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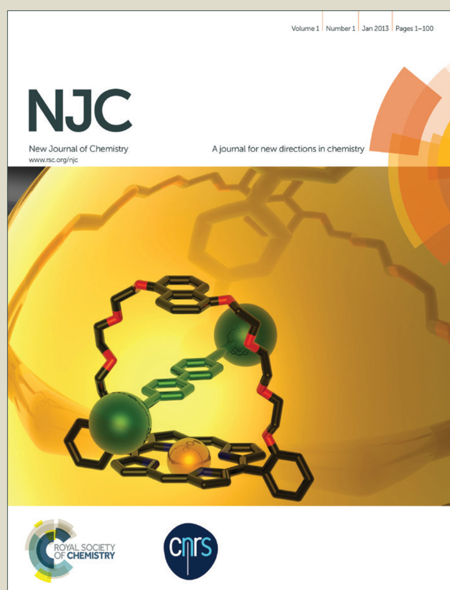


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ARTICLE

# Supramolecular immobilization of bio-entities for bioelectrochemical applications

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One constant challenge in biotechnological applications such as biosensors or biofuel cells is the immobilization of the bio-entity on respective surfaces. Beside widely used covalent binding, entrapment in polymers, or cross linking, protein or DNA grafting via supramolecular host-guest systems or metal-organic coordination, called affinity interactions, became a powerful alternative. One clear advantage of this principle is the facilitated formation of the biocomposite since the biological unit can be immobilized via self-assembly just by incubation of a surface, modified with a corresponding counterpart, in the biomolecule, bearing the other counterpart, containing solution. Most important affinity systems to immobilize bioreceptor units onto transducer surfaces are summarized. Original examples of this immobilization strategy are not only used for biosensing applications but also for other biotechnological fields like biofuel cells.

## Introduction

Biosensors are defined by a biological or biomimetic receptor unit and the transduction element that transforms the recognition event into a measurable signal. Biomolecules such as proteins or DNA strands are highly specific to the analyte and assure therefore the reliability of the sensor device. Biosensors can be classified into several types:
When the bioreceptor unit catalyzes the transformation of the analyte or an auxiliary substrate into a product that contributes to the signal capturing (e.g. electrochemical or optical signal), such biosensor setups belong to the metabolism or catalytic type. When the recognition event implies the detection based on specific interactions between the receptor and the analyte as it is for immunosensors or DNA sensors without a chemical reaction, this type is called bioaffinity sensors or affinity based biosensors [1]. The latter biosensor type is not to confuse with those where the bioreceptor unit is immobilized using affinity interactions even when these biosensors are often called "affinity biosensors" [2].

Immobilization of such bioreceptor units via such affinity interactions are based on supramolecular interactions, also including hydrophobic interactions or electrostatic interactions, and coordination chemistry with metal complexes [3]. When nature does not provide specific entities on the bioreceptors for its immobilization via affinity interactions, such functionalities have to be attached artificially. This certainly implies a supplemental step for the biosensor construction where even the recognition domain of the receptor can be affected, annihilating its biological activity. Nonetheless, up to date biotechnological biofunctionalization techniques allow a better controlled modification of such receptor units. Moreover, compared to immobilization techniques using covalent binding or cross linking with e.g. glutaraldehyde [4], the biological activity of the affinity partner bearing bioreceptors can be precisely determined after the modification step and such recognition entities can be stored under the respective ideal conditions. The final biosensor device can simply be formed via self-assembly by incubation of the transducer element in a solution containing these bioreceptors. This review focuses the principles of immobilization strategies using supramolecular interactions where some examples will present, in a non-exhaustive way, how these affinity systems can be integrated in the bioelectrochemical device.

## Biotin-(strept)avidin

The first and still most famous affinity system for the immobilization of almost all types of bioreceptors (proteins, DNA strands and bacteria) is the biotin-(strept)avidin system. All proteins of the avidin family are composed by four identic subunits, each capable to form a strong inclusion complex with biotin, known as vitamin B7 or vitamin H [5] (Fig. 1). The association constants can be up to $K_a=10^{15}$ L mol$^{-1}$ [6] and are comparable with covalent bonds. These outstanding properties of a natural product to serve as crosslinker made this biotin-(strept)avidin system the flagship for biomolecule immobilization via affinity interactions.

