemical applications. *Synthetic Metals*, 2023, 292, pp.117219. 10.1016/j.synthmet.2022.117219 . hal-03916047

HAL Id: hal-03916047

<https://hal.science/hal-03916047v1>

Submitted on 6 Feb 2024

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The versatility of pyrene and its derivatives on sp^2 carbon nanomaterials for bioelectrochemical applications.

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Abstract

Pyrene and its derivatives became a standard tool for the immobilization of bioentities on nanostructured sp^2 carbon such as graphene and carbon nanotubes (CNTs) for bioelectrochemical applications. Stable π -stacking interaction between these compounds can be formed under mild conditions without affecting sensitive anchor functions of pyrene or introducing defect groups on the carbon material. Pyrene has further beneficial properties for bioelectrochemical studies. Pyrene itself can form supramolecular inclusion complexes with β -cyclodextrin and can even interact with the hydrophobic domain of a laccase enzyme allowing oriented immobilization. Pyrene can further act as cross-linker, be electropolymerized to reinforce the formed layer, and also be electro-oxidized becoming an efficient redox mediator for the electron transfer with a FAD co-factor. This versatility of pyrene and its derivatives for bioelectrochemical applications are focused here beside some original anchoring methods for biomolecules.

Keywords: Pyrene; Carbon nanotubes; Graphene; Biosensors; Biofuel cells;

1. Introduction

Bioelectrochemical applications cover mainly biosensor and biofuel cell systems. Biofuel cells are a subcategory of standard fuel cells where the catalysts at the anode and the cathode are biological catalysts (enzymes, bacteria) instead of noble metals and alloys. Since enzymes have naturally high specificity to their substrates, the enzymatic biofuel cells can produce energy in complex media like body liquids or blood without the need of a membrane¹. A further advantage is that all components are non-toxic and to a certain extent biodegradable. Biofuel cells were therefore proposed as alternative to button cell batteries in disposable electronic devices since needed power and operational consumption match perfectly the performances of biofuel cells². Biosensors are defined by the receptor unit which is of biological origin like enzymes antibodies, antigens, or DNA strands. Synthetic but bioinspired recognition elements like aptamers, DARPins³, or other biomimetic models are now well accepted in the field of biosensor⁴. The challenge in biosensor devices is to immobilize the receptor unit on a transducer surface without altering its unique specificity and activity. Carbon nanomaterials like graphene and CNTs are commonly used for biosensors^{5,6} or biofuel cells⁷ applications. The advantage is that deposits of bulk materials on electrodes provide excellent conductivities, biocompatibility, and high accessible specific surface area. A further advantage is that several functionalization techniques exist to graft any kind of chemical groups on these carbon materials to immobilize biological macromolecules. They can be distinguished between covalent, non-covalent, and defect group functionalization where each method has its advantages and disadvantages. For instance, covalent functionalization needs quite harsh conditions and reactive species like radicals, carbenes, or nitrenes to disturb the conjugated π -system of the CNT walls⁸. One elegant method is to generate in situ diazonium salts using phenylamines who can spontaneously or via electrochemical reduction form radicals and graft onto the CNTs⁹. Many examples exist where different functions were attached to the CNT sidewalls to immobilize bioreceptors or enzymes for biosensors or biofuel cell applications^{10,11}. Another common method to graft covalently biomolecules is the use of carboxylic acid functions directly introduced on the CNTs via oxidative treatments¹². These carboxylic acid functions can then be coupled with the present amines on proteins like enzymes or

antibodies, but also electrochemical DNA sensors were formed¹³ with this method using modified DNA strands.

Here, we want to focus non-covalent (bio) functionalization of CNTs for bioelectrochemical applications. Within this strategy, pyrene and its derivatives became one of the mostly used tool due to the formation of particular efficient π -stacking interactions on CNTs and graphene¹⁴⁻¹⁶. A further advantage is that several appropriate pyrene derivatives for amide coupling with proteins, or for the modification with any chemical functions are commercially available. The modification of nanocarbons with pyrene derivatives is very simple, fast, and reproducible via drop casting or dip coating techniques under mild ambient conditions¹⁷.

The first reported use of pyrene derivatives for carbon nanotube functionalization was validated with biomolecule immobilization in 2001¹⁸. Here, the team of Hongjie Dai modified single walled carbon nanotubes (SWCNTs), deposited on TEM grids, with commercially available 1-pyrenebutanoic acid succinimidyl ester (pyrene-NHS). Such activated esters were then incubated in an aqueous solution containing a protein. As for carboxylic acid functions on oxidized CNTs, the NHS function allows coupling with amines on the protein shells forming amides without the need of further reagents. In this proof of concept work, iron containing ferritin and gold nanoparticle conjugated avidin were used for improved contrast during TEM imaging. Even when no analytical properties of these bioassemblies were presented, the authors already emphasized the appropriateness of this method for bioanalytical applications. In fact, this work had a tremendous impact in the development of nanocarbon based biosensors, biofuel cells, and other bioelectrochemical applications. For instance, Patel et al. used a pyrene moiety modified with a lysine₃(aspartic acid) polypeptide to immobilize the catalytic MoFe protein of nitrogenase on CNT electrode. Thereby, the authors have presented an electroactivity validation of the pyrene-peptide and the kinetics of MoFe nitrogenase bio-electrodes. However, the most attractive part of this work is the possibility to use the pyrene-peptide linker to graft a wide range of His-tagged proteins on based-carbon materials through the pyrene-peptide availability to chelate divalent cations of the first-row transition metal¹⁹. Recently, in a similar way, Contaldo et al. synthesized the 1-acetato-4-(1-pyrenyl)-1,4,7-triazacyclononane for the immobilization of a His-tagged recombinant CODH from *Rhodospirillum rubrum* on CNT electrode. This effective pyrene-based compound for metalloenzymes immobilization converges benefits from pyrene to form π -stacking interactions with based-carbon materials, and the availability of metal-binding from 1,4,7-triazacyclononane²⁰.