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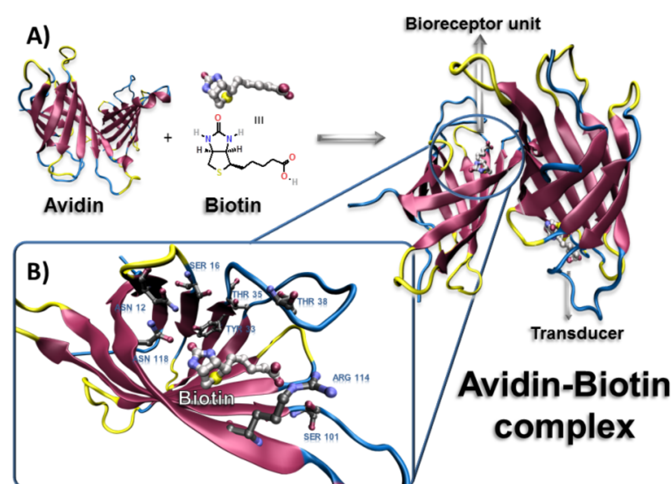


Figure 1: A) Scheme of the inclusion complex formed by biotin and avidin with two biotin guests. B) Focus on one avidin subunit and its residues interacting with the biotin molecule.

A further advantage of this affinity system is that the carboxylic acid group of the anchor biotin can easily be modified via ester or amide formations. This allows not only efficient biotin coating of surfaces but also biotinylation of proteins or amine modified DNA strands. The success of this approach lies mainly in the vast range of commercially available biotinylated biomolecules produced for the enzyme-linked immunosorbent assay (ELISA) used in clinical tests.

In terms of biotin modification of transducer surfaces, the electropolymerization of biotin labelled monomers like thiophenes [7], phenols [8], or pyrroles [9] represents a fast and controllable way to modify conductive transducers with thin biotin coatings. In this context, the anchoring of protein or oligonucleotide monolayer was thus formed via avidin bridges between electrogenerated biotin films and biotinylated enzymes, antibodies, bacteria or oligonucleotides [10-14].

Besides the biocompatibility of the procedure, the surface geometry of these films provides high accessibility of the immobilized biomolecules. In addition, the avidin proteins form a passivation layer on the transducer surface that prevents non-specific adsorption of the analytes on the surface. In contrast with conventional grafting or affinity binding, this step-by-step approach can also be applied to the preparation of assemblies containing multilayers of biological molecules [15].

The biotin monomers are usually electropolymerized on gold, platinum, glassy carbon, or carbon nanotube (CNT) electrodes providing pure biotinylated homopolymer or biotinylated copolymers by co-polymerizations with regular pyrrole, a pyrrole ammonium or a pyrrole modified tris-bipyridinyl ruthenium(II) complex [9, 16-18]. A particular exciting achievement has been the deposition of a transparent indium tin oxide (ITO) layer onto a glass fiber tip which was then used as electrode for the electrochemical formation of polypyrrole films [19]. This allowed the direct comparison between electrochemical and optical transduction. In this vein, a luminescent polypyrrole(biotin-luminol) co-polymer was successfully applied for efficient cholesterol detection using electrochemiluminescence (ECL) as transduction technique where a detection limit toward cholesterol of 1.47 $10^{-5}$ $molL^{-1}$ could be reached [20].

Another advantage of the (strept)avidin-biotin procedure is the fact that the surface of the transducer, after the recognition event, can be regenerated. It has been demonstrated that biotinylated conducting polypyrroles, used for the fabrication of DNA chips showed the capability to be regenerated after “denaturation” of the biotin/avidin links by surfactants [21]. This approach was applied to regenerate a DNA sensor chip. After DNA detection, the chips, fabricated with biotinylated polypyrroles could be reused after destruction of the biotin / avidin links. Another strategy to regenerate a biosensor based on the biotin-streptavidin affinity interaction is the controlled thermal treatment of the complex in nonionic aqueous solutions [22].