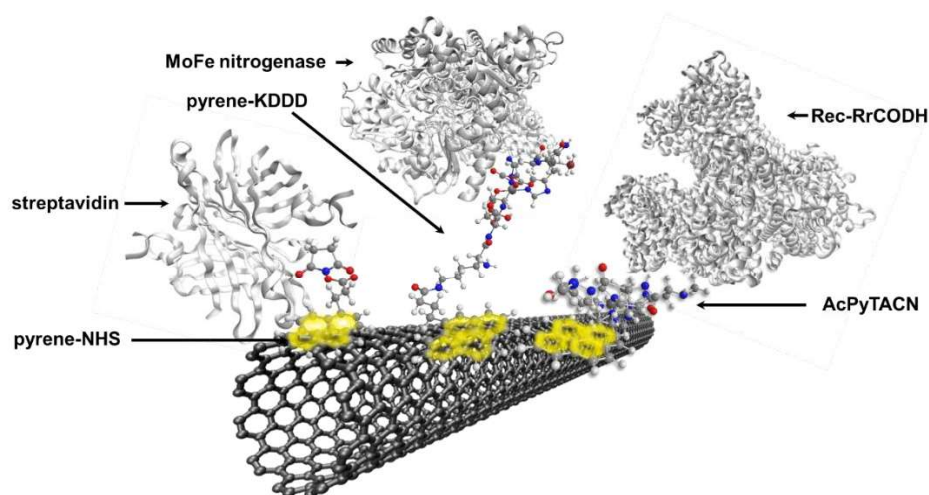


Figure 1. Representative sketch of original immobilizations of biomolecules with tailored pyrene derivatives such 1-pyrenebutanoic acid succinimidyl ester (pyrene-NHS), pyrene moiety modified with a lysine₃(aspartic acid) polypeptide (pyrene-KDDD) and 1-acetato-4-(1-pyrenyl)-1,4,7-triazacyclononane (AcPyTACN), respectively proposed by Chen et al.¹⁸, Patel et al.¹⁹ and Contaldo et al.²⁰

There are further uncountable examples in literature where pyrene derivatives serve for the immobilization of biomolecules. A pertinent selection of articles was summarized by Yan Zhou et al.²¹. Beside this versatility for biomolecule immobilization, pyrene derivatives have other interesting properties for bioelectrochemical applications which are focused here.

2. Oriented immobilization of laccase for biocathodes

Laccases belong to the multicopper enzyme family and have two catalytic sites. The so called T1 centre contains one coordinated copper ion and oxidises a wide series of phenolic compounds. The released electrons are then transferred to the trinuclear T2/T3 centre where oxygen is reduced to water²². Polyphenols, the natural substrate has some hydrophobic character and laccases have therefore a hydrophobic domain, close to the T1 centre, to provide some kind of a docking site. For the use of laccases in biocathodes for biofuel cell applications, the electric wiring of this T1 copper is of particular interest. Blanford et al. firstly reported an oriented immobilization of laccase on pyrolytic graphite using anthracene based anchor functions²³. Other polyaromatic compounds like anthraquinone, naphthalene, or chrysene resulted in excellent electrocatalytic performances of the formed biocathodes²⁴. Besides, pyrene can also interact with the hydrophobic domain of laccase from *Trametes Versicolor* enabling oriented immobilization of the enzyme²⁵. In this work, different immobilization techniques like simple adsorption onto carbon nanotubes, random covalent attachment using NHS coupling and electrosynthesized polypyrrole-pyrene were compared. The latter gave by far the highest electrocatalytic performances.

Pyrene can also be directly attached to biomolecules for their immobilization²⁶. For the investigation of electron transfer mechanism between the enzyme laccase from *Trametes sp.* and an electrode, pyrene was attached to the K₁₆₁ position, close to the T1 copper centre of this laccase variant. The sequence of the enzyme was obtained by site directed mutagenesis replacing arginine R₁₆₁ by lysine K₁₆₁. The native lysines K₄₀ and K₇₁ of the enzyme's protein shell were replaced by a methionine M₄₀ and a histidine H₇₁ function, respectively. This modified enzyme contained therefore just one free amine group on the surface for the localized attachment of an aldehyde bearing pyrene derivative via imine coupling. This mono-pyrene functionalized laccase showed excellent electrocatalytic performances towards oxygen reduction when immobilized on CNT electrodes. By the way, pyrene and its derivatives have also the capacity to form supramolecular host-guest interactions with cyclodextrins in aqueous media with an association constant of 128 L mol⁻¹ with β -cyclodextrin^{27,28}. This property was firstly used to cross link β -cyclodextrin modified glucose oxidase with a carbon nanotube electrode²⁹. The linker here was tris(bispyrene-bipyridine)iron(II), a symmetric iron complex with 6 spatially organized pyrene functions. While a part of these pyrenes adsorbs on the CNTs via π -stacking interactions, the other part remains available to form inclusion complexes with this β -cyclodextrin modified glucose oxidase leading to an efficient immobilization of the enzyme and a performing biosensor for glucose.

Pyrene – β -cyclodextrin inclusion complexes were formed with the mono-pyrene modified laccase and β -cyclodextrin modified gold nanoparticles, immobilized on pyrene adamantane functionalized CNT electrodes which led to a two-fold increase of catalytic current densities²⁶. The authors attributed this phenomenon to an increase of the surface coverage related to the additional gold nanoparticle surface and to improved direct electron transfer between gold nanoparticles and the enzyme.

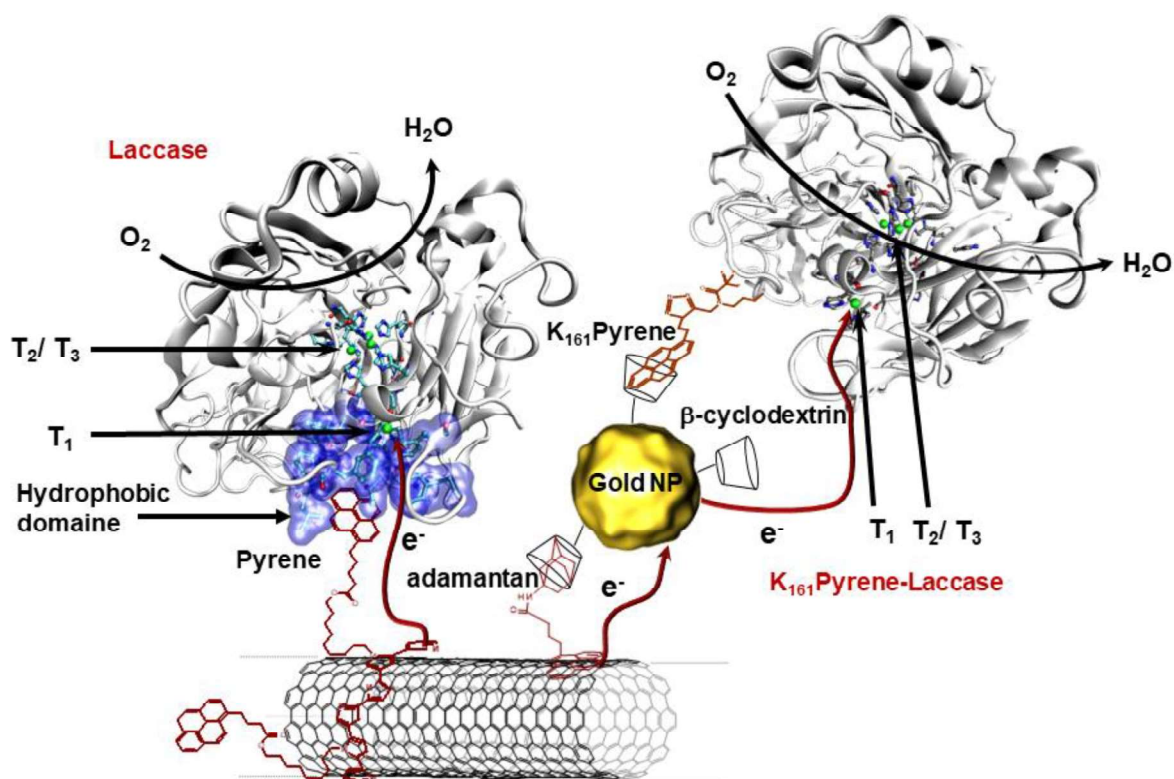


Figure 2. Examples for original wiring strategies of laccase via oriented immobilization using pyrene as supramolecular guest. (left) hydrophobic interactions of pyrene with the hydrophobic domain of laccase from *Trametes versicolor*. (right) inclusion complex of monopyrene functionalized laccase from *Trametes sp.* in β -cyclodextrin on gold nanoparticles. The particles were attached to an adamantan modified CNT electrode.

3. Pyrenes for cross linking

When at least two pyrene units are on the same molecule, this compound can act as cross linker for carbon nanomaterials leading to reinforced macroscopic morphologies. For instance, Wang et al. formed ultra-strong carbon papers using reduced graphene oxide and CNTs, cross-linked with bispyrene-diaminohexane³⁰. Few years before, the electroactive 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was functionalized with 1 butanol pyrene on each sulfonic acid group³¹. The intention was to functionalize CNTs for bioelectrochemical studies since ABTS is a perfect electron shuttle for multicopper oxidases and is commonly used as redox mediator for biocathodes³². The presence of two pyrene functions led to the formation of a free-standing CNT sheet (buckypaper) after filtration of the CNT bispyrene-ABTs mixture. With the pyrene cross linker, the obtained electroactive materials showed satisfying mechanic stability and excellent electron transfer kinetics with the multicopper enzyme laccase. After cross linking and buckypaper formation, a supplemental treatment with bispyrene-ABTS was done to saturate the surface with the intention that one pyrene function attaches to the buckypaper leaving the second pyrene group available for the oriented immobilization of laccase. Clearly improved electrocatalytic performances were obtained and could even be outperformed when laccase was present in solution.

Bispyrene-ABTS was further used to wire covalently attached laccase on calcinated electrospun carbon nanofiber felts³³. Electrospun CNT containing polyacrylonitrile fiber felts were annealed to carbonize the fibres. These carbon felts were then functionalized with 4-carboxyphenyl groups via diazonium coupling to cover the surface with carboxylic acid functions for subsequent immobilization of laccase by amide formation. The addition of bispyrene-ABTS clearly enhanced the electrocatalytic performance

of oxygen reduction since just a part of the randomly oriented laccases led to direct electron transfers. Bispyrene-ABTS acted here as molecular wire enabling mediated electron transfer by plugging the hydrophobic domain of laccase and thus wiring the T1 centre to the felt electrode.

Clearly reinforced flexible buckypapers could be obtained using a bifunctional polynorbornene copolymer containing pyrene and NHS functions³⁴. Several configurations were evaluated for laccase based buckypaper biocathode performances. The enzyme was immobilized either by simple adsorption, via covalent amide grafting thanks to the activated NHS ester, or by specific interaction with anthraquinone immobilized via NHS coupling. Anthraquinone is an excellent mimic for laccase substrates as is also commonly used for oriented immobilization of this enzyme^{35,36}. This was confirmed by these buckypaper experiments where anthraquinone modified electrodes provided most efficient electron transfers with laccase. Surprisingly, only pyrene polynorbornene without NHS functions did not result in conclusive electrocatalytic oxygen reduction. This might be related to the confinement of the pyrene functions in the polymer, principally serving for cross linking the CNTs, which makes them little available for oriented immobilization of laccase.

A similar block copolymer containing pyrene and NHS functions on a polynorbornene backbone was applied to immobilize and to wire FAD dependent glucose dehydrogenase (FAD-GDH)³⁷. After buckypaper formation cross linked by the polymer, the mediator thionine and FAD-GDH were subsequently immobilized via amide coupling. The resulting bioanode performances were compared with only pyrene-NHS functionalized buckypapers without cross linking. The polynorbornene based buckypaper provided clearly improved long term stabilities compared to the pyrene-NHS modified buckypaper and had slightly higher electrocatalytic current densities. Such improved performances were attributed to the higher available amount of NHS functions of the polynorbornene cross-linked buckypaper leading to a higher density of enzyme and mediator. Furthermore, the higher stability of FAD GDH immobilized on the electrode can also be attributed to a stronger interaction of buckypaper with pyrene-polymer than with pyrene-NHS. Hickey et al. synthesized a pyrene-modified linear poly(ethyleneimine) hydrogel to cross-link enzymes to carbon electrodes³⁸. This polymer showed not only the capacity to immobilize enzymes but also to enhance direct electron transfers of laccase with CNTs and the catalytic subunit of nitrogenase to Toray paper electrodes for the electroenzymatic reduction of N₂ to ammonia without the need of adenosine triphosphate.