However, this affinity system is not always the most appropriate immobilization methods. The fact that the protein cross-linker forms compact layers on the surface represents a disadvantage when substrates have to diffuse to the transducer. This is mostly the case for catalytic enzyme biosensors where the analyte is a small molecule which is transformed by the enzyme into a signal giving compound. For instance, glucose oxidase (GOx) catalyzes the oxidation of glucose to gluconolactone by reducing oxygen into hydrogen peroxide. The enzymatically formed $H_2O_2$ is then electro-oxidized to oxygen at the electrode surface. When GOx is immobilized via this (strept)avidin-biotin system, the diffusion of $H_2O_2$ to the electrode is hindered leading to reduced sensitivities and detection limits.

Elegant alternatives were proposed avoiding such (strept)avidin layers where also biotin can be used as anchor molecule.

## Metal ligand affinity

An alternative approach to immobilize bioreceptor units on transducer surfaces via affinity interactions is the coordination of ligands to metal centers. The proof of concept was realized in the late ‘90 where $Ni^{2+}$, $Zn^{2+}$ or $Cu^{2+}$ ions were complexed in carboxylate or imidazole containing polymer films [23]. The biosensor was formed by the coordination of histidine-tagged proteins to the metal centers. Histidine is an amino acid with imidazole as function. Such histidines can naturally be present on the protein shell or have to be attached to the receptor. A more controlled approach to immobilize biomolecules applying coordination chemistry is the use of the chelating ligand nitrilotriacetic acid (NTA) followed by the complexation of a divalent metal ions like $Cu^{2+}$ or $Ni^{2+}$. NTA functions are commonly available as N, N’ bis(carboxymethyl)-L-lysine which has the advantage that the amine group of lysine can serve for the grafting of NTA. Following this route, NTA functionalized surfaces with coordinated $Cu^{2+}$ or $Ni^{2+}$ ions were initially used in affinity columns for the isolation of histidine

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tagged proteins [24]. Haddour et al, demonstrated that this affinity system is also appropriate for the immobilization of biomolecules [25]. An immunosensor for atrazine was described using this principle. Histidine-tagged anti-atrazine was immobilized on NTA-$Cu^{2+}$ coatings and, after incubation in the analyte solution, the biosensor showed detection limits down to 10 pgmL$^{-1}$ [26]. Furthermore, polypyrrole(NTA-$Cu^{2+}$) films show an excellent permeability towards redox probes which led to a high performance impedimetric DNA sensor. Oligohistidine tagged ssDNA was immobilized on this electrogenerated polymer coating and, due to the high sensitive impedance changes combined with improved redox probe diffusion, a detection limit for the analyte ssDNA of $10^{-15}$ mol L$^{-1}$ was obtained which is still beneath the highest performances for DNA biosensors [27]. The immobilization of aptamers and the sensitive detection of model proteins such as thrombin was also investigated at polypyrrole and polypyrene electropolymerized films using either by impedimetric [28] or photoelectrochemical [29] transduction.

Particular interesting is the possibility that even biotin can be attached on NTA-$Cu^{2+}$ complex. Baur et al. adjusted more than forty years old studies about the coordination of biotin with Zn (II), Mn (II) and Cu (II) [30, 31] to develop the new affinity system NTA-$Cu^{2+}$-biotin [32]. It has been shown that the same biosensor performances were obtained using either polyhistidine or biotin as tags. The obvious advantage here is, as already mentioned, the availability of commercialized biotin tagged bioreceptor units.

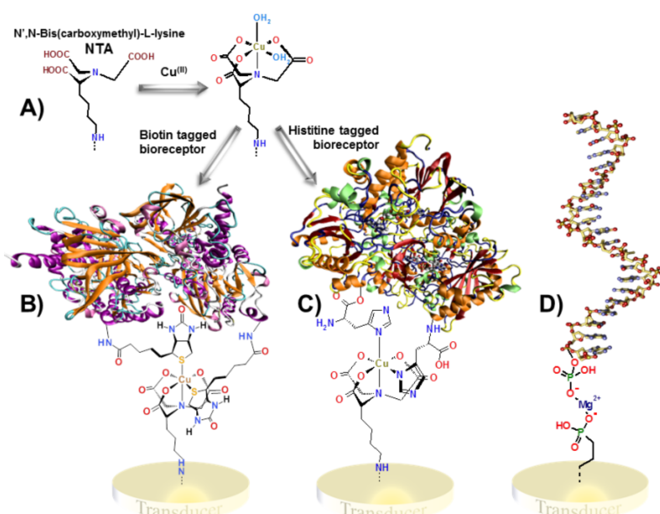


Figure 2: Immobilization of bioreceptor units by coordination of A) biotin or B) histitine tagged bioentities on NTA-$Cu^{2+}$ modified transducer surfaces. C) Coordination of DNA phosphates on phosphate group modified transducers via $Mg^{2+}$ linkage.

A further advantage of this NTA-$Cu^{2+}$ affinity system is the facilitated regeneration of the biosensor by simple addition of EDTA (ethylenediamine tetraacetic acid) or imidazole, releasing the metal free NTA ligand. [25] A similar approach was proposed by Thompson et al. [33] where $Mg^{2+}$ ions act as the linking element between a phosphonic acid-modified polymer film and the phosphonate groups of DNAs. By electrostatic modulation of a chloride ion-exchange properties of a copolymer, an extremely sensitive label-free DNA sensor with a detection limit of 1.82 $10^{-21}$ molL$^{-1}$, determined by cyclic voltammetry was obtained using $Mg^{2+}$ coordinated receptor units [34].

Among different electrode modification techniques, self-assembled monolayers (SAMs) modified with NTA groups were also investigated for the controlled immobilization of laccase [35] and hemoprotein [36]. Recently, $Zn^{2+}$ cations were used as the chelating metal center [37]. Carbon electrodes were modified by NTA groups using electrografting of a functionalized aryldiazonium salt. A histidine-tagged nitrate reductase was immobilized with NTA-$Zn^{2+}$ complex. $Zn^{2+}$ cations have the advantage not be reduced at low potentials, which is one drawback of $Cu^{2+}$ ions.

## Hydrophobic interactions : Inclusion complexes and π–stacking interactions

Within the cyclodextrin family, β-cyclodextrin (β-CD) is the mostly used version for the immobilization of biomolecules via supramolecular inclusion complexes. β-CD is the cyclic oligosaccharide composed by seven α-D-glucopyranoside units. The particular cone structure with highly hydrophilic rims and a more hydrophobic "wall" [38] form stable supramolecular complexes with a large variety of hydrophobic molecules of appropriate sizes and is frequently used in molecular sensors [39], or as solubilization agent in cosmetics and pharmaceutics [40, 41]. Villalonga et al. presented for the first time an immobilization strategy for enzymes based on the supramolecular interactions between β-CD and adamantane [42]. A gold electrode was modified with a β-CD containing polymer with incorporated gold nanoparticles to form a xanthine biosensor using adamantane tagged modified xanthine oxidase. Adamantane is a pure and extremely hydrophobic hydrocarbon with an ideal size to form an inclusion complex with β-CD with a relatively high association constant of $K_a$ = 5.2 x $10^4$ Lmol$^{-1}$ [43]. This original affinity system was further applied for the construction of an efficient glucose sensor after electropolymerization of an adamantyl-pyrrol derivative on CNT electrodes. This polymer was successfully applied to attach directly, without the need of an intermediate protein layer, β-CD modified GOx. Further improvements in sensitivity could be obtained with gold nanoparticles, where adamantane tagged GOx were anchored on a layer of β-CD modified gold nanoparticles attached to the adamantane functionalized CNTs [44].

Stable inclusion complexes with pyrene were also demonstrated. Le Goff et al. synthesized a hexa pyrene trisbipyridyl iron complex which was π-stacked to CNT electrodes. This hexagonal complex enabled the availability of free pyrene groups which could immobilize β-CD tagged GOx [45].