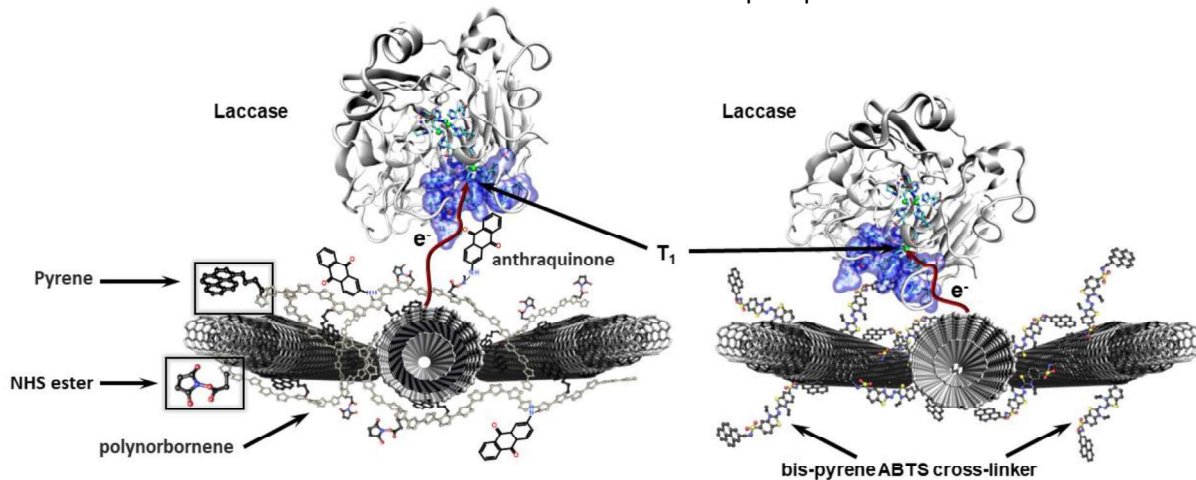


Fig. 3. Examples for the formation of reinforced buckypapers using pyrene as cross linkers. (left) bifunctional polynorbornene backbone with pyrene and NHS functions. The pyrenes served as cross linker while the NHS function served for the attachment of amino anthraquinone which also forms stable interactions with the hydrophobic domain of laccase. (right) molecular cross-linker containing two pyrenes (bispyrene ABTS). While available pyrene on the buckypaper leads to oriented immobilization and wiring of laccase, ABTS allowed additional mediated electron transfer with laccase in solution.

4. Electropolymerization of pyrenes

In a pioneering work, Waltman et al. realized the first electropolymerization of polycyclic hydrocarbons and therein pyrene³⁹. The authors revealed a radical cation coupling mechanism with similar stoichiometries as polypyrrole but pyrenes form more an agglomerate of oligomers than a homogeneous polymer film. Nonetheless, compressed pellets of these oligomers provided satisfying conductivity comparable to polypyrrole.

With the aim to profit from these electropolymerization properties, pyrenebutanol biotinester was electropolymerized on CNT electrodes⁴⁰. The cyclic voltammograms of this pyrene derivative show an irreversible oxidation wave at 0.8 V vs Ag/AgNO₃ indicating the formation of cationic radicals initiating the polymerization. While this signal decreases after several scans, another, reversible redox system appears at 0.4 V which was attributed to electroactive polypyrene. The authors further used a dip coating technique to form an as thin as possible pyrene-biotin layer on the CNTs and compared biosensor performances with and without subsequent electropolymerization. After immobilization of glucose oxidase via an avidin bridge to form a model enzyme biosensor towards glucose, the electropolymerized bioelectrodes provided clearly higher performances than the not electropolymerized version. It was assumed that despite the formation of oligomers the adhesion of pyrene-biotin was improved after electropolymerization keeping a higher amount of enzymes immobilized. Raman studies using SWCNTs with less defect functions revealed that the radical initiated polymerization of pyrenes does not affect the sp² sidewalls of the CNTs⁴¹ which should have led to an increase of the D/G band ratio. In contrast, after adsorption of the pyrene derivative, a decrease of this ratio was observed. It was concluded that π -stacked pyrenes contribute to the resonant behaviour of CNTs thus increasing the G band and decreasing the D/G ratio. The question about the formation of a monolayer around the CNTs was targeted using monolayer graphene⁴². Using high resolution XPS techniques with the graphene layer on a silicon wafer before and after dip coating and rinsing of a pyrene derivative, an average thickness of 1.4 nm could be determined which corresponds to the length of the pyrene derivative.

Pyrene could also be electrografted on amine functionalized carbon nanotubes⁴³. In this work, 3 dimensional structures were formed using electro-oxidized graphene oxide and NH₂-bearing CNTs. After electrochemical formation of secondary amines with pyrene butyric acid, laccase was immobilized via the pyrene anchor to form a biocathode. The authors claim that electro-oxidation of graphene forms supplemental hydroxyl functions of the edges which improve the adsorption of amine functionalized CNTs via hydrogen bonds. This should also reduce the intrinsic contact resistance of the composite. The performance towards electrocatalytic oxygen reduction was compared with laccase modified NH₂-CNTs alone with and without electrografting of pyrene butyric acid. The clearly lower electrocatalytic current densities emphasized the conclusion of the excellent conductivity of the graphene oxide-amino CNT composites.

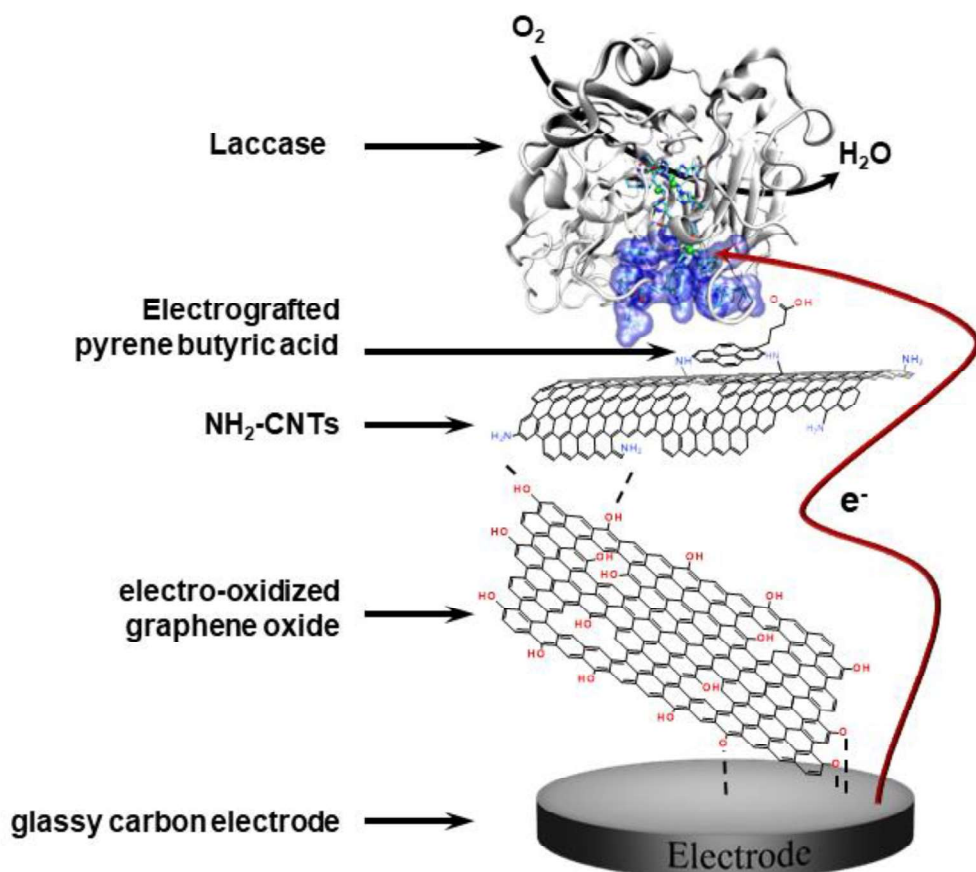


Figure 4. Illustration inspired from ⁴³ of oriented immobilization of laccase with pyrene. Here pyrene was electrografted on amine functionalized CNTs which are immobilized on an electro-oxidized graphene oxide on a glassy carbon electrode

5. Electro-oxidation of pyrene and its derivatives in aqueous media

A sophisticated procedure of increasing interest is the electro-oxidation of polycyclic aromatic hydrocarbons (PAHs) on carbon nanomaterials in aqueous medium. This easy procedure has been proven interesting through conversion PAH compounds into useful products such as for mediated bioelectrocatalysis. It is well known that many quinone derivatives are efficient redox mediators for electron transfers due to their good compromise between electron transfer kinetics and acceptable overpotentials ⁴⁴. Through an original strategy presented by Barathi et al. whereby π -stacking adsorbed pyrene on CNTs were electrochemically converted into pyrenequinone in aqueous solution ⁴⁵, Blanchard et al. evaluated the mediated electron transfer for FAD-GDH in a glucose bioanode setup ⁴⁶. In the proposed carbon nanotube-assisted electro-oxidation mechanism, the authors present controlled formation of 1,6-pyrenedione and 1,8-pyrenedione in optimised acidic conditions. The first pyrene electro-oxidation step starts *ca.* 1.0 V vs SCE forming an aromatic radical which is trapped by a water molecule while the positively polarized MWCNTs serve as a radical scavenger. The authors also suggest that the absence of 4,5 pyrenedione (ortho quinones) in the extracted products was explained by a better stabilization of pyrene's naphthalene intermediates when water traps the radical preserving Hückel's aromaticity. In pH 7 phosphate buffer solution, the π -stacking adsorbed pyrenequinones on CNTs revealed suitable redox potential ($E_{1/2} = -0.1$ V vs SCE) for mediated electron transfer with FAD-GDH from *Aspergillus sp* for catalytic glucose oxidation. Buzzetti et al. have extended the carbon nanotube-assisted electro-oxidation procedure to a series of polycyclic aromatics ⁴⁷. In this work the authors have demonstrated that naphthalene, phenanthrene, pyrene, perylene and coronene (from two to seven aromatic rings) could be electro-oxidized at 1.2 V vs SCE but with

different synthetic yields and redox potentials. Stability tests carried out via repeated voltammetric cycling in pH 7 phosphate buffer solution showed that after 100 consecutive cycles, the electro-oxidized polycyclic aromatics present different behaviours. Higher water solubility and consequent desorption of the formed hydroquinones or other side reactions might be responsible for loss of electroactivity. However, a more interesting point in this work concerns the electro-oxidation of pyrene-NHS for electron transfer between FAD-GDH and CNTs. Despite a lower synthetic yield compared to electro-oxidation of the pyrene, pyrenedione-NHS formed via electro-oxidation showed an improvement in terms of electrocatalytic current stability. The fact to having ester function able to forms amides with the enzymes clearly avoid the desorption of the enzyme. The easy nanotube-assisted electro-oxidation of pyrene in aqueous solutions is a real alternative to make a range of stable and thermodynamically suitable redox mediators and furthermore, became an efficient method for remediation and depollution of contaminated soils ⁴⁸.

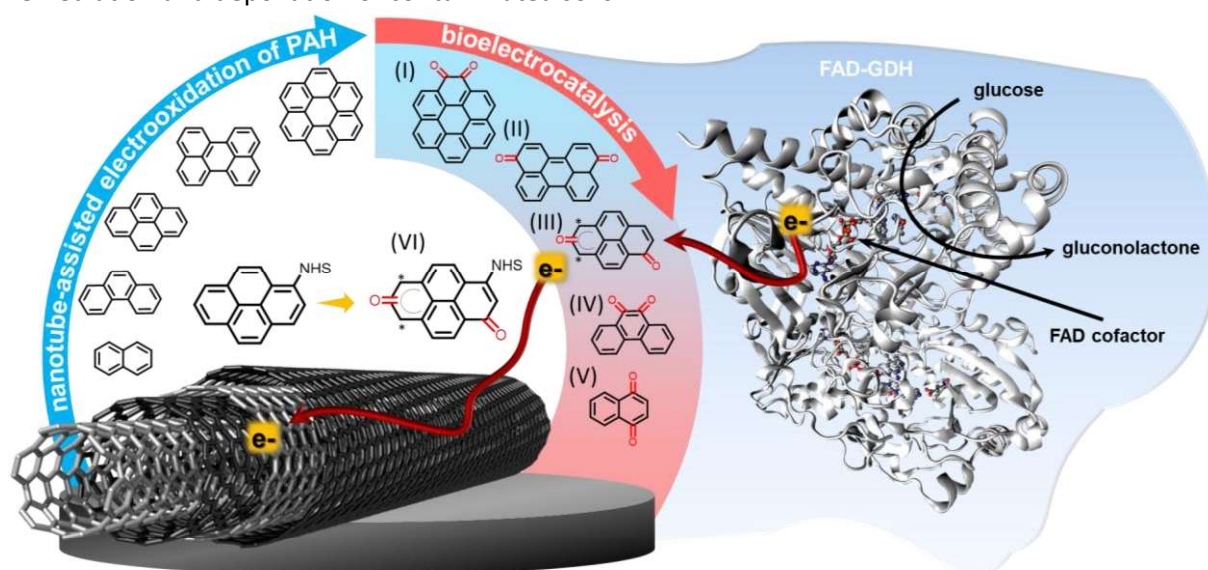


Figure 5. Illustration of carbon nanotube-assisted electro-oxidation of π -stacking adsorbed PAHs on CNTs in aqueous medium for mediated electron transfer with FAD-GDH from *Aspergillus sp* for catalytic glucose oxidation. Coronenedione (I), perylenedione (II), pyrenedione (III), phenanthredione (IV), naphthodione (V), pyrenedione-NHS (VI) show redox potentials at -0.08 ± 0.02 , -0.14 ± 0.02 , -0.07 ± 0.02 , -0.19 ± 0.02 , -0.17 ± 0.02 and -0.10 ± 0.02 V vs SCE, respectively. Furthermore, recorded catalytic current density of 0 mA cm^{-2} (I), 0 mA cm^{-2} (II), 7.5 mA cm^{-2} (III), 0.9 mA cm^{-2} (IV) 0.8 mA cm^{-2} (V) and 3.0 mA cm^{-2} (VI) were determined.