Within the vast number of possible hydrophobic entities to form host-guest complexes in order to immobilize biomolecules, biotin might be within the most appropriate ones, again, due to the availability of commercialized biotinylated

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bioreceptor units. The inclusion of biotin inside the β-CD cone was comprehensively studied where an association constant of 300 ± 12 $M^{-1}$ was determined using $^{1}$H-NMR investigations [46]. This association constant might appear low but was sufficient to immobilize biotinylated GOx and polyphenol oxidase (PPO) on a β-CD modified electrode. Additional experiments even revealed a relative high non-specific binding of GOx on β-CD which is not the case with PPO. These phenomena were attributed to a natural high amount of hydrophobic amino acids like phenylalanine or tryptophane on the GOx protein shell. These hydrophobic groups are less present in the protein shell of PPO.

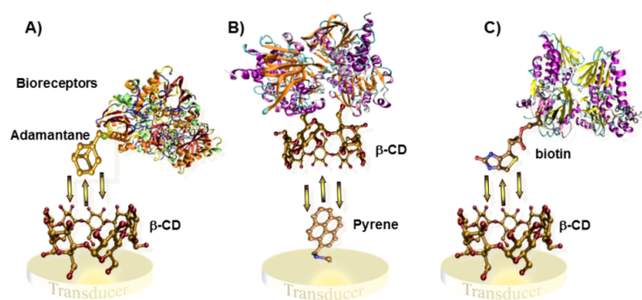


Figure 3: Bioreceptor immobilization via host guest interaction between A) β-CD and adamantane, B) β-CD and pyrene, and C) β-CD and biotin.

The use of host-guest formations for biosensing applications does not exclusively concern the receptor immobilization. Kaifer al. [47] studied a series of redox active molecules like viologen or ferrocene and their supramolecular interactions with β-CD. It has been shown that strong inclusion complexes are formed when the redox active molecule is neutral. When in oxidized form, both, viologen and ferrocene are charged and are released from β-CD.

Yumei et al. took advantage of the inclusion of ferrocene in β-CD and used it in a pyruvic acid biosensor setup as electron mediator [48].To avoid the release of charged ferrocene, all components of the bioelectrodes were crosslinked with glutaraldehyde.

Another strategy, presented for the first time by the H. Dai group [49], has widely been developed on carbon nanomaterials based on π-extended systems such as graphene and carbon nanotubes: the π-stacking of functional pyrene molecules. This approach has been successfully employed to immobilize biocatalysts by affinity interactions, as previously mentioned [50] or by amide coupling using pyrene butyric acid [51]. Using a supramolecular π-π stacking approach, pyrene-based functionalization prevents from damaging CNT sidewalls, which often occurs in the case of covalent techniques. This soft and versatile technique was used for the immobilization and direct wiring of hydrogenases and bilirubin oxidase in membraneless $H_2/O_2$ biofuel cells [52]. Using pyrene-modified proteins such as lyzozymes or protein kinases, P. G. Collins and coworkers have studied single molecule dynamics or catalysis at SWCNT Field-Effect Transistor [53, 54].

## Electrostatic - hydrophobic interactions

Under given conditions, it is possible to immobilize biomolecules via hydrophobic or electrostatic interactions by using the natural conformation of the biomolecule. Proteins are composed by hydrophobic and hydrophilic domains. When these domains are not involved in the recognition process, they can be used for the immobilization of the biomolecule. In terms of electrostatic interactions, the surface charges of the proteins are determined by their isoelectric point (pI) which indicated if the protein is positively or negatively charged at neutral pH. When a majority of peptidic carboxylic acids are present on the protein shell, the pI is in the negative range and changes to positive values in presence of a majority of amine groups. DNA strands are also composed by ionic phosphate groups and hydrophobic nucleic acids. However, the physisorption of DNA strands implies in all cases its deactivation towards its complementary DNA. One famous example is the DNA wrapping of carbon nanotubes [55] where the nucleotides of the DNA adsorb on CNTs provoking this enrolment due to the helical conformation of the DNA strand. It is clear that such wrapping DNA are biologically inactive but serve in this case as surfactant or functionalization agent [56]. DNA-CNT assemblies were nevertheless applied in bioanalytical devices. Tang et al. described an original approach for a DNA sensor device by taking advantage of the DNA wrapping of the CNT conductive channel in a field effect transistor device (NTFET) [57]. The receptor DNA was attached to the gold contacts and by hybridization with the analyte DNA, the contact resistance between the gold electrode and the CNT channel was modulated leading to a high sensitive electronic transduction of the biorecognition event. Since the CNT channel inhibited the hybridization of the wrapped DNA, the conductive channel was not affected by the recognition event and therefore, the electronic modulation. Except of such few examples of CNT based DNA sensors [58], CNT based biosensors were mainly reported for protein biosensors such as immunosensors or enzyme sensors [59].