6. Conclusion

Pyrene shows to be an ideal tool for interfacing carbon nanomaterials with biomolecules. It can simply serve as an anchor for the immobilization of bioreceptors and additionally has beneficial properties for reinforcing materials by cross-linking or electropolymerization. Pyrene can further be electro-oxidized leading to mediated electron transfer with FAD dependent enzymes. It has the perfect size and hydrophobicity to form supramolecular complexes which are sufficiently stable in aqueous media. There might be even more possibilities with multiple functionalized pyrenes which are still unexplored yet. Until now, the conjugated π -system to interact with sp^2 hybridized carbon nanomaterials remain the mostly applied property for the immobilization of biomolecules onto nanostructured carbon.

7. Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

8. Acknowledgments

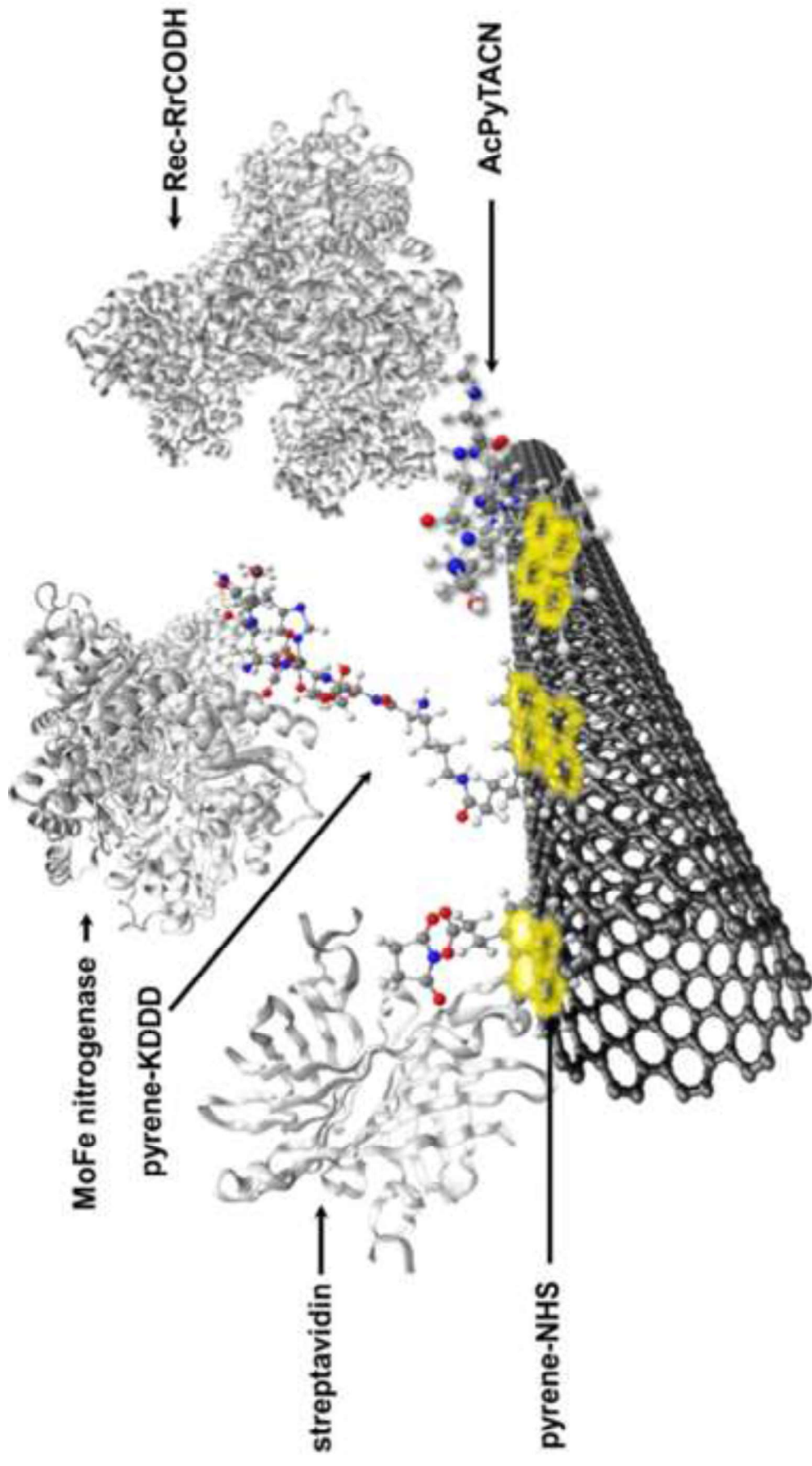
The authors wish to acknowledge the support from the platform Chimie NanoBio ICMG FR 2607 (PCN-ICMG) and from the LabEx ARCANE (ANR-11-LABX-0003-01 and CBH-EUR-GS, ANR-17-EURE-0003). The authors particularly thank the Institut Carnot PolyNat (CARN 0007-01) for financial support....