CNTs and other carbon (nano)materials like graphene [60, 61] are especially appropriate for the immobilization of bioreceptor units via hydrophobic interactions [62] where other transducer (nano)materials serve for electrostatic immobilization of biomolecules [63]. This immobilization strategy is principally used for enzyme biosensors where the random deposition of proteins does not directly affect their biological activity. In case of redox enzymes, their immobilization via hydrophobic or electrostatic interactions is in some cases particular useful when the biomolecule provides a specific domain close to its active redox center leading to enhanced electron transfer between this enzyme and the electrode [64].

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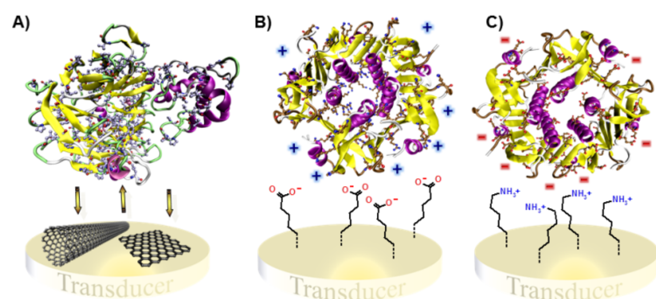


Figure 4: Immobilization strategy using A) hydrophobic interaction and electrostatic attraction of B) cationic (pI > 7) and C) anionic (pI < 7) proteins.

## Affinity interactions for improved electron transfer and redox enzymes.

Electron transfer between substrate specific redox enzymes and electrodes clearly enhances electrochemical signal capturing since enzymatically produced auxiliary redox probes randomly diffuse in the electrochemical cell and only a part is transduced by the electrode. To perform such electron transfer, two main strategies are employed:

- Mediated Electron Transfer (MET), where small redox active molecules with excellent electron transfer kinetics serve as intermediate electron donor/acceptor shuttle between the enzyme and the electrode surface.
- Direct Electron Transfer (DET), where the active site of the enzyme can directly be regenerated by the electrode after the catalytic redox reaction. Therefore, the possibility to obtain DET is strongly related to the location of the active site inside the protein, the conformational morphology of the protein, and the ability of the electrode to access the redox center.

Beside the usefulness of MET or DET in biosensor devices [64-67], electric wiring of enzymes is of steady growing interest since these biological catalysts are promising alternatives to noble metal catalysts for (bio)fuel cell applications [68-70].

The challenge to immobilize and to orientate enzymes in order to achieve DET strongly depends on the conformal structure of the enzyme. Firstly, the active center should not be situated deeply inside the protein shell or should provide in this case a physiological electron transfer pathway. Furthermore, the protein domain around the active center has to provide a concentrated amount of specific amino acids (with hydrophobic, anionic or cationic groups, respectively) in order to functionalize the electrode material with corresponding hydrophobic or ionic affinity partners.

Due to these restrictions, only few examples are reported where redox enzymes could be oriented during immobilization via affinity interactions establishing DET.

One elegant example was described by I. Willner and coworkers. They functionalized gold electrode with enzyme cofactors such as nicotinamide dinucleotide and FAD by using a boronic acid-functionalized self-assembled monolayer [71, 72]. After incubation of the electrode in an apo-enzyme solution, they have shown the possibility to reconstitute the active enzyme on the electrode surface. An efficient electron transfer between immobilized reconstituted GOx and the electrode could be observed. In another example, this group also achieved the immobilization of NADH cofactor and the contacting of NAD-dependant dehydrogenase.

Metalloenzymes were also studied. [NiFe] Hydrogenases from the Desulfovibrio species have a high amount of glutamic acids (an amino acid with two carboxylic acid groups) around the binding site of a tetrahemic cytochrome c3 group which is part of the electron transfer pathway. This strongly anionic domain led to enhanced DET after hydrogen oxidation when the electrode material was functionalized with positively charged (mostly ammonium) groups [73-77]. Other types of hydrogenases are also under constant investigations concerning its orientated immobilization via hydrophobic or again electrostatic interactions. Such sometimes weak interactions can also be reinforced by peptide couplings or cross linking reaction to assure the stability of these bioassemblies [78].

Another enzyme with one peculiar domain close to its redox center is laccase. The F. A. Armstrong group took advantage of the presence of a hydrophobic pocket near the laccase's T1 center and used it to orientate this enzyme during immobilization on anthracene modified surfaces [79]. The T1 center of the multicopper enzyme has the task to supply the T2/T3 copper centers with electrons in order to reduce oxygen. These electrons are usually obtained via oxidation of phenolic compounds, the natural substrate of laccase. When correctly oriented, this T1 center can also be regenerated by the electrode.

Several examples report the efficient immobilization, orientation, and wiring of laccase, in particular on CNTs, using polyaromatic hydrocarbons such as anthracene [80], naphthalene [81], pyrene [82], or anthraquinone [83, 84] derivatives leading to a high performance biocathodes with catalytic current densities of up to 1.85 mA in oxygen-saturated solution. [85]

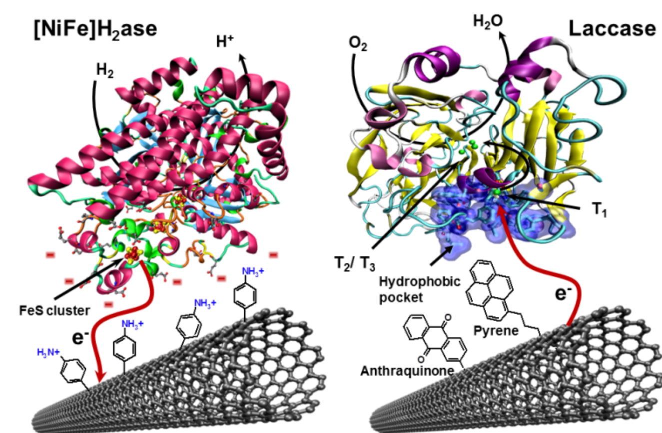


Figure 5: Schematic presentation of improved DET by oriented immobilization of A) [NiFe]H$_2$ase via targeted electrostatic interactions and B) Laccase via targeted hydrophobic interactions on functionalized carbon nanotubes.

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## Conclusion and perspectives

The immobilization of bioentities via affinity interactions is a powerful tool in bioanalytical devices and even shows in some cases clear advantages for the design of biofuel cells. Affinity systems can be attached to the biomolecules in a reproducible way at industrial scale without or barely affecting their biological activity. In particular, the affinity partner biotin has shown a strong versatility among other affinity systems and can be applied for supramolecular inclusion complexes or metal complex formation beside its well-known interaction with proteins of the avidin family. This, unfortunately, represents also a challenge when several affinity systems are used at the same time. For instance, there are examples where biotin and β-CD were combined for sensor devices, fluorescent assemblies, or for their use in cosmetics. [86-88] In these cases, particular attention has to be paid for the order of the components to immobilize on such multifunctional surfaces. Future developments in immobilization techniques using supramolecular interactions will certainly target the individual functionalities of specific biorecetors to overcome the need of chemical modifications. As already described here, the hydrophobic domain of the enzyme laccase allows its oriented immobilization, even enabling direct electron transfer with the electrode material without any supplemental modification. [82, 83] Inclusion complexes between amino acid residues on the GOx shell and β-CD were identified as non-specific interactions and therefore considered as an undesirable side effect. [46] On the contrary, such phenomena can be taken as advantage and optimized for specific residues at strategic positions on a protein shell for efficient immobilization and/or orientation of bioreceptors. Such strategy already became a standardized method in DNA sensor devices. Here, synthetic oligonucleotides of certain sequences serve as anchor groups for the corresponding DNA strand. [89] Since the defined sequence already assures the desired specificity, these anchor groups usually serve as receptors in the sensor device.

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