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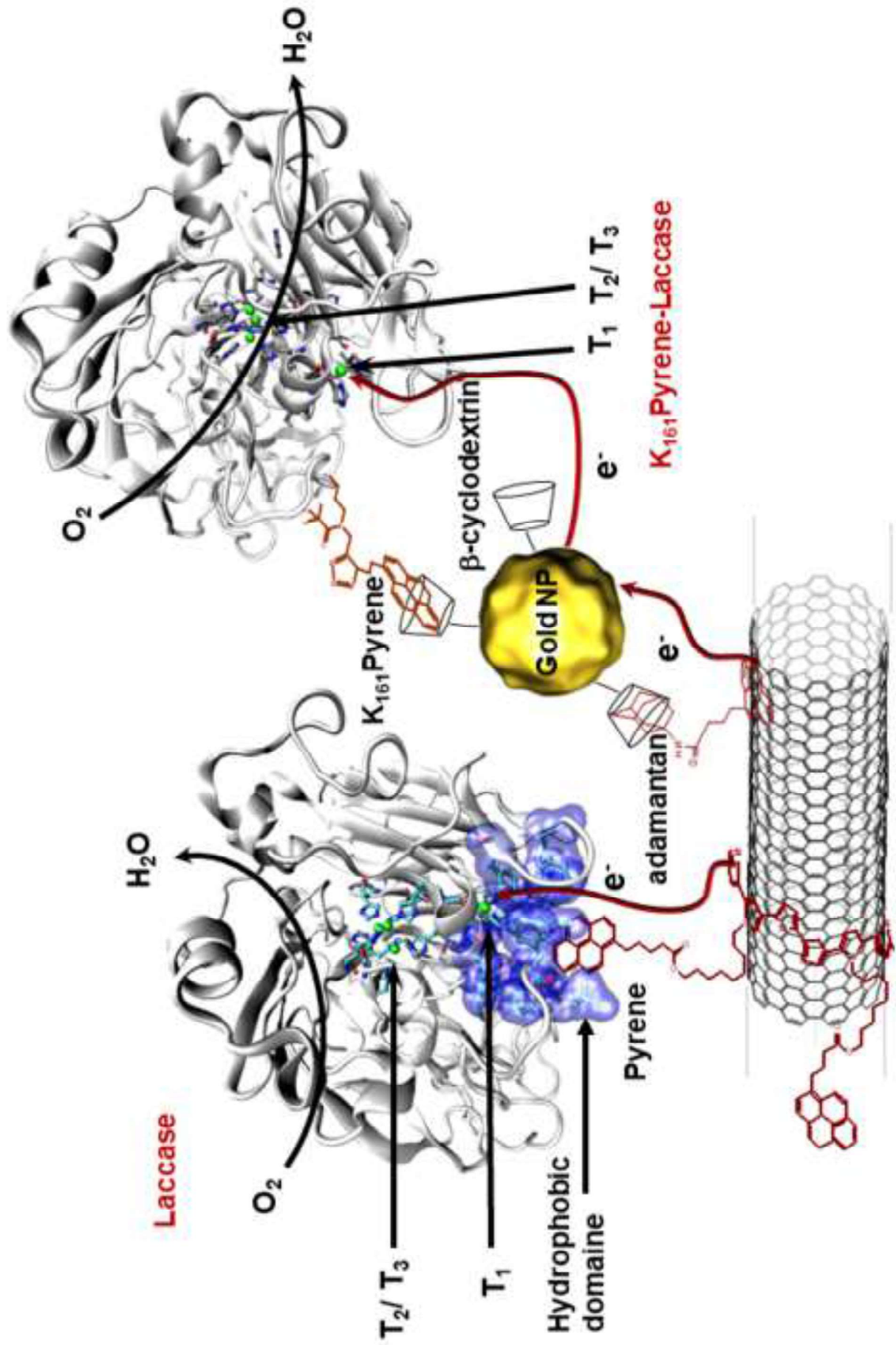


Figure 2

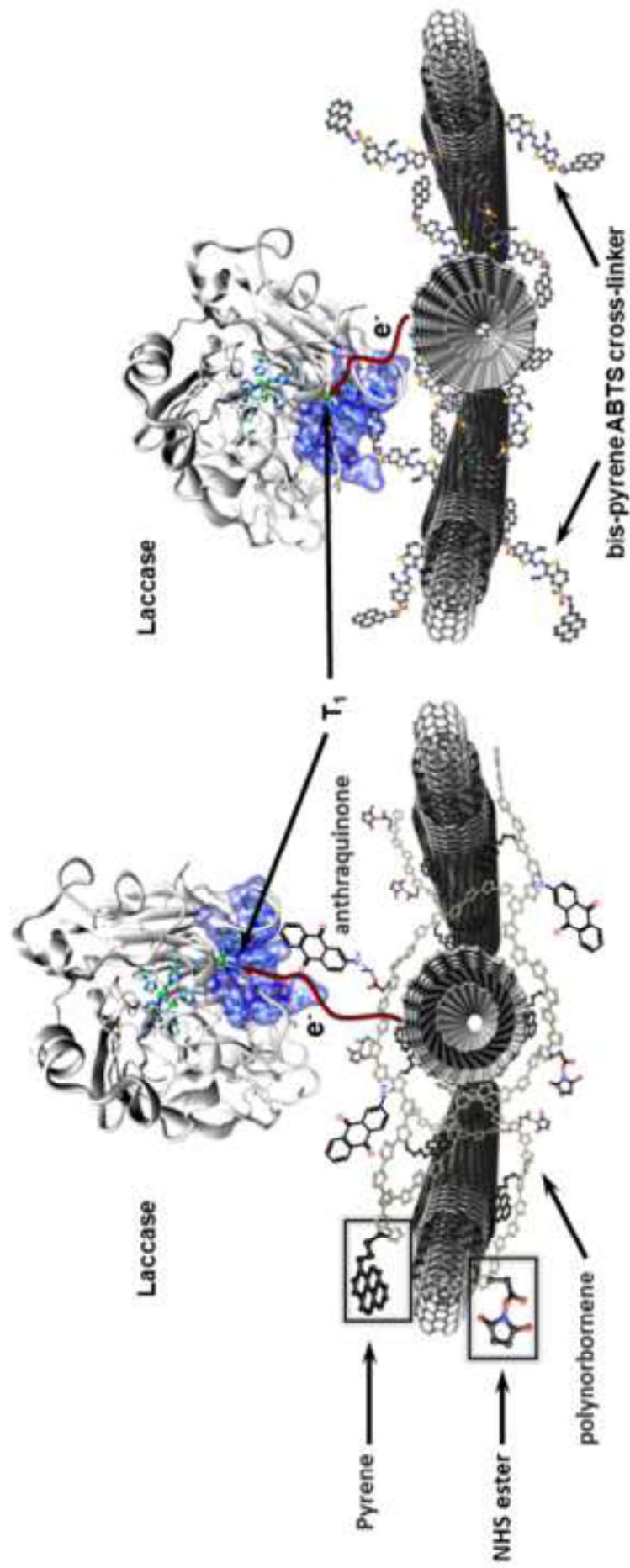
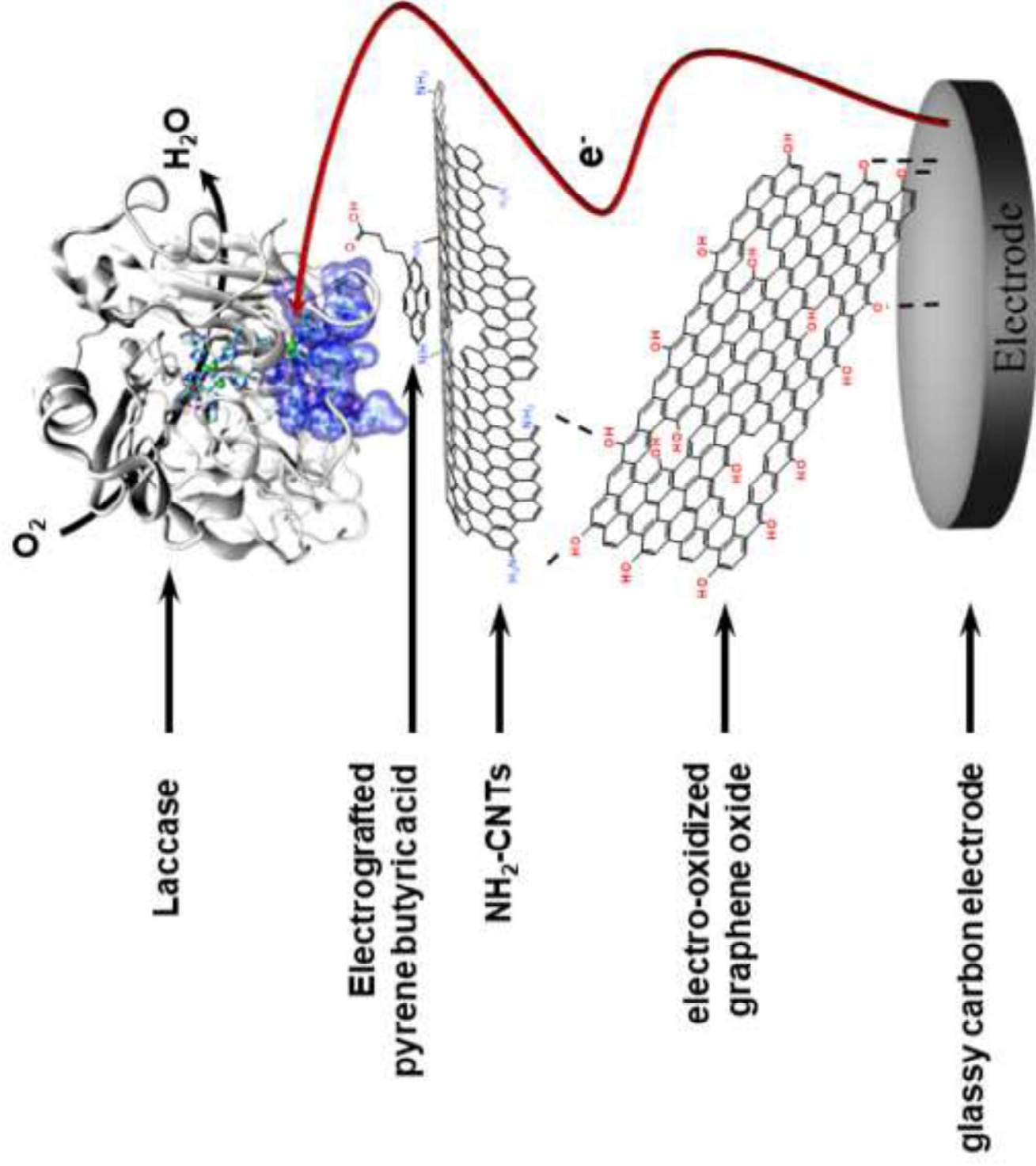


Figure 3



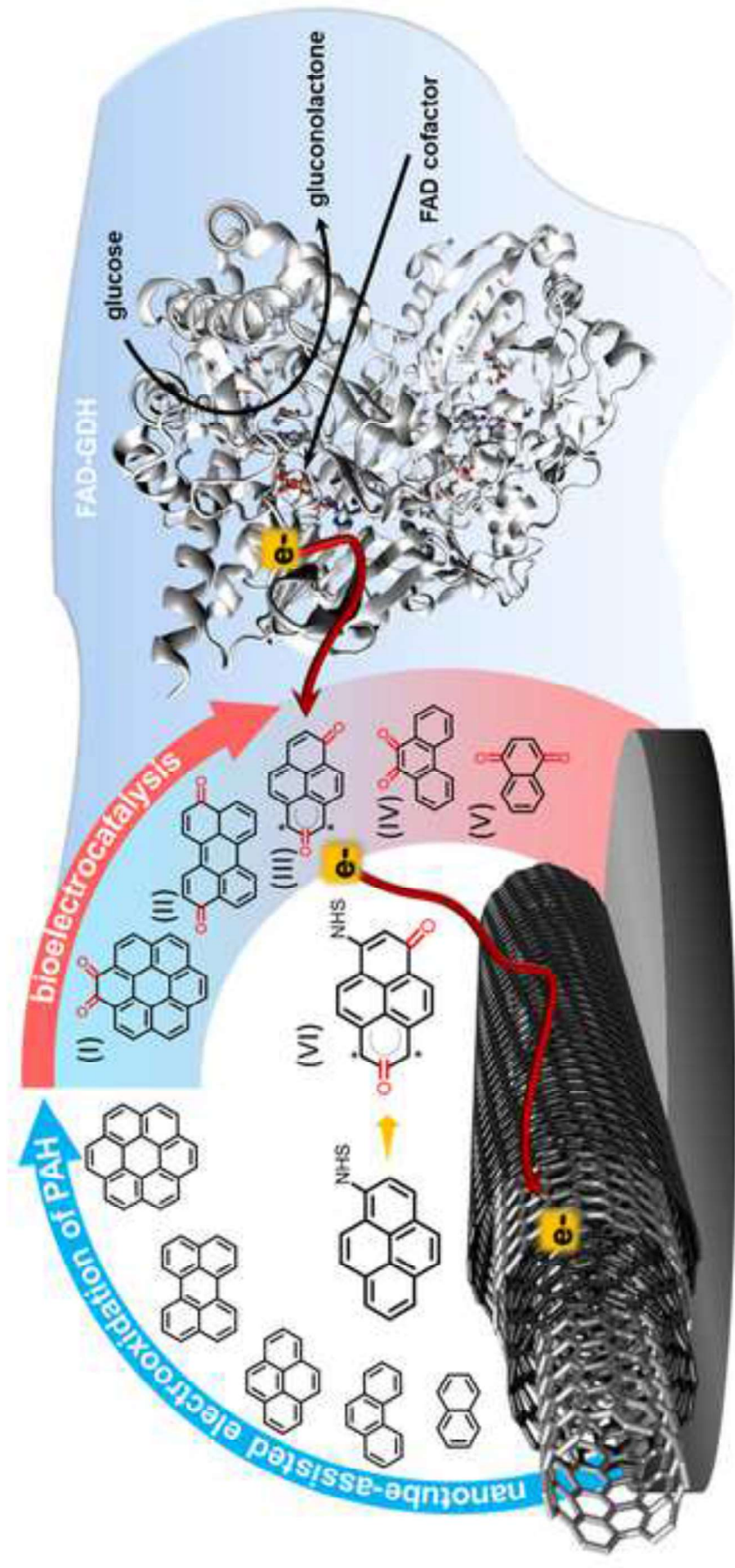


Figure 5